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EDITORIAL

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Editorial

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Tephritid fruit flies (Diptera: Tephritidae) are considered by far the most important group of horticultural pests worldwide. Female fruit flies lay eggs directly into ripening fruit, where the maggots feed causing fruit loss. Each and every continent is plagued by a number of fruit fly pests, both indigenous as well as invasive ones, causing tremendous economic losses. In addition to the direct losses through damage, they can negatively impact commodity trade through restrictions to market access. The quarantine and regulatory controls put in place to manage them are expensive, while the on-farm control costs and loss of crop affect the general well-being of growers. These constraints can have huge implications on loss in revenues and limitations to developing fruit and vegetable-based agroindustries in developing, emergent and developed nations.

Because fruit flies are a global problem, the study of their biology and management requires significant international attention to overcome the hurdles they pose. The Joint Food and Agriculture Organisation /International Atomic Energy Agency (FAO/IAEA) Programme on Nuclear Techniques in Food and Agriculture has been on the foreground in assisting Member States in developing and validating environment-friendly fruit fly suppression systems to support viable fresh fruit and vegetable production and export industries. Such international attention has resulted in the

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successful development and validation of a Sterile Insect Technique (SIT) package for the Mediterranean fruit fly.

Although demands for R&D support with respect to Mediterranean fruit fly are diminishing due to successful integration of this package into sustainable control programmes against this pest in many countries, there were increasing demands from Member States in Africa, Asia and Latin America, to address other major fruit fly pests and a related, but sometimes neglected issue of tephritid species complexes of economic importance. Any research, whether it is basic or applied, requires a taxonomic framework that provides reliable and universally recognized entities and names. Among the currently recognized major fruit fly pests, there are groups of species whose morphology is very similar or identical, but biologically they are distinct species. As such, some insect populations that are grouped taxonomically within the same pest species, display different biological and genetic traits and show reproductive isolation which suggest that they are different species. On the other hand, different species may have been taxonomically described, but there may be doubt as to whether they actually represent distinct biological species or merely geographical variants of the same species. This uncertain taxonomic status has practical implications on the effective development and use of the SIT against such complexes, particularly at the time of determining which species to mass-rear, and significantly affects international movement of fruit and vegetables through the establishment of trade barriers to important agricultural commodities which are hosts to these pest tephritid species.

A Consultants' Meeting, organized by the IAEA and held on 6-10 July 2009 in Vienna, Austria, discussed a number of major fruit fly complexes and prioritised them as to their economic importance, regional importance and potential for SIT application. Three complexes and a suspected complex were identified to be of significant importance that needed to be resolved to facilitate world agricultural trade and SIT programmes. They were:

- *Anastrepha fraterculus*
- *Bactrocera cucurbitae* (suspected complex)
- *Bactrocera dorsalis*
- *Ceratitis* FAR complex (*C. fasciventris, C. anonae, C. rosa*)

Based on this outcome, a FAO/IAEA Co-ordinated Research Project (CRP) on "Resolution of Cryptic Species Complexes of Tephritid Pests to Overcome Constraints to SIT Application and International Trade" was initiated and officially launched at the first Research Coordination Meeting (RCM) in Vienna on 2-6 August 2010. When addressing the status for each of these complexes, an integrative taxonomic approach was followed, whereby researchers used multiple, independent lines of evidence to delimit the species boundaries. These independent lines included traditional morphology, morphometrics and geomorphometics, developmental physiology, pre- and postzygotic mating incompatibility, karyology, chemoecology, and a wide range

of molecular techniques such as multi-locus markers and microsatellites among others. Over a six year period, researchers from more than 20 countries looked at a wide range of different aspects of species delimitation for the priority complexes and presented their findings and research progress during consecutive RCMs in Brisbane, Australia (February 2012), Tucumán, Argentina (August 2013) and La Réunion, France (June 2015). This volume presents the result of this collaborative and integrated approach to resolve the species complexes and clearly demonstrates the advantage of combining efforts, expertise and team-working when addressing such a complex issue as species boundaries. In total, 25 articles are included in this issue. Each paper was peer-reviewed by at least one, but usually two or more independent experts. We would like to thank the many reviewers for their valuable input and assistance in improving the contents of many of the papers. A synthesis of the findings is given in the Introductory Summary Paper, which also includes references to the many papers published elsewhere by CRP participants during the life of the project.

Regrettably, during the period that this CRP was running, we lost two prominent and leading scientists who were involved in fruit fly research for a long time and contributed to species recognition: Serge Quilici and Peter Teal.

Serge Quilici (1955–2015) was a senior researcher at CIRAD (Centre de Coopération International en Recherche Agronomique pour le Développement) based in La Réunion, France. He was well known in the world of fruit fly research, in particular for his involvement in research on fruit fly behaviour, invasive species, interspecific competition, host selection and the role of semiochemicals among others. As a longstanding member of the fruit fly community he coordinated several international projects, supervised many students (in particular from Africa), and kept a number of official functions in international bodies and organisations.

Peter Teal (1953-2015) was a research leader of the chemistry research unit at the Center for Medical, Agricultural and Veterinary Entomology (CMAVE) at USDA-ARS Gainesville, Florida (USA) and Acting Station Director at the Subtropical Horticulture Research Station in Miami. He was mainly known for his outstanding work in the field of isolation and identification of naturally produced compounds that can affect the behaviour and reproduction of insects, including fruit flies. His research on insect physiology was of great importance in the development of control and monitoring strategies for pest species and Peter received international recognition for his research outputs and leadership.

Both researchers were also very warm and kind persons willing to assist and guide fellow scientists in their research. Their guidance but also their friendliness and companionship will be sorely missed by all of us. We, therefore, dedicate this issue to the memory of these two scientists who have shown the path to several of us and upon whose work we have continued to explore the boundaries of these species complexes.

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FORUM PAPER

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Resolving cryptic species complexes of major tephritid pests

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Abstract

An FAO/IAEA Co-ordinated Research Project (CRP) on "Resolution of Cryptic Species Complexes of Tephritid Pests to Overcome Constraints to SIT Application and International Trade" was conducted from 2010 to 2015. As captured in the CRP title, the objective was to undertake targeted research into the systematics and diagnostics of taxonomically challenging fruit fly groups of economic importance. The scientific output was the accurate alignment of biological species with taxonomic names; which led to the applied outcome of assisting FAO and IAEA Member States in overcoming technical constraints to the application of the Sterile Insect Technique (SIT) against pest fruit flies and the facilitation of international agricultural trade. Close to 50 researchers from over 20 countries participated in the CRP, using coordinated, multidisciplinary research to address, within an integrative taxonomic framework, cryptic species complexes of major tephritid pests. The following progress was made for the four complexes selected and studied:

Anastrepha fraterculus complex – Eight morphotypes and their geographic and ecological distributions in Latin America were defined. The morphotypes can be considered as distinct biological species on the basis of differences in karyotype, sexual incompatibility, post-mating isolation, cuticular hydrocarbon, pheromone, and molecular analyses. Discriminative taxonomic tools using linear and geometric morphometrics of both adult and larval morphology were developed for this complex.

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Bactrocera dorsalis complex – Based on genetic, cytogenetic, pheromonal, morphometric, and behavioural data, which showed no or only minor variation between the Asian/African pest fruit flies *Bactrocera dorsalis, B. papayae, B. philippinensis* and *B. invadens*, the latter three species were synonymized with *B. dorsalis*. Of the five target pest taxa studied, only *B. dorsalis* and *B. carambolae* remain as scientifically valid names. Molecular and pheromone markers are now available to distinguish *B. dorsalis* from *B. carambolae*.

Ceratitis FAR Complex (*C. fasciventris, C. anonae, C. rosa*) – Morphology, morphometry, genetic, genomic, pheromone, cuticular hydrocarbon, ecology, behaviour, and developmental physiology data provide evidence for the existence of five different entities within this fruit fly complex from the African region. These are currently recognised as *Ceratitis anonae, C. fasciventris* (F1 and F2), *C. rosa* and a new species related to *C. rosa* (R2). The biological limits within *C. fasciventris* (i.e. F1 and F2) are not fully resolved. Microsatellites markers and morphological identification tools for the adult males of the five different FAR entities were developed based on male leg structures.

Zeugodacus cucurbitae (formerly *Bactrocera* (*Zeugodacus*) *cucurbitae*) – Genetic variability was studied among melon fly populations throughout its geographic range in Africa and the Asia/Pacific region and found to be limited. Cross-mating studies indicated no incompatibility or sexual isolation. Host preference and genetic studies showed no evidence for the existence of host races. It was concluded that the melon fly does not represent a cryptic species complex, neither with regard to geographic distribution nor to host range. Nevertheless, the higher taxonomic classification under which this species had been placed, by the time the CRP was started, was found to be paraphyletic; as a result the subgenus *Zeugodacus* was elevated to genus level.

Keywords

Anastrepha fraterculus, Bactrocera carambolae, Bactrocera dorsalis, Ceratitis anonae, *Ceratitis fasciventris*, *Ceratitis rosa*, *Zeugodacus cucurbitae,* integrative taxonomy, Sterile Insect Technique, sibling species

Introduction

Tephritid fruit flies (Diptera: Tephritidae) are among the world's worst pests of agriculture, being of major economic importance in nearly all tropical, subtropical and temperate countries (Cavalloro 1983, White and Elson-Harris 1994). By laying their eggs directly into fruit, where the maggots feed and develop, these pest species cause enormous devastation to both food production and international trade in spite of often intensive insecticide applications. They are among the primary causes of poverty, malnutrition and poor production and trade in fresh horticultural commodities in large areas of tropical developing countries, impeding the development of lucrative and labour-intensive fruit and vegetable-based agroindustries in rural areas (Waterhouse 1993, Allwood and Leblanc 1996).

The study of the biology and management of tephritids requires significant international attention to overcome transboundary hurdles and to assist the global community in developing and validating more environment-friendly fruit fly suppression systems to support viable fresh fruit and vegetable production and export industries. Such international attention has resulted in the successful development and validation of a Sterile Insect Technique (SIT) package for the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann, 1824) (Dyck et al. 2005). R&D support for this pest species

is diminishing due to successful integration of the SIT into area-wide integrated pest management (AW-IPM) programmes to manage *C. capitata* populations (Enkerlin 2005). On the other hand there is increased demand from Africa, the Asia-Pacific and Latin America to address other major tephritid species or groups of economic importance. Some of these major pest fruit fly species occur within cryptic species complexes that include taxonomically described species that may actually be geographical variants of the same species. Conversely, some fruit fly populations grouped taxonomically within the same pest species display different biological and genetic traits, including reproductive isolation, which suggest that they are different species (Clarke and Schutze 2014). This uncertain taxonomic status has important practical implications on the effective development and use of the SIT against such pest complexes where the species under mass-rearing is not the same as the population occurring in the target area. Uncertainty of taxonomic status can also result in the incorrect establishment of trade barriers for agricultural commodities that are hosts of pest tephritids.

The resolution of some of the taxonomic uncertainties that surround major cryptic species complexes is therefore critical both for integrated SIT application and for subtropical and tropical countries to overcome non-tariff trade barriers, enabling them to export their fresh fruit and vegetable commodities to international markets. In particular, it is essential that the sterile males from such species complexes produced in regional fruit fly rearing facilities and destined for release in different countries or regions are behaviourally fully compatible with the target native fruit fly pest populations in the various recipient regions (Cayol et al. 2002). If the taxomomic status of species complexes remained unresolved, it would be difficult or impossible to achieve this desirable goal.

To address these issues, a major international collaboration was initiated in 2010 under the auspices of the Joint Food and Agriculture Organization / International Atomic Energy Agency (FAO/IAEA) Programme on Nuclear Techniques in Food and Agriculture. This paper summarises the goals, achievements and results of this coordinated research project that are compiled in this special issue of Zookeys (2015, Special Issue 540).

Approach

During a Consultants' Meeting, held from the 6th to 10th of July 2009 in Vienna, Austria, the potential for conducting co-ordinated R&D in this area was assessed, and the major tephritid pest complexes were discussed and prioritised in terms of economic importance and potential for SIT application. Three complexes, the *Anastrepha fraterculus* complex (Latin America), the *Bactrocera dorsalis* complex (Asia and Pacific, Africa), and the *Ceratitis* FAR (= *C. anonae* Graham, 1908, *C. fasciventris* (Bezzi, 1920), *C. rosa* Karsch, 1887) complex (Africa) were confirmed to be of priority. The possibility that *Bactrocera cucurbitae* (Coquillett, 1899) (Asia and Pacific, Africa) also represents a species complex was evaluated and considered a lower, but still important, priority. In

each of these groups (Figure 1), questions were raised concerning the validity of some of the described species, the capacity to diagnose described species, or the strong *a priori* evidence that unrecognised sibling taxa may occur.

A proposal for an FAO/IAEA Co-ordinated Research Project (CRP) on "Resolution of Cryptic Species Complexes of Tephritid Pests to Overcome Constraints to SIT Application and International Trade" was formulated and approved for the period 2010-2015. The specific objectives of this CRP were to define, using an integrative taxonomic approach (Schlick-Steiner et al. 2010), the species limits within the target complexes, and to develop robust species-specific diagnostic tools.

This international research network was operated under the IAEA Research Contract Programme and included 22 research teams. Other research teams also par-

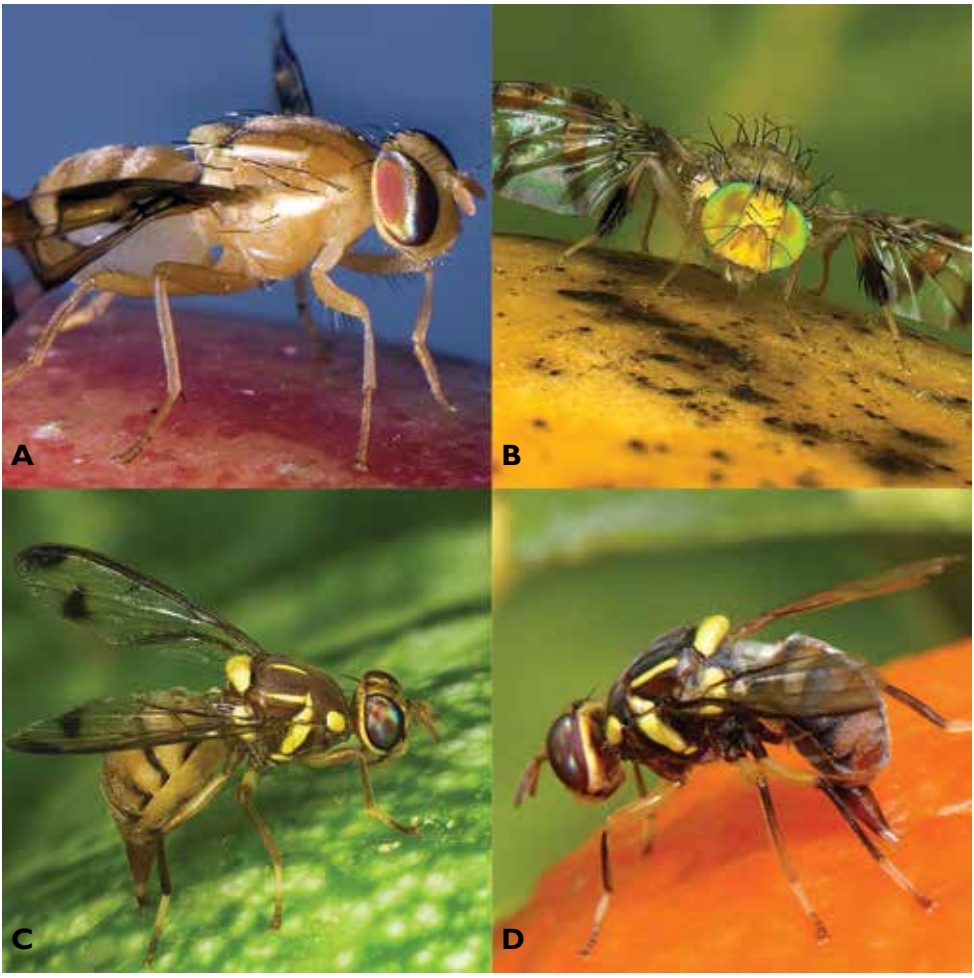

Figure 1. Habitus image of a representative of the four cryptic species complexes. **A** *Anastrepha fraterculus* **B** *Ceratitis rosa* (R2 type) **C** *Zeugodacus cucurbitae* **D** *Bactrocera dorsalis.* Photo credits: **A** Michal Hoskovec, **B** and **C** Antoine Franck, **D** Ana Rodriguez.

ticipated directly or indirectly and were fully funded by their institutions and governments. Overall close to 50 researchers from over 20 countries from all continents participated at one time or another during the six years of the CRP (2010-2015).

Research networks were established to (1) encourage close collaboration among institutes from developed and developing countries, (2) provide a forum for information exchange between scientists, and (3) embrace a focused approach to the development, capacity building and technology transfer of environment-friendly technologies. A worldwide network of partners provided representative samples of the fruit fly populations in order to assess the genetic diversity throughout the distributional ranges of the members of each complex. A generic protocol for collection and shipment of live and dead insects for vouchering, rearing, morphological and morphometric studies, chemical ecology and molecular assays was developed at the start of the CRP and used for distribution of material between the participating research units. Whenever possible, colonies of populations were established at the FAO/IAEA Agriculture and Biotechnology Laboratories in Seibersdorf, Austria, to be able to carry out field cage cross-mating studies that would not have been acceptable at other locations due to quarantine regulations and the risk of pest establishment.

During the implementation of the CRP, four Research Co-ordination Meetings (RCMs) were held to review research progress and to agree on future research directions and activities: the first RCM in Vienna, Austria from 2 - 6 August 2010, the second RCM in Brisbane, Australia from 31 January - 3 February 2012, the third RCM in Tucumán, Argentina from 26 - 31 August 2013 and the fourth and final RCM in La Réunion, France from 1 - 5 June 2015.

Situation Analysis at the Start of the CRP and Ouputs for Each Complex

1.1 *Anastrepha fraterculus* Complex Situation Analysis

The South American fruit fly, *Anastrepha fraterculus* (Wiedemann, 1830) s.l., is present in most countries of the Americas from the USA to Argentina (Hernández and Aluja 1993, Steck 1999, Zucchi 2007). Its centre of diversity is the South American subcontinent, where formerly it was thought to occurr in two, possibly unconnected bands: one along the western edge, including both highland and lowland areas of the Andean range, and the other along the east coast. However, recent data indicate its presence in parts of the Brazilian Amazon basin (Zucchi et al. 2011). It has been reported to infest about 110 host plants including major fruit crops (Norrbom and Kim 1988, Zucchi 2000, 2015, Norrbom 2004). The presence of this highly destructive pest results in quarantine restrictions for fruit export to many countries (Steck 1999).

The high levels of variability found among different populations throughout the geographical range of *A. fraterculus* led to the conclusion that it is a complex of cryptic species rather than a single biological entity (Stone 1942, Morgante et al. 1980, Malavasi and Morgante 1982, Solferini and Morgante 1987, Steck 1991, Steck and

Sheppard 1993, Selivon 1996, Hernández-Ortiz et al. 2004). Differences have been reported based mainly on morphology, pest status and genetics (including karyotype, isozyme and molecular analyses); these are reviewed in Steck (1999) and some aspects discussed in subsequent studies (McPheron et al. 1999, Norrbom et al. 1999, Gomes Silva 2000, Smith-Caldas et al. 2001, Aluja et al. 2003, Hernández-Ortiz et al. 2004, Barr et al. 2005, Selivon et al. 2005, Goday et al. 2006, Selivon and Perondini 2007, Silva and Barr 2008, Prezotto 2008, Cáceres et al. 2009). However, in order to establish how much of this variation reflects population level variation, and how much reflects unrecognised cryptic species diversity, it is necessary to systematically correlate these genetic and morphological differences with the existence of reproductive isolation and other life history related traits (hosts, demography, etc).

Reproductive incompatibility has been reported both at pre- and post-zygotic levels between some *A. fraterculus* populations. At the pre-zygotic level, mating compatibility was evaluated among different populations from South America, involving lowland (Peru) and highland (Colombia) areas from the Andean region, and the south-eastern part of the continent (Brazil and Argentina). Most of the populations were shown to have some level of incompatibility with each other and thus appeared sexually isolated. Flies of different populations were often sexually active at different times of the day suggesting different sexual behaviour (Selivon 1996, Vera et al. 2006, Cáceres et al. 2009).

Post-zygotic studies between two populations from Brazil (Selivon et al. 1999) and between one Argentinean population and one Peruvian population (Cáceres et al. 2009) found partial hybrid inviability and sex ratio distortion confirming the existence of post-zygotic barriers. In the former case, cytological, isozyme and molecular studies revealed differences among groups (Malavasi and Morgante 1982, Selivon 1996, Selivon et al. 2005, Goday et al. 2006); while for the latter case, differences between groups were also found in terms of male sex pheromones and karyotypes (Cáceres et al. 2009).

The combined results of these studies suggested the existence of seven different biological entities referred to as: *Anastrepha* sp. 1 *aff. fraterculus* (Brazilian 1 morphotype) (Yamada and Selivon 2001), *Anastrepha* sp. 2 *aff. fraterculus* (Brazilian 2 morphotype) (Yamada and Selivon 2001), *Anastrepha* sp. 3 *aff. fraterculus* (Brazilian 3 morphotype) (Selivon et al. 2004), *Anastrepha* sp. 4 *aff. fraterculus* (Peruvian morphotype) (Selivon et al. 2004), *A. fraterculus* Mexican morphotype (Hernández-Ortiz et al. 2004), *A. fraterculus* Andean morphotype and *A. fraterculus* Venezuela coastal lowlands (Steck 1991).

Although previous studies provided strong evidence supporting the existence of several biological species, major knowledge gaps still existed in 2010. In particular, the described studies used different methodologies, did not use the same identified biological material and, most importantly, did not include all of the morphotypes. Therefore, in order to be able to formally describe and name these putative species, it was considered critical to apply a standardised, complete set of methodologies to all populations from the entire geographic distribution range in a comprehensive integrative taxonomy study. This would allow the characterization of each putative species and would provide sound diagnostic tools for addressing the related management and trade issues.

Definition of species limits and formal naming of these putative species will be relevant for plant protection authorities in determining which of them may or may not be quarantine pests. This would immediately allow some countries to gain access to international fresh fruit markets for those countries and commodities which can be determined to be outside the geographic and host range of correctly delimited *A. fraterculus* s.s. In addition, detailed studies on pest status, host range, economic impact and distribution would minimize any possible impact on trade between South American countries. Furthermore, knowing species boundaries and their levels of sexual compatibility within the complex would enable the implementation of the SIT.

1.2 Outputs on the *Anastrepha fraterculus* complex

Colonies from five *A. fraterculus* morphotypes (Mexican, Andean, Peruvian, Brazilian 1 and Brazilian 3) were established and used for behavioural, chemical, cytological, molecular and larval morphology studies. Linear and geometric morphometry were validated as tools for morphotype discrimination. Comprehensive morphometric studies supported the existence of eight morphotypes: the seven reported previously and a new Equadorian morphotype (Hernández-Ortiz et al. 2012, 2015).

Guidelines for performing mating compatibility field cage tests were developed. Reproductive compatibility studies were performed among the five morphotypes. Among the combinations studied, morphotypes were incompatible at the pre- and post-zygotic level (Rull et al. 2012, 2013, Devescovi et al. 2014). Male calling and courtship behaviour were recorded for four morphotypes. Sexual behaviour studies helped to identify behavioural characteristics that allowed distinct morphotype descriptions such as time of sexual activity, acoustical signals and sequence of courtship behaviour (Rull et al. 2012, 2013, Devescovi et al. 2014, Dias et al. in press). One post-copulatory study revealed differences in sperm storage and remating propensity between the Peruvian and Brazilian 1 morphotypes (Abraham et al. 2014). Hybrid females tended to mate with hybrid males and as a result Segura et al. (2011) suggested that hybridization is a possible speciation mechanism. In all combinations analysed, post-zygotic isolation was found to be weaker than pre-zygotic (Devescovi et al. 2014). *Wolbachia* was detected in several morphotypes (Cáceres et al. 2009, Lima 2015, Lima et al. unpublished data) and more in-depth studies aiming to characterize these strains are in progress.

The chemical profiles of the male pheromones and cuticular hydrocarbons of these five morphotypes were characterized as complex blends that were qualitatively and quantitatively unique for the different morphotypes (Břízová et al. 2013, Gonçalves et al. 2013, Vaníčková et al. 2012, 2015a,c,d, Milet-Pinheiro et al. 2015). The description of the mitotic karyotypes from the Mexican, Colombian and Equadorian morphotypes, from which information was absent or incomplete, allowed confirming that karyotypes are unique for each morphotype (Canal et al. unpublished data). The polytene chromosome map for one morphotype was constructed (Gariou-Papalexiou

et al. unpublished data). Internal Transcriber Spacer 1 (ITS 1) was found to be a good molecular marker to identify different groups (Sutton et al. 2015). Microsatellites were developed (Lanzavecchia et al. 2014) and proved to be successful to discriminate among morphotypes (Lima et al. unpublished data, Manni et al. 2015). The phylogenetic relationships of Andean-Ecuadorian populations were determined with other molecular markers (Ludeña et al. 2011).

Based on all the collected evidence it is now possible to describe four morphotypes as new species with the exception of the three Brazilian morphotypes for which it is still necessary to solve problems with the unknown origin of the holotype male of *Anastrepha fraterculus* (Wiedemann) and the new Ecuadorian morphotype from which further studies are required for an integrative description (Hernández-Ortiz et al. 2015). A manuscript with the description of the new species is in preparation (V. Hernández-Ortiz personal communication) and improved diagnostic tools are now available based on morphology, molecular markers, chemical profiles, cytology and sexual behaviour.

The knowledge and gaps identified at the start of the CRP, as well as the progress made by the end of the CRP in addressing the gaps identified for the *A. fraterculus* complex are summarized in Table 1.

2.1 *Bactrocera dorsalis* Complex Situation Analysis

Across Asia and the Pacific the fruit fly subfamily Dacinae contains some 47 recognised pest species (Drew and Romig 2013). Of these, eight were recognized within the *Bactrocera dorsalis* complex, with some being the most economically damaging of all pest species within the subfamily (Drew and Hancock 1994, Clarke et al. 2005). Losses caused by *B. dorsalis* complex species include destruction of crops, restriction of international trade, and the establishment of a range of quarantine and regulatory activities carried out by various regional governments.

Background research on these flies has generated data on diagnostics, field surveillance, quarantine strategies, field pest control, and market access protocols (e.g. Tan and Nishida 1996, 1998, Muraji and Nakahara 2002, Naeole and Haymer 2003, Smith et al. 2003, Armstrong and Ball 2005). But the key knowledge gap of the *B. dorsalis* complex was a lack of consensus on species limits of the major pest species in the complex, particularly *B. dorsalis* s.s. (Hendel, 1912), *B. papayae* (Drew & Hancock, 1994), *B. philippinensis* (Drew & Hancock, 1994), *B. carambolae* (Drew & Hancock, 1994) and *B. invadens* (Drew, Tsuruta & White, 2005) (Clarke et al. 2005, Drew et al. 2005, Wee and Tan 2005, Ebina and Ohto 2006, Drew et al. 2008). Failure to resolve the taxonomic status of the members of this complex prevented further development towards SIT integration into AW-IPM programmes against these pest insects and limited international horticultural trade.

Background research on the taxonomy of the *B. dorsalis* complex has been unable to provide definitive identification of some species (Clarke et al. 2005). This has confounded collecting associated host plant records and defining geographic distributions. It was

Table 1. Baseline and progress on the *Anastrepha fraterculus* complex.

Method	Knowledge at CRP start and gaps identified	Progress in addressing gaps identified	Output References
DNA Analysis	Only three gene regions studied for representatives of *A. fraterculus* s.l. populations (COI, 16S, and period genes). Other molecular markers have not been applied. There are no data for several of the morphotypes and the total number of specimens analysed so far is small. Microsatellite data are not yet available.	Extensive sampling from more than 70 populations from ten Latin American countries. COI data showed Colombian populations to be related to each other; Brazilian populations clustered in three clades, one of which included the population from Argentina. Mexico formed a separate clade. Ribosomal ITS 1 studies performed on populations from Andean, Brazilian and Mesoamerican regions. For the Andean region a total of 6 ITS 1 sequence variants in 4 groups were identified. Several microsatellite loci isolated and validated as markers in populations pertaining to at least three different morphotypes.	Ludeña et al. 2011, Lanzavecchia et al. 2014, Manni et al. 2015 this issue, Sutton et al. 2015 this issue, Lima 2015, Canal et al. (unpubl.), Lima et al. (unpubl.)
Cytology	Karyotypes described for Brazilian 1, 2, 3, Mexican and Peruvian morphotypes. No karyotype available for Andean, Ecuadorian and Venezuelan morphotypes. No polytene chromosome banding patterns described.	Karyotypes were described for the Andean and Ecuadorian morphotypes and completed for the Mexican karyotype. New detailed photographic polytene chromosome maps of Brazil 1 morphotype constructed; these maps can be the reference for comparative polytene chromosome analysis among different morphotypes).	Canal et al., (unpubl.); Giardini, Gariou-Papalexiou et al. (unpubl.)
Morphology	At least three morphotypes recognized (Andean, Brazilian 1 and Mexican) based on discriminant function analysis of aculeus, wing and mesonotum. Indications of four additional morphotypes. Egg morphology only described for three Brazilian morphotypes. None of the larval stages have been thoroughly described and compared among morphotypes.	Morphometric analysis involving 8 populations from Ecuador, 11 from Colombia and 23 from Brazil. Description of seven adult morphotypes using a multivariate approach published. A new 8th morphotype was recognized from Ecuador. Linear and geometric morphometric analysis of mouthparts of third instar larvae from five morphotypes (Andean, Brazilian 1, Ecuadorian, Mexican and Peruvian) allowed discrimination among morphotypes. SEM observations on third instar larvae of Brazilian 1, Mexican, and Peruvian morphotypes. Egg morphology of several *Anastrepha* species from the *fraterculus* group described.	Dutra et al. 2011, 2012, Hernández-Ortiz et al. 2012, this issue, Perre et al. 2014, Canal et al. 2015 this issue
Sexual Behaviour	Male courtship and female responses within and among morphotypes only partially known. Mating behaviour described for only 3 morphotypes, with very different mating times during day in field cage trials and segregated leks. Pre-zygotic isolation detected among some populations, but mating compatibility not evaluated among all morphotypes. Pre-zygotic isolation factors largely unknown.	High levels of sexual isolation among Andean, Brazilian 1, Mexican and Peruvian morphotypes; the Andean mated at dusk, the Brazilian 1 and Mexican early in the morning, and the Peruvian around midday. Populations from southern Brazil and Argentina compatible, while southern, southeastern and northeastern Brazilian populations partially or completely incompatible among each other. Detailed behavioural analysis including calling and sound analysis showed corresponding differences between these populations. Hybrids presented an intermediate calling behaviour. Remating propensity and duration of the refractory period independent of male origin. Tendency for Peruvian females mated with Brazil 1 males not to allow sperm transfer.	Dias 2012, Rull et al. 2012, 2013, Abraham et al. 2014, Cladera et al. 2014, Devescovi et al. 2014, Dias et al. in press, Juárez et al. 2015 this issue, Vaníčková et al. 2015d this issue

Method	Knowledge at CRP start and gaps identified	Progress in addressing gaps identified	Output References
Post-zygotic compatibility	Crosses between populations from Peru and Argentina and Brazil 1 and Brazil 2 resulted in reduced egg hatch, larval viability and distorted sex ratio. *Wolbachia* presence was confirmed for some populations, but its role in post-zygotic incompatibility not determined.	Compatibility among one population from Argentina and two from Brazil demonstrated, as well as among six Colombian ones. Cross-mating among Brazilian 1, Brazilian 3, Colombian, Mexican and Peruvian morphotypes resulted in no or unviable eggs, or with significantly lower hatch rate and sex ratio distortion than those laid by females mated with homotypic males. The crosses of some F_1 x F_1 males and females resulted in high fertility levels. *Wolbachia* studies expanded to additional populations.	Rull et al. 2012, 2013, Devescovi et al. 2014, Lima et al. (unpubl.)
Pheromone Components	Chromatograms of male borne volatiles for two morphotypes (Brazilian 1, Peruvian) and their hybrids available. The function of identified chemicals either alone or combined in eliciting attraction of conspecific females unknown.	The male borne volatiles from Andean, Brazilian 1 and Brazilian 3 morphotypes were characterized. Gas chromatography – electroantenography (GC-EAD) of females from Andean, Brazilian 1, Brazilian 3 and Peruvian morphotypes showed some similarities but also specificities among morphotypes in the antennal active compounds.	Břízová 2011, Vaníčková 2012, Břízová et al. 2013, Zykova 2013, Vaničkova et al. 2015a this issue, Kalinova (unpubl.)
Cuticular hydrocarbons	Cuticular hydrocarbon composition known only from one Argentine and one southern Brazil population.	Cuticular hydrocarbons from males and females from various populations of Andean, Brazilian 1, Brazilian 3, Mexican and Peruvian morphotypes were characterised, showing significant differences.	Vaníčková 2012, Vaničkova et al. 2012, Vaničkova et al. 2015a,c this issue
Distribution	*A. fraterculus* s.l. is widely distributed from southern Texas to northern Argentina, but the detailed distributions of morphotypes largely unknown. Also elevational transects in Andean countries lacking and needed to determine limits of highland and lowland morphotypes.	Mexican morphotype extends from Mexico to Central America; Venezuelan morphotype occurs only in the Caribbean lowlands of Venezuela; Andean morphotype in the Venezuelan and Colombian highlands (above 900 m elevation); the Peruvian morphotype in coastal areas of Ecuador and Peru, the Ecuadorian morphotype in the inter Andean Valleys in Ecuador and the southeastern Andean Valleys in Peru; Brazilian 3 morphotype in the northeastern coastal and southeastern regions from Brazil; Brazilian 1 morphotype in Argentina and southern Brazil.	Zucchi et al. 2011, Hernández-Ortiz et al. 2012, Perre et al. 2014, Hernández-Ortiz et al. 2015 this issue
Host Range	Host lists for *A. fraterculus* s.l. have been published for several countries, but these have not been associated with the various morphotypes. Host ranges for most morphotypes are largely unknown.	In areas with only one morphotype (Argentina, Mexico and Central America, highland Colombia-Venezuela), host ranges are being updated based on existing records. A host list was established for Colombia and new host plant information obtained in Bolivia, Ecuador and Peru. The host list for Brazilian morphotypes was updated to 110 hosts.	Castañeda et al. 2010, Zucchi 2015

considered absolutely essential that the species be accurately identified to be able to apply AW-IPM field programmes that include a SIT component. Because the trade implications and response systems to detections and/or incursions are different for all members in the complex, "near-enough" identification is, unfortunately, not good-enough. Consequently countries have difficulty overcoming the phytosanitary barriers to export-trade to major importers such as Australia, Japan, Europe, New Zealand, South Africa, and the USA. Another severe problem would arise if one member of the complex is detected or becomes established in a country, but is unable to be differentiated from others in the complex. In this case that country would then be forced to admit that all members of the complex may in fact be present, which would result in extended trade embargoes.

Therefore a comprehensive integrative taxonomy approach involving biological, morphological, chemo-ecological and molecular studies of the various members of the *B. dorsalis* complex were needed to: (1) resolve species limits by seeking a consensus result from different tests; (2) examine congruence between data from the different approaches to either support taxonomic revision or retain existing species status; and (3) develop robust diagnostic tools for the identified species.

2.2 Outputs on Five Priority Species in the *Bactrocera dorsalis* Complex

Quantified cross-species field-cage mating trials, as described in the FAO/IAEA/USDA Quality Control Manual (FAO/IAEA/USDA 2014), were completed for *B. dorsalis* s.l. populations from China, Kenya, Malaysia, Pakistan, the Philippines, Suriname and Thailand. Results presented in Schutze et al. (2013), Bo et al. (2014), and Chinvinijkul et al. (2015) demonstrated pre- and post-zygotic mating compatibility between all target taxa except for crosses involving *B. carambolae*, which always showed some level of sexual isolation from the other taxa. Post-zygotic isolation tests up to three generations were carried out for crosses involving *B. dorsalis* s.s. (China and Pakistan) and *B. invadens*; no evidence for hybrid incompatibility was detected (Bo et al. 2014).

Chemical components and ratios of sex pheromone stored in male rectal gland and emitted during courtship after feeding on methyl eugenol (ME) were determined qualitatively and quantitatively for *B. dorsalis* s.s., *B. invadens, B. papayae* and *B. philippinensis.* The four ccomplex members had identical volatile emission profiles and rectal pheromonal components consisting of 2-allyl-3,4, dimethoxyphenol (DMP) and E-coniferyl alcohol (*E*-CF) (Tan et al. 2011, 2013), and the ratios of DMP: *E*-CF were not significantly different between the different members. Probit analysis showed that the responsiveness of these four members to ME was similar as their ED_{50} values (= dose at which 50 % of the population responded) were not significantly different (Hee et al. 2015a). However, differences were found for *B. carambolae*.

Wing shape variation analysed through geometric morphometrics was used for the first time in the *B. dorsalis* complex (Schutze et al. 2012a,b, Krosch et al. 2013). Variation in wing shape proved to be extremely informative in interpreting variation within the *B. dorsalis* complex.

Genetic, cytogenetic and molecular analyses of *B. dorsalis* complex specimens collected across the geographical range were carried out in participating laboratories in Asia, Australia and Europe:

Cytogenetics: One of the objectives was to identify and evaluate cytogenetic tools that could help to resolve the taxonomic status of the five taxa under study, focusing on chromosomal rearrangements, especially inversions. For this purpose, mitotic and polytene chromosomes were analysed from colonized specimen representing *B. dorsalis* s.s., *B. papayae, B. philippinensis, B. invadens* and *B. carambolae*. Analysis of mitotic karyotypes could not detect any differences among these five taxa (Yesmin and Clyde 2012, Yesmin 2013, Augustinos et al. 2014, Augustinos et al. 2015), showing that all had the typical *B. dorsalis* s.s. karyotype as previously described by Hunwattanakul and Baimai (1994). Polytene chromosome maps were developed for the first time of a member of the *B. dorsalis* complex, i.e. *B. dorsalis* s.s. (Zacharopoulou et al. 2011a). Subsequent analysis showed that the five members of the complex do not present any chromosomal rearrangements that could be used as diagnostic characters and therefore these taxa can be regarded as homosequential (Augustinos et al. 2014, 2015). Although *B. carambolae* presented the same mitotic and polytene chromosome karyotype as the other members of the complex, the presence of a high number of minor asynapses in F_1 hybrids of *B. dorsalis* s.s. *x B. carambolae* crosses may indicate the presence of small differences in the chromosomal organization among the parental entities. However, these observations cannot be regarded as diagnostic at the species level (Augustinos et al. 2014).

Microsatellites: Microsatellite DNA markers derived from *B. dorsalis* s.s. were tested on populations of *B. dorsalis* s.s. from Bangladesh, Cambodia, China (multiple populations), Hawaii (two populations), Laos, Malaysia, Taiwan, and Thailand (multiple populations) (Shi et al. 2012, Krosch et al. 2013, Aketarawong et al. 2011, 2014a). The same set of markers combined with microsatellite markers derived from *B. papayae* were used to compare populations of *B. dorsalis* and *B. papayae* from the Thai/Malay Peninsula (Krosch et al. 2013, Aketarawong et al. 2014b). No genetic isolation was found between the *B. dorsalis* and *B. papaya* populations, supporting the hypothesis that both are the same entity. On the other hand, microsatellite markers, which amplify for *B. carambolae* and *B. dorsalis*, showed different genetic clusters between these two species, although admixture populations were observed. Admixture is evidence that some gene flow (i.e. hybridisation) may occur in the field between these species (Aketarawong et al. 2015).

Haplotype analysis: CO1 haplotype networks showed that common haplotypes were shared between *B. dorsalis, B. papayae, B. philippinensis* and *B. invadens*, but not with *B. carambolae* (Schutze et al. 2012b, 2015a). This supports the hypothesis that the first four taxa are a single biological species, while *B. carambolae* is distinct.

Phylogenetic analysis: A phylogenetic study using six neutral genetic markers found that *B. carambolae* could be resolved as a monophyletic clade from the other four target species, which were mixed together as an unresolved comb (Boykin et al. 2014, Schutze et al. 2015a).

Based on genetic, cytogenetic, pheromonal, morphometric and behavioural data, which repeatedly showed no or only minor variation between *B. dorsalis, B. invadens, B. papayae,* and *B. philippinensis*, formal taxonomic name changes were made. *B. philippinensis* was made a junior synonym of *B. papayae* by Drew and Romig (2013). Subsequently, also *B. papayae* and *B. invadens* were synonymised with *B. dorsalis* (Schutze et al. 2015b, Hee et al. 2015b), while the status of *B. carambolae* has not been altered. This means that only *B. dorsalis* and *B. carambolae* remain as scientifically valid names. The name changes have been widely accepted by national and regional plant protection organizations around the world, the Secretariat of the International Plant Protection Convention and the FAO (http://www.fao.org/news/story/en/item/262972/icode/).

In the works of Drew and Romig (2013), San Jose et al. (2013) and Schutze et al. (2015b) new morphological descriptions of the target taxa are provided. However, the use of morphology alone is not sufficient for definitive diagnosis of *B. carambolae* and *B. dorsalis*, but molecular and pheromone markers are now available to distinguish them. Molecular protocols using neutral genetic markers to distinguish the two species from each other, and from other closely related taxa, are provided in Boykin et al. (2014). Microsatellite markers which amplify for both species and which are used in population genetic studies, are provided in Aketarawong et al. (2015). The Y-specific marker will also separate the two species. For adult flies, the presence of the ME metabolites DMP and *E*-CF in the male rectal gland following ME feeding can be used to discriminate *B. dorsalis* from *B. carambolae* (which produces only *E*-CF) (Tan and Nishida 1996, Wee et al. 2007, Wee and Tan 2007).

The knowledge and gaps identified at the start of the CRP, as well as the progress by the end of the CRP in addressing the gaps identified for the *Bactrocera dorsalis* complex are summarized in Table 2.

3.1 *Ceratitis* FAR Complex Situation Analysis

The Afro-tropical fruit flies *Ceratitis fasciventris, C. anonae* and *C. rosa* (i.e. the *Ceratitis* FAR complex), together with *C. capitata* and *C. cosyra*, are considered major horticultural pests of that region (White and Elson-Harris 1994, De Meyer 200la). These species are of quarantine significance (EPPO/CABI 1997) as they are highly polyphagous and damage a wide range of unrelated wild and cultivated crops (De Meyer et al. 2002), resulting in enormous economic losses wherever they occur (Barnes 2000, De Meyer 2001b). They have different distribution patterns that partially overlap, resulting in sympatric occurrence in particular areas.

Ceratitis rosa, C. fasciventris and C. anonae were considered the three members of the *Ceratitis* FAR species complex (Virgilio et al. 2007a, 2007b). Taxonomically, *C. fasciventris* was initially considered a variety of *C. rosa* (Bezzi 1920) but has recently been recognized as a different entity with species status (De Meyer 2001a).

Unlike *C. capitata*, which has over the last century spread from its home range in East Africa and attained an almost world-wide distribution (Fletcher 1989, White

Table 2. Baseline and progress on the *Bactrocera dorsalis* complex.

Method	Knowledge at CRP start and gaps identified	Progress in addressing gaps identified	Output References
DNA Analysis	There is no adequate and consistent sample coverage for the five target species. Nuclear ribosomal ITS1+2 diagnostic for separating *B. carambolae* from remaining four species. Mitochondrial DNA markers show no clear distinction between currently defined species. Microsatellite sequences available as well as nuclear coding gene data for 16 loci. Lack of discriminatory characters means that either they are yet to be discovered, or such characters do not exist and the species are the same.	Significantly improved sample coverage, including from China, Indian subcontinent, Myanmar, Indo/Malay Archipelago, and African populations. Additional diagnostic genetic markers (mitochondrial, ribosomal and nuclear genes) developed, but they do not discriminate between four of the five species; *B. carambolae* forms a monophyletic group according to COI and ITS1. A full multigene phylogenetic analysis published. Microsatellites indicate origin of *B. dorsalis* is China. Y specific primers have been developed and differences of the amplified sequences have been found between *B. dorsalis* and *B. carambolae*, but not for *B. dorsalis, B. papayae, B. philippinensis* and *B. invadens*. Combined genetic results cannot consistently separate *B. dorsalis, B. papayae, B. philippinensis* and *B. invadens*. Unique markers have been identified for *B. carambolae*.	Aketarawong et al. 2014a, 2014b, 2015 this issue, Boykin et al. 2014, Leblanc et al. 2015 this issue
Cytology	Studies on mitotic karyotypes identified several forms within the *B. dorsalis* complex, but did not definitively distinguish between putative species. Further polytene maps are needed to allow distinguishing between putative species. Polytene mapping for these species could be linked with genomic data.	Cytological evidence, neither on mitotic nor on polytene chromosomes can discriminate between the five target taxa, therefore these taxa can be regarded as homosequential. Hybrid analysis also shows no chromosomal structural differences; only minor asynapses were observed in a few hybrids between *B. dorsalis* and *B. carambolae*. *In situ* hybridization of the six unique sequences shows no evidence for the presence of chromosomal rearrangements. However, analysis of mitotic and polytene chromosomes from both *B. kandiensis* and *B. tryoni* clearly differentiates these taxa from *B. dorsalis* s.s.	Zacharopoulou et al. 2011a, Zacharopoulou and Franz 2013, Augustinos et al. 2015 this issue
Genomics	Unpublished Hawaiian *B. dorsalis* s.s. genome. Transcriptomics under way for *B. dorsalis* s.s., *B. philippinensis* and *B. carambolae,* potential of some markers for these species.	Public web portal for accessing the current scaffold and contig structure of the Hawaiian *B. dorsalis* s.s. genome. The genome for *Bactrocera tryoni* and draft genomes for *B. neohumeralis* and *B. jarvisi* have been published as reference genomes. Comparative transcriptome data for *B. dorsalis* s.s., *B. papayae, B. philippinensis, B. carambolae* and *B. invadens* have been analysed for 'species' specific SNPs. No specific SNPs could be identified.	Gilchrist et al. 2014, Armstrong et al. (unpubl.)
Morphology	No consensus on species limits of the major *B. dorsalis* complex pest species. Explicit intra-specific population-level variation in both external and internal morphological characters. Therefore for some species unable to provide definitive identification of specimens. Egg and immature morphology not investigated for *B. dorsalis* complex species.	*B. invadens vs B. dorsalis* s.s. colour morphs described and the range of morphological variation assessed from across their geographic ranges. Crosses between colour morphs (pale brown and black scutum) of *B. dorsalis* from Pakistan confirm that scutum colour morph is a simple qualitative genetic trait. Morphological pattern variation in *B. dorsalis* s.s has been assessed for flies fed varying quantities of food (standard lab diet). No clear correlation was observed. Egg and immature material has been gathered and is being included as part of a major project describing the immature stages of tephritids.	Leblanc et al. 2013, Leblanc et al. 2015 this issue, Schutze et al. 2015a
Morphometrics	Large number of morphometric studies for *B. dorsalis* complex species; often impossible to separate between and within population variation in morphometric traits. Insufficient understanding of relative environmental/genetic influences on morphometric phenotype. Morphometric approaches have rarely been linked adequately with other morphological or genetic approaches.	Geometric morphometric wing shape data are consistent with *B. dorsalis* s.s., *B. invadens, B. papayae* and *B. philippinensis* representing the same species which displays strong isolation-by-distance patterns within SE Asia. All 'outgroup' species resolve strongly from the *B. dorsalis* complex species. Aedeagi from a latitudinal gradient from northern Thailand to Peninsular Malaysia for *B. dorsalis* s.s. and *B. papayae* show a significant and continuous latitudinal cline from north to south, with northern Thailand flies the shortest and Malaysian flies possessing the longest aedeagi. Morphometrics of genitalia, head width and wings have been undertaken for *B. dorsalis* s.s. reared under different larval densities.	Schutze et al. 2012a, Schutze et al. 2012b, Krosch et al. 2013, Schutze et al. 2015a

Method	Knowledge at CRP start and gaps identified	Progress in addressing gaps identified	Output References
Sexual Behaviour	Knowledge of some mating compatibility for individual species crosses from isolated studies. Lack of comparative mating compatibility studies across populations/species from across their geographic range acquired under equivalent semi-natural conditions.	Comparative field cage studies demonstrating complete pre- and post-zygotic compatibility among *B. dorsalis* s.s., *B. invadens, B. papayae* and *B. philippinensis*; hybrid offspring obtained between crosses are also viable. *B. carambolae* shows partial pre-and post-zygotic incompatibility with *B. dorsalis* s.l.. Following methyl-eugenol (ME) feeding by males *B. dorsalis* and *B. carambolae,* relatively sexual incompatibility remained in cross-mating field cage studies. No compatibility under same conditions with outgroups.	Chinvinijkul et al. 2015 this issue; Schutze et al. 2015c, this issue
Sensitivity to methyl-eugenol (ME)	Male responsiveness to ME varied between *B. dorsalis*, *B. papayae* and *B. carambolae*. Initial feeding on high-concentration ME reduced response to subsequent exposure in *B. dorsalis* s.s. The male mating advantage seen following ME-feeding begins later for *B. carambolae* relative to *B. dorsalis*. Lack of specific knowledge of the ME response of *B. philippinensis* and *B. invadens*. Species sensitivity to ME across the four species has not been compared.	Probit analysis on the males' sensitivity to ME showed no significant differences in the ED_{50} between *B. dorsalis, B. papayae, B. philippinensis* and *B. invadens.* Electrophoretic analyses of the male antennal protein extracts for *B. dorsalis, B. papayae, B. philippinensis* and *B. invadens* also showed no differences in the protein electropherogrammes. This also included that of males exposed to ME.	Hee et al. 2015a this issue
Pheromone Components	Phermononal components following ME consumption identical in *B. dorsalis* s.s. and *B. papayae*. Endogenous pheromone components different between *B. carambolae* and both *B. dorsalis* and *B. papayae*. Pheromone components of *B. philippinensis* and *B. invadens* so far not been characterised. ME metabolites (i.e. DMP and *E*-CF) presence in male rectal gland derived from ME feeding can be used to discriminate against *B. carambolae* (which produces only *E*-CF).	Pheromone components post-ME feeding for *B. invadens* and *B. philippinensis* males identical to those recorded for *B. dorsalis* and *B. papayae*, but different to those of *B. carambolae.* Pheromone components in male rectal glands and volatile emissions virtually identical among *B. dorsalis, B. papayae, B. philippinensis* and *B. invadens*, but distinctive as compared to *B. carambolae*. Ratios of pheromonal components (DMP:*E*-CF) quantified from male rectal gland (in storage) and volatile emission following ME consumption were not significantly different between *B. dorsalis, B. papayae, B. philippinensis* and *B. invadens*. Endogenously produced pheromone constituents confirmed as unique marker for *B. carambolae* (i.e. 6-oxo-1-nonanol) to enable species separation from *B. dorsalis* s.l.	Hee et al. 2015a this issue, Tan et al. 2011, Tan et al. 2013
Cuticular hydrocarbons	No cuticular hydrocarbons of the *B. dorsalis* complex so far studied.	The identification and quantification of cuticular hydrocarbons of males and females of *B. philippinensis*, *B. papayae*, *B. dorsalis*, *B. invadens* and *B. carambolae* was performed on the GC×GC/TOFMS. Quantitative as well as qualitative CHC profile differences were found between sexes. Female profiles show high amount of short-chained hydrocarbons, not male profiles.	Kalinova et al. (unpubl.)
Distribution	Collection locality considered 'species character-state' in many operational keys. For example, *B. dorsalis* within Thailand is restricted to central/northern Thailand and *B. papayae* to southern Thailand, but zone of transition between *B. dorsalis* s.s. and *B. papayae* on the Thai/Malay Peninsula not confirmed. Endemic range of *B. invadens* is not known.	Two independent studies show there is no zone of transition between *B. dorsalis* s.s. and the former *B. papayae* on the Thai/Malay Peninsula based on population genetic data (e.g. Fst values and demonstrated gene flow) and morphological data, supporting a continuum rather than different species. Morphological and wing shape analysis suggests that the native range of invasive African *B. dorsalis* (formerly *B. invadens*) need not have been restricted to Sri Lanka but may have been more widely distributed across the Indian subcontinent. *B. dorsalis* is now recognised as a naturally wide-spread and highly invasive species which occurs across sub-Saharan Africa, across the Indian sub-continent to Asia and into the South Pacific.	Krosch et al. 2013, Aketarawong et al. 2014b, Schutze et al. 2015a
Host Ranges	Well documented for most pest populations being tested, but not yet fully for *B. philippinensis* and *B. invadens*.	A single host list is being consolidated, which covers *B. dorsalis* and the former *B. papayae*, *B. invadens* and *B. philippinensis*.	Luc Leblanc et al. (unpubl.)

and Elson-Harris 1994, http://www-naweb.iaea.org/nafa/news/images/Distribution-Mediterranean-fruit-fly-Ceratitis-capitata.jpg), *C. rosa, C. fasciventris* and *C. anonae* have so far not been reported outside the African mainland (except for La Réunion and Mauritius) but are potentially invasive.

Due to the difficulty in distinguishing morphologically some members of the complex, especially females, a number of molecular approaches for species recognition were used (Baliraine et al. 2004, Barr et al. 2006, Virgilio et al. 2008). However, these diagnostic tools remained inadequate for quarantine purposes and much more robust molecular markers were needed.

3.2 Outputs for *Ceratitis* FAR Complex

Development of molecular diagnostics, using microsatellites (Delatte et al. 2013), revealed a more complex structure than the mere existence of three entities within the *Ceratitis* FAR complex. Five genotypic groups were identified (Virgilio et al. 2013) and later confirmed by morphological differences of the males (De Meyer et al. 2015a). Morphological diagnostics for male specimens of the five entities, called R1, R2 (*C. rosa* type 1 and 2), F1, F2 (*C. fasciventris* type 1 and 2) and A (*C. anonae*) were developed. Morphometric diagnostics using wing landmarks were developed for both sexes to a certain extent (Van Cann et al. 2015). Microsatellites allowed distinction between the five entities. Cytological studies were restricted to one representative (F2) acting as a reference dataset (Drosopoulou, unpublished data).

Adult morphology and morphometry, pheromone, cuticular hydrocarbon and distributional data were collected that provide evidence for the specific status of all three formerly recognized taxonomic entities within the FAR complex (i.e. *C. fasciventris*, *C. anonae*, *C. rosa*) (Vaníčková et al. 2014, Břízová et al. 2015, De Meyer et al. 2015, Van Cann et al. 2015). More detailed studies were conducted for the two *C. rosa* types (R1, R2) (adult morphology and morphometry, pheromone, cuticular hydrocarbon, developmental physiology, behavioural, and ecological data), which provided evidence that the two *C. rosa* types represent two separate species (Tanga et al. 2015, Van Cann et al. 2015, Vaníčková et al. 2015b), of which one (currently referred to as R2) will be formally described. An altitudinal transect in Tanzania, where R1 and R2 occur in sympatry, confirmed that R1 is more tolerant to higher temperatures and R2 better adapted to colder environments (Mwatawala et al. 2015a). For the two *C. fasciventris* types (F1, F2) these additional studies could not be conducted as laboratory colonies of one of the two types could not be established, preventing experiments on developmental physiology and mating compatibility. Larval morphology did not provide evidence with regard to the specific status, except for *C. fasciventris* (F2) to some extent (Steck and Ekesi 2015). Moreover, as a spin-off of this research it was shown that characters previously considered diagnostic for differentiation between species and even between the genera *Ceratitis* and *Bactrocera*, proved to be variable.

The knowledge and gaps identified at the start of the CRP, as well as the progress made by the end of the CRP in addressing the gaps identified for the *Ceratitis* FAR complex are summarized in Table 3.

4.1 *Zeugodacus cucurbitae* Situation Analysis

The melon fly, *Zeugodacus curcurbitae* (initially referred to as *Bactrocera* (*Zeugodacus*) *cucurbitae*), is a major pest of cucurbit crops that has spread from its area of origin (South East Asia) across Africa, Hawaii, the Indian Ocean, Papua New Guinea and the Solomon Islands (Severin et al. 1914, Dhillon et al. 2005). In particular, it causes severe losses in food crops and restrictions to trade for some cucurbit crops.

Some populations were identified in Africa, islands in the Indian Ocean, Hawaii and South East Asia with different host use, which could indicate the existance of very closely related species. Although the SIT has been effectively applied against the melon fly in certain regions (Koyama et al. 2004), this issue needed to be resolved to enable the application of species-specific treatments such as the SIT against all populations in all regions.

4.2. Outputs for *Zeugodacus cucurbitae*

In spite of earlier observations and indication of different host-use by *B. cucurbitae* in different geographic regions, genetic studies using mitochondrial and nuclear markers indicated very low intraspecific variability worldwide. Population genetic studies using microsatellites were able to distinguish five major groups worldwide: African mainland and Seychelles, Réunion and Mauritius, Central Asia, SE Asia, and Hawaii (Virgilio et al. 2010). However no phylogeographic patterns could be discerned using cytogenetics analyses (Zacharopoulou et al. 2011b, 2013) or mitochondrial and nuclear gene fragments (total of 2764 bp) (Virgilio, unpublished data). The invasion history for the species on the African mainland was also reconstructed (Delatte et al. unpublished data).

Cross-mating experiments were conducted at the start of the CRP between populations of Mauritius, Seychelles and a genetic sexing strain of Hawaii and these indicated no mating incompatibility or sexual isolation (Sookar et al. 2013). Given this fact and the genetic assessments, it was decided there was no need for additional cross-mating studies.

Further studies, including host preference and microsatellite markers, did not show any relation between genetic structure and host plants (Virgilio et al. 2010, Sookar et al. 2013). It was concluded that there is no evidence of the existence of host races or cryptic species within *B. cucurbitae*. However, as a spin-off of the conducted research, recent studies on the higher phylogeny of dacines have shown that the higher taxonomic classification under which *B. cucurbitae* is placed, is a paraphyletic grouping, requiring a taxonomic change in generic placement (Krosch et al. 2012, Virgilio et

Table 3. Baseline and progress on the *Ceratitis* FAR Complex.

Method	Knowledge at CRP start and gaps identified	Progress in addressing gaps identified	Output References
DNA Analysis	Attempts to develop specific diagnostic markers had been made but were so far ineffective. Need for further exploration for markers, especially microsatellites.	Microsatellites were developed for the *Ceratitis* FAR complex. Population genetic structure for the complex revealed five clearly distinguishable clusters: *C. fasciventris*: F1, F2; *C. anonae*; *C. rosa*: R1, R2. Restriction site associated DNA sequencing (RAD-seq) confirmed the robustness of the five genotypic clusters. There is still the need for a diagnostic marker.	Delatte et al. 2013, De Meyer et al. 2015a this issue, Virgilio et al. 2013
Cytology	No data available. Cytology has the potential to provide a diagnostic tool.	Mitotic karyotype and polytene chromosome analysis of *C. fasciventris* from Kenya showed rearrangements in two polytene arms and differences in the size of mitotic sex chromosomes.	Drosopoulou, (unpubl.)
Morphology	Males of three taxa can be distinguished to some extent, however, separation of females is not possible, and larval morphology so far not studied.	After recognition of five clusters (based on molecular work), adult characters were re-examined. Consistent morphological differences were found to distinguish male *C. rosa* (R1 and R2) and *C. fasciventris* (F1 and F2). SEM studies for all immature stages were conducted, including several populations of *C. rosa* (R1 and R2) from different geographical regions. No diagnostic characters could be found to differentiate the different entities (except for *C. fasciventris* F2).	De Meyer et al. 2015a this issue, Steck and Ekesi 2015 this issue
Morphometrics	No data available, but might have potential for separation of females, in view that adult females cannot be differentiated on morphological characters.	Morphometric studies on all five genotypes allowed resolving to a large extent morphospecies and genotypic clusters. Wing landmarking might represent a possible tool for the diagnosis for species within the FAR complex.	De Meyer et al. 2015 a this issue, Van Cann 2013, Van Cann et al. 2015 this issue
Male Lure Response	FAR complex males apparently attracted to trimedlure, however, response of representatives of the *Ceratitis* FAR complex to male lures to be investigated.	EGOlure shown to be a stronger attractant for *C. rosa* (R1 and R2) than trimedlure. In addition, EGOlure shown to be able to attract *C. cosyra* in a significantly stronger way than terpinyl acetate.	Mwatawala et al. 2012, 2015a this issue
Developmental Physiology	Need to determine whether there are developmental/ physiological differences between the entities recognized.	Marked difference in development and survival in relation to different temperature ranges in the two *C. rosa* types both in Kenya and South Africa. *Ceratitis rosa* R1 being more tolerant to higher temperature and *C. rosa* R2 better adapted to colder environments.	Tanga et al. 2015 this issue

Method	Knowledge at CRP start and gaps identified	Progress in addressing gaps identified	Output References
Sexual Behaviour	Only some behavioural work on *C. rosa,* limited for the other entities. Need to determine any behavioural differences between the entities recognized.	Pre-zygotic mating incompatibility studies under semi-natural conditions using populations of *C. rosa* R1, R2 and *C. fasciventris* F2 showed clear evidence of reproductive isolation between the two *C. rosa* types R1 and R2, similar to the reproductive isolation observed between each of them and *C. fasciventris* F2.	Ekesi et al. (unpubl.)
Pheromone Components	Pheromones of taxonomic entities recognized so far not studied.	Composition of investigated pheromones is different from that of *C. capitata*, and confirmed differences among the five FAR taxa.	Břízová et al. 2015 this issue
Cuticular hydrocarbons	Cuticular hydrocarbons not studied, but can contribute to resolving the specific status of the taxa within the complex.	Differences detected between the five taxa recognized in the FAR complex. Sexual differences were also found in each species.	Vaničkova et al. 2014, 2015b this issue
Distribution	Need to re-assess whether there are distributional differences between the entities recognized.	*Ceratitis rosa* R1 largely absent from southern part of the African continent and from higher altitudes. *Ceratitis fasciventris* F1-F2 largely separated (western-central versus eastern Africa), although isolated populations of 'western' type also found in Angola, Malawi, Tanzania, Zambia.	De Meyer et al. 2015a this issue, Mwatawala et al. 2015a this issue
Host Ranges	Need for additional studies to determine whether there are host range differences between the entities recognized.	*Ceratitis rosa* R1 and R2 do not show major differences in host range except that the hosts from temperate climates (*Pyrus*, *Rubus*, *Coffea*) are predominantly infested by *C. rosa* R2. Information for the *C. fasciventris* entities is inconclusive because lack of F1 data.	De Meyer et al. (unpubl.)

Table 4. Baseline and progress on *Zeugodacus cucurbitae*.

Method	Knowledge at CRP start and gaps identified	Progress in addressing gaps identified	References
DNA Analysis	General information on phylogeography / population genetics available on a worldwide basis. Also information available on population genetics with regard to the African mainland and La Réunion. More data on potential host races needed, both within Cucurbitaceae (cultivated versus wild) and cucurbits versus non-cucurbits.	Detailed DNA studies on population genetics in La Réunion, and to a lesser extent in Tanzania, have shown that there is no relation between genetic structure and host plants. The same observation was made from extensive sampling of potential hosts (including non-cucurbits) in Thailand. Mitochondrial variation in *Z. cucurbitae* populations from Hawaii were studied and little variation detected. Population sampling for African continent increased to retrace the invasion routes and invasion history within and towards/from Africa. The results show that there is a single recent (20th century) invasion event and that the western African populations are the result of a subsequent invasion from eastern Africa.	De Meyer et al. 2015b this issue, Jacquard et al. 2013, Clarke et al. (unpubl.), Delatte et al. 2015 (in press)
Cytology	Work has been carried out on two *Z. cucurbitae* populations regarding karyotyping and polytene maps.	No cytogenetic differences found between two populations of *Z. cucurbitae* (genetic sexing strain Hawaii and Bangladesh wild type). Karyotyping reveals that *Z. cucurbitae* is significantly different from other *Bactrocera* (i.e. position of subgenus *Zeugodacus* in relation to *Bactrocera* and *Dacus*).	Zacharopoulou et al. 2011b, 2013
Morphology	*Z. cucurbitae* can be morphologically differentiated from other species within the subgenus. Some populations appear to indicate very closely related species.	No other valid taxa have been identified that could cause possible confusion with *Z. cucurbitae*. Also no differences identified among populations. Therefore no need for further studies. Subgenus *Zeugodacus* erected to genus level.	De Meyer et al. 2015b this issue, Virgilio et al. 2015
Sexual Behaviour	Good knowledge of mating behaviour through studies in Japan, Hawaii and recently in Austria.	Compatibility studies under semi-natural conditions investigating cross-mating among three populations from Hawaii, Mauritius and Seychelles. No sexual isolation was discovered.	Sookar et al. 2013
Host Range	Well documented as a whole, although possibility of host races and different host range reported in different parts of distribution range	Reared from 17 plant species comprising 10 families covering Cucurbitaceae, Solanaceae, Anacardiaceae, Rutaceae and Myrtaceae. Host ranges were studied in relation to genetic structure. Populations of *Z. cucurbitae* vary in their preference to host plants. A tomato population exclusively preferred tomato compared to the other host plants.	Mwatawala et al. 2010, 2015b
Cuticular hydrocarbons	No studies yet specifically with regard to *Z. cucurbitae* cuticular hydrocarbons have been conducted	The composition of the cuticle of virgin males and females - ages 5, 15, 20, 30 after emergence - was analysed by GCxGCMS. The preliminary data demonstrate sex- and age-specific differences.	Vaníčková (unpubl.)

al. 2015). A nomenclatorial act has raised the subgenus *Zeugodacus* (as well as other subgenera belonging to the *Zeugodacus* group, sensu Drew 1989) to genus level. As a result, *Bactrocera cucurbitae* was put in a new generic combination: *Zeugodacus cucurbitae*, and should be referred to by this name from now onwards (Virgilio et al. 2015).

The knowledge and gaps identified at the start of the CRP, as well as the progress made by the end of the CRP in addressing the gaps identified for *Z. cucurbitae* are discussed in De Meyer et al. (2015b) and summarized in Table 4.

Conclusion

Following an integrative taxonomic approach, the biology, cytogenetics, ecology, morphology, genetics, and physiology of major pest tephritid cryptic species complexes is now much better understood. This increased knowledge has resulted in formal decisions on the species status of some taxa within these complexes, thus facilitating international horticultural trade and simplifing SIT application against pest species of these complexes. In the case of *Anastrepha fraterculus* it was shown that it consists of a complex of a number of different species of no monophyletic origin, with distinct geographic and ecological distributions in Latin America. Also for the *Ceratitis* FAR complex evidence has been provided for the existence of five different entities within this complex from the African region, i.e. *Ceratitis anonae*, *C. rosa* (R1 and a new species referred to as R2), while for *C. fasciventris* the biological limits between F1 and F2 are not fully resolved. On the other hand the Asian/African pest fruit flies *B. papayae, B. philippinensis* and *B. invadens* were shown to represent populations of *B. dorsalis*, while only *B. carambolae* remains a valid species for which molecular and pheromone markers are now available to distinguish it from *B. dorsalis*. Finally studies among populations throughout the geographic range of *Bactrocera cucurbitae* in Africa and the Asia/Pacific region showed no evidence for the existence of host races. However, the higher taxonomic classification under which *Bactrocera cucurbitae* is placed was found to be a paraphyletic grouping, requiring the elevation of the subgenus *Zeugodacus* to genus level. As a result, *Bactrocera cucurbitae* was put in a new generic combination: *Zeugodacus cucurbitae*.

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RESEARCH ARTICLE

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Morphometric study of third-instar larvae from five morphotypes of the *Anastrepha fraterculus* cryptic species complex (Diptera, Tephritidae)

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Abstract

The occurrence of cryptic species among economically important fruit flies strongly affects the development of management tactics for these pests. Tools for studying cryptic species not only facilitate evolutionary and systematic studies, but they also provide support for fruit fly management and quarantine activities. Previous studies have shown that the South American fruit fly, *Anastrepha fraterculus*, is a complex of cryptic species, but few studies have been performed on the morphology of its immature stages. An analysis of mandible shape and linear morphometric variability was applied to third-instar larvae of five morphotypes of the *A. fraterculus* complex: Mexican, Andean, Ecuadorian, Peruvian and Brazilian-1. Outline geometric morphometry was used to study the mouth hook shape and linear morphometry analysis was performed using 24 linear measurements of the body, cephalopharyngeal skeleton, mouth hook and hypopharyngeal sclerite. Different morphotypes were grouped accurately using canonical discriminant analyses of both the geometric and linear morphometry. The shape of the mandible differed among the morphotypes, and the anterior spiracle length, number of tubules of the anterior spiracle, length and height of the mouth hook and length of the cephalopharyngeal skeleton were the most significant variables in the linear morphometric analysis. Third-instar larvae provide useful characters for studies of cryptic species in the *A. fraterculus* complex.

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Keywords
South American fruit fly, immature, taxonomy, geometric morphometry, linear morphometry, morphotypes

Introduction

Some species within the Tephritidae family are among the most important pests for agriculture because of their direct effects on fruit production and the quarantine restrictions imposed to prevent the transfer of foreign species from one region to another (Schutze et al. 2012, Norrbom et al. 2013). In this family, there are species of agricultural importance that are, in reality, complexes of cryptic species (Kitthawee and Dujardin 2010, Hernández-Ortiz et al. 2012, Ruiz-Arce et al. 2012, Schutze et al. 2012, Krosch et al. 2013, Vaníčková et al. 2014). The occurrence of cryptic species among economically important fruit flies strongly affects the development of management tactics for these pests. Their economic importance is variable from one region to another, which makes the establishment of management practices more difficult. Detailed knowledge of the biology and taxonomy of these species is essential for the application of methods such as the sterile insect technique (SIT), the use of pheromones, the determination of pest-free or low-prevalence areas and quarantine measures or risk analysis (Frías et al. 2006, Schutze et al. 2012, Krosh et al. 2013, Norrbom et al. 2013, Perre et al. 2014).

The definition and determination of species is one of the most important topics in modern systematics. Traditionally, the description of species has been based on the study of morphological characteristics. In recent decades, other biological, ecological, genetic and evolutionary tools have been integrated with morphology to find new species, particularly within cryptic species complexes (Baylac et al. 2003, Bickford et al. 2007, Wiens 2007, de Queiroz 2007, Yeates et al. 2011, Krosh et al. 2013). Tools for studying cryptic species not only facilitate evolutionary and systematic studies, but they also provide support for fruit fly management and quarantine activities.

The South American fruit fly, *Anastrepha fraterculus* (Wiedemann), is a species of great economic importance within the genus and is subject to quarantine restrictions. It is widely distributed in America and is associated with a large number of host fruits (Hernández-Ortiz et al. 2012, Norrbom et al. 2013). In fact, this nominal species comprises a cryptic species complex, as has been demonstrated by genetic (Steck 1991, Steck and Sheppard 1993, Smith-Caldas et al. 2001) and cytogenetic (Selivon et al. 2004, 2005, Goday et al. 2006) studies, reproductive isolation tests (Selivon et al. 1999, Vera et al. 2006, Cáceres et al. 2009, Devescovi et al. 2014), chemo-taxonomy (Cáceres et al. 2009, Břízová et al. 2013, Vaníčková et al. 2015) and morphological (Selivon and Perondini 1998, Selivon et al. 2005, Hernández-Ortiz et al. 2004, 2012) analysis. Based on adult morphology, Hernández-Ortiz et al. (2012) identified seven morphotypes within this complex: Mexican, Andean, Venezuelan, Peruvian, and three morphotypes from the Brazilian territory, one of which extends to Argentina. In addition to these, Hernández-Ortiz et al. (2015) recently identified the Ecuadorian morphotype.

Studies of the immature stages may be informative for the definition of species limits as well as for studies of phylogeny and evolution (Norrbom et al. 1999, Dujardin et al. 2014). In addition, in the case of fruit flies these studies could be important for quarantine actions because this is the stage that damages fruits (Steck et al. 1990, Frías et al. 2008, Dutra et al. 2012) and the one that is mostly intercepted during trade. According to Frías et al. (2008), larvae of only 7% of Tephritidae species have been described in 17% of the genera. Studies on the larval morphology of *Anastrepha* have been performed by Steck and Malavasi (1988), Steck and Wharton (1988), Carroll and Wharton (1989), Steck et al. (1990), Frías et al. (2006, 2008, 2009) and Dutra et al. (2012). However, previous studies have barely covered the morphological descriptions of the studied species, except that of Steck et al. (1990), who used multivariate analysis to find traits which could separate 13 species. Frías et al. (2006, 2008) also studied larval differentiation among the genera *Anastrepha, Ceratitis, Bactrocera, Rhagoletis* and *Toxotrypana*; further, they differentiated the larvae of some species of *Rhagoletis* that occur in Chile. In larvae of fruit flies, only allometric studies have been performed. These studies have shown that several structures, such as the cephalopharyngeal skeleton and the mouth hook may have taxonomical importance for the group. However, the results have not been completely satisfactory.

The study of larvae would benefit from more sophisticated tools for measuring the extant morphologic variability, as could be the case of shape analysis of certain structures, since forms are among the features that show differences in the speciation processes (Jirakanjanakit et al. 2008, Schutze et al. 2012, Dujardin et al. 2014). Shape analysis through the study of outlines has been successfully applied to delimit cryptic species of mosquitoes and ticks (Dujardin et al. 2014) and to study the effect of hybridization in mandibles of stag beetles (Tatsuta et al. 2011). However, in spite of its capacity to detect minimal morphological variation, measurement errors can be introduced in geometric morphometric studies due to observer error, common in many works, photographing and collecting landmarks (Dujardin et al. 2010, Toro et al. 2010). Several solutions to this have been proposed (Arnqvist and Martensson 1998) with modern techniques of digital photography providing an adequate resolution for these liabilities (Dujardin et al. 2010).

The aim of this study was to perform a comparative analysis of third instar larvae of representatives of five morphotypes of the *A. fraterculus* complex (Mexican, Andean, Peruvian, Brazilian-1 and Ecuadorian). Through the use of geometric morphometry of the shape of the mouth hook and linear morphometry of larvae, we tested several variables and determined their usefulness in the differentiation of these morphotypes.

Methods

Biological material. The taxonomic identity of all larvae used in this study was fully known from associated reared adults and the diagnoses developed by Hernández-Ortiz et al. (2004, 2012, 2015) (Table 1). The samples from Mexico and Ecuador derived

Table 1. Data on collection of third-instar larvae of five morphotypes of the *Anastrepha fraterculus* complex.

Morphhotype	Country	State	Municipality	Host	Latitude	Longitude	Altitude
Andean	Colombia	Boyaca	Duitama	Guava feijoa (*Acca sellowiana*)	5°49'29,9"N	73°04'29,7"W	2569
Brazil sp1	Brazil	São Paulo	Itaquera	Guava (*Psidium guajaba*)	23°30'S	46°40'W	700
Ecuadorian	Ecuador	Pichincha	Quito	Custard apple (*Annona cherimola)*	00°06'47"S	78°25'33"W	1861
Mexican	Mexico	Veracruz	Teocelo	Guava	19°23'8"N	96°58'20"W	1190
Peruvian	Peru	Lima	La Molina	Custard apple	12°00'03"S	76°57´00"W	255

from natural populations. The samples from Colombia and Brazil came from colonies reared for a few generations on host fruit in laboratory conditions. The sample from Peru came from a laboratory colony maintained on an artificial diet since 2002 at the laboratories of the International Atomic Energy Agency, in Seibersdorf, Austria. The sample of the Brazilian-1 morphotype was collected in the field and identified by one of the co-authors (DS) in the same location at which previous genetic, cytogenetic and morphometric studies were conducted with adults of this morphotype (Yamada and Selivon 2001). For each population we studied a total of 20 individuals.

Preparation of larvae. Larvae were prepared following methods described by Frías et al. (2006) as follows: third-instar larvae were killed in boiling water for one minute in groups of up to 20 individuals and then put in a 75% alcohol solution for storage. Larvae specimens were photographed in dorsal view before proceeding with their preparation. Next, the larvae were left for one night in a 10% KOH solution, and the internal body content was withdrawn. Later, the cephalopharyngeal skeleton was carefully separated, removing the adhering tissue as much as possible. This structure was positioned in lateral view on a concave glass slide, slightly immersed in glycerin, covered and photographed. Digital images were also taken of the anterior spiracles by placing the cuticle on a glass slide with glycerin. The larval cuticle and the cephalopharyngeal skeleton were stored in Eppendorf tubes with glycerin and deposited in the Museum of the Laboratory of Entomology at the University of Tolima.

The left mouth hook was carefully separated, and the remaining tissue was removed as much as possible. Permanent slides were made with Canada balsam, putting the mandible in lateral view, and were deposited in the Museum of the Laboratory of Entomology at the University of Tolima. The mounting were done placing small amounts of Canada balsam each time to keep the mouth hooks in the best position to minimize the error.

Image capture. All pictures were taken with a Moticam10 digital camera, coupled to an Advance Optical stereoscopic microscope for digital images of the body, and a Carl Zeiss Primo Star Trinocular microscope was used for pictures of the mouthparts. In both cases, the camera had a 10X lens. The cephalopharyngeal skeleton and the anterior spiracle were photographed with a 10X objective, and the

hypopharyngeal sclerite and mouth hook were photographed with a 40X objective. All digital images were taken at high resolution (3,664 × 2,748 pixels). The mouth hook at 400X magnification resulted in a 3D figure with blurred edges; therefore, multiple shots (between six and 10) were taken at different focal planes and later assembled with the software Helicon Focus 6.0.18 (2013). All the images were edited with Adobe Photoshop CS5 Extended 12.0 x64 (Adobe 2010). The third dimension can be ignored in geometric morphometry when it is not important compared to the other two, and if the imaged structure is in approximately the same position and of good quality (Zelditch et al. 2004, Dujardin et al. 2014). These methods minimized the variability of the data.

Outline Geometric Morphometry. The assessment of the shape variation of the mouth hook among the samples was performed using an elliptical Fourier analysis (EFA) (Tatsuta et al. 2011, Dujardin et al. 2014), for which points were marked on the image, making a complete outline description. Several modules of the CLIC software, version 84 (Dujardin 2013) were used in the analyses. The COO module was used for collecting the outlines, TET for concatenating the files, FOG for analysis and validation of classifications, and PAD to estimate the repeatability of the size and shape. Landmark captures were performed four times by a single observer (NA Canal) following Dujardin et al. (2010).

Linear morphometry. Samples were compared with a discriminant function analysis (DFA) applied over either linear measures between two points or the ratio between them. Measurements suggested by Steck and Wharton (1989), Steck et al. (1990) and Frías et al. (2006, 2008) were followed, and additional variables were included, which were deduced from the geometric morphometry study. We follow the terminology used by White et al. (1999) and Frías et al. (2008).

The mouth hook morphology was observed carefully. Its shows a medial nub in the ventral curve, where the cuticle and muscles attaches, with a front and a rear notches next to it that extend to the top; a posterior apodema, like a neck, is also found. The anterior part of the dorsal apodema could be found where the slope turns greater (Figure 1).

All measurements were done on the digitized images of the structures. After variables were defined, measurements were performed three times by a single observer (NA Canal), but no differences in outcomes were found. Twenty-four variables were used, 15 of which corresponded to linear measurements, and nine to the ratios between various pairs (Figure 2).

Abbreviations of the variables used are as follows

BL: body length; **BW**: body width at the sixth abdominal tergite; **CSL**: cephalopharyngeal skeleton length, from the anterior apex of the mandible to the end of the ventral cornua, at lower end of the dorsal cornua; **HSL**: hypopharyngeal sclerite length, from mouth hook joint to the rear distal point; and **HSH**: height of the hypopharyngeal

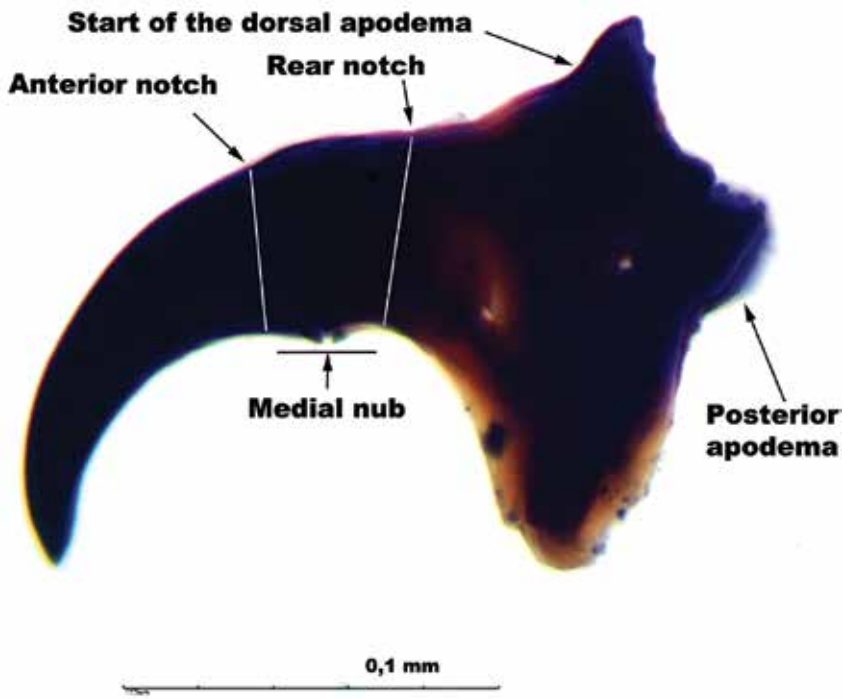

Figure 1. Lateral view of mouth hook of the third instar larvae of *Anastrepha fraterculus* complex.

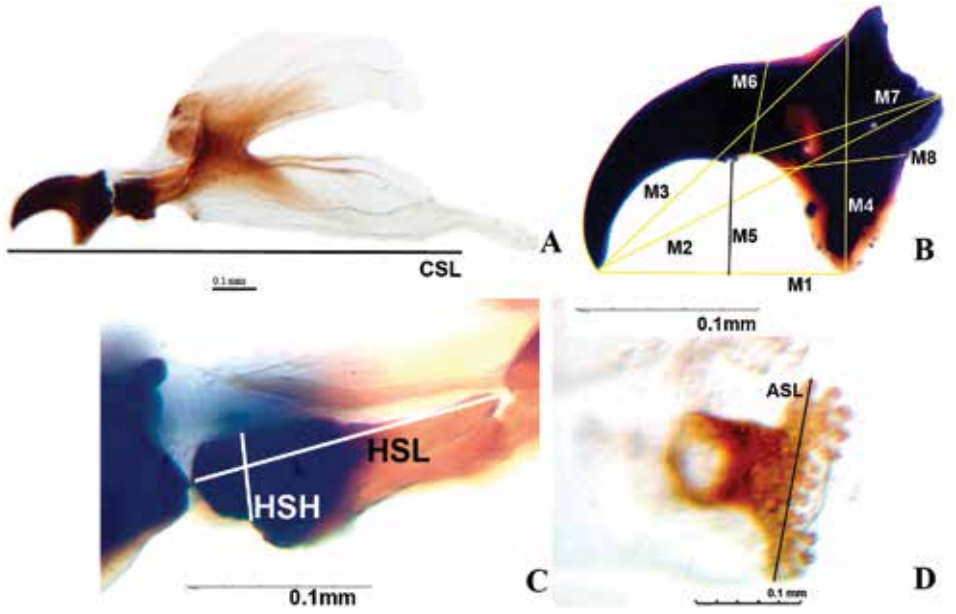

Figure 2. Linear variables measured in the third-instar larvae of the *Anastrepha fraterculus* complex. **A** cephalopharyngeal skeleton **B** mouth hook **C** hypopharyngeal sclerite **D** anterior spiracle. Variables are defined in the text.

sclerite at the anterior base of the hypopharyngeal bridge, perpendicular to the upper edge.The measurements of the mouth hook were **M1**: length from the apex to the ventral apodeme, **M2**: length from the apex to the dorsal most tip of neck, **M3**: length from the apex to the anterior base of the dorsal apodeme, **M4**: height from the apex of the ventral apodeme to the anterior base of the dorsal apodeme, **M5**: depth of ventral concavity from line M1 to tip of nub, **M6**: thickness of mouthhook at posterior base of nub by the posterior notch, **M7**: distance between the posterior base of nub and dorsal most tip of neck, and **M8**: width of the ventral apodeme at the base of the neck, in a line parallel to M1. **ASL**: width of the left anterior spiracle between the apices of the most extreme tubules, **AST**: number of tubules of the anterior spiracles, **X1**: BL/BW, **X2**: M1/M4, **X3**: M2/M4, **X4**: M1/M5, **X5**: M2/M5, **X6**: M3/M4, **X7**: CSL/HSL, **X8**: CSL/M3, and **X9**: CSL/M1.

Data analysis. The shape of the mouth hook was studied with an outline analysis in a two-dimensional plane, for which an EFA (Tatsuta et al. 2011, Dujardin et al. 2014) was used. Briefly, the outline curve was decomposed into a series of ellipses based on their sine and cosine; each one was referred to as a harmonic, and each harmonic was represented by four coefficients (Fourier coefficients). Based on the coefficients of the first harmonic, the rest of the coefficients were standardized to be used in later analyses. EFAs require doing principal components analysis (PCA) on the standardized coefficients. Based on the first principal components obtained, a DFA was performed, and afterwards, each individual was reclassified through a Jackknife procedure.

For the linear morphometry, a multivariate analysis was performed. The mean and standard deviations were calculated, and normality and homogeneity of variance tests were run for each of the variables. To assess the probability of individuals being classified into the predicted groups defined by the morphotypes and the contribution of each of the variables for group discrimination, a DFA was performed on the complete dataset, with the forward stepwise method. A canonical analysis was done to determine the canonical variables and their significance through a Chi-squared test. All analyses were performed using Statistica 12 (StatSoft 2014).

Results

Mouth hook shape. The discriminant function analysis showed that all the samples studied differed in the shape of the mouth hook (Figure 3). The analysis of reclassification of the individuals correctly included 100% of the individuals into the expected morphotype. The allometric analysis showed a 0% influence of the size on Canonical Factor 1, and a 3% influence on Canonical Factor 2, indicating that the size of the individuals did not influence the results on the shape of the mouth hook (Figure 4).

The mouth shape outlines for each individual were aligned, rotated and grouped to build the representative shapes of the morphotypes (Figure 5). The morphotypes

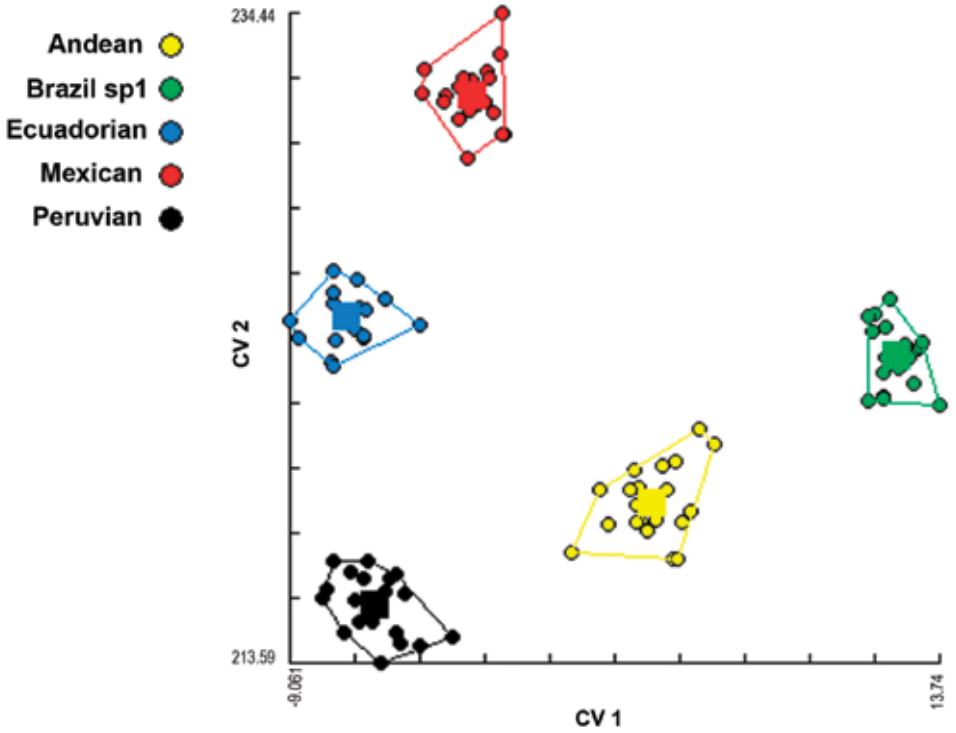

Figure 3. Grouping analysis of five morphotypes of the *Anastrepha fraterculus* complex, according to the shape of the mouth hook of third-instar larvae based on the values of the first two canonical factors in the discriminant analysis. The contribution of the first factor was 44%, and that of the second was 27%.

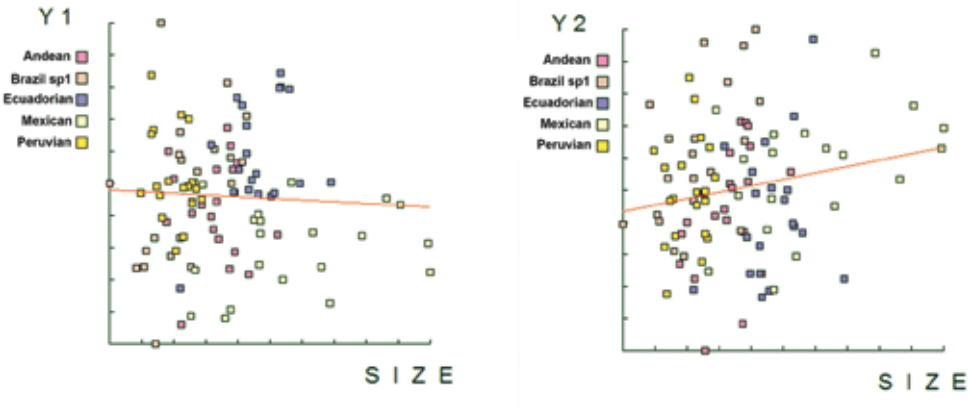

Figure 4. Allometric study indicating the influence of the mouth hook size in grouping five morphotypes of the *Anastrepha fraterculus* complex, studied with an elliptical Fourier analysis.

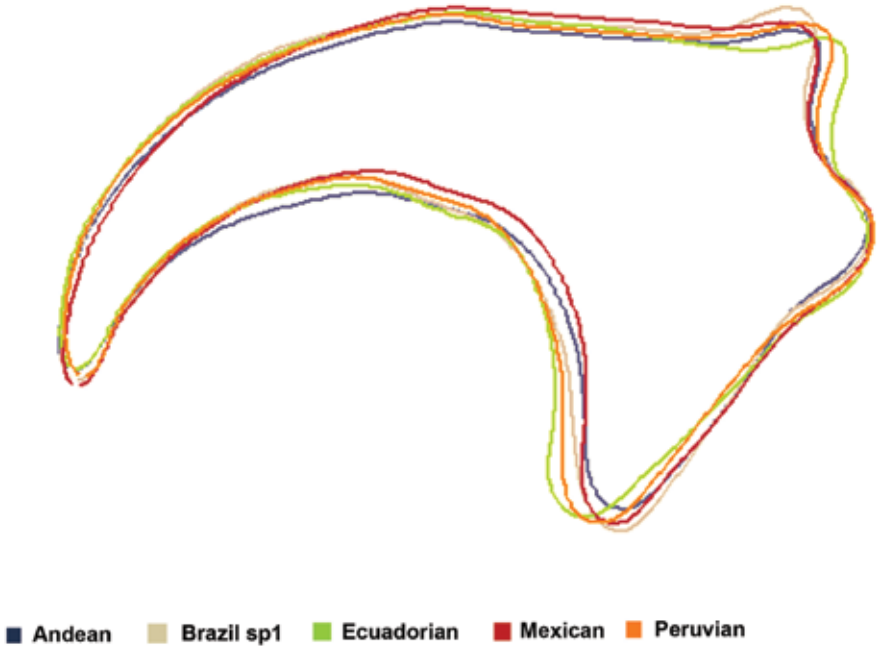

Figure 5. Representative outline of the mouth hook shape in third-instar larvae of five morphotypes of the *Anastrepha fraterculus* complex, obtained through an elliptical Fourier analysis.

showed variability mainly over the dorsal and ventral apodemes and, less noticeably, over the width of the middle part.

Size variability of the individuals. The variability of the individual sizes was studied through the morphometry of the larvae. The DFA included all 18 variables of the model (excluding CSL, M2, X4, X6, X7, and X8); 10 of the variables resulted in statistically significant differences for the segregation of the morphotypes (Wilks' Lambda: 0.005 approx. $F(72,309)=12.224$, $p<0.0001$) (Table 2). The statistically significant variables were the body length and width of the larvae, three measurements of the mouth hook, the hypopharyngeal sclerite, and the size and number of the anterior spiracle tubules. The average body length (BL) was greater in the Peruvian, Ecuadorian and Mexican morphotypes, and body width (BW) was greater in the Andean morphotype; the ratio between these two measurements (X1) was lower in the Andean population. The HSL was, on average, longer in the Andean and Ecuadorian morphotypes. The mouth hook length between the apex and the ventral apodeme (M1) was shorter for the Peruvian morphotype; the mean width in the middle part of the mouth hook (M6) was greater for individuals of the Ecuadorian morphotype and smallest for the Peruvian morphotypes. The average basal width of the ventral apodeme (M8) was narrower in specimens of the Brazilian-1 and Peruvian morphotypes. The anterior spiracle

Table 2. Independent contribution to the discriminant model of each of the variables measured from third-instar larvae of five morphotypes of the *Anastrepha fraterculus* complex. * < 0.05= statistically significant.

Variables	Wilks' Lammbda	F-remove (4.78)	p-value
ASD	0.006756	6.85482	<0.0001*
M5	0.005493	1.92970	0.113783
ASL	0.008777	14.74068	<0.0001*
X9	0.006039	4.05858	0.004*
M6	0.005942	3.67924	0.008*
X1	0.006071	4.18342	0.004*
M3	0.005450	1.76149	0.145094
X3	0.005190	0.74581	0.563734
M1	0.005842	3.29114	0.015*
HSL	0.005989	3.86235	0.006*
HSH	0.005509	1.99152	0.103994
X2	0.005472	1.84521	0.128604
M8	0.006221	4.76893	0.001*
M4	0.005597	2.33372	0.062912
M7	0.005471	1.84152	0.129291
BL	0.006709	6.67357	0.0001*
BW	0.006513	5.90854	0.0003*
X5	0.005293	1.14799	0.340365

was longer in specimens of the Andean morphotype, and the number of spiracle tubules was smaller in the Brazilian-1 and Peruvian morphotypes. The X9 ratio between the CSL and the length of the mouth hook from the apex to the ventral apodeme (M1) was greater for Peruvian individuals and smaller for Mexican individuals (Table 3). The standard deviations of the measurements were low (Table 3), indicating that accuracy of measurements was high and repeatable.

The canonical analysis resulted in four canonical roots, and the Chi-squared test showed statistical significance for all the roots. CV-1 had 51.6% of the discrimination power, CV-2 had 24.2%, CV-3 had 19.3% and CV-4 had 4.9%. In the first root, variables with major contribution to the separation of the groups were the anterior spiracle length (ASL) and the number of tubules of the anterior spiracle (AST), followed in importance by the hypopharyngeal sclerite length (HSL), body width (BW), and dimensions of the mouth hook M3 (length from the apex to the most distal and dorsal point) and M8 (width of the ventral apodeme). The most important variables for CV-2 were the ASL, BL/BW (X1) and mouth hook length/width (X2), followed by the BL and HSL (Table 4). The 3D graph of the morphotype centroids, including the first three canonical roots, shows the separation of the different populations (Figure 6).

Table 3. Means (mm) and standard deviations of 24 morphometric variables of third-instar larvae of five morphotypes of the *Anastrepha fraterculus* complex.

Population	BL	BW	HSL	HSH	CSL	M1	M2	M3	M4	M5	M6	M7
Andean	9.22±0.97	2.09±0.19	0.2±0.015	0.07±0.006	1.14±0.07	0.17±0.01	0.25±0.01	0.23±0.01	0.15±0.016	0.08±0.007	0.06±0.008	0.1±0.007
BrasilSp1	8.77±0.74	1.71±0.29	0.17±0.012	0.07±0.007	1.05±0.04	0.15±0.02	0.23±0.02	0.21±0.02	0.14±0.016	0.08±0.006	0.05±0.007	0.09±0.006
Peruvian	9.48±0.38	1.87±0.14	0.18±0.007	0.07±0.005	1.09±0.04	0.14±0.01	0.22±0.01	0.21±0.01	0.14±0.008	0.08±0.005	0.05±0.004	0.09±0.007
Ecuador	10.02±0.37	1.89±0.13	0.21±0.013	0.07±0.008	1.15±0.04	0.16±0.01	0.26±0.01	0.25±0.01	0.16±0.012	0.08±0.006	0.06±0.006	0.1±0.006
Mexico	9.7±0.73	2±0.17	0.19±0.026	0.07±0.009	1.15±0.11	0.18±0.03	0.27±0.03	0.25±0.03	0.17±0.023	0.09±0.008	0.06±0.011	0.11±0.014
All Grps	9.44±0.79	1.91±0.23	0.19±0.02	0.07±0.007	1.12±0.08	0.16±0.02	0.25±0.03	0.23±0.02	0.15±0.019	0.08±0.009	0.06±0.009	0.1±0.011

Population	M8	X1	X2	X3	X4	X5	X6	ASL	ASD	X7	X8	X9	Valid N
Andean	0.06±0.008	4.41±0.31	1.14±0.10	1.69±0.15	2.21±0.24	3.26±0.32	1.55±0.10	0.34±0.07	14.45±0.89	5.79±0.32	4.96±0.39	6.72±0.49	20
BrasilSp1	0.05±0.005	5.23±0.72	1.07±0.08	1.59±0.11	2.03±0.26	3±0.31	1.44±0.09	0.19±0.02	10.55±0.83	6.09±0.45	5.14±0.36	6.91±0.70	20
Peruvian	0.06±0.006	5.1±0.34	1±0.07	1.56±0.09	1.9±0.17	2.94±0.21	1.46±0.07	0.2±0.02	11.55±1.0	6.09±0.25	5.19±0.28	7.56±0.45	20
Ecuador	0.07±0.007	5.32±0.31	1.01±0.09	1.59±0.11	1.98±0.16	3.12±0.23	1.51±0.09	0.24±0.02	14.1±1.12	5.47±0.28	4.68±0.24	7.02±0.48	20
Mexico	0.06±0.010	4.86±0.32	1.07±0.05	1.6±0.07	1.93±0.22	2.89±0.23	1.49±0.09	0.23±0.03	12.75±1.02	5.98±0.31	4.55±0.24	6.34±0.51	20
All Grps	0.06±0.009	4.98±0.54	1.06±0.09	1.61±0.12	2.01±0.24	3.04±0.29	1.49±0.10	0.24±0.06	12.68±1.77	5.88±0.40	4.9±0.39	6.91±0.66	100

Table 4. Correlation between the variables and canonical roots from the discriminant analysis for 24 measurements of third-instar larvae of five morphotypes of the *Anastrepha fraterculus* complex.

Variable	Root 1	Root 2	Root 3	Root 4
ASD	-0.545921	-0.100158	-0.344710	0.172453
M5	0.041755	-0.071879	-0.653270	0.146808
ASL	-0.513976	0.354166	-0.104138	-0.004608
X9	0.036323	-0.175930	0.385942	0.368080
M6	-0.119046	-0.177843	-0.293162	-0.329549
X1	0.159367	-0.335778	0.090498	-0.231802
M3	-0.183020	-0.159496	-0.634346	0.078811
X3	-0.118893	0.134014	-0.037045	-0.103410
M1	-0.106642	0.111731	-0.475412	-0.141087
HSL	-0.254104	-0.211098	-0.239891	-0.017785
HSH	-0.071814	0.014497	0.099759	0.055553
X2	-0.103959	0.305811	-0.053167	-0.234816
M8	-0.189002	-0.150179	-0.247975	0.111262
M4	-0.038899	-0.119367	-0.407167	0.016389
M7	-0.077578	-0.007283	-0.429200	0.136471
BL	-0.075040	-0.250882	-0.204123	0.306914
BW	-0.182774	0.132194	-0.198878	0.344580
X5	-0.177914	0.039586	0.090199	-0.187916
Eigenvalue	6.9315	3.239	2.599	0.653
Cumulative proportion	0.5164	0.758	0.951	1.000

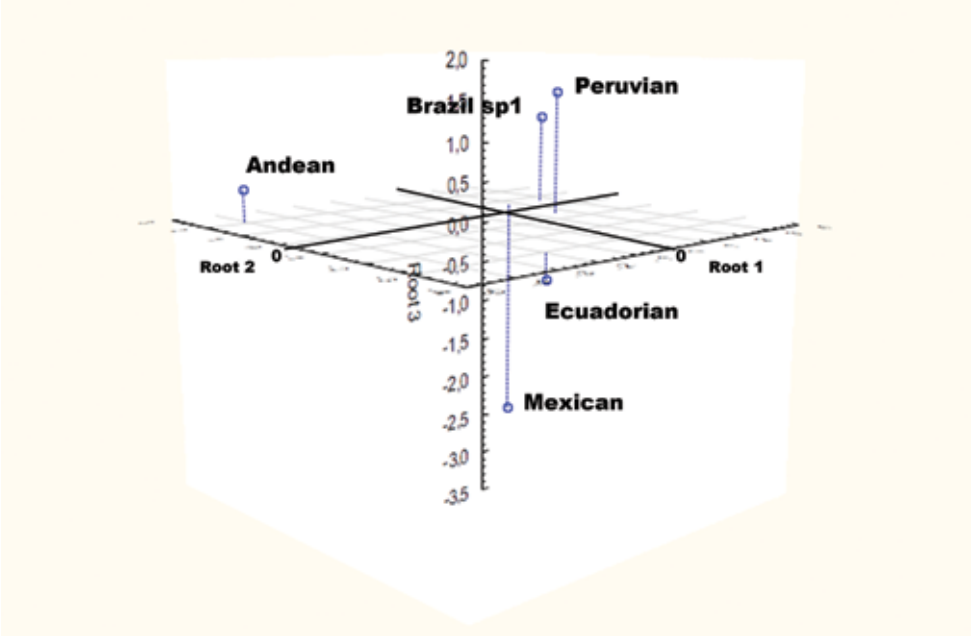

Figure 6. 3D scatterplot of discriminant function analysis applied to the centroid values of 24 measurements in third-instar larvae of five morphotypes of the *Anastrepha fraterculus* complex.

Table 5. Classification matrix of individuals according to a predictive model of third-instar larvae of five morphotypes of the *Anastrepha fraterculus* complex. Rows: Observed classifications; Columns: Predicted classifications. Same probabilities for all the groups.

Group	Percent	Andean	Brasil sp1	Peruvian	Ecuador	Mexico
Andean	100.0000	20	0	0	0	0
BrasilSp1	90.0000	0	18	2	0	0
Peruvian	95.0000	0	1	19	0	0
Ecuadorian	100.0000	0	0	0	20	0
Mexican	95.0000	0	1	0	0	19
Total	96.0000	20	20	21	20	19

The prediction model indicated that 96% of the individuals were correctly placed in their respective morphotypes; all of the Andean and Ecuadorian specimens were properly classified, and only two individuals from Brazilian-1, one from Mexico and one from Peru were incorrectly classified (Table 5).

Discussion

The results obtained from our study of the mouth hook shape of the third-instar larvae established that variation exists in the shape of this structure that usefully separates the five morphotypes. Moreover, it was possible to confirm the presence of variability in the dorsal and ventral apodeme areas. Geometric morphometry is a sensitive tool to study the presence of cryptic species (Adams et al. 2004, Dujardin 2008, Dujardin et al. 2010, 2014), and recently, outline geometric morphometry has facilitated the study of complex structures, for example, in immature stages of insects (Dujardin et al. 2014). To the extent of our knowledge, this is the first study to use outline-based morphometrics for immature stages of tephritid fruit flies.

Geometric morphometry has been used for differentiation of fruit flies (Kitthawee y Dujardin 2010, Yee et al. 2011, Schutze et al. 2012, Perre et al. 2014, Hernández-Ortiz et al. 2015), in all cases on adult structures, leading to the possibility of differentiating formal species or cryptic species complexes. Unfortunately, geometric morphometry does not generate characters that can be used in traditional taxonomic keys, and further studies are needed to determine how to use this information (Dujardin et al. 2010). An alternative is, for example, the development of automatic systems for identification, as suggested by Faria et al. (2014), based on the geometric morphology of adults from several species of *Anastrepha*. The results obtained from our study suggest that the methods proposed here could be used for developing an identification system of this type that extends to larvae.

The results of the linear morphometry were also highly satisfactory, reaching a 96% accuracy of the predicted classification for the studied individuals. According to what was previously reported regarding size variation of insects through generations

of laboratory rearing (Jaramillo et al. 2002, Jirakanjanakit et al. 2008), it is possible that these results were especially influenced by the long breeding of the Peruvian morphotype. Repeatability of results could be supposed based on the low variance of the variables.

Steck et al. (1990) used morphometric and morphological variables to differentiate 13 species of *Anastrepha* based on the study of third-instar larvae. The accuracy of the key produced was high for species differentiation, despite the high variability in some of the species. Samples of the nominal species *A. fraterculus* from various parts of Latin America were included in that study, and the authors found that it was one of the species with highest variability in the characters analyzed. Currently it is well known that, in reality, the authors studied different cryptic species, hence the difficulty in identifying *A. fraterculus* species. However, these authors could establish differences between species through normalization of variables by transformation and construction of linear discriminant functions. Our study included new variables to differentiate the cryptic species of *A. fraterculus*, which could complement the study of Steck et al. (1990) for recognizing species, by including additional morphological characters.

Still these techniques have some difficulties. The most common errors made in this analysis result from poor mounting of structures, digital imaging and determination of landmarks (Dujardin et al. 2010). The principal error in our study could be in the imprecision of the landmarks; however, after some testing, our approach resulted in a minimized error and in a consistent and reliable data for analysis. Outcomes of the classification of individuals and the low variance on linear variables of the model supported this conclusion.

Specimens studied here derive from different sources, either from wild samples reared on natural hosts or from lab strains reared on artificial diets, however we do not know the effects of this on the measured structures, and further studies are needed. Some authors have suggested that developmental conditions affect the size of insects (Jaramillo et al. 2002, Schutze et al. 2012). Jaramillo et al. (2002) and Jirakanjanakit et al. (2008) found variability in the head and wing size of insects reared in the laboratory for several generations, but not in the shape until after at least 10 generations. Our sample of the Peruvian morphotype came from a colony artificially reared since 2002, and may deviate from wild specimens. However the low values resulting from the allometric studies (CV-1=0%, CV-2=3%, Figure 4) confirm that our results are due to the shape but not the size of the individuals.

In many cases, the use of morphological characters of immature stages of insects for phylogenetic studies has helped to improve the understanding of relationships among groups (see revision in Meier and Lim 2009). Even though, immature stages have been widely ignored in studies of taxonomy, systematics and phylogeny, perhaps due to the difficulty of associating them with the adults and of determining stable morphological characters for them (Meier and Lim 2009). We suggest that further effort should be made in rearing specimens and revising methods and characters.

Conclusions

Outline geometric morphometry and linear morphometry proved to be useful tools for the study of cryptic species of the *A. fraterculus* complex. The results obtained from this work with third-instar larvae should be expanded to include additional populations to strengthen the dataset and advance our tools to study cryptic species of economically important fruit flies.

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Morphometric divergence in populations of *Anastrepha obliqua* (Diptera, Tephritidae) from Colombia and some Neotropical locations

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Abstract

The West Indian fruit fly, *Anastrepha obliqua*, is one of seven species of quarantine importance of its genus and is one of the most economically important fruit fly pests in Colombia. The taxonomic status of this species is a key issue for further implementation of any pest management program. Several molecular studies have shown enough variability within *A. obliqua* to suggest its taxonomic status could be revised; however, there are no morphological studies supporting this hypothesis. The aim of this work was to describe the morphological variability of Colombian populations of *A. obliqua,* comparing this variability with that of other samples from the Neotropics. Measurements were performed on individuals from 11 populations collected from different geographic Colombian localities and were compared with populations from Mexico (2), Dominica Island (1), Peru (1) and Brazil (2). Linear morphometric analyses were performed using 23 female morphological traits, including seven variables of the aculeus, three of the thorax, and six of the wing; seven ratios among them were also considered. Discriminant function analyses showed significant morphological differentiation among the Colombian populations, separating them into two groups. Furthermore, in the comparisons between Colombian samples with those from other countries, three clusters were observed. The possibility of finding more than one species within the nominal *A. obliqua* population is discussed.

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Keywords
West Indian fruit fly, taxonomy, linear morphometry

Introduction

Anastrepha Schiner is the most diverse and economically important genus of fruit flies in the Neotropics, with more than 250 described species (Norrbom et al. 2012). *Anastrepha obliqua* (Macquart), known as the West Indian fruit fly, has quarantine status and is one of the most harmful species within the genus (Norrbom et al. 2012, Ruíz-Arce et al. 2012). It is distributed from northern Mexico to southeast Brazil including several Caribbean islands (Ruíz-Arce et al. 2012). Norrbom (2004) lists 104 species within 27 host plant families for *A. obliqua*; however, the species most affected by this pest are mango (*Mangifera indica*) and *Spondias* in Mexico (Orozco-Dávila et al. 2014), Colombia (Nuñez 1981, Mangan et al. 2011), the southern Caribbean (Mangan et al. 2011) and Brazil (Zucchi 2000).

The West Indian fruit fly belongs to the *fraterculus* group, which involves 34 species (Norrbom et al. 2012) from which one complex of cryptic species has been recognized (Hernández-Ortiz et al. 2012). The taxonomic status of *A. obliqua* has been questioned, and the existence of a group of cryptic species within the nominal species is presumed (Ruíz-Arce et al. 2012). Hernández-Ortiz et al. (2004, 2012, 2015) studied Latin American populations of *Anastrepha fraterculus* (Wiedemann) using linear morphometry of the aculeus and the wings and found eight different morphotypes, showing that the use of morphological characters and linear morphometry is a useful tool for the study of cryptic species in the genus *Anastrepha*, especially in the *fraterculus* group.

Smith-Caldas et al. (2001) studied the COI mitochondrial gene sequences of 15 species of *Anastrepha*, 12 of which were from the *fraterculus* group. The sample included eight populations of *A. obliqua*: five from Brazil, two from Mexico and one from Colombia. The results showed two well-separated groups, the first of which included the Mexican population, the Colombian population and one Brazilian population while the remaining Brazilian populations formed a second group. Therefore, the authors indicated the need to study this species more extensively. Ruiz-Arce et al. (2012) studied 54 Latin American populations of *A. obliqua* with COI and ND6 genes and concluded that there are six genetic types that require taxonomic revision (Mesoamerica, Central America, Caribbean, Western Mexico, South American Andes and Eastern Brazil).

Other studies have also suggested variability within the species. Karyotype descriptions of Brazilian and Mexican samples reflect the existence of a constriction at the apical end of the X chromosome (Solferini and Morgante 1987, Selivon et al. 2005, Ibañez-Palacios et al. 2010), while Bush (1962) studied individuals that were clearly devoid of that chromosome constriction. Additionally, morphological descriptions of eggs (Emmart 1933, Norrbom 1985, Murillo and Jiron 1994, Selivon and Perondini

2000, Figuereido et al. 2011) and larvae (White and Elson-Harris, 1992, Frías et al. 2006) also reported morphological variability in the populations studied.

In Colombia, mango is the second most important fruit product based on its planting area and it is cultured at low altitudes throughout the country. *Anastrepha obliqua* is one of the biggest limiting factors of its production (MADR 2006, Sosa et al. 2011), and therefore, the development of sustainable management systems has become a priority. This species of fruit fly is widely distributed at altitudes less than 1,500 m, following the distribution of mango and the species of *Spondias*, which are its main hosts (Castañeda et al. 2010). Because the Andes of Colombia are divided into three high altitude mountain ranges, extending east to Venezuela with two deep valleys separating them, the populations of *A. obliqua* located in lowlands could be isolated in the different areas of the country.

The Sterile Insect Technique (SIT), for the control of the West Indian fruit fly" has been implemented in Mexico, and this fly is one of the priority species for the development of this technique in other regions (Cáceres et al. 2014, Orozco-Dávila et al. 2014). Studies to identify more efficient specific attractants are also being developed (Cruz-López et al. 2006). However, the precise taxonomic knowledge of the pest is a fundamental requirement for these techniques, as well as other quarantine measures, risk analysis and free or low prevalence areas (Hernández-Ortiz et al. 2004, Norrbom et al. 2012).

The study of the interpopulation variability of *A. obliqua* in Colombia or other areas of the Neotropics is particularly relevant to determine if this nominal species is composed by a cryptic species complex considering the implications this has for fruit international trade. These studies are also important for the implementation of pest management systems in different regions, such as the SIT. The aim of this study was to describe the morphologic variability of *A. obliqua* through the use of multivariate morphometric analyzes among Colombian populations distributed throughout the country and through comparisons with populations coming from its distribution range such as Mexico, Peru, Brazil and Dominica Island. The hypotheses of this study is that if there is genetic variation in *A. obliqua* suggesting the existence of more than a simple biological entity (Ruiz-Arce et al. 2012), this variability must be observed in the morphology of the species.

Methodology

Insect collection

The individuals studied were collected from 11 localities of Colombia along the inter-Andean valleys of the Magdalena River (4) and Cauca River (3) and in the Eastern Plains (2) and the Caribbean Plains (2) (Figure 1). These populations were compared with two samples from Mexico (East and West), two from southeastern Brazil (Sao Paulo), one from Peru and one from Dominica Island (Table 1). Specimens were collected from infested fruits or McPhail traps placed in the field or obtained from labora-

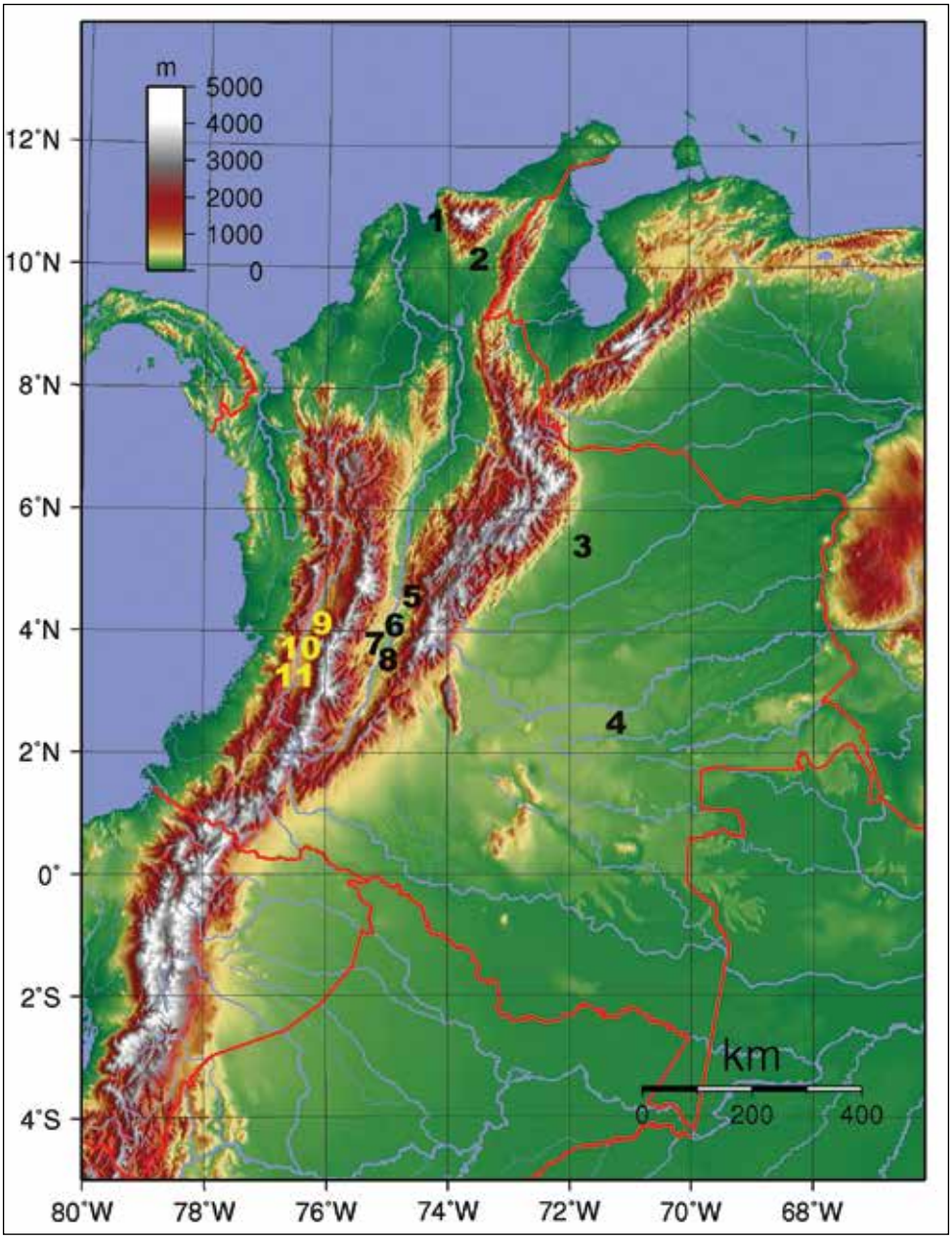

Figure 1. Geographic distribution of populations of *Anastrepha obliqua* collected in Colombia for morphometric analysis: **1** Cienaga **2** Huacachi **3** Villanueva **4** Puerto Colombia **5** Anolaima **6** Coello **7** Espinal, 8 Guamo **9** La Tebaida **10** Zarzal, and **11** La Unión. (Image from https://elpaisdelcafe.files.wordpress.com/2012/02/200px-colombia_topography.png)

Table 1. Collection data for *Anastrepha obliqua* populations.

Country	Population	State	City	Host	Data	Latitude	Longitude	Altitude	n
Colombia	Anolaima	Cundinamarca	Anolaima	*Mangifera indica*	6/8/2010	4°43'23"N	74°25'4"W	972	20
	Coello	Tolima	Coello	*Spondias mombin*	8/28/2010	4°16'55"N	74°54'16"W	309	20
	Guamo		Guamo	*Spondias purpurea*	Lab rearing	4°04'35"N	74°59'35"W	345	20
	Espinal		Espinal	*Mangifera indica*	Lab rearing	4°11'59"N	74°58'0.7"W	380	20
	La Union	Valle del Cauca	La Unión	*Mangifera indica*	11/12/2013	4°33'53"N	76°05'22"W	954	14
	Zarzal		Zarzal	*Mangifera indica*	11/11/2013	4°25'33"N	76°03'43"W	954	19
	La Tebaida	Quindio	La tebaida	*Spondia* sp.	7/10/2010	4°29'42"N	75°41'36"W	1409	20
	Villanueva	Casanare	Villanueva	*Spondia* sp.	3/22/2013	4°22'13"N	72°46'17"W	160	20
	PuertoC	Guaviare	Purto Colombia	McPhail Traps	10/09/09	2°36'13"N	72°39'W	189	20
	Huacachi	Cesar	Huacachi	*Mangifera indica*	8/6/2013	10°30'25"N	73°0.9'47"W	136	20
	Cienaga	Magdalena	Cienaga	McPhail Traps	8/1/2005	11°58'92"N	74°12'18"W	N.R	18
México	Mex-Pacific	Guerrero	Los Ayutlas	McPhail Traps	06/28/2008	16°59'17"N	99°04'57"W	340	20
	Mex-Gulf	Veracruz	Los Tuxtlas	*Spondias sp*	10/19/2012	18°26'36"N	95°02'46"W	397	20
Brasil	Brazil-1	São Paulo	USP	*Spondias mombin*	Lab rearing	23°33'55"S	46°44'04"W	780	9
	Brazil-2	São Paulo	USP	*Spondias mombin*	Lab rearing	23°33'55"S	46°44'04"W	780	10
Peru	Peru-Pacific			McPhail Traps	ND	ND	ND	ND	20
Isla Dominica	Caribe			McPhail Traps	ND	15°31'24"N	61°21'56"W	ND	17

tory colonies. Samples of mango fruit (*Mangifera indica*) and several species of *Spondias* were collected. The fruits were collected in plastic trays with damp vermiculite and transferred to the laboratory, where they remained for the separation of larvae/pupae and subsequent acquisition of adults. The imagos were kept in cages and fed for at least five days for the fixation of their morphological characteristics. In order to obtain native populations of insects, fruit sampling was conducted in non-commercial areas. Specimens from McPhail traps were collected from only one trap in the same date. Samples from Espinal and Guamo (Colombia) were obtained from a laboratory colony maintained, in artificial diet, since 2005 with periodical introductions of wild flies. In these cases, specimens were obtained three generations after field specimens were introduced in the colony. Samples from Brazil were obtained from specimens stored in alcohol from reared insects at two different times and came from colonies reared in fruits for a few generations.

Morphological variables

Morphometric studies were conducted on the aculeus, right wing and mesonotum of randomly selected five-day-old females following methods for the study of the *A. fraterculus* complex described by Hernández-Ortiz et al. (2004, 2012). The ovipositor was cleaned with 10% sodium hydroxide for 24 hours and the wing and the aculeus were mounted on permanent slides with Canad balsam. The abdomen and thorax were preserved in 70% alcohol. The aculeus was photographed with a Canon Powershot G10 digital camera, adapted to a Carl Zeiss Primo Star microscope.

The wing and the mesonotum were photographed with a Moticam10X camera adapted to a stereomicroscope. The images were measured with the software Motic Image Plus 2.0 (Motic 2013); all measurements were performed by only one observer (MRC).

For the study, 23 variables were used (Figure 2): *Aculeus*: A1, total length of the aculeus; A2, width at the end of the sclerotized margin on the ventral side; A3, width at the beginning of the serrated section, measured between the apices of the second pair of teeth; A4, length of the basal end of the aculeus (from the margin of the sclerotized area on the ventral side at the beginning of the serrated section); A5, length of the apex of the aculeus (length of the serrated section); A6, length of the lateral right side from the base of the sclerotized area; A7, average number of teeth per side; A8, length of the apex (A4+A5); A9: proportion of the non-serrated and serrated areas of the apex (A4/A5); A10, proportion of the length of the tip of the aculeus and total aculeus length (A8/A1); and A11, proportion of the non-serrated area of the apex and total apex length (A4/A8). *Wing*: W1, length of the wing from the basal extreme of the costal margin to the apex; W2, wing width at the apex of the R1 vein; W3, width of the S band from the union of the S band and the R4+5 vein perpendicular to the costa; W4, width of the base of the proximal branch of the V band; W5, S- and V-band connection between R_{2+3} and R_{4+5} (1= present, 2= absent);

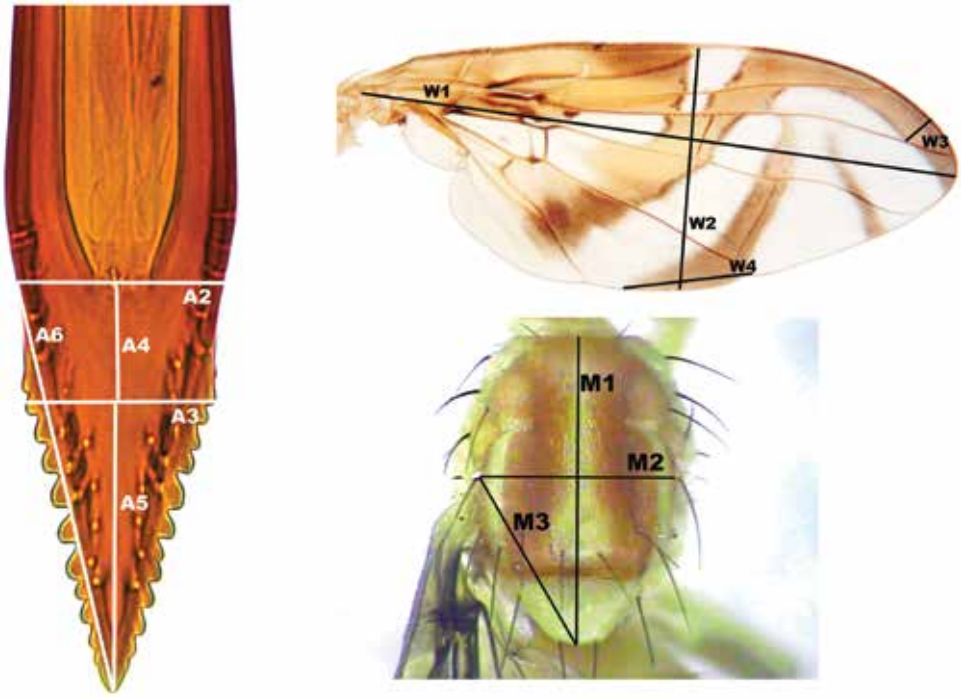

Figure 2. Diagram of the structures and variables used in the morphometric analysis of *Anastrepha obliqua* females in the aculeus (ventral view), mesonotum (dorsal view) and wing. The variables are described in the text.

W6, V-band anterior connection of proximal and distal arms between R_{4+5} and M (1= present, 2= absent). *Thorax*: M1, maximum length; M2, width at the level of the postsutural supra-alar seta; and M3, diagonal distance from the postsutural-supra-alar seta to the apex of the scutellum. In addition, ratios were used, X1, proportion of the total length of the aculeus and maximum length of the thorax (A1/M1); X2, proportion of the total aculeus length and wing length from the basal extreme of the costal vein to the apex (A1/W1); and X3, proportion of the maximum thorax length and maximum wing length (M1/W1).

Statistical analysis

The samples were grouped according to their geographical origin to discern the degree of variability or possible differentiation between populations. The mean and standard deviation were calculated for each of the variables. A discriminant function analysis (DFA) was applied to the set of variables. Measures of the mean distances of the data were derived from the comparison of pairs of centroids, expressed as the Mahalanobis distance (MD). The functions were evaluated by applying a canonical correlation

analysis to determine the significance and discrimination power of the model and the specific variables responsible for the segregation of the groups. All statistical analyses were conducted using the software Statistica 12 (StatSoft Inc. 2014). The *voucher* specimens of the Colombian, Peruvian and Brazilian populations were deposited in the Entomology Collection Laboratory of the University of Tolima, Ibagué –Tolima, and the samples from Mexico and Dominica were deposited in the Entomological Collection of the Institute of Ecology (INECOL), Xalapa - Mexico.

Results

Colombian populations

The results of the DFA applied to 11 Colombian populations of *Anastrepha obliqua* showed significant differences among them (Wilks' Lambda: 0.00444, F (180,1498)= 7.0895, p< 0.0001). The exploratory model included 23 variables assessed, and 15 of them had a significant contribution to the model (P<0.05): aculeus (A2, A3, A4, A7, A8, A9 and A10), wing (W1, W3, W4 and W5) and mesonotum (M1 and M2); significant differences in the ratios of X1 and X2 were also observed (Table 2).

The Chi-squared tests indicated that the first eight, of the resulting ten canonical roots, were significant. Based on the standardized coefficients for the significant morphological variables, the first canonical root (CV-1) represented 63.1% of the model discrimination, CV-2 represented 13.5% and CV-3 represented 7.2% (Table 3).

The scatterplot obtained with the first two discriminant functions (Root 1 and Root 2) indicates the formation of two distinct groups. The first group was composed by nine populations distributed along the middle Valley and lower Magdalena River (Anolaima, Coello, Espinal, Guamo, Cienaga, and Huacachi), one sample in the Andean basin of the Cauca River (La Tebaida), and two in the eastern Plains (Villanueva and Puerto Colombia). The second group was composed by two populations located in the Cauca River Valley (La Unión and Zarzal) (Figure 3). The 3D scatterplot based on the first three canonical roots shows the mean distances between the centroids of each of the populations (Figure 4), where La Unión and Zarzal are located distally on canonical axis 1 (positive coefficient), while other populations are located basally to this axis (negative coefficient).

The largest Mahalanobis distances were found between Zarzal and La Unión with respect to the other populations (MD= 45.38–76.04 and MD= 32.89–58.3, respectively); the other nine populations had distances between 5.47 and 23.27 (Fig 5).

The grouping of 11 Colombian populations based on the means of all of the variables resulted in the formation of two large clusters (Euclidian Distance=142): one included the Zarzal and La Union populations, and the second comprising the remaining nine populations (Figure 5).

Table 2. Discriminant function analysis summary of 11 Colombian populations of *Anastrepha obliqua*. Only significant variables in the model are included.

Variables	Wilks' Lambda	F-remove 10,167	p-level	R-Square
W4	0.006767	8.825243	< 0.0001	0.280973
A2	0.005525	4.120675	< 0.0001	0.521245
A9	0.005423	3.736215	0.000151	0.990530
A4	0.005394	3.627670	0.000215	0.995470
W3	0.005309	3.305551	0.000614	0.101540
M2	0.005235	3.023529	0.001528	0.631673
M1	0.005153	2.713091	0.004108	0.987195
A8	0.005138	2.655682	0.004922	0.995699
W5	0.005116	2.572283	0.006392	0.118329
X1	0.005097	2.502542	0.007942	0.988528
A3	0.005069	2.394307	0.011094	0.644490
X2	0.005058	2.353962	0.012554	0.996985
W1	0.005055	2.343680	0.012954	0.996412
A7	0.005029	2.243795	0.017544	0.278160
A10	0.004946	1.927770	0.044553	0.986000

Table 3. Standardized coefficients for canonical variables resulting from the discriminant function analysis of 11 Colombian population of *Anastrepha obliqua*. All canonical roots were significant.

Variables	Root 1	Root 2	Root 3	Root 4	Root 5	Root 6	Root 7	Root 8
A2	0.391	-0.131	-0.453	-0.417	-0.136	0.526	0.020	-0.392
A8	0.970	2.180	2.065	-5.627	-2.515	-5.268	-4.700	-1.553
M2	-0.396	0.555	0.263	0.021	-0.230	-0.093	0.094	-0.243
W4	0.021	-0.272	-0.887	0.073	0.103	-0.369	0.060	0.352
A10	-0.798	-1.386	-0.290	3.342	-1.684	0.533	0.084	-0.658
W3	0.084	0.006	-0.446	0.271	-0.064	0.049	-0.442	-0.009
A4	0.346	-0.572	-2.466	4.026	5.605	6.379	6.264	2.871
A9	0.480	0.467	-1.652	2.147	4.136	4.178	4.718	2.849
W2	0.165	-0.241	0.056	-0.037	0.317	0.647	0.377	-0.686
W5	0.038	0.072	0.022	0.088	0.334	0.333	-0.522	0.410
A3	0.382	-0.141	0.422	-0.092	-0.384	-0.286	0.126	0.669
A7	-0.252	0.222	-0.064	0.011	0.318	0.099	0.314	0.207
M3	0.274	0.574	-0.672	-0.455	0.127	0.629	-0.436	0.095
X2	-0.038	-5.365	1.610	3.953	-1.078	-2.621	-1.080	-9.026
W1	0.030	-4.933	1.722	3.171	-0.856	-2.958	-1.380	-8.032
M1	-1.661	2.220	-1.475	-1.368	-0.243	-0.342	3.662	0.908
X1	-1.273	2.550	-1.813	-1.511	-0.398	0.184	3.680	0.877
A1	0.657	1.293	0.231	-0.453	0.740	0.977	-0.878	3.841
Eigenvalue	7.660	1.637	0.891	0.618	0.488	0.350	0.233	0.141
Cummulative %	0.631	0.766	0.839	0.890	0.930	0.959	0.978	0.990

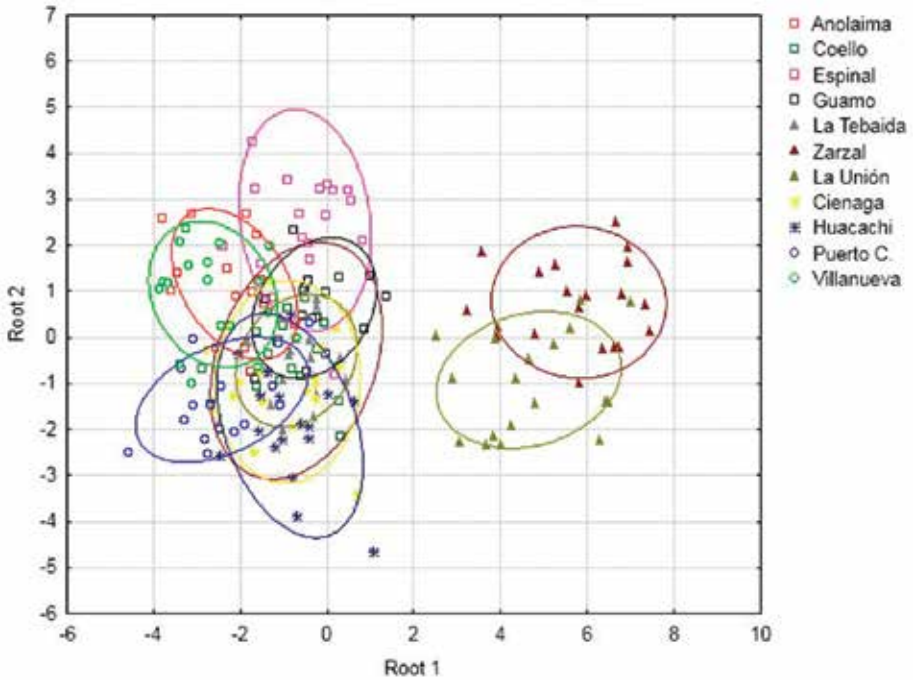

Figure 3. Discriminant function analysis applied to 11 Colombian populations of *Anastrepha obliqua*. Centroids with 95% prediction ellipses.

Colombian and external populations

The comparison of the Colombian populations with the external populations was conducted by sorting the Colombian populations into two groups according to our previous results. The first (Colombia-1) included the populations of the Magdalena Valley, the Coast and the eastern Plains, and the second (Colombia-2) included the individuals of La Unión and Zarzal. Discriminant analysis was performed for all 23 variables; however the W5 and W6 put too far the Brazilian populations and hindered the variability among other groups. Hence, a DFA was applied with 21 morphometric variables (excluding W5 and W6), and a significant differentiation was found among the groups (Wilks' Lambda: 0.00596, F (119,1724)= 17.300; $p< 0.0001$). This model was constructed with the 17 variables resulted of significance (Table 4). The analyses of the standardized coefficients produced seven significant discriminant functions and the first three represented 82.9% of the discrimination among groups (Table 5).

The grouping of samples based on the means of all the variables and using the first two canonical roots showed the divergence between the Colombian groups. Colombia-1

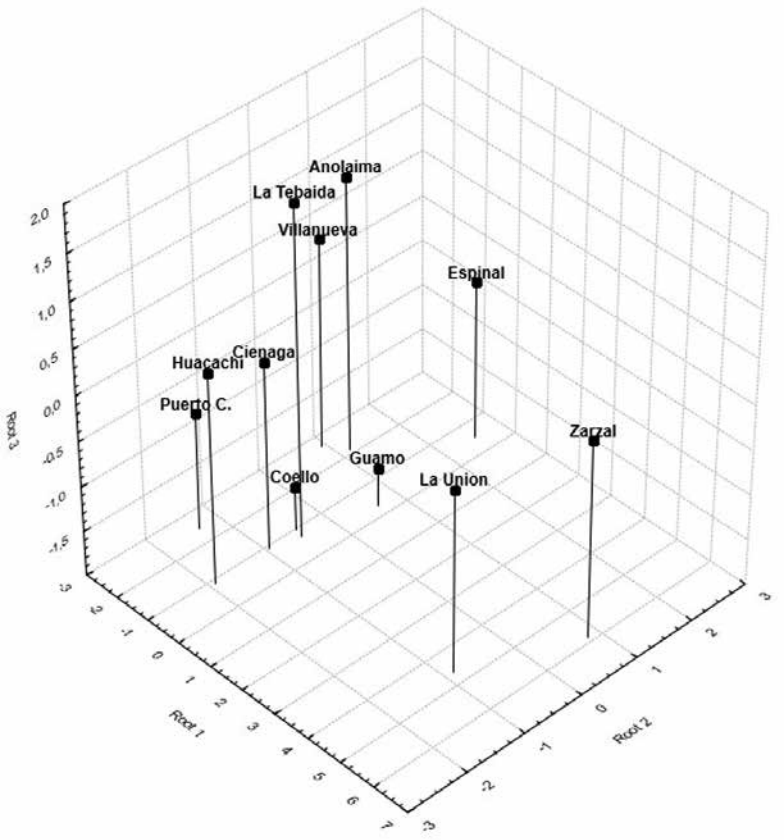

Figure 4. 3D scatterplot of discriminant function analysis of 11 Colombian populations of *Anastrepha obliqua*.

was placed in a group with the Mex-Pacific, Peru-Pacific and Caribbean populations; Colombia-2 formed a group with the Mex-Gulf population, and the Brazilian samples remained separate (Figure 6).

The scatterplot obtained by the comparison of the first two discriminant functions (Root 1 and Root 2) and the 3D scatterplot based on the first three canonical roots are shown in Figure 7.

The prediction model was able to correctly assign 93.4% of the individuals in their corresponding groups; all of the Brazilian individuals were classified correctly, and the success rates of the other groups were between 89.5 and 97.4% (Table 6). The model indicates that the Colombian samples were correctly separated into two groups, as the incorrectly assigned individuals in each of the Colombian groups were located in groups from other countries.

The means and standard deviations for each of the variables studied are shown in Table 7.

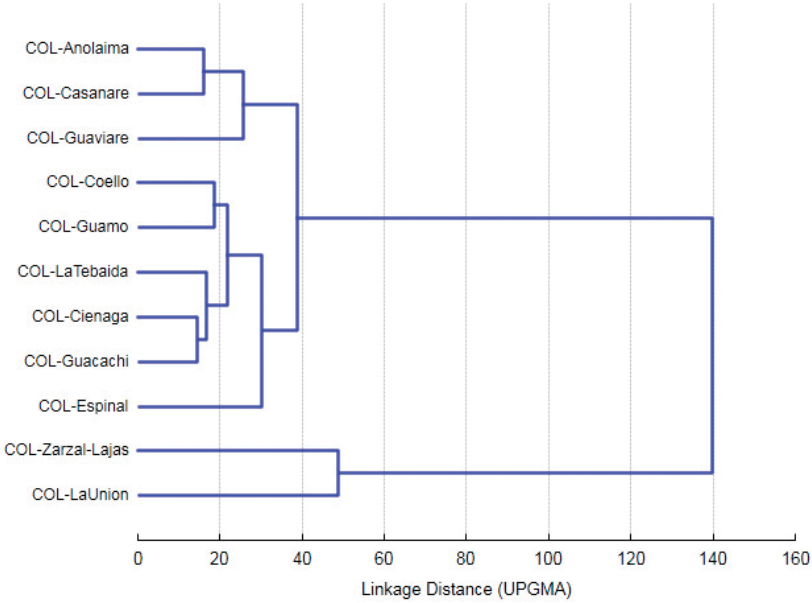

Figure 5. Dendrogram of morphological similarities based on the distance matrix between 11 Colombian populations of *Anastrepha obliqua*.

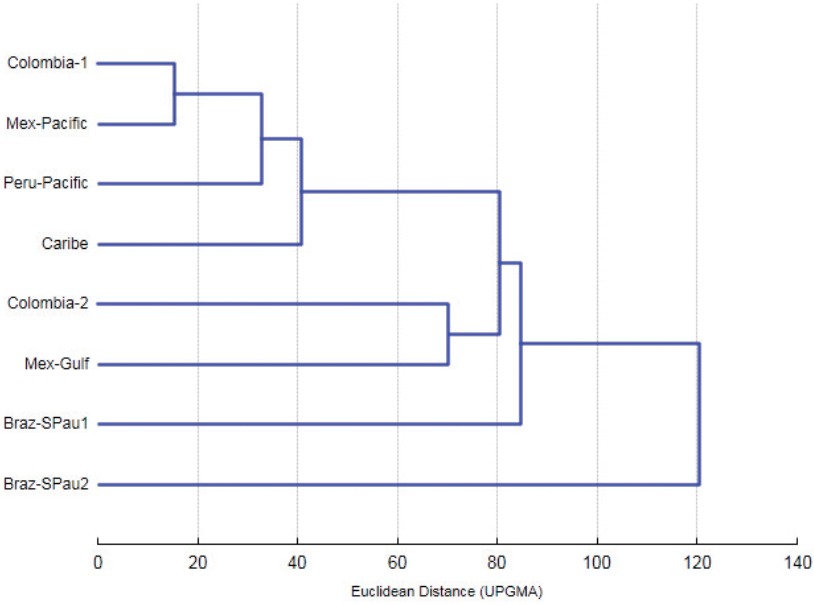

Figure 6. Dendrogram based on the Euclidean distance of Colombian groups of *Anastrepha obliqua* compared with six Neotropical populations using the UPGMA method.

Table 4. Discriminant function analysis summary of *Anastrepha obliqua* grouped by Colombian morphos and other Neotropical samples. Only significant variables in the model are included.

Variables	Wilks' Lambda	F-remove (7,263)	p-level	R-Square
W3	0.009257	20.74111	< 0.0001	0.160514
W1	0.008741	17.49202	< 0.0001	0.997393
A1	0.008487	15.89532	< 0.0001	0.987234
X2	0.008162	13.84414	< 0.0001	0.996066
W4	0.007055	6.87049	< 0.0001	0.188851
A10	0.006982	6.41526	< 0.0001	0.959241
M1	0.006709	4.69552	0.000056	0.997781
X3	0.006672	4.46242	0.000104	0.996826
A7	0.006670	4.44466	0.000109	0.248941
A8	0.006569	3.80834	0.000586	0.993947
W2	0.006541	3.63394	0.000927	0.735419
A3	0.006487	3.29340	0.002249	0.637386
M3	0.006421	2.87547	0.006557	0.830076
M2	0.006406	2.78386	0.008263	0.630219
A2	0.006381	2.62708	0.012231	0.586167
A9	0.006371	2.56574	0.014242	0.987284
A5	0.006291	2.05764	0.048489	0.993312

Table 5. Standardized coefficients for canonical variables resulting from the discriminant function analysis of two Colombian population groups and other samples from the Neotropics of *Anastrepha obliqua*. All canonical roots were significant.

Variables	Root 1	Root 2	Root 3	Root 4	Root 5	Root 6	Root 7
W3	-0.14546	0.57827	-0.48720	-0.13891	-0.27142	-0.10810	-0.3763
W1	2.34202	8.07865	1.55013	10.35805	7.48457	-1.07338	6.4754
A1	0.23325	-5.10279	-1.59967	-2.67578	-1.76714	-0.32702	0.0577
X2	0.14063	8.22717	1.60284	6.11910	3.52443	0.54381	-1.0280
W4	0.05219	-0.04958	-0.45978	-0.15183	-0.04098	0.49208	0.0617
A10	-0.26785	-0.34056	-1.26399	1.73401	1.06799	0.34111	-2.5677
M1	-2.99532	-0.23069	0.69352	-5.98948	-6.33550	2.14080	-10.3391
X3	2.23303	0.27413	-0.47150	5.02686	4.85777	-2.58086	8.6129
A7	-0.21248	-0.17495	-0.31814	0.07865	-0.22900	-0.15917	0.1443
A8	-0.32696	-0.54133	3.10445	-3.08459	-3.72604	-1.88189	1.5067
W2	0.05675	-0.09495	-0.36750	-0.16253	-0.36755	-0.80740	0.5985
A3	0.39987	0.27838	-0.02253	-0.16642	0.18564	0.16871	-0.2246
M3	0.06434	-0.25025	-0.11419	-0.25789	0.99853	0.42983	-0.1847
M2	-0.21312	0.03360	-0.09815	0.52113	-0.19375	0.09046	0.1040
A2	0.34622	-0.05214	0.04938	-0.01043	-0.37470	0.23335	0.0871
A9	0.70756	0.89411	-1.52496	0.14320	2.86986	0.96121	1.2761
A5	1.00339	1.01537	-1.71105	1.29513	3.46539	1.19083	0.5293
Eigenvalue	5.18751	2.05775	1.23376	0.89666	0.40178	0.31538	0.1344
Cummulative %	50.7	70.8	82.9	91.7	95.6	98.7	100.0

Table 6. Classification matrix of individuals according to a predictive model of two Colombian groups and six Neotropical samples of *Anastrepha obliqua*. Rows: Observed classifications; Columns: Predicted classifications. Same probabilities for all the groups.

	% Correct	Colombia-1	Colombia-2	Mex-Pacific	Mex-Gulf	Peru-Pacific	Brazil-1	Brazil-2	Caribe	N
Colombia-1	91.8	**145**	0	10	0	3	0	0	0	158
Colombia-2	97.4	0	**37**	0	1	0	0	0	0	38
Mex-Pacific	94.7	1	0	**18**	0	0	0	0	0	19
Mex-Gulf	94.4	0	0	1	**17**	0	0	0	0	18
Peru-Pacific	93.8	0	0	1	0	**15**	0	0	0	16
Brazil-1	100.0	0	0	0	0	0	**9**	0	0	9
Brazil-2	100.0	0	0	0	0	0	0	**10**	0	10
Caribe	89.5	1	0	0	0	1	0	0	**17**	19
Total	**93.4**	147	37	30	18	19	9	10	17	**287**

Table 7. Means and standard deviations (mm) of morphometric variables of two Colombian groups of *Anastrepha obliqua* females and six other populations from the Neotropics. Values of A9, A10, A11, X1, X2, X3 represent ratios of two variables, W5 and W6 refer to presence/absence.

Variables	Colombia-1	Brazil-1	Brazil-2	Mex-Pacific	Mex-Gulf	Peru-Pacific	Caribe	Colombia-2
A1	1.52±0.04	1.70±0.02	1.72±0.04	1.56±0.05	1.56±0.03	1.60±0.05	1.5±0.03	1.62±0.04
A2	0.08±0.00	0.09±0.01	0.10±0.01	0.08±0.01	0.10±0.01	0.08±0.00	0.09±0.01	0.10±0.01
A3	0.07±0.00	0.08±0.01	0.09±0.01	0.07±0.00	0.08±0.01	0.06±0.00	0.07±0.01	0.08±0.00
A4	0.05±0.01	0.04±0.01	0.06±0.00	0.05±0.01	0.05±0.01	0.06±0.01	0.06±0.01	0.06±0.01
A5	0.11±0.01	0.12±0.01	0.14±0.01	0.12±0.01	0.13±0.00	0.11±0.01	0.11±0.01	0.14±0.01
A6	0.17±0.01	0.17±0.01	0.21±0.01	0.17±0.01	0.19±0.01	0.18±0.01	0.17±0.01	0.20±0.01
A7	10.6±0.98	11.56±0.39	11.03±0.64	11.47±0.66	10.78±0.89	10.5±1.08	10.05±0.76	10.39±0.89
A8	0.16±0.01	0.16±0.01	0.20±0.01	0.17±0.01	0.19±0.01	0.17±0.01	0.17±0.01	0.19±0.01
A9	0.42±0.08	0.36±0.07	0.41±0.05	0.39±0.05	0.39±0.05	0.57±0.07	0.53±0.06	0.42±0.06
A10	0.12±0.01	0.12±0.01	0.14±0.01	0.12±0.01	0.14±0.01	0.13±0.01	0.13±0.01	0.14±0.01
A11	1.11±0.01	1.12±0.01	1.14±0.01	1.12±0.01	1.13±0.00	1.11±0.01	1.11±0.01	1.14±0.01
W1	6.62±0.31	6.9±0.41	7.27±0.15	6.86±0.16	7.44±0.19	6.68±0.27	6.76±0.33	6.84±0.27
W2	2.7±0.14	2.77±0.18	3.01±0.06	2.90±0.07	3.03±0.11	2.78±0.15	2.83±0.14	2.77±0.12
W3	0.45±0.05	0.43±0.1	0.45±0.05	0.57±0.04	0.60±0.07	0.52±0.05	0.61±0.05	0.47±0.06
W4	1.31±0.14	1.54±0.05	1.48±0.09	1.45±0.1	1.44±0.11	1.40±0.11	1.54±0.09	1.35±0.11
W5	1.03±0.18	1.11±0.33	1±0	1±0	1±0	1±0	1±0	1±0
W6	1.07±0.26	2±0	2±0	1.05±0.23	1±0	1±0	1±0	1.05±0.23
M1	3.07±0.2	2.95±0.2	3.05±0.11	3.24±0.11	3.18±0.16	3.25±0.13	3.00±0.17	2.96±0.18
M2	1.95±0.13	1.98±0.05	1.93±0.07	2.01±0.06	2.04±0.1	2.04±0.12	1.89±0.13	1.87±0.12
M3	1.93±0.13	1.89±0.06	1.95±0.09	2.01±0.08	2.02±0.1	2.09±0.11	1.92±0.13	1.88±0.13
X1	0.50±0.04	0.58±0.04	0.57±0.02	0.48±0.02	0.49±0.03	0.49±0.02	0.50±0.03	0.55±0.04
X2	0.23±0.01	0.25±0.02	0.24±0.01	0.23±0.01	0.21±0.01	0.24±0.01	0.22±0.01	0.24±0.01
X3	0.46±0.02	0.43±0.02	0.42±0.01	0.47±0.02	0.43±0.02	0.49±0.02	0.44±0.01	0.43±0.03
n	158	9	15	19	18	16	19	38

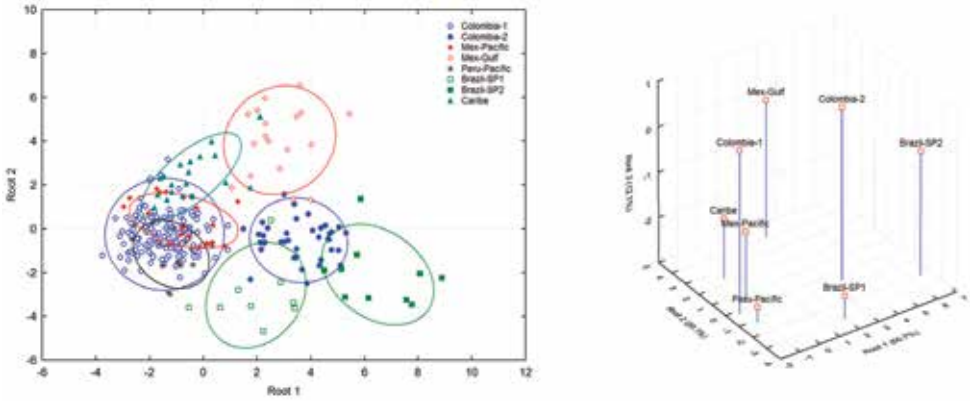

Figure 7. Discriminant function analysis applied to morphometric variables of females of Colombian *Anastrepha obliqua* populations compared with six external populations. **A** Scatterplot of individuals showing centroids with 95% prediction ellipses **B** Mean distance among centroids (3D scatterplot).

Discussion

The morphometric analysis of natural populations of *A. obliqua* from Colombia resulted in the separation of individuals into two groups. The Zarzal and La Unión populations had the greatest values for ovipositor width at the end of the oviduct (A2, 0.09–0.11 mm); width at the beginning of the serrated section (A3, 0.08–0.09 mm); length of the tip of the aculeus (A4, 0.12–0.15 mm); length of the apex of the aculeus (A4+A5, 0.18–0.21 mm) and the proportion of the length of the tip of the aculeus and total aculeus length (A10, 0.13–0.15mm). The remaining populations had smaller values: A2, 0.075–0.083 mm; A3, 0.06–0.08 mm; A4, 0.09–0.12 mm; A4+A5, 0.14–0.18 mm and A10, 0.11–0.13mm. The tip of the aculeus is one of the most important taxonomic characters for species separation within the genus *Anastrepha* (Zucchi 2000, Hernández-Ortiz et al. 2004) and contributed here to separate the Colombian populations into two groups. The usefulness of the linear morphometry was shown for *A. fraterculus* collected from different countries in Latin America (Hernández-Ortiz et al. 2004, 2012, 2015) and, together with additional cytogenetic studies (Goday et al. 2006), reproductive isolation (Vera et al. 2006, Devescovi et al. 2014) and chemotaxonomy (Vaníčková et al. 2015) have demonstrated that it is a cryptic species complex.

The populations of the Sabana province (to the east) have no apparent morphological separation, although they form a clade with one Magdalena River population (Anolaima) that is slightly separated from the Magdalena river populations and the Magdalena (Cienaga) and Guajira (Huacachi) provinces (Euclidean distance≈ 40). These populations are also geographically isolated and should be studied more extensively.

Populations from the Cauca River Valley presented higher levels of variability. Two of them segregated into one group and La Tebaida population grouped with the other populations from the Magdalena River and the Caribbean and eastern region. This population is at the highest altitude surveyed and is isolated by the mountains

from the smaller valleys that run into the Cauca River. Two of the authors (N. Canal and M.R. Castañeda) traveling along the Cauca River Valley found roadside mango markets without any type of sanitary control and whose product came from Tolima crops in the Magdalena Valley. One possible explanation for the similarity found, would be that these markets move infested fruit from the Magdalena valley to the Cauca river valley and some populations established in specific areas such as for La Tebaida population. In contrast the Zarzal and La Union populations were already collected in the Valley plains and may represent the local variability.

By including the six external populations in the discriminant analysis, the predictive model indicated that two Colombian groups remain isolated, reinforcing the result that there may be two groups in Colombia.

Genetic studies conducted by Ruíz-Arce et al. (2012) using COI and ND6 markers in Latin American and Caribbean populations of *A. obliqua* indicated that the Colombian and Peruvian populations formed a single genotype, which the authors called the Andean South American type; however, those authors included four Colombian populations, all belonging to the geographical Magdalena River Valley. The morphometric analysis also indicates that all the populations of the Magdalena River Valley are similar; divergent populations were those collected in the Cauca River Valley.

Predictive model showed in addition that the Mex-Pacific and Peru-Pacific populations are close to the Magdalena River populations (Colombia-1), forming a relatively compact group. Also, the Gulf of Mexico population (Mex-Gulf) is grouped with the Colombian populations of the Cauca River (Colombia-2), however, there is one important divergence represented by the high Euclidean distance between them (≈80), suggesting that it may correspond to a different group in accordance with the findings of Smith-Caldas et al. (2001) and Ruíz-Arce et al. (2012).The Caribbean (Dominica Island) population is grouped with the clade of Colombia-1 but was also slightly divergent. The Brazilian populations represent a group that is morphometrically different from all the other populations studied (Euclidean distance ≈120); however, the distance between them is high (≈85).

Genetic studies conducted previously indicate the existence of those groups and established some relationship among them. Smith-Caldas et al. (2001) found that the populations of *A. obliqua* studied with COI were combined into two groups; the population of Colombia was included in a group with a population of Brazil (northeast) and a sample of Los Tuxtlas (Gulf of Mexico); the second group included populations of Brazil. This finding was similar to the results of the linear morphometrics. Ruíz-Arce et al. (2012) found that eastern populations of the Sierra Madre in Mexico were genetically similar to the populations of Colombia (four from the Magdalena River) and Peru and that the populations of the eastern Sierra Madre (Tuxtlas) were separate from them. The morphometric results confirm these findings. Likewise, Ruiz-Arce et al. (2012) found that the samples that came from the Caribbean Islands had variability with respect to the other samples.

Ruíz-Arce et al. (2012) included nine populations of southeastern and northeastern Brazil, and all the haplotypes found corresponded to the same group. In contrast, Smith-Caldas et al. (2001) found that the genotypes of populations of southeastern

Brazil formed a very strong, separate group from the other group that included a northeastern population. Our studies included only two populations of southeastern Brazil that had a morphometric divergence. Morphometric studies of *A. obliqua* should be expanded to include a greater number of populations across a wider range of the species distribution.

The morphometric analyses of *A. obliqua* females indicate that in Colombia there could be two different morphotypes and also that the external samples could be divergent and several groups may exist. Larger studies should be performed to confirm this hypothesis.

Traditionally, linear measurements have been valid tools to separate species within the existing diversity; however, the development of modern tools in morphometry, genetics, behavior and ecology has allowed the recognition of a wide variability within the existing nominal species, suggesting the existence of greater diversity in nature and hampering the definition and delimitation of the species (Baylac et al. 2003, Bickford et al. 2007, Wiens 2007, de Queiroz 2007, Yeates et al. 2011, Krosch et al. 2013). De Queiroz (2007) recognizes that there are many existing definitions of species and that many of them exist due to the tools and particular interests of the researchers. In some cases, as when speciation is more incipient, the existing tools are more inefficient. Thus, in the modern delimitation of species, the iterative or integrative proposal of taxonomy appears as the best approach (Yeates et al. 2011).

Following the proposal by De Queiroz (2007) and Yeates et al. (2011), the studies by Smith-Caldas et al. (2001), Ruíz-Arce et al. (2012) and our group with molecular markers and morphometry suggest the presence of seven groups within the species, whose denomination may be a modification of that proposed by Ruíz-Arce et al. (2012), Mesoamerica, Central America, Caribbean, western Mexico, Magdalena, Cauca and Brazil, which must be at the very least, separate metapopulations lineages. Other biological, ecological and morphological studies are needed to define the definitive limit of these lineages as species.

Conclusions

The results of this work, in conjunction with previous studies, indicate that in Colombia, two groups exist that could be under a divergence process which could lead to speciation. Further, the same seems to be occurring in different Neotropical regions. Larger studies are required to define the taxonomic status of the species, which could be relevant for pest management.

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Sex chromosomes in mitotic and polytene tissues of *Anastrepha fraterculus* (Diptera, Tephritidae) from Argentina: a review

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Abstract

Cytogenetics, which is considered a fundamental tool to understand basic genetic and genomic issues of species, has greatly contributed to the description of polymorphisms both at inter- and intra-specific level. In fact, cytogenetics was one of the first approaches used to propose *Anastrepha fraterculus* (Diptera: Tephritidae) as a complex of cryptic species. Different morphological variants of sex chromosomes have been reported among Argentinean populations of *A. fraterculus*. However, since this high structural variability in sex chromosomes does not pose a reproductive barrier, their role in speciation is yet to be unveiled. This review provides an update on general aspects of cytogenetics in Argentinean *A. fraterculus* populations, focused on the prevalence of X-Y arrangements.

Keywords

Cytogenetics, karyotype variants, South American fruit fly, heterochromatin, centromeres, ribosomal genes

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General background

Cytogenetic studies have provided significant information about intra- and inter-species genetic variation (Sumner 2003). Cytogenetic studies focus mainly on sex chromosomes, which present unusual features relative to autosomes (Traut et al. 2008). In organisms with the classical X-Y systems of sex determination, Y chromosomes lack genetic recombination, are male limited, and show an abundance of genetically inert heterochromatic DNA containing few functional genes, whereas X chromosomes also show sex-biased transmission, and are hemizygous in the heterogametic sex (Kaiser and Bachtrog 2010). In particular, in some insect species, sex chromosomes show high structural variability (see Traut 1999, Kaiser and Bachtrog 2010, Palacios-Gimenez et al. 2013). In the genus *Anastrepha*, Solferini and Morgante (1987), Selivon et al. (2005a), Goday et al. (2006) and Garcia-Martinez et al. (2009) compared different species and reported specific sex-chromosome banding patterns. In addition, they described variability in the length, number, size, and position of heterochromatic blocks in 'the South American fruit fly', *Anastrepha fraterculus* Wiedemann (Diptera: Tephritidae). This species is distributed from southern United States to Argentina (Salles 1995, Steck 1999) and constitutes an economically important pest. Currently, it is considered a complex of cryptic species (for reviews and references, cf. Selivon et al. 2004, 2005a, Cáceres et al. 2009, Hernández-Ortiz et al. 2012, Cladera et al. 2014). Multivariate morphological studies including samples from different regions of the American continent have characterized seven distinct morphotypes (Hernández-Ortiz et al. 2012). Studies based on genetic differentiation, karyology, morphology, reproductive compatibilities combined with bionomic parameters, eggshell morphology and some aspects of early embryogenesis of samples from northern and southern Brazil have identified at least four entities of the *A. fraterculus* complex: *A*. sp.1 *aff. fraterculus*, *A*. sp. 2 *aff. fraterculus*, *A.* sp. 3 *aff. fraterculus*, and *A.* sp.4 *aff. fraterculus*. The first three entities have been reported in different regions of Brazil, whereas *A.* sp. 4 *aff. fraterculus* has been described in Guayaquil, Ecuador (Selivon and Perondini 1997, 1998, Selivon et al. 1997, 1999, 2004, 2005 a, 2005b, Goday et al. 2006).

Reproductive incompatibilities between *A*. sp.1 *aff. fraterculus* and *A*. sp. 2 *aff. fraterculus* living in sympatry were first described by Selivon et al. (1999, 2005a). Later, Vera et al. (2006) showed pre-mating isolation between flies from Peru and Argentina, Brazil and Colombia, as well as between flies from Piracicaba (São Paulo, Brazil) and Argentina. Cáceres et al. (2009) found that hybrids between strains from Peru and Argentina carried the expected mix of sex chromosome cytotypes, but presented sex ratio distortion and high rates of sterility or inviability. High levels of mating isolation have also been reported among Mexican, Peruvian and the Brazilian-1 morphotypes (Rull et al. 2013). Reproductive isolation between the four morphotypes of *A. fraterculus* complex and flies from "the Andean morphotype" were also found by Devescovi et al. (2014). These and other factors analyzed by the authors are indicative of incipient speciation, providing a strong evidence for a taxonomic revision of this species complex (Selivon et al. 2005a, Cáceres et al. 2009).

Polytene chromosome analysis and the availability of polytene maps of different genera of the family Tephritidae, as *Ceratitis* (Zacharopoulou, 1990), *Bactrocera* (Mavragani-Tsipidou et al. 1992, Augustinos et al. 2014), *Dacus* (Drosopoulou et al. 2011), *Rhagoletis* (Kounatidis et al. 2008) and *Anastrepha* (Garcia-Martinez et al. 2009), have allowed identifying differences between closely related species. Moreover, several groups of cryptic species were initially identified using sequences of the polytene chromosomes as genetic markers and later confirmed by molecular markers studies (Ramirez and Dessen 2000).

Karyotype and sex chromosome configurations

Karyological studies performed in wild populations of *A. fraterculus* from Argentina have shown structural variability in the sex chromosomes. Lifschitz et al. (1999) described an acrocentric X chromosome and a small submetacentric Y chromosome (see also Basso and Manso 1998, Basso et al. 2003). Lifschitz et al. (1999) and Manso and Basso (1999) also reported four morphological variants of the X chromosome (named X_1, X_2, X_3 and X_4) and six variants of the Y chromosome (named Y_1, $Y_2 \ldots Y_6$) at low frequency (Figure 1). Basso and Manso (1998) and Basso et al. (2003) also studied the viability and survival of individuals with different karyotype configurations under laboratory conditions, and showed that the cytogenetic differences found among these Argentinean populations do not represent evidence of reproductively separate species, but seem to be examples of intra-species chromosome polymorphisms.

In an experiment under field cage conditions Petit Marty et al. (2004a, 2004b) confronted *A. fraterculus* flies from extreme regions (NOA and NEA) inside Argentina and compared the frequency of homotypic and heterotypic crosses. No evidence of sexual incompatibility was found, either pre-zygotic (Petit Marty et al. 2004a) or post-zygotic (Petit Marty et al. 2004b). These studies confirmed the presence of a single *A. fraterculus* biological entity in Argentina.

After a revision of *A. fraterculus´* chromosomes studies we concluded that the most frequent karyotype found in Argentina consists in five pairs of acrocentric autosomes, a submetacentric X chromosome (named X_1, Lifschitz et al. (1999) (CI average: 31.23) and a metacentric Y chromosome (named Y_5, Basso 2003) smaller than the X chromosome (Figure 2) (preliminary reported in Giardini et al. 2009b). It is important to highlight that the size and the patterns obtained with the different banding techniques for the X chromosome are the same as the one described by Lifschitz et al. (1999) as X_1. The only difference between them is the position assigned to the centromere and for that reason we kept on the same name. This difference in the centromere position could probably be explained by the lower resolution in the old pictures obtained by Lifschitz et al. (1999). This karyotype also corresponds to the one that was characterized by Selivon et al. (2005a) using samples from the southern Brazil (*A.* sp1 *aff. fraterculus*) although without specifying the centromere position.

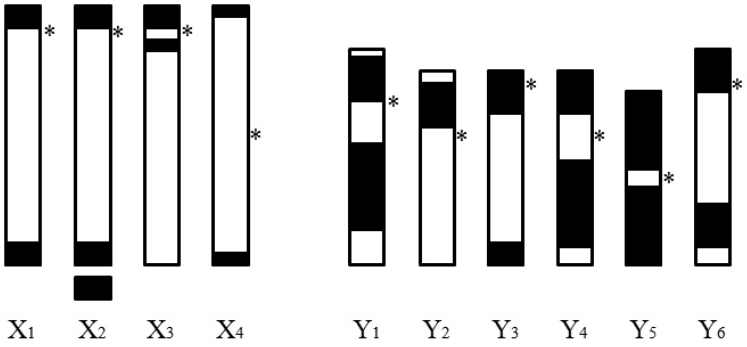

Figure 1. C-Band Ideogram. Sex chromosomes configurations of *A. fraterculus* found in Argentina (redrawn from Basso 2003). * Position of centromeres in each chromosome.

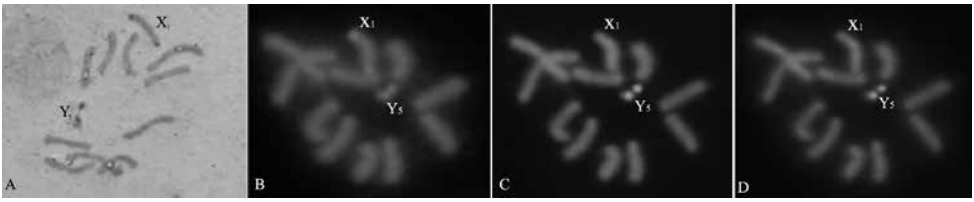

Figure 2. The most frequent karyotype of *A. fraterculus* found in Argentina. Mitotic chromosome preparations from third instar larvae of *A. fraterculus* male. **A** C-Bands **B** DAPI stain **C** CMA stain **D** Merged DAPI/CMA images.

After C-band staining (Figure 2A), the X_1 chromosome shows two prominent and different-sized blocks of heterochromatin located at the terminal region, whereas the Y_5 chromosome also shows two heterochromatin blocks positioned in different arms, one on the proximal end and the other in the sub-median region (Figure 2B). Both chromosomes show DAPI-positive signals in the same position as the heterochromatic blocks (Figure 2B). No CMA-positive band is distinguishable on the X_1 chromosome (Figure 2C). However, on the Y_5 chromosome, CMA-positive bands are observed at the same position as C-bands and DAPI-positive bands (preliminary reported in Giardini et al. 2009b). These observations indicate that the sex chromosomes analyzed in these populations of *A. fraterculus* differ in the nucleotide composition of the heterochromatic regions: the heterochromatic regions on the X_1 chromosome are AT-rich, whereas those of the Y_5 chromosome are AT+CG-rich DNA sequences (preliminary reported in Giardini et al. 2009b). These findings are in agreement with those of Goday et al. (2006).

Anastrepha fraterculus has also been cytogenetically characterized by means of fluorescence *in situ* hybridization (FISH), to locate the ribosomal genes on the chromosome complement. The first studies carried out by Basso and Manso (1998) on cytological preparations of *A. fraterculus* from Argentina using a heterologous probe from *Drosoph-*

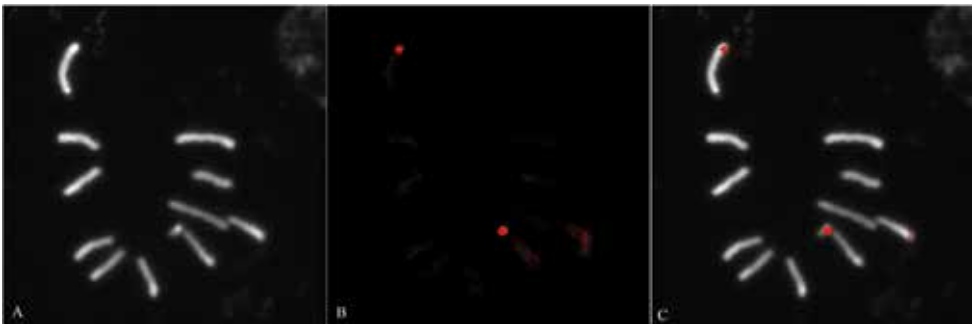

Figure 3. 18S rDNA FISH analysis. Mitotic chromosome preparations from third instar larvae of *A. fraterculus* male. **A** DAPI stain **B** rDNA hybridization signal (autologous probe) **C** Merged images.

ila hydei rDNA described two positive signals at the terminal positions in the X-chromosome. Later, Goday et al. (2006) carried out studies using a *Drosophila melanogaster* probe in a comparative analysis of the *fraterculus* complex using samples from Brazil. In *A.* sp.1 *aff. fraterculus* individuals, these authors found an rDNA positive signal that co-localized with a DAPI-positive band at a distal position in the X_1 chromosome, and a second signal at the DAPI/CMA-positive regions of the Y_5 chromosome. By using a specific probe designed from a region of *A. fraterculus* 18S rDNA (Figure 3), in our lab we have observed a pattern of signals equivalent to the one previously described by Goday et al. (2006) (preliminary reported in Giardini et al. 2009b). These two last studies confirmed the general tendency observed for the rDNA of reside on the heterochromatic regions of the sex chromosomes other than centromeres (Drosopoulou et al. 2012).

Chromatin characteristics

As a first attempt to study histone modifications in *A. fraterculus* chromosomes, we performed immunodetection assays with specific antibodies in mitotic preparations of Argentinean *A. fraterculus* to analyze the presence of histone H3 phosphorylated at positions 10 or 28 (preliminary reported in Giardini et al. 2011). Both variants of histone H3 serve as markers for chromosomal condensation and segregation during mitosis and meiosis (Goto et al. 1999). Using the H3S28ph antibody, we found positive signals in all centromeres (Figure 4), whereas using the H3S10ph antibody, we found characteristic positive signals of chromosome condensation in all the complement, showing the expected behavior of chromosomes during the mitosis (data not shown) (preliminary reported in Giardini et al. 2011). Considering that histone modification patterns are a particularly informative feature in relation to chromatin characterization, our results represent the first epigenetic characterization of *A. fraterculus* mitotic chromosomes. Although preliminary, these studies allow confirming the acrocentric nature of the autosomes.

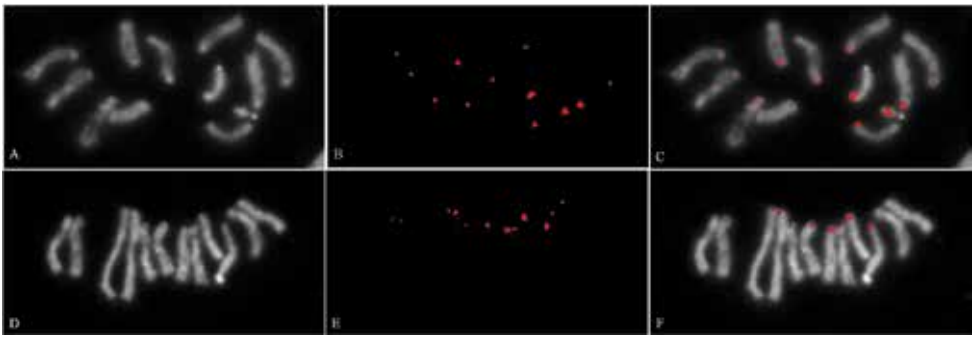

Figure 4. Immunodetection analysis with H3S28ph antibody. Mitotic chromosome preparations of male (**A, B, C**) and female (**D, E, F**) individuals from *A. fraterculus* **A, D** DAPI stain **B, E** anti-H3S28ph hybridization signal **C, F** Merged images. Arrow heads indicate sex chromosome position.

Sex chromosomes in polytene tissues

The existence of polytene chromosomes in the salivary glands of *A. fraterculus* was first reported by Mendes (1958). Our group characterized these chromosomes and published the first polytene pictures of this species (Giardini et al. 2009a). These chromosomes show homogeneity in chromosome length, similar banding and puffing patterns between sexes, and the absence of a typical chromocentre, resulting in the observation of complete individual chromosomes. We have described each chromosome on the basis of constant morphological structures (landmarks) and specific features (e.g., puffing pattern) and performed an approximation to a linear map following a customary labeling system (see details in Giardini et al. 2009a). Currently, a detailed map of *A. fraterculus* is in progress (M. Cecilia Giardini, Antigone Zacharopoulou, in preparation).

We have also performed a simultaneous analysis of mitotic and polytene nuclei of Argentinean *A. fraterculus*, and observed that neither the number of polytene chromosomes nor their banding patterns differentiate males from females (Giardini et al. 2009a). This suggests that in *A. fraterculus*, as well as in other tephritid flies (Zacharopoulou 1987, Mavragani-Tsipidou et al. 1992, Zacharopoulou et al 2011a, 2011b, Garcia-Martinez et al. 2009), the sex chromosomes do not form polytene chromosomes. This finding was tested by FISH experiments using the specific 18S rDNA probe in polytene chromosomes, which revealed a hybridization signal in a region of granular and uncondensed heterochromatin (Figure 5) that corresponded to the non-polytene sex chromosomes (Giardini et al. 2012).

Conclusion and remarks for the future

This review summarizes the cytogenetic information available from Argentinean *A. fraterculus*, focused on sex chromosome variation. Figure 6 shows an ideogram illustrating the results of all the techniques applied so far in the cytogenetic characteriza-

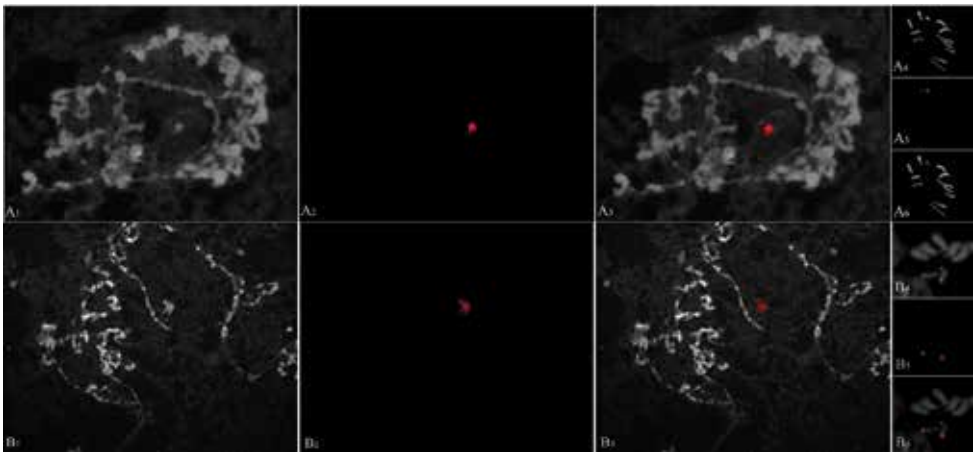

Figure 5. 18S rDNA FISH analysis in polytene and mitotic tissues. Polytene and mitotic chromosome preparations obtained from third instar larvae of male (**A**) and female (**B**) of *A. fraterculus*. In each case: **1** Polytene chromosomes DAPI stain **2** Polytene chromosomes 18S rDNA hybridization signa (FISH) **3** Polytene chromosomes merged image (DAPI/FISH) **4** Mitotic chromosomes DAPI stain **5** Mitotic chromosomes 18S rDNA hybridization signal (FISH) **6** Mitotic chromosomes merged image (DAPI/FISH).

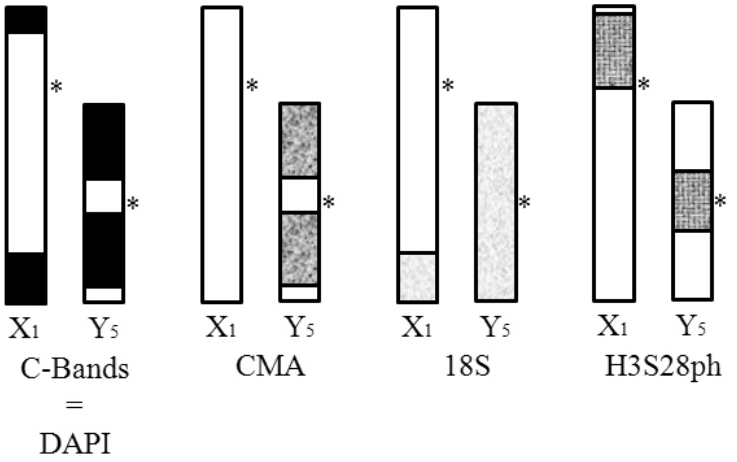

Figure 6. Cytogenetic summary of sexual chromosome pair. Ideogram of sex chromosomes of *A. fraterculus* from Argentina (most frequent karyotype). Relative location of C-Bands, DAPI/CMA bands, 18S and anti-H3S28ph hybridization signals.

tion of the sex chromosome pair from mitotic preparations of *A. fraterculus*. Several structural polymorphisms have been described in sex chromosomes from wild and laboratory Argentinean populations. In contrast to that observed in Brazilian populations, these polymorphisms do not act as reproductive barriers between individuals of different populations. Deeper characterization of the *A. fraterculus* karyotype by FISH allowed the identification and location of ribosomal genes in terminal position on the

sex chromosomes. Chromatin characteristics were also explored, and allowed the specific detection of centromeric regions and chromosomal condensation status in mitotic chromosomes of this species. The first characterization of polytene chromosomes in this species provided the description of landmarks and specific features on this type of chromosomes, and the detection of sex chromosomes as granular and uncondensed heterochromatin in polytene tissues.

All the results described here represent valuable information to be further used in the identification of genetic entities in the *A. fraterculus* complex of cryptic species. Deeper characterization of the structural variation of the sex chromosomes and polytene chromosome needs to be addressed to have a complete genetic picture of this species, which represents one of the most destructive fruit flies of economic importance in Argentina and the South American region. A detailed taxonomic revision of *A. fraterculus* and the accurate elucidation of the complexity displayed by this species in South America are of uttermost importance to develop environment-friendly autocidal control methods as is the Sterile Insect Technique (SIT), ensuring its specificity and effectiveness.

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Taxonomy and phenotypic relationships of the *Anastrepha fraterculus* complex in the Mesoamerican and Pacific Neotropical dominions (Diptera, Tephritidae)

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Abstract

Previous morphometric studies based on linear measurements of female structures of the aculeus, mesonotum, and wing revealed the existence of seven morphotypes within the *Anastrepha fraterculus* cryptic species complex along the Neotropical Region. The current research followed linear and geometric morphometric approaches in 40 population samples of the nominal species *A. fraterculus* (Wiedemann) spread throughout the Meso-American and Pacific Neotropical dominions (including Mexico, Central America, Venezuela, Colombia, Ecuador, and Peru). The goals were to explore the phenotypic relationships of the morphotypes in these biogeographical areas; evaluate the reliability of procedures used for delimitation of morphotypes; and describe their current distribution. Findings determined that morphotypes previously recognized via the linear morphometrics were also supported by geometric morphometrics of the wing shape. In addition, we found an eighth morphotype inhabiting the highlands of Ecuador and Peru. Morphotypes are related into three natural phenotypic groups nominated as Mesoamerican-Caribbean lineage,

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Andean lineage, and Brazilian lineage. The hypothesis that lineages are not directly related to each other is discussed, supported by their large morphological divergence and endemicity in these three well-defined biogeographic areas. In addition, this hypothesis of the non-monophyly of the *A. fraterculus* complex is also supported by evidence from other authors based on molecular studies and the strong reproductive isolation between morphs from different lineages.

Keywords
Cryptic species complex, linear morphometrics, geometric morphometrics, distribution

Introduction

Phylogenetic relationships stated that infrageneric classification based on morphology of the genus *Anastrepha* Schiner recognizes nearly 21 species groups (Norrbom et al. 1999, 2012). One of them is the "*fraterculus* species group" consisting of 34 species, with some widely distributed in the Neotropics (e.g., *A. ludens* (Loew), *A. obliqua* (Macquart), *A. suspensa* (Loew), and *A. fraterculus*). One of these nominal species, the "South American fruit fly" *Anastrepha fraterculus* (Wiedemann) occurs from south of the USA (Texas) through Mexico, Central America to Argentina and represents a cryptic species complex (hereafter denoted as the *Af* complex).

First evidence of the *Af* cryptic species complex appeared in the comprehensive taxonomic revision of the genus *Anastrepha* made by Stone (1942). Since then, other findings from distinct populations along its distributional range were reported supporting this hypothesis, such as differences of karyotypes (Mendes 1958, Bush 1962, Solferini and Morgante 1987, Selivon et al. 2005b); isozyme divergence (Morgante et al. 1980, Steck 1991); DNA sequences (Steck and Sheppard 1993, Smith-Caldas et al. 2001); or studies with multiple approaches including karyotype, isozymes, and morphology (Selivon and Perondini 1998, Selivon et al. 2004, 2005a). Moreover, differences in host range and pest status (Baker 1945, Aluja et al. 2003, Hernández-Ortiz and Morales-Valles 2004, Zucchi 2007); reproductive isolation and sexual incompatibilities (Selivon et al. 1999, 2005a, Vera et al. 2006, Cáceres et al. 2009, Rull et al. 2013, Devescovi et al. 2014); or data on pheromone composition and cuticular hydrocarbon profiles (Břízová et al. 2013, Vaníčková et al. 2015).

Morphometric analyses have been a useful technique in detecting morphological differences among organisms to distinguish closely related species of fruit flies (Adsavakulchai et al. 1999, Khamis et al. 2012, Schutze et al. 2012). Based on adult morphology of the *Af* complex, Hernández-Ortiz et al. (2004) developed a morphometric technique using linear measurements of the aculeus, wing, and mesonotum for the full recognition of the Mexican morphotype, separating it from other South American samples from Colombia, Brazil and Argentina. Further linear morphometric studies applied to 32 populations from Mexico, Central America, and South America (including Venezuela, Colombia, Ecuador, Peru, Brazil and Argentina) confirmed previous findings, and added the fact that seven morphotypes could be distinguished within the *Af* complex throughout the Neotropical region (Hernández-Ortiz et al. 2012).

Despite all evidence gathered by different sources, it is still difficult to set out the taxonomic status of the morphotypes mainly due to two reasons. The first one is that other methodological approaches, such as DNA sequences or sexual compatibility have shown large interpopulation divergences, without allowing full identification of interspecific boundaries; and the second one is that information about the overall distribution of the cryptic species still remains uncertain. This is especially true for morphotypes occurring in the North and Central Andes, and for the Brazilian morphotypes.

According to Daly (1985), multivariate methods of morphometric analysis (e.g., DFA, PCA) can be widely applied in biology. However, two general kinds of problems may be encountered in canonical variate analysis of morphometric data: a) linear dependence when two or more variables are highly correlated; and b) heteroscedasticity of the covariance matrices (inequality of dispersion matrices). In this sense, ratios have been used for scaling morphometric variables to remove variation in general body size; to express shape by finding the proportion of one dimension of a structure to another; and to express growth in the size of some structure from one instar to the next. Additionally, because linear distance measurements usually are highly correlated with size, much effort was spent in developing methods for size correction, so that size-free shape variables could be extracted and patterns of shape variation elucidated (Bookstein et al. 1985, Sundberg 1989). The most widespread approach of the geometric morphometrics, is to represent each specimen by the relative positions of morphological landmarks, that can be located precisely and establish a one-to-one correspondence among all specimens included in the analysis (Klingenberg 2010). Shape is defined as all the geometric information about a configuration of landmarks and it is extracted by a procedure called Procrustes superimposition, which removes variation in size, position and orientation from the data on landmark coordinates, and which is at the core of geometric morphometrics (Goodall 1991, Dryden and Mardia 1998, Zelditch et al. 2012).

Another crucial issue for the resolution of this cryptic species complex is understanding the distributional patterns of their morphotypes. Morrone (2014) recently revised the biogeographic regionalization of the Neotropical region. The Mesoamerican dominion comprises lowlands of central and southern Mexico, and most of Central America (Guatemala, Belize, Honduras, El Salvador and northern Nicaragua). The Pacific dominion encompasses southern Central America (southeastern Nicaragua to Panama) and northwestern South America (including western Colombia, Ecuador, Peru, northwestern Venezuela, Trinidad and Tobago, and the Galapagos Islands). Contiguous to these dominions, the Mexican Transition Zone (MTZ) occupies an area where the Neotropical and Nearctic regions overlap, corresponding basically to the mountainous areas of central and southern Mexico and northern Central America; and the South American Transition Zone (SATZ) represented by highlands of the Andes between western Venezuela and northern Chile, and central western Argentina (*sensu* Morrone 2006). In this sense, correlating the occurrence of the different morphotypes to biogeography will add valuable information to delimit the distribution of the species involved.

Given this scenario, systematic studies that identify the incidence areas of the different *Af* morphotypes throughout the Neotropical region are needed. Increasing the number of samples from Colombia, Ecuador and Peru will confirm previous evidence that suggests that biogeographical and ecological factors in these countries, contribute to the understanding of the distributional patterns of the morphotypes. As such, the goals of this study were to explore phenotypic relationships among different morphs of the *Af* complex in the Mesoamerican and Pacific biogeographical dominions; to make comparisons of the usefulness of the linear morphometrics and geometric morphometry of the wing shape for delimitation of the morphotypes; and to describe their distributional patterns throughout the biogeographical provinces currently recognized.

Methods

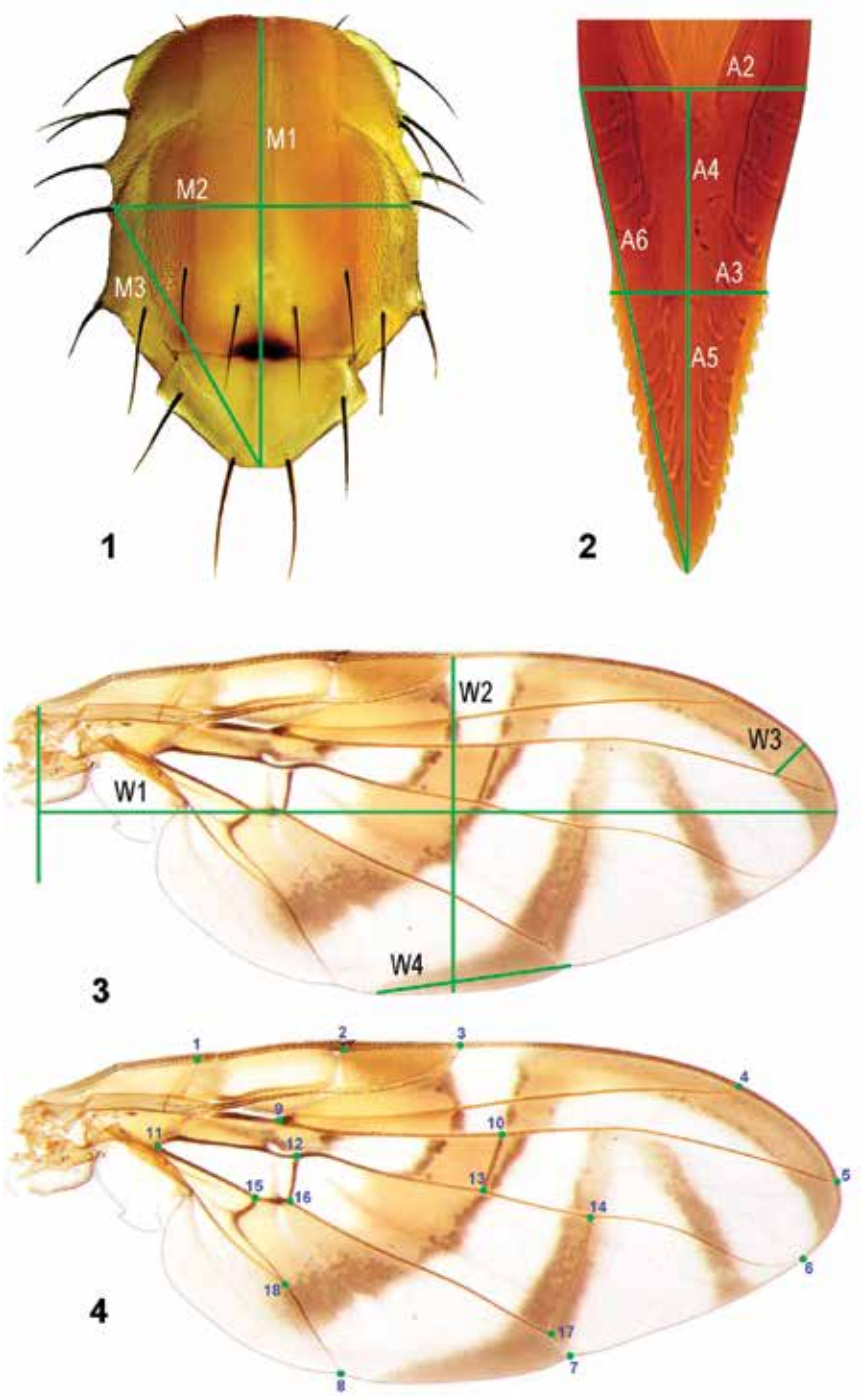
Biological material

We used samples from forty populations obtained from different sources. Most of them were collected from nature directly on their hosts and afterwards reared to adult specimens in the laboratory. Others were collected in McPhail traps baited with hydrolyzed protein, and in few cases, we analyzed samples from laboratory strains established for long time at the Seibersdorf facilities of the FAO/IAEA Agriculture and Biotechnology Laboratories (Austria). Specific data of collection, country, location, and specimens examined are listed in Table 1.

Permanent mounting slides were made prior to observations. Female aculeus was cleaned in a boiling solution, consisting of 10% sodium hydroxide, for approximately 15-20 min; in addition the right wing of each specimen was cut from its base. After that, structures were washed with distilled water and further dehydrated by gradual alcohol series (50, 70, 100% by holding them for 20 min at each step), placed in xylene 2-3 min, and immediately mounted with Canada balsam. Digital images of the mesonotum and wing were made with a digital camera (Olympus C5050) adapted to stereomicroscope (Olympus SZX7); and images of the aculeus were performed using an optical microscope (Olympus BX41) with objective 40X. Permanent slides and pinned voucher specimens of the studied samples were deposited at the Entomological Collections of the Instituto de Ecología AC (Xalapa, Mexico), Universidad del Tolima (Ibagué, Colombia), and the Universidad de las Fuerzas Armadas – ESPE (Quito, Ecuador).

Linear Morphometrics

In total, 612 female specimens were examined, considering 27 morphometric traits of structures such as mesonotum, aculeus, and wing. Variables as linear distances between two points, as ratios of two variables, and qualitative features of wing pattern were assessed following methods described by Hernández-Ortiz et al. (2004, 2012):



Figures 1–4. Morphological structures and variables used for morphometric assessment of the *Anastrepha fraterculus* complex: **1** Thorax in dorsal view **2** Aculeus tip in ventral view **3** Measurements for the linear morphometrics of wing **4** Landmarks used for the geometric analysis of the wing shape.

Table 1. List of samples examined of the *Anastrepha fraterculus* along the Neotropics, showing data of location, georeferentiation, and source of sample

Sample- Key	Country	Locality	Altitude (m)	Coordinates	Source	N Linear	N Geometrics
MEX-Jica	Mexico	La Jicayana	400	19°21'44"N, 96°39'23"W	McPhail trap	10	10
MEX-Teoc	Mexico	Tejería	980	19°23'14"N, 96°36'59"W	*Psidium guineense*	10	10
MEX-Apaz	Mexico	Apazapan	250	19°17'00"N, 96°39'23"W	McPhail trap	10	10
MEX-Coat	Mexico	Coatepec	1200	19°27'25"N, 96°57'29"W	*Syzygium jambos*	10	10
MEX-Tuxt	Mexico	Los Tuxtlas	160	18°35'06"N, 95°04'12"W	*Psidium guajava*	10	10
MEX-Chis	Mexico	San Vicente	1400	16°11'50"N, 92°02'57"W	*Psidium guajava*	10	10
MEX-Tap	Mexico	Tapachula	150	ND	Seibersdorf Lab-strain	6	6
MEX-QRoo	Mexico	Chunhuhub	30	19°37'39"N, 88°38'56"W	McPhail trap	10	10
GUA-City	Guatemala	Guatemala City	1500	14°36'51"N, 90°32'22"W	*Psidium guajava*	15	15
PAN-LCam	Panama	La Campana	61	08°44'16"N, 79°51'29"W	*Psidium guajava*	15	15
PAN-BCol	Panama	Barro Colorado Is.	125	09°09'08"N, 79°50'47"W	*Eugenia uniflora*	17	17
VEN-Corr	Venezuela	Corrales	40	10°44'35"N, 71°21'10"W	McPhail trap	15	15
VEN-LMit	Venezuela	Loma Mitimbís	1570	09°16'57"N, 70°14'59"W	*Rubus glaucus*	15	15
VEN-DDiaz	Venezuela	Diego Díaz	1640	ND	*Eriobotrya japonica*	15	15
VEN-SDom	Venezuela	Santo Domingo	2500	08°57'37" N, 71°02'54"W	*Coffea arabica*	15	15
VEN-Tig	Venezuela	Tiguanín	1900	ND	*Psidium caudatum*	15	15
COL-Cund	Colombia	La Mesa	1350	04°38'09"N, 74°27'21"W	McPhail trap	10	10
COL-Tol	Colombia	Vereda Gamboa	1600	04°26'11"N, 75°11'29"W	Seibersdorf Lab-strain	15	15
COL-Bar	Colombia	Barbosa	1880	05°55'57"N, 73°37'16"W	*Psidium guajava*	13	16
COL-Cach	Colombia	Tocarema alto	1850	04°45'01"N, 74°23'01"W	*Coffea arabica*	20	20
COL-Duit	Colombia	Duitama	2569	05°49'29"N, 73°04'29"W	*Acca sellowiana*	20	20
COL-Rold	Colombia	La Aguada	1764	04°23'05"N, 76°13'20"W	*Coffea arabica*	20	20
COL-Lun	Colombia	El Guabo	1704	01°36'53"N, 77°07'53"W	*Coffea arabica*	20	20
COL-Pen	Colombia	Pensilvania	2091	05°22'03"N, 75°09'29"W	*Acca sellowiana*	20	21
COL-Sev	Colombia	Sevilla	1556	04°17'19"N, 75°54'23"W	*Coffea arabica*	20	20
COL-Sibu	Colombia	Fatima	2136	01°12'05"N, 76°54'48"W	*Ps. acutangulum*	20	20
COL-Ibag	Colombia	Ibagué	1433	04°24'53"N, 75°18'50"W	*Lab colony-U Tolima*	20	20

Sample- Key	Country	Locality	Altitude (m)	Coordinates	Source	N Linear	N Geometrics
ECU-Agro	Ecuador	Km39 via la Costa	7	01°57'15"S, 79°55'17"W	McPhail trap	17	20
ECU-Guay	Ecuador	Guayaquil	80	02°12'13"S, 79°53'50"W	*Ps. guajava*	14	15
ECU-Bab	Ecuador	Recinto Tauín	91	01°45'29"S, 79°26'50"W	McPhail trap	20	20
ECU-Chac	Ecuador	Chacras	370	03°26'51"S, 79°49'53"W	McPhail trap	20	20
ECU-Chot	Ecuador	Ambuquí	1550	00°26'50"N, 78°00'18"W	McPhail trap	20	20
ECU-Per	Ecuador	Perucho	1861	00°06'48"N, 78°25'33"W	McPhail trap	20	20
ECU-Pich	Ecuador	Guayllabamba	2176	00°03'47"S, 78°20'56"W	McPhail trap	20	20
ECU-Pat	Ecuador	Patate	2034	01°19'04"S, 78°30'44"W	McPhail trap	20	20
PER-Piu	Peru	Piura	35	05°12'00"S, 80°37'00"W	Seibersdorf Lab-strain	15	15
PER-LMol	Peru	La Molina	300	12°05'21"S, 76°55'41"W	Seibersdorf Lab-strain	15	15
PER-Chon	Peru	Chongona	1502	12°45'49"S, 72°36'15"W	McPhail trap	7	10
PER-Echa	Peru	Puente Echarate	941	12°46'10"S, 72°34'37"W	McPhail trap	11	14
PER-VSag	Peru	Valle Sagrado	2859	13°19'00"S, 72°05'21"W	McPhail trap	17	17

Mesonotum (Figure 1). M1) mesonotal length; M2) mesonotal width at level of postsutural supra-alar seta; M3) length from the apex of scutellum to the left postsutural supra-alar seta.

Aculeus (Figure 2). A1) total aculeus length; A2) basal width of the aculeus tip; A3) width at beginning of serrated section; A4) basal tip length of non-serrated section; A5) apical tip length of serrated section; A6) length from basal left side to aculeus apex; A7) mean number of lateral teeth; A8) aculeus tip length (A4+A5); A9) ratio of the length of nonserrated section/length of serrated section (A4/A5); A10) ratio of aculeus tip length/aculeus length (A8/A1); A11) ratio of length of non-serrated section/aculeus tip length (A4/A8).

Wing (Figure 3). W1) wing length; W2) wing width at R_1 apex; W3) width of apical section of S-band (from juncture of S-band and vein R_{4+5} perpendicular to Costal vein); W4) distance from proximal end of proximal arm of V-band on posterior wing margin to apex of vein Cu1; W5) S- and V-band connection between R_{2+3} and R_{4+5} (1= present; 2= absent); W6) V-band anterior connection of proximal and distal arms between R_{4+5} and M (1= present; 2= absent); W7) ratio of wing width/wing length (W2/W1).

Additional variables of ratios between two measurements were assessed as follows: X1) aculeus length/mesonotum length (A1/M1); X2) aculeus length/wing length (A1/W1); X3) mesonotum length/wing length (M1/W1); X4) mesonotum length/mesonotum width (M1/M2); X5) width at beginning of serrated section/length of serrated section (A3/A5); X6) mesonotum width/wing length (M2/W1).

Geometric morphometrics

Eighteen homologous landmark coordinates were digitized on the wings. A total as 626 females belonging to 40 populations distributed from Mexico through Central America, Venezuela, Colombia, Ecuador and Peru were examined.

Landmarks were determined by the intersection or termination of wing veins as follows:

1) junction of humeral and costal veins; 2) subcostal break along costal vein; 3) apex of vein R_1; 4) apex of vein R_{2+3}; 5) apex of vein R_{4+5}; 6) apex of vein M; 7) apex of vein CuA_1 on posterior margin; 8) apex of vein CuA_2 on posterior margin; 9) basal bifurcation of R_{2+3} and R_{4+5}; 10) junction of R_{4+5} and cross vein r-m; 11) basal angle of cell bm; 12) junction of M and cross-vein dm-bm; 13) junction of M and cross vein r-m; 14) junction of M and cross-vein dm-cu; 15) junction of CuA_1 and Cu_2; 16) junction of CuA_1 and cross vein bm-cu; 17) junction of CuA_1 and dm-cu; 18) junction of A and Cu_2 (= apex of cell bcu) (Figure 4).

Data analyses

Linear measurements and the landmark coordinates were acquired from digitized images of wing, aculeus and mesonotum using the TPS DIG software package (Rohlf

2010a, 2010b). Canonical Variate Analyses (CVA) were executed to explore the morphological similarities among the 40 populations of the *Af* complex, and to test the reliability of the predictive model of morphotypes as well. The model based on linear morphometry was constructed by the forward stepwise analysis method, which reviews all variables and evaluates which ones will contribute further to the discrimination between groups. From linear morphometric data, a dendrogram of the relationships among samples was constructed, based on Mahalanobis distances computed from the CVA by the unweighted pair group average method (UPGMA), using Statistica (Statsoft 2006). Statistical validation of morphotypes and lineages was made through multivariate analysis of variance (MANOVA) of the scores from the CVA's, and their pairwise comparisons by Hotelling's test with Bonferroni correction using R software (R Core Team 2014). Additional tests on the feasibility of the prediction model were performed through the classification function analysis of individuals grouped by morphotypes and lineages.

The wing shape information was extracted by the generalized Procrustes superimposition analysis, which is used to remove non-shape variation by scaling all specimens to unit size, translating to a common location and rotating them to their corresponding landmarks lined up as closely as possible (Goodall 1991). To test the accuracy of morphotypes and lineages established *"a priori"*, we conducted CVA's and the classification of individuals by group using SPSS v.13 program. To prove their statistical significance, we also executed MANOVA tests and their pairwise comparisons (R Core Team 2014). To evaluate the effect of wing size, a multivariate regression of the wing shape (dependent variable) *vs.* log-centroid size (independent variable) with permutation tests (10,000 iterations) were performed. Differences of the wing shape were visualized using wireframe comparisons along the first two canonical variates. Procrustes superimposition analysis, wing size analysis, and drawing of wireframes were executed with MORPHOJ (Klingenberg 2011).

Results

Morphotypes

The exploratory canonical variate analysis (CVA) of linear morphometrics, applied to 40 populations along the Mesoamerican and Pacific Neotropical dominions showed significant differences among them ($F = 9.40$; Wilk's lambda < 0.0001; DF = 39/547; $p < 0.0001$). The tree of similarities computed from the Squared Mahalanobis distance matrix, supported the presence of six well-differentiated morphotype clusters: the Mexican, Venezuelan, Andean, and Peruvian (previously established by Hernández-Ortiz et al. 2004, 2012), a new cluster designated as Ecuadorian morphotype, and a single population from East-Peru (Figure 5).

The predictive model of linear morphometrics showed that centroid means for the Andean, Peruvian and Ecuadorian morphotypes were mainly differentiated by the CV-1 scores, which contributed with 61.5% of the differentiation. The CV-2 accounted for

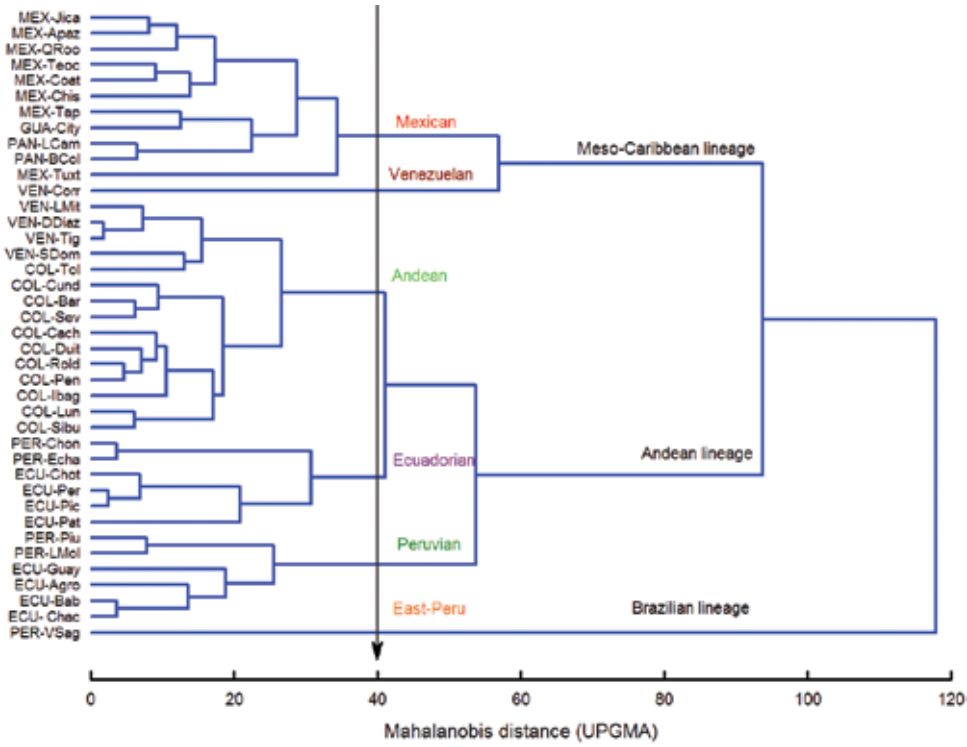


Figure 5. Phenogram showing the linear morphometric relationships among 40 population samples of the *Af* complex, based on Mahalanobis distances computed from the CVA. Clustering method UPGMA.

19.6%, distinguishing the East-Peru sample. The CV-3 scores accounted for only 8.4% of the variation among groups (Table 2, Figure 6). Variables with greatest statistical significance were represented by characters of the aculeus (A1, A2, A7, A9), mesonotum (M1, M3), wings (W3, W4, W5), and ratios between two variables (X2, X3, X5) (Table 3).

MANOVA tests showed significant overall differences among morphotypes based on linear morphometry (F = 366.73; Wilk's lambda = 0.0024; DF = 25/2237; p < 0.0001), and all Hotelling's pairwise comparisons (*post hoc*) also led to significant differences (p < 0.0001). Furthermore, reliability of morphotypes based on the wing shape also proved to be statistically significant (F = 159.93; Wilk's lambda = 0.0235; DF = 25/2289; p < 0.0001), as well as all Hotelling's pairwise comparisons between each other (p < 0.0001).

Morphological similarities through the Squared Mahalanobis Distance matrix (SMD) were assessed by pairwise comparisons among morphotypes. For example, closer distances were noted between morphs such as Ecuadorian *vs.* Andean (SMD = 15.2), and Peruvian *vs.* Andean (SMD = 19.7); or moderate distances between Peruvian *vs.* Ecuadorian (SMD = 37.9), and Mexican *vs.* Venezuelan (SMD = 37.7) (Table 4). The overall rate of reliability to identify individuals within expected morphotypes was very

Table 2. Chi-Square tests with successive roots removed. Results of five significant variates produced by two morphometric models.

Model	Function	Eigenvalue	Canonical R	% Variance	% Cumulative
Linear morphometrics	1	10.451	0.955	61.5	61.5
	2	3.333	0.877	19.6	81.1
	3	1.442	0.768	8.4	89.5
	4	1.156	0.732	6.8	96.3
	5	0.624	0.620	3.7	100
Geometric morphometrics	1	2.996	0.866	43.5	43.5
	2	2.171	0.827	31.5	75.0
	3	1.295	0.751	18.8	93.8
	4	0.309	0.486	4.5	98.3
	5	0.120	0.328	1.7	100.0

Table 3. Means and Standard Deviations for all measurements of the morphotypes encountered. Linear measures are in mm, except qualitative traits (W5, W6, A7), and ratios (A9, A10, A11, W7, X1, X2, X3, X4, X5, X6). See methods for explanations.

	Mexican	Venezuelan	Andean	Peruvian	Ecuadorian	East-Peru
A1	1.773 ± 0.10	1.945 ± 0.05	1.801 ± 0.10	1.68 ± 0.07	1.905 ± 0.12	1.727 ± 0.06
A2	0.123 ± 0.01	0.131 ± 0.01	0.123 ± 0.01	0.120 ± 0.01	0.136 ± 0.01	0.116 ± 0.01
A3	0.087 ± 0.01	0.093 ± 0.01	0.080 ± 0.01	0.079 ± 0.01	0.083 ± 0.01	0.077 ± 0.01
A4	0.117 ± 0.01	0.142 ± 0.01	0.120 ± 0.01	0.114 ± 0.01	0.128 ± 0.01	0.122 ± 0.01
A5	0.161 ± 0.01	0.178 ± 0.01	0.126 ± 0.01	0.132 ± 0.01	0.132 ± 0.01	0.143 ± 0.01
A6	0.284 ± 0.02	0.328 ± 0.02	0.253 ± 0.02	0.252 ± 0.01	0.268 ± 0.02	0.271 ± 0.02
A7	11.83 ± 1.52	14.13 ± 0.77	10.97 ± 1.16	13.11 ± 1.12	10.80 ± 0.97	9.65 ± 0.63
A8	0.277 ± 0.02	0.32 ± 0.01	0.250 ± 0.08	0.246 ± 0.01	0.260 ± 0.02	0.265 ± 0.02
A9	0.730 ± 0.10	0.803 ± 0.06	0.954 ± 0.14	0.866 ± 0.10	0.974 ± 0.14	0.856 ± 0.11
A10	0.157 ± 0.01	0.165 ± 0.01	0.139 ± 0.04	0.146 ± 0.01	0.137 ± 0.01	0.153 ± 0.01
A11	0.420 ± 0.03	0.445 ± 0.02	0.485 ± 0.04	0.463 ± 0.03	0.491 ± 0.04	0.460 ± 0.03
W1	6.287 ± 0.51	7.033 ± 0.26	6.653 ± 0.50	6.383 ± 0.31	7.089 ± 0.35	7.521 ± 0.41
W2	2.681 ± 0.24	2.903 ± 0.12	2.837 ± 0.23	2.785 ± 0.17	3.002 ± 0.15	3.126 ± 0.20
W3	0.441 ± 0.04	0.411 ± 0.03	0.314 ± 0.04	0.366 ± 0.03	0.300 ± 0.03	0.454 ± 0.03
W4	1.401 ± 0.13	1.459 ± 0.10	1.317 ± 0.17	1.429 ± 0.18	1.749 ± 0.11	1.936 ± 0.13
W5	1.16 ± 0.37	1.93 ± 0.26	1.98 ± 0.12	2.00 ± 0.00	2.00 ± 0.00	1.59 ± 0.51
W6	1.00 ± 00	1.00 ± 0.00	1.70 ± 0.46	1.77 ± 0.42	1.62 ± 0.49	1.00 ± 0.00
W7	0.426 ± 0.01	0.413 ± 0.01	0.427 ± 0.02	0.436 ± 0.01	0.424 ± 0.01	0.415 ± 0.01
M1	2.884 ± 0.24	3.159 ± 0.12	2.879 ± 0.26	3.061 ± 0.17	3.083 ± 0.20	3.005 ± 0.22
M2	1.900 ± 0.16	2.103 ± 0.09	1.856 ± 0.19	1.987 ± 0.11	2.036 ± 0.13	1.984 ± 0.14
M3	1.815 ± 0.15	2.007 ± 0.08	1.792 ± 0.19	1.925 ± 0.12	1.992 ± 0.12	1.910 ± 0.15
X1	0.617 ± 0.04	0.616 ± 0.02	0.628 ± 0.04	0.550 ± 0.03	0.621 ± 0.06	0.576 ± 0.04
X2	0.283 ± 0.02	0.277 ± 0.01	0.271 ± 0.01	0.263 ± 0.01	0.269 ± 0.02	0.231 ± 0.02
X3	0.459 ± 0.02	0.449 ± 0.01	0.433 ± 0.02	0.479 ± 0.02	0.434 ± 0.02	0.399 ± 0.02
X4	1.520 ± 0.09	1.503 ± 0.04	1.555 ± 0.06	1.541 ± 0.06	1.518 ± 0.08	1.515 ± 0.05
X5	0.540 ± 0.04	0.523 ± 0.04	0.635 ± 0.05	0.598 ± 0.04	0.630 ± 0.07	0.537 ± 0.04
X6	0.303 ± 0.02	0.299 ± 0.01	0.279 ± 0.01	0.311 ± 0.01	0.287 ± 0.01	0.264 ± 0.01
Valid N	**123**	**15**	**258**	**101**	**98**	**17**

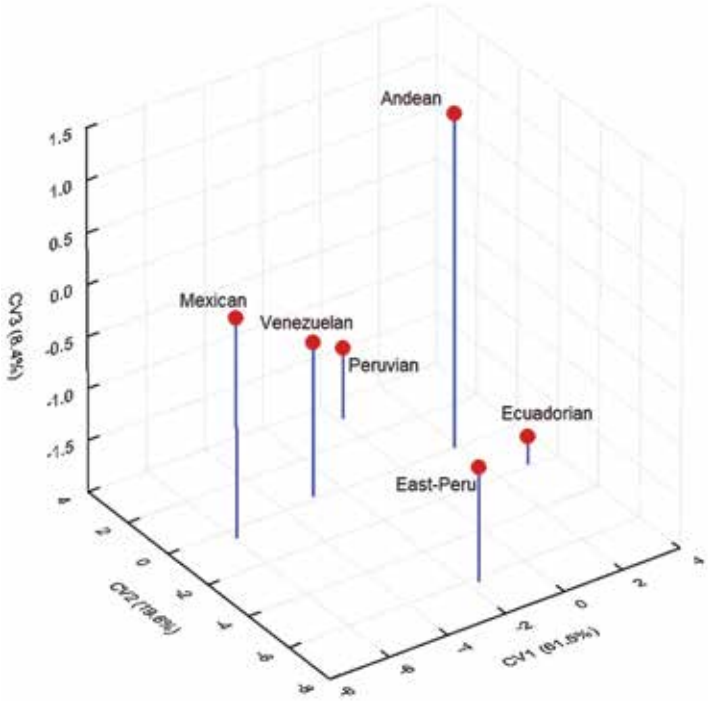


Figure 6. Scatter plot of means of centroids morphotypes from first three Canonical Variates resulted from the CVA applied to linear morphometric model. Percentage in parenthesis indicates the contribution to the differentiation of the groups.

Table 4. Squared Mahalanobis Distances from linear morphometric data produced by pairwise comparisons among morphotypes from Mesoamerica and Pacific Neotropical dominions.

	Mexican	Venezuelan	Andean	Peruvian	Ecuadorian	East-Peru
Mexican	0	37.7	62.8	54.6	87.0	84.3
Venezuelan		0	50.8	48.1	67.0	100.7
Andean			0	19.7	15.2	81.2
Peruvian				0	37.9	108.0
Ecuadorian					0	80.1
East-Peru						0

high (96.1%). The correct classification of the specimens according to cross-validation of the CVA was 93.5 and 100% for Mexican and Venezuelan morphotypes, respectively; a range from 96.1–97% for the Andean, Peruvian and Ecuadorian morphotypes; while 100% of individuals from the East-Peru location (Per- Valle Sagrado) were correctly classified (Table 5).

Moreover, the predictive model based on the CVA of the wing shape showed that 43.5% of the variability can be explained by the first canonical variable (CV-1), which

Table 5. Classification matrix of individuals by morphotypes according to tested models: Above line: Linear morphometrics. Below line: Geometric morphometrics. Rows: observed classifications; Columns: Predicted classifications.

	% Correct	Mexican	Venezuelan	Andean	Peruvian	Ecuadorian	East-Peru	N
Mexican	93.5	**115**	7	1	0	0	0	123
Venezuelan	100.0	0	**15**	0	0	0	0	15
Andean	96.1	0	0	**248**	1	9	0	258
Peruvian	97.0	0	0	3	**98**	0	0	101
Ecuadorian	96.9	0	0	3	0	**95**	0	98
East-Peru	100.0	0	0	0	0	0	**17**	17
Linear model	**96.1**	**115**	**22**	**255**	**99**	**104**	**17**	**612**
Mexican	87.8	**108**	9	0	4	0	2	123
Venezuelan	100.0	0	**15**	0	0	0	0	15
Andean	87.4	6	5	**229**	4	18	0	262
Peruvian	95.2	0	0	1	**100**	4	0	105
Ecuadorian	88.5	0	0	11	1	**92**	0	104
East-Peru	100.0	0	0	0	0	0	**17**	17
Geometric model	**89.6**	**114**	**29**	**241**	**109**	**114**	**19**	**626**

recognizes the closely linked Andean and Ecuadorian morphotypes, and in turn, is clearly divergent from others. The second canonical variable (CV-2) described 31.5% of differences, recognizing the Mexican and Venezuelan morphotypes near each other, but differing from the Peruvian morphotype. The third canonical variable (CV-3) accounted for only 18.8% of the variability among groups. These wing shape variations are represented by the wireframes of morphotypes encountered, showing the change of the shape expected along the first two canonical variables (Table 2, Figure 7).

The allometric variation of the wing shape assessed by multiple regression of log-centroid size *vs.* shape scores, revealed significant differences ($p < 0.0001$), proving that wing size predicted for only 2.26% of the total shape variation. However though this test proved to be significant it is considered relatively minor given the low percentage shown (Figure 8). *A priori* allocation of individuals into each of the morphotypes resulted in an overall rate of 89.6% with some differences respect to linear model; the Andean and Ecuadorian morphotypes exhibited identification rates of 87.4% and 88.5%, respectively; the Mexican 87.8%, the Peruvian 95.2%, while in the Venezuelan and the East-Peru samples 100% of the specimens were correctly classified (Table 5).

Phenotypic relationships

In accordance with the results from previous cluster analysis of the 40 populations examined, morphotypes were linked at higher distance forming three different phenotypical groups herein called the Meso-Caribbean, Andean and Brazilian phenotypic

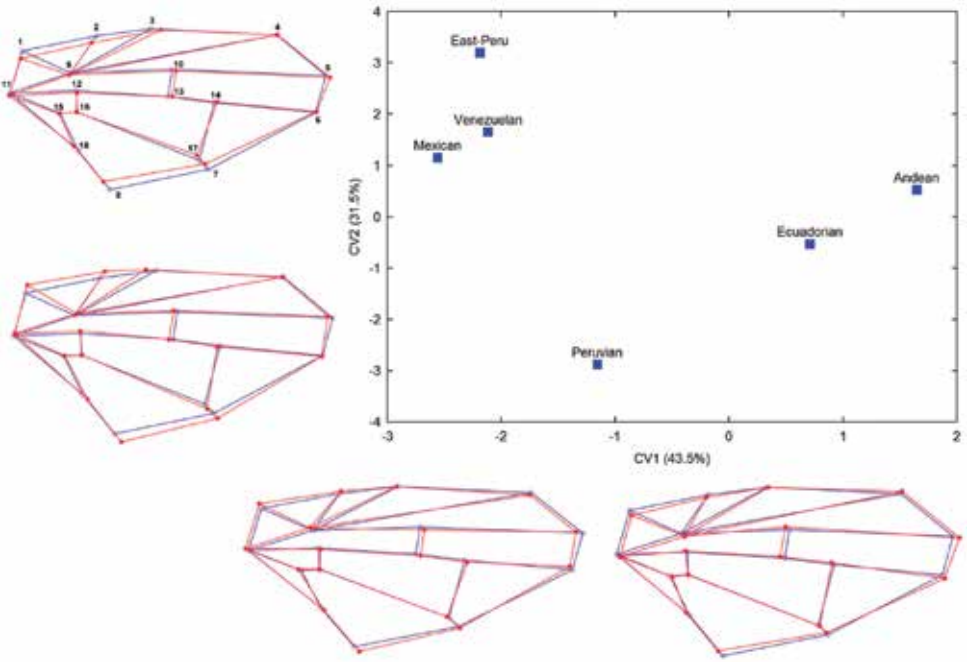


Figure 7. Average scores for the first two canonical variates (CV1 and CV2) derived from CVA for the total variation of wing shape between morphotypes of the *Af* complex. Wireframes showing the shape changes (red lines) from the consensus configuration of landmarks (blue lines) to each extreme negative and positive of CV scores.

lineages. Multivariate regression analysis (MANOVA) applied to scores obtained from the CVA, proved that accuracy of lineages was highly significant. The linear model showed highly significant differences between lineages (F = 1150.8; Wilk's lambda = 0.0437; DF = 4/1216; p < 0.0001), and among all pairwise comparisons (Hotelling's test p < 0.0001). The predictive model, using the geometric method, also demonstrated highly significant differentiation between lineages (F = 433.3; Wilk's lambda = 0.1746; DF = 4/1244; p < 0.0001) and all paired comparisons among them as well (Hotteling's test, p < 0.0001). Mahalanobis distances exhibited remarkable divergence when contrasting morphotypes from distinct lineages; for instance, pairwise comparisons between East-Peru (Brazilian lineage) with all other morphotypes (SMD = 80.1–108), or distances among samples from the Andean lineage *vs.* the Meso-Caribbean lineage (SMD = 48.1–87.0) (Table 4).

Mesoamerican-Caribbean lineage (shortly named *Meso-Caribbean*). It clustered all samples from Mexico, Central America, and the Caribbean coast of Venezuela. This lineage consisted of the two vicariant Mexican and Venezuelan morphotypes (*sensu* Hernández-Ortiz et al. 2012). The former occurs in the territories of Mexico and Central America, and a single population from the Caribbean coast of Venezuela (Zulia state) distinguished the latter. The linear model showed close similarities among all

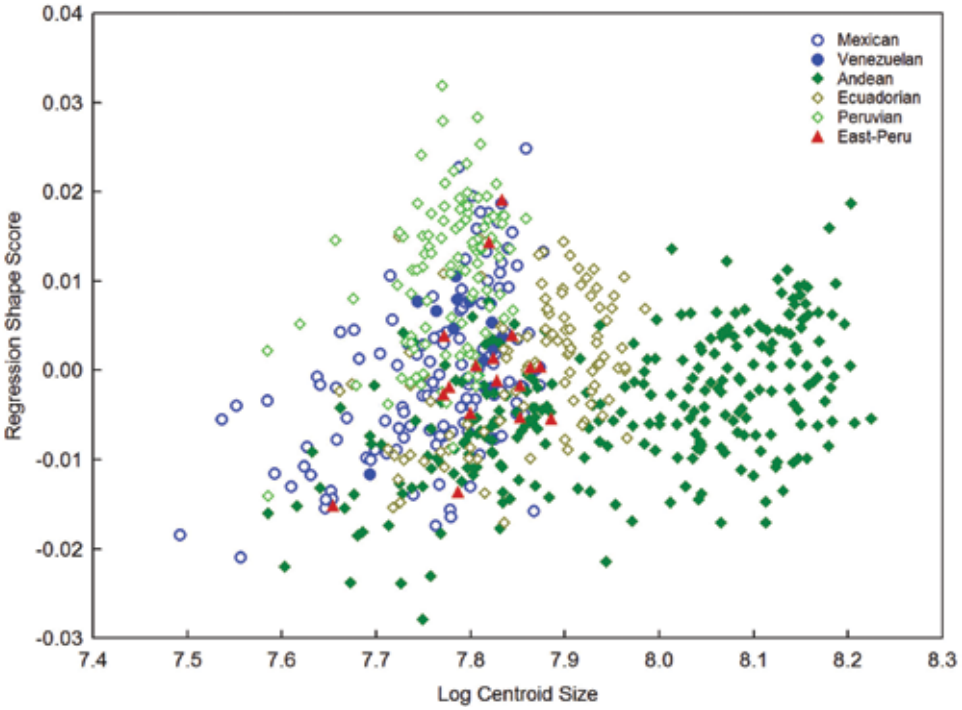
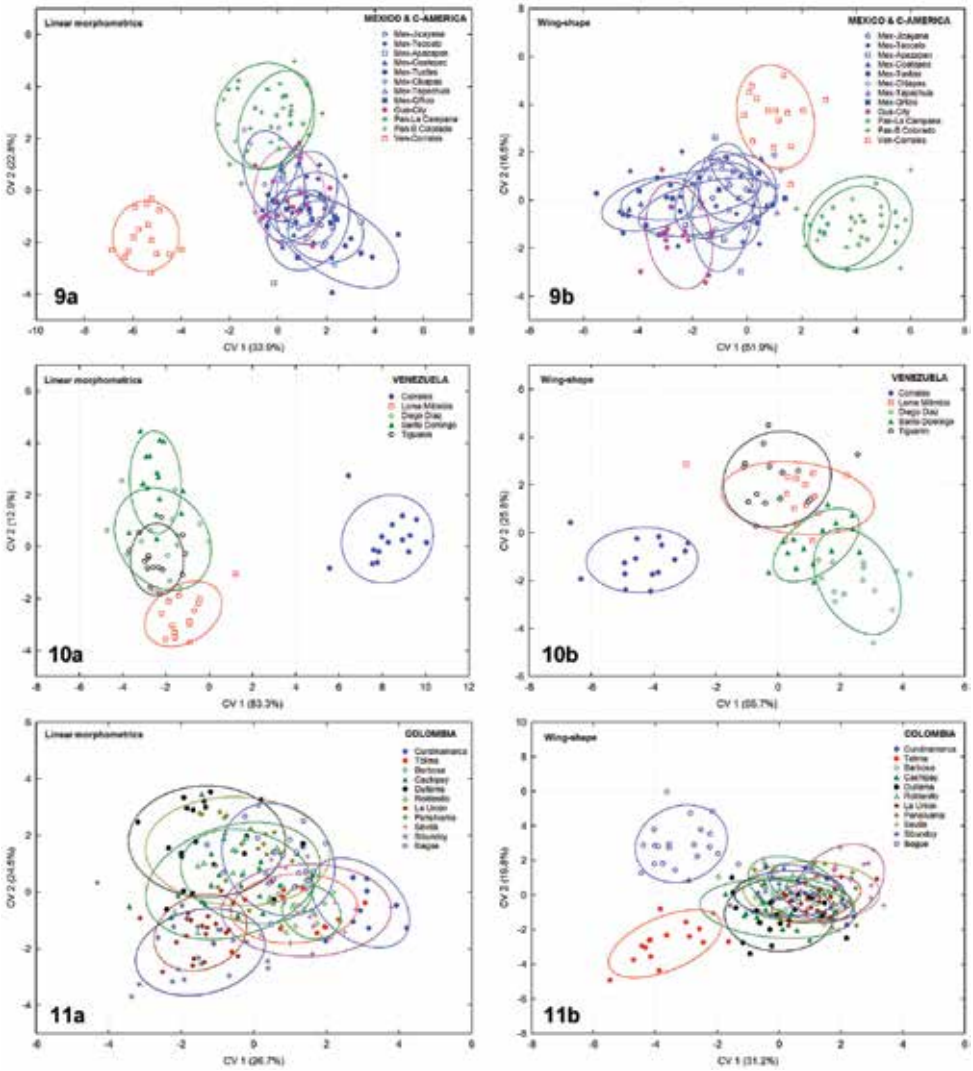


Figure 8. Analysis of allometry among morphotypes in the *Anastrepha fraterculus* complex. Multivariate regression performed from Procrustes coordinates against log-Centroid size values for the wings.

samples from Mexico, Guatemala and Panama, and a clear segregation of the Caribbean population (Ven-Corrales). The wing shape model also exhibited similar results, nevertheless keeping separated the Panamanian populations (Figure 9a–b).

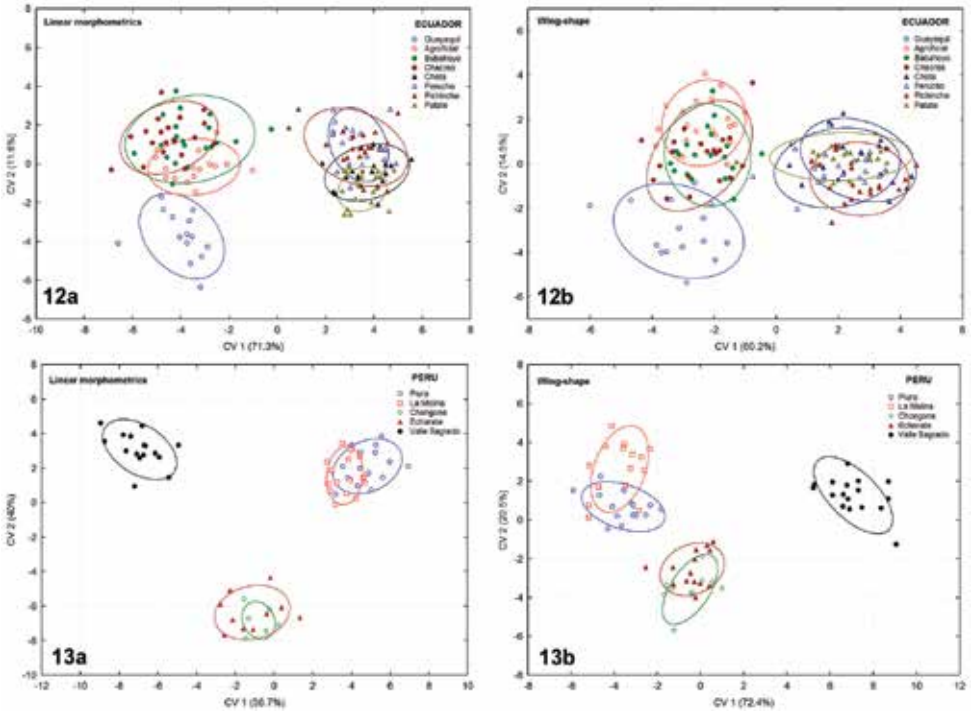
This lineage exhibited distinctive morphological features such as the aculeus length (A1 = 1.77–1.95 mm); wider aculeus tip at beginning of serrated section (A3 = 0.087–0.093 mm); longer serrated section (A5 = 0.161–0.178 mm); ratio of non-serrated section/aculeus tip (A11 = 0.420–0.445); and lowest ratio of width/length of serrated section (X5 = 0.523–0.540), like specimens of the Brazilian lineage. Remarkable qualitative features in the wing pattern were also recorded: the typical Costal, S- and V- bands are broad and heavily colored; the upper connection between arms of V- band (W6) in nearly 100% of specimens examined; and wider apical section of S- band (W3 = 0.411–0.441 mm). In the Mexican morphotype, aculeus tip constriction at beginning of serrated section is almost unnoticeable, and connection between S- and V- bands is always present; whereas in the Venezuelan morphotype S- and V- band connection is typically absent in most specimens, and the aculeus tip wider with numerous marginal teeth (A7 = 14.1 teeth per side) (Figures 14–17, 26–29).

Andean lineage. It comprises three clusters of samples: a) the Andean morphotype grouped all 15 populations coming from high mountains of Venezuela and Colombia;



Figures 9–11. Scatter plots of individuals tested by CVA grouping samples by distributional areas: **9a–b** Mesoamerican-Caribbean lineage represented by 12 populations from Mexico, Guatemala, and Panama, including the single Venezuela lowland for comparisons **10a–b** Five populations from Venezuela **11a–b** Eleven populations from Colombia **a** linear morphometrics **b** geometric morphometrics of wing-shape. Confidence ellipses 95%.

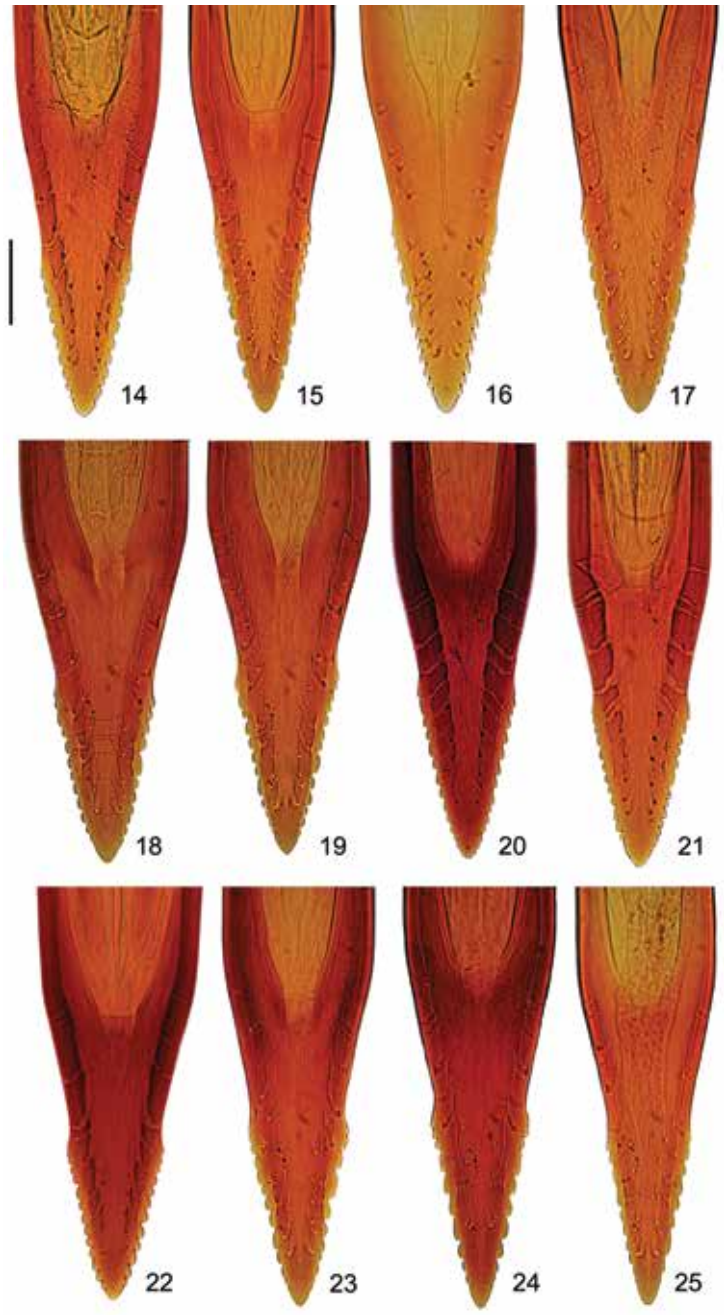
b) the Peruvian morphotype clustered six lowland populations along the Pacific coast of Ecuador and Peru; and c) the Ecuadorian morphotype, here recognized by the first time, including six highland populations from Ecuador and Peru. We highlight some variables, which may distinguish the morphs of this lineage from others: the apical section of S- band extremely narrow (W3 = 0.300–0.366 mm); S- and V- band con-



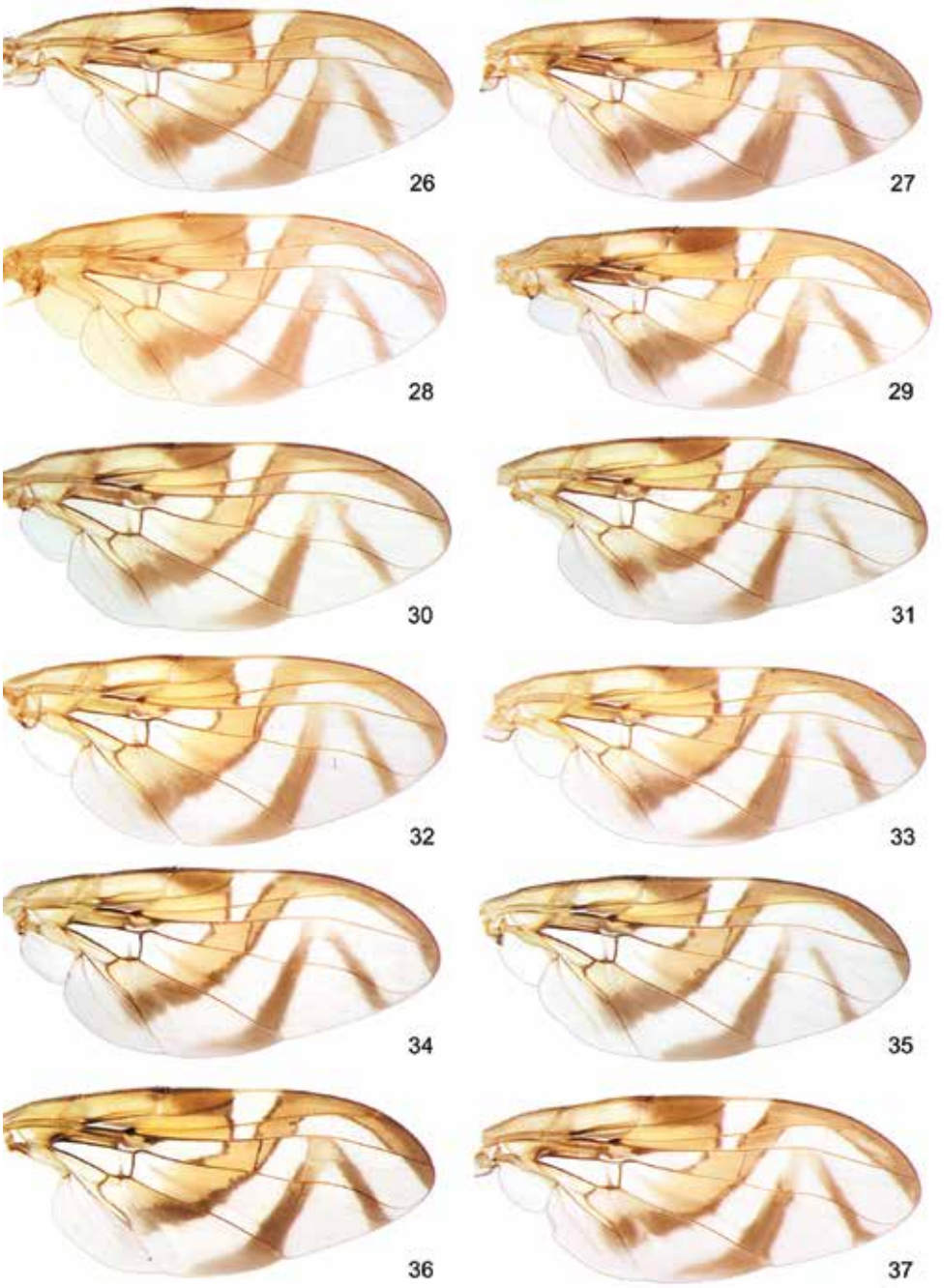
Figures 12–13. Scatter plots of individuals tested by CVA grouping samples by distributional areas: **12a–b** Eight populations from Ecuador **13a–b** Five populations from Peru. **a** linear morphometrics **b** geometric morphometrics of wing-shape. Confidence ellipses 95%.

nection (W5) missing in near 97% of the specimens examined; V- band arms upper connection (W6) absent in nearly one half of the specimens; and higher ratio between width/length of serrated section (X5 = 0.598–0.635). The Peruvian morph exhibited higher average teeth on the aculeus tip (A7 = 13.1 teeth per side) when compared to Ecuadorian and Andean morphotypes (A7 = 10.8, 10.9 teeth per side, respectively). The Andean morph showed a strong narrowing of apical section of S- band, in addition to distal arm of V- band diffuse and reduced (Figures 18–23, 30–35).

Brazilian lineage. It was recognized by a single population from the high mountains of the East-Andean region in Peru, which showed a clear differentiation from all other samples studied, and a preliminary analysis placed it closely related to the Brazilian morphs (*sensu* Hernández-Ortiz et al. 2012). We have not fully characterized this lineage, since other Brazilian morphotypes were not reviewed under this perspective. However, we made some comparative inferences from other lineages here described. The sample from East-Peru (Per-Valle Sagrado) exhibited particular morphological traits as follows: shorter aculeus length; aculeus tip narrow and longer in proportion to total length; lowest number of marginal teeth (A7 = 9.6 teeth per side), this last one probably a common feature in populations inhabiting southern Brazil and Argentina (see Hernández-Ortiz et al. 2012). Ratios of the aculeus/wing length (X2 = 0.231), and



Figures 14–25. Typical shape of the aculeus tip in morphotypes from the Meso-American and Pacific dominions. Mexican morphotype: **14** Mexico-Apazapan **15** Guatemala-City **16** Panama-La Campana. Venezuelan morphotype: **17** Venezuela-Corrales. Andean morphotype: **18** Venezuela-Loma Mitimbis **19** Colombia-Barbosa. Peruvian morphotype: **20** Ecuador-Agroficial **21** Peru-La Molina. Ecuadorian morphotype: **22** Ecuador-Chota **23** Peru-Echarate. Brazilian lineage: **24** Peru-Valle Sagrado **25** Argentina-Tucuman. Scale bar 0.05 mm.



Figures 26–37. Typical wing patterns in morphotypes from the Mesoamerican and Pacific dominions. Mexican morphotype: **26** Mexico-Apazapan **27** Guatemala-City **28** Panama-La Campana. Venezuelan morphotype: **29** Venezuela-Corrales. Andean morphotype: **30** Venezuela-Loma Mitimbis **31** Colombia-Barbosa. Peruvian morphotype: **32** Ecuador-Agroficial **33** Peru-La Molina. Ecuadorian morphotype: **34** Ecuador-Chota **35** Peru-Echarate. Brazilian lineage: **36** Peru-Valle Sagrado, **37** Argentina-Tucuman.

mesonotum/wing length (X3 = 0.399) showed the lowest values among all samples. This means that wing length is larger than aculeus and mesonotum respect to other morphotypes examined (Figures 24–25, 36–37).

Distribution

The dendrogram of morphometric similarities also provided evidence that more than one morphotype could occur in some South American countries located in the Pacific dominion. Therefore, further discriminant analyses were performed separately.

Venezuela. Samples from five locations were considered for the analyses and the results from both linear and geometric morphometry were almost identical. The single population examined of the Caribbean coast (Ven-Corrales) belonged to the Venezuelan morphotype (Meso-Caribbean lineage), and it was distinguished from a second group comprising all four populations coming from the highlands, identified as the Andean morphotype (Andean lineage) (Figure 10a–b).

Colombia. Linear morphometric analysis grouped all 11 Colombian populations under the Andean morphotype. Nevertheless, the wing shape analysis revealed three partially differentiated groups: one cluster with individuals from 9 populations, a second sluster with individuals from the laboratory strain of the Vienna facilities (Col-Tolima), and the other one from Ibagué (Col-Ibagué) (Figure 11a–b).

Ecuador. The linear morphometrics and wing shape analyses applied to eight populations from Ecuador yielded identical results, forming two distinct morphological clusters inhabiting this country. The four lowland samples were closely related to each other within the Peruvian morphotype (*sensu* Hernández-Ortiz et al. 2012), while the four other samples from the highlands were classified under a new Ecuadorian morphotype, here described for the first time (Figure 12a-b).

Peru. Both morphometric techniques applied to five populations analyzed of this country revealed the presence of three different morphological clusters. The first one comprised two lowland samples classified into the Peruvian morphotype (Per-Piura, Per-La Molina). The second cluster was represented by two samples from the highlands (Per-Echarate, Per-Chongona) and belonged to the Ecuadorian morphotype. The third morphological entity, consisting of a single population from the East-region of the Andes (Per-Valle Sagrado), proved to be distinct from all other samples examined, tentatively related to the Brazilian-1 morphotype within the Brazilian lineage (Figure 13a–b).

Distributional patterns based in the current classification of the Neotropical biogeographic provinces (*sensu* Morrone 2014), showed that the Mexican morphotype occurs in areas from 30–1400 m altitude in the Veracruzan, the Pacific lowlands, and the Yucatan peninsula provinces (Mesoamerican dominion); also in the Chiapas highlands province (Mexican Transition Zone), and in Central America recorded from the Guatuso-Talamanca province (north of the Pacific dominion), and probably spread to the Puntarenas-Chiriquí province (Table 6). The Venezuelan morphotype was recorded

Table 6. Distribution of the morphotypes through biogeographical provinces of the Mesoamerican and Pacific dominions (*sensu* Morrone 2014).

Morphotype	Biogeographical Sub-region	Biogeographical Province	Country	Sample-Key
Mexican	Mesoamerica	Veracruzan	Mexico	MEX-Jica
			Mexico	MEX-Teoc
			Mexico	MEX-Apaz
			Mexico	MEX-Coat
			Mexico	MEX-Tuxt
		Pacific Lowlands	Mexico	MEX-Tap
		Yucatan Peninsula	Mexico	MEX-QRoo
	Mex Tran Zone	Chiapas Highlands	Mexico	MEX-Chis
			Guatemala	GUA-City
	Pacific	Guatuso-Talamanca	Panama	PAN-Lcam
			Panama	PAN-Bcol
Venezuelan	Pacific	Guajira	Venezuela	VEN-Corr
Andean	Pacific	Magdalena	Venezuela	VEN-Lmit
			Venezuela	VEN-DDiaz
			Venezuela	VEN-Sdom
			Venezuela	VEN-Tig
			Colombia	COL-Cund
			Colombia	COL-Tol
			Colombia	COL-Bar
			Colombia	COL-Cach
			Colombia	COL-Duit
			Colombia	COL-Pen
			Colombia	COL-Ibag
		Cauca (north)	Colombia	COL-Rold
			Colombia	COL-Lun
			Colombia	COL-Sev
			Colombia	COL-Sibu
Ecuadorian	Pacific	Cauca (south)	Ecuador	ECU-Chot
			Ecuador	ECU-Per
			Ecuador	ECU-Pich
			Ecuador	ECU-Pat
	South Brazilian	Yungas	Peru	PER-Chon
			Peru	PER-Echa
Peruvian	Pacific	Western Ecuador	Ecuador	ECU-Guay
			Ecuador	ECU-Agro
			Ecuador	ECU-Baba
			Ecuador	ECU- Chac
		Ecuadorian	Peru	PER-Piu
	S-Am Tran Zone	Desert	Peru	PER-LMol
Brazilian complex	S-Am Tran Zone	Puna	Peru	PER-VSag

from the Guajira province (40 m); however, it could reach out other nearby lowlands along the Caribbean coast into the Venezuelan province, as represented by lowland samples near Caracas examined by Steck (1991), and Steck and Sheppard (1993).

The Andean morphotype only occurs in the Pacific dominion along the Magdalena province, occupying the highlands of Venezuela (from 1570–2500 m altitude) and Colombia (from 1350–2569 m); it was also found in several Colombian locations in the north of the Cauca province (Roldanillo, La Union, Sevilla, and Sibundoy). However, in the Colombian Pacific lowlands represented by the Chocó-Darién province, we did not record any sample of the *Af* complex so far.

The Peruvian morphotype was distributed throughout the Pacific Coastal lowlands from Ecuador (7–370 m) and Peru (35–300 m), into the Western-Ecuador and Ecuadorian provinces (Pacific dominion), and the Desert province of the South American Transition Zone. The Ecuadorian morphotype exhibited a distribution along the mountains of the south of Cauca province in the inter-Andean valleys from Ecuador (1550–2176 m), together with two other Peruvian highland samples (Per-Chongona, Per-Echarate) located at 941–1502 m, respectively, in the East-side of the Andes within the Yungas province (South Brazilian dominion). A single population sample was characterized as belonging to the Brazilian lineage, and it was collected in Cusco at the Inca region called Sacred Valley (2859 m), located in the East-side of the Andes into the Puna province of the South American Transition Zone.

Discussion

Results showed that the nominal species *Anastrepha fraterculus* (Wiedemann) includes several cryptic species in concordance with previous morphometric findings (Hernández-Ortiz et al. 2004, 2012). Specifically, the *Af* complex consists of eight morphotypes throughout the Neotropics, and in turn, these are related to each other within at least three phenotypic lineages. Major similarities were seen between morphotypes belonging to the same lineage; for example, closer distances were observed between the Mexican and Venezuelan morphotypes (Meso-Caribbean lineage), or between the Andean and the Ecuadorian morphotypes (Andean lineage). The phenotypic proximity between the Ecuadorian and the Andean morphotypes, together with the fact that individuals were partially classified within each other, means that they could have a partial and incomplete isolation.

Linear and geometric morphometric analyses showed similar results, both demonstrating to be useful for diagnosis and recognition of morphotypes presumably representing the cryptic species of the *Af* complex. However, we should also mention that some differences were noted. For instance, differences between samples reared from laboratory colonies, originally stemmed from the same area in Colombia (Col-Tolima, Col-Ibagué) proved to be divergent in wing shape between each other. This is probably due to laboratory strains facing phenotypic selection under artificial conditions over many generations. Therefore, it is advisable to use wild samples for identification of natural morphs, especially if geometric morphometrics is applied. Wing shape analysis

also differentiated two Panamanian samples (Pan-La Campana, Pan-B Colorado) from other populations belonging to the Meso-Caribbean lineage, even though they belonged to field collections. This highlights the need to further investigate other samples from that region to assess natural variation.

It could be argued however, that other factors may have influenced the ultimate morphological phenotype of the wing shape of flies. In particular, altitude has been found to have an impact on the wing shape of the potato moth (Hernández et al. 2010); wing shape differences between the fruit flies *Rhagoletis pomonella* and *R. zephyria*, were hypothesized to have changed in relation to host associations (Yee et al. 2009). However, we consider this might not be the case within the *Af* complex for several reasons. Firstly, the nominal species *A. fraterculus* is highly polyphagous, and host usage, albeit not the same, is highly overlapping among morphotypes (Norrbom 2004, Hernández-Ortiz and Morales-Valles 2004, Zucchi 2007). For example, *Psidium guajava* L. is a host widespread along its geographic distribution and altitudinal range in Mexico, Central America, the Andean countries, Brazil and Argentina. Secondly, the morphometric analysis performed on *A. fraterculus* in Mexico distinguished a single Mexican morphotype, even though eight populations from a wide altitudinal range, and belonging to three distinct host species were examined (Hernandez-Ortiz et al. 2004). Thirdly, in the Brazilian territory there are proofs of the occurrence of three morphotypes (Hernández-Ortiz et al. 2012), and evidence of karyotype differentiation and reproductive isolation supporting the existence of distinct species (Selivon et al. 2004, 2005a, 2005b); however all of them feed on guava, among other hosts.

Species boundaries are related with the extent and limits of gene flow, the selection intensities on ecologically or reproductively functional phenotypes across the species range, and their genetic architecture, all indispensable pieces of information for predicting the course of early lineage divergence and the origins of new species (Shaw 1998). In the biological species concept defined as "groups of interbreeding natural populations that are reproductively isolated from other such groups" (*sensu* Mayr 1969), it is not clear that in all sexually reproducing species, reproductive ties such as gene flow between demes provide the major cohesive force. The concept of interbreeding is a rather complex idea, because hybridization can be discussed in terms of reproductive modes but also in terms of speciation, that is, hybridization as "*prima facie*" evidence for incomplete speciation (Wiley 1981).

By contrast, in the evolutionary species concept defined as "a single lineage of ancestor – descendant populations, which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate" (*sensu* Wiley 1978, modified from Simpson 1961), all terminal lineages are evolutionary species or descendant of higher taxa represented by their ancestral evolutionary species. Each branch therefore is the result of a speciation event; however, the concept does not preclude a particular ancestral species from surviving a speciation event. In this regard, De Queiroz (1998) noted that the term lineage is used for a single line of direct ancestry and descent, while a clade is a unit consisting of an ancestral species and its descendants, and are monophyletic in terms of their component species, however, lineages can be paraphyletic or even polyphyletic in terms of their lower level components.

In the broad sense, the monophyly of the *fraterculus* species group has been accepted based on morphology (Norrbom et al. 1999, Norrbom et al. 2012). In several papers it is assumed that reproductive isolation between cryptic species of the *Af* complex has recently evolved, leaving implicit the idea that those morphospecies had direct relationships, and at some time of its evolutionary history there was an interpopulation divergence among them (Cáceres et al. 2009, Segura et al. 2011, Rull et al. 2013, Devescovi et al. 2014). This is probably true among morphospecies related within each phenotypic lineage that could have a common origin and most likely a more recent evolution. However, from a theoretical perspective, the monophyly of the *Af* complex has never been tested so far. Conversely, other studies from various methodological sources stated that this species complex is not monophyletic. This assumption is supported by extreme allele differences found between highland and lowland Venezuelan samples (presumably from distinct lineages), being the largest genetic divergence found among samples studied by Steck (1991). The phylogenetic relationships inferred from mtDNA sequences of COI supported the presence of multiple gene pools and the non-monophyly among samples of the nominal species *A. fraterculus* (Smith-Caldas et al. 2001). In the same way, a phylogenetic relationship analysis based on the nuclear gene period of *Anastrepha* (Barr et al. 2005) with samples from Venezuela (Mérida and Caracas), Mexico, and Brazil (Sao Paulo) found them to be related in different clades.

The occurrence of strong sexual incompatibility between distinct phenotypic lineages also supports the non-monophyly hypothesis. For instance, high levels of pre- and post-zygotic isolation, karyotypic and polytene chromosome differences, and qualitative and quantitative differences in male pheromones were found in two laboratory strains from Argentina and Peru (Cáceres et al. 2009, Segura et al. 2011) which belong to the Brazilian and Andean lineages, respectively. In addition, pre-zygotic reproductive isolation resulted in strong assortative mating to gene flow among the Mexican morphotype and other populations classified in the Brazilian-1 and Peruvian morphotypes (Rull et al. 2013), all of them belonging to three distinct phenotypic lineages described herein. Moreover, there is strong pre-zygotic isolation through temporal partitioning of mating activity of a Colombian population (Andean morphotype) compared with four other morphotypes spanning from Mexico to Argentina (Devescovi et al. 2014).

In fact, the current study reveals that the *Af* complex is integrated by eight morphotypes, which are related into three phenotypic lineages that are virtually endemic, as they are restricted to certain regions, and there is no evidence of contact zones among them so far. The Meso-Caribbean lineage is restricted to the Mesoamerican dominion, to part of the Mexican Transition Zone, and also to the northern of Pacific dominion in Central America and the Caribbean coast of Venezuela. The Andean lineage essentially occupies most of provinces in the Pacific dominion and some parts of the South American Transition Zone; while the Brazilian lineage would be distributed along the Parana dominion in the eastern part of Brazil, and the Chacoan dominion in southern Brazil and northern Argentina.

In this regard, there are also historical processes associated to each biogeographical dominion that cannot be neglected, since they explain the own history of the

biota they inhabit. According to Hoorn et al. (2010) plate subduction along the Pacific margin caused uplift in the Central Andes (Peruvian and Bolivian Andes) during the Paleogene (65 to 34 Ma). The posterior plate breakup in the Pacific, and subsequent collision with the South American and Caribbean plates, resulted in intensified mountain building in the Northern Andes (Venezuelan, Colombian and Ecuadorian Andes) by the late Oligocene to early Miocene (~ 23 Ma); while plate reorganization ultimately resulted in closing of the Panama Isthmus during the Pliocene (at ~ 3.5 Ma). These data sustain that the origin of the Northern and Central Andes, and their current connection with Mesoamerica, occurred in remarkable different times. This would mean prolonged periods of isolation between morphotypes inhabiting those geographical areas. In this sense, Drew (2004) stated that high levels of endemism in an area would indicate that speciation has occurred in relative isolation over a considerable time.

The relationship between morphological structure and genotype is complex and poorly understood for most characters, since we need to know if there is a relationship between the morphological characterizations and the real units of evolution (Shubin and Marshall 2000). This idea is particularly relevant when large numbers of sibling species occur. Therefore, from a practical point of view, it is necessary to understand the mechanisms of reproductive isolation between morphotypes, also as an essential precondition for applying control methods such as the sterile insect technique (SIT). However, from an evolutionary perspective, implications of the non-monophyly of the *Af* complex prevent making direct inferences about mechanisms of genetic or reproductive divergence among populations, since morphotypes belonging to distinct phenotypic lineages might have evolved independently in different clades.

Conclusions

In this research, the presence of eight morphotypes is established within the *Anastrepha fraterculus* (Wiedemann) complex, including the first characterization of the Ecuadorian morphotype with samples coming from the mountains of Ecuador and Peru. The morphotypes clustered into three phenotypic lineages we called Meso-Caribbean, Andean, and Brazilian. Based upon their morphological divergence and the current distributional areas, we suggest that these lineages would not have a direct connection with each other and might have evolved separately in these biogeographical regions. In terms of distributional areas or countries, the Mesoamerican dominion was only occupied by the Mexican morphotype. In other countries from the Pacific dominion such as Colombia and Venezuela, two morphotypes were encountered, the Venezuelan inhabiting the Caribbean lowlands of Venezuela, and the Andean in the highlands of both countries. In the territories from Ecuador and Peru, the Peruvian morphotype was found in the lowlands, and the Ecuadorian morphotype in the highlands. Furthermore, in the Eastern side of the Andes in Peru, another morphotype was detected that appears closely related to the morphotypes of the Brazilian lineage.

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Evaluating mating compatibility within fruit fly cryptic species complexes and the potential role of sex pheromones in pre-mating isolation

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Abstract

The study of sexual behavior and the identification of the signals involved in mate recognition between con-specifics are key components that can shed some light, as part of an integrative taxonomic approach, in delimitating species within species complexes. In the Tephritidae family several species complexes have received particular attention as they include important agricultural pests such as the *Ceratitis fasciventris* (Bezzi), *Ceratitis anonae* (Graham) and *Ceratitis rosa* Karsch (FAR) complex, the *Bactrocera dorsalis* (Hendel) complex and the *Anastrepha fraterculus* (Wiedemann) complex. Here the value and usefulness of a

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methodology that uses walk-in field cages with host trees to assess, under semi-natural conditions, mating compatibility within these complexes is reviewed, and the same methodology to study the role of chemical communication in pre-mating isolation among *A. fraterculus* populations is used. Results showed that under the same experimental conditions it was possible to distinguish an entire range of different outcomes: from full mating compatibility among some populations to complete assortative mating among others. The effectiveness of the methodology in contributing to defining species limits was shown in two species complexes: *A. fraterculus* and *B. dorsalis*, and in the case of the latter the synonymization of several established species was published. We conclude that walk-in field cages constitute a powerful tool to measure mating compatibility, which is also useful to determine the role of chemical signals in species recognition. Overall, this experimental approach provides a good source of information about reproductive boundaries to delimit species. However, it needs to be applied as part of an integrative taxonomic approach that simultaneously assesses cytogenetic, molecular, physiological and morphological traits in order to reach more robust species delimitations.

Keywords

species delimitation, field cages, Tephritidae, *Anastrepha fraterculus, Bactrocera dorsalis, Ceratitis fasciventris*, *Ceratitis anonae, Ceratitis rosa*

Relevance of studying sexual behavior and mating compatibility in complexes of pest species

The biological species concept proposes the occurrence of reproductive isolation barriers that prevent interbreeding and hybridization between species (Mayr 1942, Dobzhansky 1970). According to Dobzhansky (1937, 1970), reproductive isolation can take place in different forms and can be classified as pre-zygotic barriers that act before fertilization, and as post-zygotic barriers that act after fertilization or even after the formation of hybrids. Pre-zygotic barriers comprise those that prevent mating (pre-mating) such as behavioral (also referred as sexual, but see Coyne and Orr (2004) for a discussion), ecological (i.e. habitat, temporal and pollinator isolation) and mechanical isolation barriers, and those that prevent gene flow and occur after mating (post-mating), such as copulatory behavioral isolation, cryptic female choice (Eberhard 1996, 2015) and gametic isolation. Post-zygotic barriers can be extrinsic and related to the environment (i.e. ecological inviability and behavioral sterility) or intrinsic reflecting developmental problems in the hybrids independently of the environment (i.e. hybrid inviability and sterility) (Coyne and Orr 2004). Determining the evolutionary forces that lead to speciation and the mechanisms (i.e. type of reproductive barriers) by which populations initially diverge and are kept isolated afterwards is one of major challenges in evolutionary biology and speciation studies (Marie Curie SPECIATION Network, 2012). Behavioral isolation, which includes every behavior or signaling trait that affects recognition between con-specific mates, attractiveness and mate choice (Panhuis et al. 2001), has been considered a key component in speciation initiation (Coyne and Orr 2004).

The elucidation of the mechanisms that underlie reproductive incompatibility among species within cryptic species complexes is relevant to accurately delimit species and to understand mate recognition systems. According to Bickford et al. (2007), cryptic species are defined as "two or more distinct species that are erroneously classified (and hidden) under one species name". Following the biological species concept, demonstrating the occurrence of reproductive incompatibility should be sufficient to delimit species (Mayr 1942); however this is not universally accepted (see de Queiroz 2005). The current recommendation is to use integrative taxonomy and combine evidence from molecular, behavioral, physiological and morphological traits (Dayrat 2005). Schlick-Steiner et al. (2010) suggest that at least three different, independent criteria should be used to delimit species. Delimiting species is particularly relevant in those cases where clear taxonomic identification and species delimitation have implications for biogeography, conservation, international trade, and pest management strategies.

One paradigmatic example of the need to resolve species complexes comes from Tephritidae fruit flies. This family is composed of approximately 5,000 species of fruit flies (Norrbom et al. 1999, 2004), some of which are destructive pests of fruit and vegetable production (White and Elson-Harris 1994). Some major pest species complexes include taxonomically described "species" that are in fact geographical variants of the same species. Other complexes are composed of populations that are taxonomically grouped within the same pest species but display different biological and genetic traits and show reproductive isolation which is strong evidence that they are indeed different species. The uncertain taxonomic status has at least two important practical implications. The first is related to fruit commercialization as international movement of fruits and vegetables is conditioned by pest presence in the exporting and importing countries. The second is related to pest management. Several species within this family are managed using area-wide integrated pest management (AW-IPM) (Klassen and Curtis 2005, Vreysen et al. 2007) approaches that include a sterile insect technique (SIT) component (Knipling 1955). The SIT is based on the repeated release in the targeted area of mass-reared male flies of the pest species that are sterilized by using ionizing radiation. Wild females that mate with sterile males are inseminated with sterile sperm that induces embryonic lethality; therefore they do not produce offspring or produce offspring that is unviable. The resulting reduction in population replacement will cause a gradual decline in the population size of the targeted species (Dyck et al. 2005). Sexual compatibility between females from the target wild population and the released sterile males is absolutely essential for the SIT to be an effective control method.

Here we aimed to review the value and usefulness of a methodology that assess mating compatibility under standard semi-natural conditions. We focused on the Tephritidae family in general, and on the *Anastrepha fraterculus* (Wiedemann) cryptic species complex and the *Bactrocera dorsalis* complex (Drew and Hancock 1994) in particular. In addition, the use of walk-in field cages was evaluated as a method for studying the role of chemical communication in pre-mating isolation within the *A. fraterculus* cryptic species complex.

Mating compatibility field cage tests in the Tephritidae

The international manual for Product Quality Control for Sterile Mass-Reared and Released Tephritid Fruit Flies (FAO/IAEA/USDA 2014) provides a standard Mating Performance Field Cage Test to be carried out under semi-natural conditions in walk-in field cages (Box 1). The protocol has improved over the years, within the framework of SIT applications, and provides information on the mating behavior of the used strains and insect populations, with particular focus on mating competitiveness and mating compatibility. In the case of mating competitiveness tests, the goal is to evaluate the ability of sterile males to compete with wild males for mating with the wild females of the target population. In the case of mating compatibility, the goal is to determine the degree of sexual compatibility between the target wild population and any available strain already adapted to laboratory mass-rearing conditions. The compatibility should be measured before any large-scale rearing or release operations are initiated, while the competitiveness needs to be assessed at regular intervals during implementation of such a program. Cases of unsatisfactory male competitiveness or even mating incompatibility should either lead to the selection of a different strain or the colonization of a new strain from the target area.

Mating Performance Field Cage Tests can be applied to assess mating compatibility among two or more populations/species from a given species complex in studies aiming to clarify the taxonomic relationships within species complexes. Data derived from these tests can generate simple, reproducible, meaningful indices of sexual compatibility that can be used to make comparisons between different populations by observing the components of the courtship and mating behavior and any other intra- and inter-sexual interactions during the time of sexual activity. Mating compatibility studies using this standard test gave relevant results for different fruit fly species, including *Ceratitis capitata* (Wiedemann), species within the FAR complex, species within the *B. dorsalis* complex, *Zeugodacus cucurbitae* (Coquillett) and the *A. fraterculus* cryptic species complex.

For *C. capitata*, Cayol et al. (2002) studied the mating compatibility among eight populations that originated from different geographical areas of the world, i.e. Australia, France (La Réunion island), Greece, Guatemala, Israel, Kenya, Portugal (Madeira island) and South Africa. The authors concluded that there was no evidence of mating incompatibility and in addition, full compatibility was shown between these populations and four genetic sexing strains (Franz 2005) with a different genetic background. These results proved that only very few genetic sexing strains are needed across the world when applying the SIT against *C. capitata*. These data are consistent with Lux et al. (2002) who analyzed in detail the courtship behavior of several wild populations and mass-reared strains of *C. capitata* and found no qualitative or quantitative differences.

The *Ceratitis* FAR complex is composed of three species, *C. anonae* (Graham), *C. rosa* Karsch and *C. fasciventris* (Bezzi) that occur in certain areas of Africa. Due to their highly invasive potential and some difficulties in distinguishing some members of the complex morphologically, a number of different approaches for species recognition

Box 1. Mating compatibility using walk-in field cage test procedures

The standardized Mating Performance Field Cage Test (FAO/IAEA/USDA 2014) involves the release of males and females from two different populations into a cylindrical cage (2 m tall and 3 m in diameter) (Calkins and Webb 1983). The cage contains one tree that provides a courting and mating substrate for the flies. Prior to the experiments, flies are sorted by sex on the day of emergence, the sexes kept in separate rooms, and provided with water and food until sexual maturation. Releasing flies from two different populations in a field cages requires a marking system to make them distinguishable. This can be achieved either with a dot of water-soluble paint applied on the notothorax, feeding the adults with a diet that contains a food colorant or with fluorescent dyes applied to the pupae before adult emergence. The colors should be applied randomly for each replicate to avoid any potential interference of the color in mate selection. On the day of the test, males from the two selected populations are released into the field cage. After the release, males are given time to acclimatize (15 – 30 min), establish territories and initiate their sexual displays. The time of the day to initiate the test depends on the temporal sexual activity pattern of the populations under investigation. Females are released after males begin to display behaviors related to sexual activity. An observer, who is located inside the field cage, screens the tree and the inside of the cage (netting, poles) and searches for mating couples. Once a couple is detected, the observer removes the couple by gently coaxing it into a small vial and records the origin or type of the male and the female, the time of onset of copulation, and the location of the couple, i.e. on the poles of the cage, the cage netting or in the tree. The location on the tree can be further specified in terms of height (upper, middle or low), cardinal axes (north, east, south or west), and location within the canopy (in the peripheral layer of the canopy or in the core of the canopy). The vial is kept inside the cage (placed under the shadow of the tree) until the couple disengages and the time at which copulation ends is recorded. The test is completed when sexual activity ceases. Only those experiments in which at least 20% of the males and females from each strain engaged in matings are considered as part of the data set and a minimum of nine replicates are required for each treatment of the field cage test. The data are used to derive several mating indices such as the Relative Isolation Index (RII) (McInnis et al 1996), the Stalker's Index (I) (Stalker 1942), and the Index of Sexual Isolation (ISI) (Cayol et al. 1999) that provide indications on the level of mating compatibility. The ISI ranges from -1 to 1 and when the confidence interval includes zero, the tested combinations are considered as sexually compatible. In addition, the Male and Female Relative Performance indices (MRPI and FRPI, respectively) (Cayol et al. 1999) estimate the relative participation of each sex of a given population (regardless of the origin of its partner). The comparison of the time to start copula for each particular mating combination (latency), the time spent *in copula* and the location of the couples provide additional cues to better understand possible isolation mechanisms. The combined analysis of the different indices (ISI, MRPI and FRPI) and the other recorded variables (or even a more detailed analysis of courtship components and other behaviors, see Briceño and Eberhard 1998, Lux et al. 2002) provide a complete and reliable description of the sexual compatibility between populations (FAO/IAEA/USDA 2014).

were used (reviewed in De Meyer et al. 2015). An in-depth molecular study (Virgilio et al. 2013) revealed greater genetic differentiation than expected at the intra-species level. In particular, the analysis showed the presence of five genotypic groups, involving two *C. rosa*, two *C. fasciventris* and one *C. anonae*, suggesting that *C. rosa* and *C. fasciventris* may each include more than one species. In contrast, *C. anonae* did not show a clear intra-specific population genetic structure. Two different *C. rosa* populations, lowland and highland, with different biological attributes such as developmental rates were characterized along an altitudinal transect (Mwatawala et al. 2015). Recently, a field cage mating compatibility study using *C. rosa* and *C. fasciventris* showed that the level of isolation between low and highland populations of *C. rosa* was as high as that found

between the two species, irrespective of whether the *C. rosa* populations being compared with *C. fasciventris* originated from the low- or the highlands (S. Ekesi pers. comm.). These significant levels of mating isolation could be correlated not only with the strong population structuring found at the molecular level (Virgilio et al. 2013) but with the differences found in pheromones and cuticular hydrocarbons (CHCs) (Vaníčková et al. 2014). In all, the evidence gathered confirmed cryptic speciation and the existence of more than three species within the FAR complex (De Meyer et al. 2015).

A whole body of evidence has been collected that supports the hypothesis that the nominal species *Bactrocera dorsalis* (Hendel), *Bactrocera papayae* Drew and Hancock, and *Bactrocera philippinensis* Drew and Hancock, all belonging to the *B. dorsalis* complex, are in fact one biological species with geographical variation. Schutze et al. (2013) evaluated for the first time the mating compatibility among all possible combinations of these members using the Mating Performance Field Cage Test and found no mating isolation among *B. dorsalis*, *B. papayae* and *B. philippinensis*. In contrast when any of these entities were tested with *B. carambolae*, a certain degree of pre-mating isolation was detected. This was not only revealed by the differences in the numbers of mating types, but also in the location of the couples in the field cage: most couples involving *B. carambolae* females were found in the tree and those involving *B. papayae* and *B. dorsalis* females were found against the roof of the field cage. Schutze et al. (2013) concluded that *B. dorsalis*, *B. papayae* and *B. philippinensis* belonged to a single species. Similar mating compatibility studies between *Bactrocera invadens* from Kenya and *B. dorsalis* from China and Pakistan were carried out by Bo et al. (2014) using the same field cage methodology. Full mating compatibility was demonstrated also between both *B. dorsalis* populations and *B. invadens*, and hybrid offspring obtained between both crosses were viable for several generations. These results, in combination with findings from another field cage mating study (Chinvinijkul et al. 2015), as well as morphological, molecular genetics, cytological, sexual compatibility and chemo-ecology studies carried out over the past 20 years, led to the conclusion that these members of the *B. dorsalis* complex belonged to the same single species and their synonymization was recently published (Schutze et al. 2015a and references therein). Only *B. carambolae* was maintained as a valid biological species.

The different host use pattern of *Z. cucurbitae* observed in Mauritius and some African locations suggested the possibility of a sibling species complex, i.e. species that are the closest relative of each other and have not been distinguished from one another taxonomically (Bickford et al. 2007). If this were true, the use of the genetic sexing strain developed at the United States Department of Agriculture, Agricultural Research Services (USDA-ARS) facilities in Hawaii by McInnis et al. (2004) for sterile male releases in Mauritius would have been at risk. Mating compatibility studies between populations from Mauritius, Seychelles and the genetic sexing strain from Hawaii using the Mating Performance Field Cage Test showed, however, that the flies were fully compatible with no evidence for incipient speciation in spite of the differences in host use (Sookar et al. 2013).

Anastrepha ludens (Loew) is a species that belongs to the *fraterculus* group and is a major pest in Mexico and other Central American countries (Aluja 1994). A mass-

rearing facility in southern Mexico provides sterile flies to different regions of Mexico. Orozco-Dávila et al. (2007) using the same Mating Performance Field Cage Test evaluated the compatibility between the mass-reared strain and six wild populations from these regions and found that this strain was fully compatible with all the wild populations tested and that the sterile males were also competitive with wild males. However, some subtle differences in the time of male calling activity were detected in the wild populations with some calling earlier than others; differences that could be the result of adaptations to the local environments in different latitudes as seen for the case of *Bactrocera* (Schutze et al. 2013). Studies including both *A. ludens* and *A. obliqua* (Macquart) on a field-caged mango tree (larvae of both species can be found infesting mango) showed distinct temporal pattern of sexual activities for each species, although there was a small overlap of male calling activities, during which the formation of mixed leks was observed (Aluja et al. 1983).

Anastrepha fraterculus is a species with a wide geographical range (Steck 1999) and high levels of variability in morphology, cytology and genetics, suggesting that it consists of a cryptic species complex (Stone 1942, but see Steck 1999 for a review) that includes different species (Selivon 1996, Yamada and Selivon 2001, Selivon et al. 2004) or morphotypes (Hernández et al. 2004, 2012, 2015). In order to assess whether this variability is accompanied with mating incompatibility among different populations, studies involving several morphotypes and geographical scales were performed using the Mating Performance Field Cage Test (Table 1). Petit-Marty et al. (2004a) evaluated the mating compatibility among four populations from Argentina, two from the northwest and two from the northeast of the country. All pairwise mating combinations were compatible and additional studies on post-zygotic barriers confirmed full compatibility among populations (Petit-Marty et al. 2004b). The genetic (Alberti et al. 2002) and morphometric (Hernández-Ortiz et al. 2012) characterization of these populations confirmed that they all belonged to the Brazilian-1 morphotype (Hernández-Ortiz et al. 2012). The study of Petit-Marty et al. (2004a) was extended on a regional scale by Vera et al. (2006) who evaluated the mating compatibility among six populations of *A. fraterculus* from three different morphotypes (Brazilian-1, Peruvian and Andean). These authors found all levels of mating isolation among different populations, ranging from full compatibility to high incompatibility. Within morphotypes, the results ranged from full compatibility to moderate incompatibility (as in the case of Tucumán from Argentina and Piracicaba from Brazil, Table 1). However, among different morphotypes, mating isolation was always significant. The study also revealed the occurrence of temporal differences in sexual activity patterns of some of the populations: while the populations from the Brazilian-1 morphotype mated early in the morning, the Peruvian ones mated at midday and the Andean ones mated in the evening. Interestingly, the cases of moderate isolation observed within some morphotypes were accompanied by differences in the location of the mated couples. More recently, Rull et al. (2012) evaluated the compatibility of three strains belonging to the Brazilian-1 morphotype, i.e. two laboratory strains from southern Brazil and one strain from Argentina, and

Table 1. Summary of sexual isolation indices from field cage tests carried out for the *Anastrepha fraterculus* complex.

Reference	Population - mating combination	Morphotypes combination	ISI	Isolation level
Petit-Marty et al. 2004	Tucumán (Arg) - Entre Ríos (Arg)	Brazilian-1 - Brazilian-1	–0.01 ± 0.17	Random mating
	Tucumán (Arg) - Misiones (Arg)	Brazilian-1 - Brazilian-1	–0.03 ± 0.05	Random mating
	Jujuy (Arg) - Tucumán (Arg)	Brazilian-1 - Brazilian-1	– 0.01 ± 0.05	Random mating
	Jujuy (Arg) - Entre Ríos (Arg)	Brazilian-1 - Brazilian-1	– 0.04 ± 0.17	Random mating
	Jujuy (Arg) - Misiones (Arg)	Brazilian-1 - Brazilian-1	–0.09 ± 0.09	Random mating
	Misiones (Arg) - Entre Ríos (Arg)	Brazilian-1 - Brazilian-1	–0.09 ± 0.13	Random mating
Vera et al. 2006	La Molina (Peru) - Entre Ríos (Arg)	Peruvian - Brazilian-1	0.92 ± 0.03	High
	Tucumán (Arg) - Piura + La Molina (Peru)	Brazilian-1 - Peruvian	0.83 ± 0.06	High
	Tucumán (Arg) - La Molina (Peru)	Brazilian-1 - Peruvian	0.82 ± 0.03	High
	La Molina (Peru)- Ibague (Col)	Peruvian – Andean	0.78 ± 0.02	High
	La Molina (Peru) - Piracicaba (Br)	Peruvian - Brazilian-1	0.55 ± 0.06	Moderate
	Tucumán (Arg) - Piracicaba (Br)	Brazilian-1 - Brazilian-1	0.43 ± 0.08	Moderate
	Tucumán (Arg)- Entre Ríos (Arg)	Brazilian-1 - Brazilian-1	0.12 ± 0.10	Random mating
	La Molina (Peru) - Piura + La Molina (Peru)	Peruvian – Peruvian	0.10 ± 0.12	Random mating
Cáceres et al. 2009	Tucumán (Arg) - La Molina (Peru)	Brazilian-1 - Peruvian	0.77 ± 0.05	High
	Tucumán (Arg) - La Molina $(Peru)_{Unisex\ Arg}$	Brazilian-1 - Peruvian	0.73 ± 0.05	High
	Tucumán (Arg) - La Molina $(Peru)_{Unisex\ Peru}$	Brazilian-1 - Peruvian	0.86 ± 0.04	High
	Hybrid ArgPeru – $Arg_{UnisexArg}$	Hybrid Brazilian-1/ Peruvian - Brazilian-1	0.30 ± 0.12	Moderate
	Hybrid PeruArg – $Arg_{UnisexArg}$	Hybrid Peruvian/ Brazilian-1 - Brazilian-1	0.15 ± 0.11	Random mating
	Hybrid ArgPeru – $Peru_{UnisexPeru}$	Hybrid Brazilian-1/ Peruvian – Peruvian	0.10 ± 0.10	Random mating
	Hybrid PeruArg – $Peru_{UnisexPeru}$	Hybrid Peruvian/ Brazilian-1 – Peruvian	0.13 ± 0.09	Random mating
Rull et al. 2012	Tucumán (Arg) - Vacaria (Br)	Brazilian-1 - Brazilian-1	0.12 ± 0.06	Random mating
	Tucumán (Arg) - Pelotas (Br)	Brazilian-1 - Brazilian-1	0.14 ± 0.09	Random mating
	Pelotas (Br) - Vacaria (Br)	Brazilian-1 - Brazilian-1	0.14 ± 0.08	Random mating
Dias 2012	Pelotas (Br) - Bento Gonçalves (Br)	Brazilian-1 - Brazilian-1	0. 14 ± 0.07	Random mating
	São Joaquim (Br) - Vacaria (Br)	Brazilian-1 - Brazilian-1	0.04 ± 0.04	Random mating
	São Joaquim (Br) - Bento Gonçalves (Br)	Brazilian-1 - Brazilian-1	0.14 ± 0.07	Random mating
	Bento Gonçalves (Br) - Vacaria (Br)	Brazilian-1 - Brazilian-1	0.03 ± 0.05	Random mating
	Piracicaba (Br) - São Joaquim (Br)	Brazilian-1 - Brazilian-1	0.55 ± 0.09	Moderate
	Piracicaba (Br) - Bento Gonçalves (Br)	Brazilian-1 - Brazilian-1	0.56 ± 0.05	Moderate
	Piracicaba (Br) - Vacaria (Br)	Brazilian-1 - Brazilian-1	0.53 ± 0.10	Moderate

Reference	Population - mating combination	Morphotypes combination	ISI	Isolation level
Rull et al. 2013	Xalapa (Mex) - Tucumán (Arg)	Mexican - Brazilian-1	0.82 ± 0.06	High
	Xalapa (Mex) - Vacaria + Pelotas (Br)	Mexican - Brazilian-1	0.89 ± 0.02	High
	Xalapa (Mex) - La Molina (Peru)	Mexican – Peruvian	0.74 ± 0.03	High
Devescovi et al. 2014*	Tucumán (Arg) - Ibague (Col)	Brazilian-1 – Andean	1	High
	Xalapa (Mex) - Ibague (Col)	Mexican – Andean	0.94	High
	La Molina (Peru) - Ibague (Col)	Peruvian – Andean	0.65	Moderate-High

*ISI values were estimated from Table 1.

found them fully compatible. This result was later confirmed by a study with wild flies covering five populations from south and southeast Brazil that also belonged to the Brazilian-1 morphotype (Dias 2012). On the other hand, the evaluation of the compatibility between populations from the Mexican morphotype and those from other morphotypes (Peruvian and Brazilian-1) showed strong incompatibility with no difference of the time of sexual activity when compared to the Brazilian-1 morphotype (Rull et al. 2013). Devescovi et al. (2014) expanded the evaluation of the Andean morphotype, confirmed the mating isolation with populations of the Peruvian morphotype and extended it to those of the Brazilian-1 morphotype. Two recent reviews of this subject can be found in Cladera et al. (2014) and Vaníčková et al. (2015a). Cladera et al. (2014) discuss the implications of mating compatibility for SIT application against *A. fraterculus*, while Vaníčková et al. (2015a) discuss the data obtained from mating incompatibility among Brazilian populations in a broader perspective, including sexual behavior, post-zygotic studies, and chemical ecology.

Are results from walk-in field cage tests reliable?

There is no doubt that mating compatibility studies carried out in small cages under laboratory conditions can result, because of the high densities and close proximity of flies due to the limited space, in interspecific matings that normally would not occur under natural or even semi-natural conditions. The unreliability of small cage mating tests and the need to develop a field cage method that reflects more the natural situation was realized at the early stages of fruit fly SIT programs (Boller 1977, Prokopy and Hendrichs 1979, Aluja et al. 1983). In addition, Japanese *Z. cucurbitae* researchers found that the mating competitiveness of mass-reared vs. wild melon flies varied depending on the size of laboratory cages and fly density. As size increased, the competitiveness of wild flies increased, whereas that of the laboratory-reared flies decreased, confirming the need to carry out these assessments under walk-in field cage conditions to eliminate this distortion (Koyama 1982, Kuba et al. 1984).

Since then, the field cage test that is routinely used in SIT programs around the world has gradually evolved (FAO/IAEA/USDA 2014). After several decades of experience with the Mating Performance Field Cage Test a wealth of information confirmed its reliability. Despite this wealth of data, some still might question the usefulness of walk-in field cages as reliable tools to evaluate mating compatibility within the Tephritidae. Nevertheless, all evidence points towards a positive answer with the most convincing arguments being that a) under the same experimental conditions, using a field-caged host tree, an entire range of outcomes can be obtained: from full mating compatibility among some populations to complete assortative mating among others, b) these outcomes can be replicated by different research teams, and c) they have been fully endorsed from evidence obtained following simultaneously molecular, morphological and other approaches. The capacity of the field cage test to measure the degree of sexual compatibility was again shown in these recent studies described above, involving the *A. fraterculus* cryptic species and *B. dorsalis* species complexes, helping to define species limits and even leading to synonymization in the case of the *B. dorsalis* complex (Schutze et al. 2015a).

As a standard index, the Index of Sexual Isolation (ISI, see Box 1) has been found adequate to provide a measure of the level of mating isolation between populations (Table 1). Based upon the accumulated data, the following scale is proposed: ISI between -0.2 and 0.2 for random mating (or higher range provided that the 95% confidence interval includes zero), ISI between 0.2 and 0.5 for moderate yet statistically significant isolation (when the 95% confidence interval does not include zero), ISI between 0.5 and 0.7 for moderate to high isolation and ISI above 0.7 for strong isolation (Table 1). In addition, other relevant measures, such as, Relative Isolation Index (RII), Male Relative Performance Index (MRPI), Female Relative Performance Index (FRPI) (see Box 1), mating location and latency to mate also contribute to understand the mechanisms involved in mating isolation. For instance, the RII was shown to be more sensitive to slight changes in the number of mating pairs obtained from the different mating combinations (FAO/IAEA/USDA 2014); MRPI and FRPI provide information on the readiness of each sex from the two populations to mate and this has proven to be a reliable indicator of how populations and strains can be more or less competitive (males) or receptive (females); a different mating location of the mating pairs has been explained by the occurrence of spatial isolation; and differences in the timing of sexual activity of the male and female flies has reflected temporal isolation.

How to further refine and obtain additional information from field cage tests?

Although mating compatibility studies in walk-in field cages have been used to delimit species boundaries, there are variants to the standard protocol that can contribute to complete the picture of the potential acting isolation mechanisms. Here we discuss some of these possible variants and provide examples in which these modifications were found to be beneficial.

Changing the sex ratio or replacing flies

The general recommendation for the Mating Performance Field Cage Test is to release the flies at a 1:1 male:female ratio. However, this can bias measures of pre-zygotic compatibility for populations which show subtle differences in the timing of mating. For instance, the already mentioned study of Schutze et al. (2013) revealed slight differences in the time of sexual activity of the populations and species under study. Therefore the authors were concerned about the possibility that males and females of the population that engaged in earlier matings deprived males of the population that engaged in sexual activities later from a potential mating with a heterotypic female (as these would have been removed already as part of the procedure to extract formed mating pairs from the field cage, see Box 1), even in the absence of sexual incompatibility. Therefore, the authors proposed a protocol to use a 1:2 male:female ratio in the field cage to ensure an excess of females allowing heterotypic crosses independent of timing of mating. Alternatively, mating pairs can be replaced with virgin flies of the same population as they form. This way, the cage always would contain the same number of males and females of each population or origin, independent of the number of mating pairs that formed and have been removed. However, the latter mentioned protocol may disturb the flies in the field cage and newly released males will lack the acclimatization period. Therefore, our recommendation is to keep the male:female ratio at 1:1 and only if differences in time of mating are revealed, then consider the possibility of changing the sex ratio.

Switching the time of sexual activity

One alternative to avoid the effect of a different timing of sexual activity on mating compatibility is to attempt matching the mating time of flies from different populations. This can potentially be achieved by maintaining the flies during their pre-copulatory period, which depending on the species can vary between 2–30 days, under different light regimes to synchronize the peaks of their mating times. This approach was used by Vera et al. (2006) when they evaluated an *A. fraterculus* population from Peru and one from Argentina, which showed mating incompatibility due to differences in their timing of sexual activity. While Argentinean flies mated early in the morning (mean latency to mate < 1h), Peruvian flies became sexually active 3–4 h later. To synchronize their sexual activity periods, Peruvian flies were held after emergence for two weeks in a room in which the lights were turned on 4 h earlier than in the room that contained the Argentinean flies. Flies were released in the field cages at sunrise under natural light conditions. Results showed that although Peruvian flies advanced their mating peak period by approximately one hour, and hence, the overlap of the timing of sexual activity between the two populations increased, the number of heterotypic matings remained low, confirming the presence of strong sexual isolation. Further research with other morphotypes and species complexes is needed to confirm whether this approach can be more widely applied to dissect and better understand mating incompatibility.

What material to select for the evaluations?

In many cases, sourcing of the flies can be problematic as often populations, morphotypes, or species can be hard to find in nature or for which collection, transport, export or import permits are difficult to obtain. In those cases, compatibility studies require the establishment of laboratory colonies and this raises concerns on the extent, degree and impact of laboratory adaptation on the sexual behavior of the flies. Reduction of male competitiveness, changes in male courtship, and increase in female receptivity associated to mass-rearing are frequently documented in the literature (Iwahashi 1996, McInnis et al. 1996, Briceño and Eberhard 1998, Rull et al. 2005). To avoid this, the use of wild flies or strains only recently introduced into the laboratory and maintained using natural fruit as oviposition and larval rearing substrate under relaxed conditions (i.e. "wildish" strains) is recommended.

The wide range of mating compatibility studies performed over the last years has generated data that allow assessing the impact of laboratory rearing on mating compatibility. Two populations of *B. dorsalis* originating from central and southern Thailand showed initially significant positive assortative mating as a result of differences in latency to mate and the degree of male and female fly participation in mating. After one year of rearing under the same laboratory conditions, flies from the same populations mated at random and no differences in latency or female receptivity were detected (Schutze et al. 2015b). The change from positive assortative to random mating was attributed by the authors to changes in mating latency and relative participation of the sexes in mating. The change in mating time was associated with adapting the populations originating from different latitudes to the same local environmental conditions, whereas the reasons for differential mating participation remain to be resolved. The authors concluded that for the purpose of species delimitation, taking alone the results of the wildish colonies would have resulted in wrong conclusions. As such, they recommended the use of laboratory cultures after the initial adaptation to the same laboratory conditions but not exceeding the sixth generation. Different results however, were obtained with populations of *A. fraterculus*. After three years of identical laboratory rearing at the same facility, populations from two different morphotypes remained isolated even though populations originated from different latitudes (Cáceres et al. 2009). Differences in the timing of mating and in the chemical profiles of male-borne volatiles were, among others, possible explanations for this mating isolation. Taken together, results showed that subtle differences in mating time of the *B. dorsalis* populations probably originated from some kind of local adaptation of the flies to particular environmental conditions in the area of origin, can be removed under identical rearing conditions, while for the case of *A. fraterculus* the initial isolation barriers between the two morphotypes remained even after a longer period of identical rearing conditions. It could be argued in the *B. dorsalis* case, that the differences were not strong enough to keep the populations isolated when maintained under similar environmental conditions, while in the *A. fraterculus* case they were strong enough to persist. Since it is always very difficult to know in advance the strength of isolating mechanisms, it is safer

to use wild flies or populations recently introduced into the laboratory. We concur also with the recommendation of Schutze et al. (2015b) to use recently colonized populations that have been given some time to adapt to similar environmental conditions when subtle adaptations to the environment are expected. However, one should not neglect colonies that have been cultured for a longer time if wild material is scarce or difficult to obtain.

Releasing females from one origin only

Wee and Tan (2000) used a variant of the Mating Performance Field Cage Test in which females from one population were released rather than females from two populations. This approach was evaluated with *B. papayae* and *B. carambolae* and the data indicated that *B. papayae* females were selective and preferred to mate with con-specific males, while *B. carambolae* females did not discriminate between males of the two populations. The same no-choice approach was used by Cáceres et al. (2009) with *A. fraterculus* populations from Peru and Argentina. High ISI values were obtained both when females from two origins were used and when females from one origin were used. Although releasing both sexes is closer to the natural situation, the use of females from one population may be justified in well-defined cases when they do not seem to bias the results obtained. This variant avoids also that females from one population sequester the males from their own origin. On the other hand, using only wild females of the target population is closer to the situation when mass-rearing and releasing sterile males of a genetic sexing strain in an SIT program. Therefore when assessing the competitiveness of males of genetic sexing strains, the preferred option is to have laboratory males (non-irradiated or irradiated) compete with wild males from a target population in the absence of laboratory females.

Evaluating hybrids

Hybrids derived from populations or morphotypes that show sexual isolation under walk-in field cage conditions, but that can produce progeny under high-density conditions using small laboratory cages, can be a fertile ground to understand speciation processes. For example, male hybrids obtained under such conditions from matings between *A. fraterculus* populations from Peru and Argentina produced a pheromone that was a mix of the parental pheromones (Cáceres et al. 2009), and the hybrid females preferred mating with hybrid males rather than with males of the parental populations (Segura et al. 2011). Interestingly, parental females did not discriminate between the males of their own morphotype and the hybrid males (Cáceres et al. 2009), probably indicating that parental females from both morphotypes use the compounds present in the pheromones of their con-specific males to recognize them, even when hybrid males' pheromone had different proportions of these compounds and, what is more,

the presence of additional compounds that are not part of the parental pheromone. The marked preference of hybrid females for hybrid males induced Segura et al. (2011) to postulate that hybrid speciation has the potential to occur in nature and could be one of the reasons for the high number of taxonomic entities that comprise this *A. fraterculus* cryptic species complex. In addition, it showed the probable significance of chemical cues for species recognition within the *A. fraterculus* cryptic species complex, highlighting the need for specific research on this topic.

The role of pheromones in species recognition

The ability to find a mate depends on the recognition between members of the same species. The sensory system is a significant component of sexual communication across many insect taxa and plays an important role in pre-mating isolation (Symonds and Elgar 2008, Smadja and Butlin 2009). These sensory systems consist of sensory neuronal receptors and neuronal pathways that allow discrimination of individual sensory modalities and their mutual integration. In many species, visual, acoustic, tactile and chemical signals are all involved in the pre-mating communication process. If stabilizing selection acts on the sexual communication system, any change made can lead to a process of evolutionary divergence which may be the initial step towards behavioral isolation within a process of speciation (Symonds and Elgar 2008, Smadja and Butlin 2009). Consequently, understanding the role of each signal on the evolution of reproductive barriers is likely to be important for understanding speciation.

Anastrepha fraterculus mating system and chemical communication

As in many others, but not all, tephritid flies, *A. fraterculus* has a lek mating system (Malavasi et al. 1983) in which males aggregate and emit different visual, acoustical and chemical signals to attract females to a courting arena (a behavior known as "calling"). Once in the lek, the female fly assesses the multimodal calling behavior of a number of calling males before selecting a mate among the available ones. There is no data available about what modality is crucial in female choice. In general, chemical signals are supposed to play an important role both in female attraction and female choice. Long range (pheromones) and close range (cuticular hydrocarbons) signals are involved. Pheromones are produced in salivary and anal glands (Nation 1989) and are released by expanding the lateral pouches of the pleural abdominal cuticle, and the periodic protrusion of anal tissue which forms a pouch. Usually, this behavior is accompanied by rapid wing movements (fanning) that produce vibrations and a flux of air over the body surface, thought to enhance the diffusion of pheromone (Nation 1989, Segura et al. 2007, Lima-Mendonça et al. 2014, Bachmann et al. 2015). Although the exact factors of mate selection by the females are not fully understood, male copulatory success seems to be related, at least in part, to pheromone calling activity (Bachmann et

al. 2015), the male location within the lek (Segura et al. 2007), and specific morphological traits such as wing width and thorax length (Sciurano et al. 2007) as well as eye length (Segura et al. 2007). Female mating preference seems therefore to be the result of the integration of multiple stimuli. Although there are no formal recordings, observations in walk-in field cages revealed that males belonging to different morphotypes called in the same tree or even formed mixed leks (for *A. ludens* and *A. obliqua* see also Aluja et al. 1983) from which males courted females from the other morphotypes, suggesting that species recognition occurs during courtship and probably the females play a key role at the time of mate selection to avoid cross-mating. As such *A. fraterculus* females possibly use all the signals emitted by the males during courtship for species recognition, especially those that present differences among morphotypes.

The volatiles emitted by the males seem to vary both in quality and quantity across morphotypes within the *A. fraterculus* complex (Cáceres et al. 2009, Břízová et al. 2013, Vaníčková et al. 2015b, Table 2), and appear to be playing a role in more long-range interactions and courtship, while the less volatile signals and cuticular hydrocarbons mediate close-range chemical interactions (Vaníčková et al. 2015b). In addition, females from the different morphotypes showed differences in their antennal response (Břízová et al. 2013, Milet-Pinheiro et al. 2014), i.e. while the antenna of Tucumán, Argentina (Brazilian-1 morphotype) females responded to (*Z*,*E*)-a-Farnesene, (*E*,*E*)-a-Farnesene, Epianastrephin, (*Z*)-3-Nonen-1-ol and (*E*,*Z*)-3,6-Nonadien-1-ol (Břízová et al. 2013), the antenna of Alagoas (Brazilian-3 morphotype) females responded to a- Pinene, Limonene, (*Z*)-3-Nonen-1-ol and (*E*,*Z*)-3,6-Nonadien-1-ol, (S,S)-(-)-Epianastrephin (Milet-Pinheiro et al. 2014). Although it has been shown that these compounds attracted females of the Brazilian-3 morphotype and that the females responded equally to an artificial blend of the synthetic compounds and to volatiles collected from males, it is still not known whether these differences observed at the chemical and electrophysiological level are translated into different behaviors, since the behavioral response to the pheromone of the Argentinean populations is not known yet. Therefore, in order to elucidate this, it is necessary to determine the degree of intra- and inter-specific variability in males' pheromone and in female' pheromone perception and especially to conduct behavioral tests in which females are faced with the pheromones of con-specific and hetero-specific males simultaneously to determine the role of pheromone signaling for species recognition. The same would be interesting to perform with cuticular hydrocarbons that are supposed to mediate the close-range chemical recognition.

The response of *Anastrepha fraterculus* females to calling males

Walk-in field cages can also be useful to explore the role of chemical communication in mate finding and species recognition and the Manual for Product Quality Control for Sterile Mass-Reared and Released Tephritid Fruit Flies (FAO/IAEA/USDA 2014) provides a protocol to carry out pheromone attraction tests in field cages. This protocol

Table 2. Chemical profiles of the male-borne volatiles from different populations from the *Anastrepha fraterculus* complex.

Compound		Morphotype									
		Brazilian-1									
		Tucumán	Tucumán	Bento Gonçalves	Bento Gonçalves	Pelotas	Pelotas	São Joaquim	São Joaquim	Piracicaba	Vacaria
	RI	1	2	2	3	2	3	2	3	2	2
3-Hexanone	791	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
2-Hexanone	796	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
Hexanal	801	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
α-Pinene	938	wi	wi	wi	+	wi	++	wi	++	wi	wi
Camphene	956	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
β-Pinene	985	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
Myrcene	991	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
Ethyl hexanoate	996	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
p-Cymene	1022/1030	wi	+	+	wi	+++	wi	+	wi	+	+
2-Ethylhexan-1-ol	1029/1030	wi	++	++	wi	+	wi	+	wi	+	+++
Limonene*[2]	1041/1035	-	++	++	+++	+++	+++	+++	+++	+	++
5-Ethenyldihydro-5-methyl-2(3H)-furanone	1044	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
Indane	1046	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
(Z)-β-Ocimene	1050/1035	wi	++	+	wi	+	wi	+	wi	++	tr
(E)-β-Ocimene	1059	+	wi	wi	wi	wi	wi	wi	wi	wi	wi
Linalool	1101	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
(Z)-Nonanal	1107	+	tr	++	wi	+	wi	+	wi	+	+++
Camphor	1141	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
(Z)-3-Nonen-1-ol*[12]	1159/1158	+++	+	++	++	tr	++	+	++	+	+
(E,Z)-3,6-Nonadien-1-ol*[12]	1161/1160	wi	++	+++	+++	tr	++	++	+++	+++	+
Decenal	1210	wi	tr	+	wi	+	wi	+	wi	+	+
Bornyl acetate	1293	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
(E)-α-Bergamontene	1435	-	wi	wi	wi	wi	wi	wi	wi	wi	wi
(Z)-β-Farnesene	1448	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi

Compound		Morphotype									
		Brazilian-1									
		Tucumán	Tucumán	Bento Gonçalves	Bento Gonçalves	Pelotas	Pelotas	São Joaquim	São Joaquim	Piracicaba	Vacaria
	RI	1	2	2	3	2	3	2	3	2	2
(Z,E)-α-Farnesene*[12]	1495/1492	+++	+	+	+	+	—	+	+	+	+
Germacrene D	1498/1502	wi	tr	+	wi	+	wi	+	wi	+	+
Suspensolide	1506/1509	+++	+	++	wi	+	wi	+	wi	++	+
(E,E)-α-Farnesene*[12]	1512/1510	+++	+++	++	+++	++	+++	+++	+++	+++	+
Anastrephin	1617/1610	+++	+	+	wi	+	wi	+	wi	+	+
Caryophyllene oxide	1606	wi	wi	wi	wi	wi	wi	wi	wi	wi	wi
Epianastrephin*12	1621/1625	+++	+	+	+	+	+++	++	+	+	+
Benzoic acid	wi	+++	wi	wi	wi	wi	wi	wi	wi	wi	wi
β-Bisaboline	wi	-	wi	wi	wi	wi	wi	wi	wi	wi	wi

1- Cáceres et al. 2009; 2- Břízová et al. 2013; 3- Vaníčková et al. 2015; 4- Milet-Pinheiro et al. 2014

*1 2= Břízová et al. 2013 + Milet-Pinheiro et al. 2014- antennally active; *2 = Milet-Pinheiro et al 2014- antennally active

+++ = large amounts (^{3} 19%); ++ = medium amounts (7-19%); + = small amounts (1-7%); tr = traces (<0.1%); - = no detectable amounts; wi = without information

Table 2. Continued.

		Morphotype							
Compound		**Brazilian-3**			**Peruvian**	**Andean**			
		Alagoas	**Alagoas**	**Alagoas**	**La Molina**	**Ibague**	**Sibundoy**	**Duitama**	**Cachipay**
	RI	**2**	**4**	**3**	**1**	**3**	**3**	**3**	**3**
3-Hexanone	791	wi	tr	wi	wi	wi	wi	wi	wi
2-Hexanone	796	wi	+	wi	wi	wi	wi	wi	wi
Hexanal	801	wi	+	wi	wi	wi	wi	wi	wi
α-Pinene	938	wi	+	+	wi	+++	+++	+++	+++
Camphene	956	wi	tr	wi	wi	wi	wi	wi	wi
β-Pinene	985	wi	+	wi	wi	wi	wi	wi	wi
Myrcene	991	wi	+	wi	wi	wi	wi	wi	wi
Ethyl hexanoate	996	wi	+	wi	wi	wi	wi	wi	wi
p-Cymene	1022/1030	++	+	wi	wi	wi	wi	wi	wi
2-Ethylhexan-1-ol	1029/1030	tr	+	wi	wi	wi	wi	wi	wi
Limonene*[2]	1041/1035	+++	+++	+++	+	+++	+++	+++	+++
5-Ethenyldihydro-5-methyl-2(3H)-furanone	1044	wi	+	wi	wi	wi	wi	wi	wi
Indane	1046	wi	+	wi	wi	wi	wi	wi	wi
(Z)-β-Ocimene	1050/1035	—	+	wi	wi	wi	wi	wi	wi
(E)-β-Ocimene	1059	wi	+	wi	+++	wi	wi	wi	wi
Linalool	1101	wi	tr	wi	wi	wi	wi	wi	wi
(Z)-Nonanal	1107	+	wi	wi	+	wi	wi	wi	wi
Camphor	1141	wi	tr	wi	wi	wi	wi	wi	wi
(Z)-3-Nonen-1-ol*[12]	1159/1158	+	tr	++	+	+	+	+	+
(E,Z)-3,6-Nonadien-1-ol*[12]	1161/1160	+++	+	+++	wi	+	+	+	+
Decenal	1210	+	wi	wi	wi	wi	wi	wi	wi
Bornyl acetate	1293	wi	+	wi	wi	wi	wi	wi	wi
(E)-α-Bergamontene	1435	wi	+	wi	+++	wi	wi	wi	wi
(Z)-β-Farnesene	1448	wi	+	wi	wi	wi	wi	wi	wi
(Z,E)-α-Farnesene*[12]	1495/1492	+	+	+	-	+	+	+	+

	Morphotype							
Compound		**Brazilian-3**			**Peruvian**	**Andean**		
		Alagoas	**Alagoas**	**Alagoas**	**La Molina**	**Ibague**	**Sibundoy**	**Duitama**	**Cachipay**
	RI	**2**	**4**	**3**	**1**	**3**	**3**	**3**	**3**
Germacrene D	1498/1502	+	tr	wi	wi	wi	wi	wi	wi
Suspensolide	1506/1509	++	+	wi	+++	wi	wi	wi	wi
(E,E)-α-Farnesene*[12]	1512/1510	+	+++	—	+++	+	+	+	+
Anastrephin	1617/1610	+	+	wi	+++	wi	wi	wi	wi
Caryophyllene oxide	1606	wi	tr	wi	wi	wi	wi	wi	wi
Epianastrephin*12	1621/1625	+	+	+	+++	+	+	+	+
Benzoic acid	wi	wi	wi	wi	wi	-	wi	wi	wi	wi
β-Bisaboline	wi	wi	wi	wi	wi	+++	wi	wi	wi	wi

1- Cáceres et al. 2009; 2- Břízová et al. 2013; 3- Vaníčková et al. 2015; 4- Milet-Pinheiro et al. 2014

*1 2= Břízová et al. 2013 + Milet-Pinheiro et al. 2014- antennally active; *2 = Milet-Pinheiro et al 2014- antennally active

+++ = large amounts (³ 19%); ++ = medium amounts (7-19%); + = small amounts (1-7%); tr = traces (<0.1%); - = no detectable amounts; wi = without information

has the advantage that it can provide information on both relatively long and close distance recognition. Several examples of the use of walk-in field cages to assess tephritid attraction to different odor sources can be found in the literature (Webb et al. 1983, Shelly 2000a, 2000b, López-Guillén et al. 2011, Liendo et al. 2013, Milet-Pinheiro et al. 2014). However, the usefulness of walk-in field cages to assess preference of female flies for pheromones of con-specific vs hetero-specific males has not been evaluated yet.

To determine whether *A. fraterculus* male pheromones from two different populations were equally attractive to con-specific and hetero-specific females, the methodology of Liendo et al. (2013) was adapted, which in turn is an adaptation of the methodology proposed by Shelly (2000a) for *C. capitata* (Box 2). The experiments were carried out in a greenhouse of the FAO/IAEA Insect Pest Control Laboratory (IPCL) in Seibersdorf, Austria and involved one population from Piracicaba, São Paulo, Brazil and one from Tucumán, Argentina. Obtained results show that orientation and location of the female flies in the field cages was affected by the presence of calling males (Table 3). In the control cages in which only one type of male was calling (the other containers were empty controls), it was found that Piracicaba females preferred the tree and the leks with calling males instead of those with no males. In a similar way, Tucumán females preferred the artificial leks with males instead of the empty containers. In the cages in which males of the two populations were calling, females showed no particular preference; leks of the two origins were visited at equal rates. This is in agreement with the fact that these populations have been both identified as belonging to the Brazilian-1 morphotype. However, considering previous chemical and behavioral data, some differences could have been expected. Quantitative differences were found in the pheromone compounds of these populations. (Břízová et al. 2013, Table 2). In addition, some mild, yet significant, mating isolation associated with some spatial distribution within the field cage (homotypic couples were found in different areas of the cage) was found between Tucumán and Piracicaba (Vera et al. 2006) and between Piracicaba and other Brazilian-1 morphotype populations from Southern Brazil (Dias 2012). It can be argued that these differences could have affected the frequency of female visits to a lek; however our results suggest that mate recognition may have occurred later, when the females were already in closer vicinity of the males and other signals (visual, vibratory or chemical related to differences in the cuticular hydrocarbons [Vaníčková et al. 2012]) might have been important.

The results presented above showed that walk-in field cages can be used to measure the response of *A. fraterculus* females towards volatiles emitted by *A. fraterculus* calling males and this opens opportunities to better understand the mechanisms behind mating isolation between morphotypes. The experimental protocol, however, entails two issues that need to be resolved: first, the comparisons are restricted to populations that have the same timing of sexual activity; second, it is not possible to control the amount of chemical stimuli released as the number of males that are calling at any particular time within the artificial lek cannot be controlled. The former can be solved by changing the photoperiod of those populations that have different time of sexual activity, while for the latter it is advisable to monitor the number of males calling during the test, as for

Box 2. Female orientation to male pheromone.

For the purpose of evaluating the response of females towards the male pheromone, an indicator of intra-specific recognition in lek-forming tephritids, field cages are set up with two potted trees inside, which are virtually divided into two sectors. Each sector contains one tree. The test involves two steps. In the first, it is determined whether females orient to the pheromone of con-specific males and 25 mature virgin females of a given population are released into the field cage during the period of sexual activity. Fifteen minutes later, 3 "artificial leks" consisting of cylindrical metal wire-mesh containers (3 cm diam., 7 cm long) with 7 sexually mature males inside (Figure 1) are hung in one of the trees while 3 containers without males are hung in the other tree. Once the females and the containers are placed in the cage, an observer scores the number of females in each sector, in each tree and those on the artificial lek. These parameters are recorded every 15–20 minutes, with at least six observations made during the period of mating activity in each cage. If significant differences in preference towards male-containing leks are found (see below), it is possible to continue with the second step, which involves the evaluation of the ability of the females to distinguish between male pheromones of different populations, morphotypes or species. To do so, 25 mature virgin females from two different populations are released inside each field cage, into which artificial leks with males from the same two populations are hung from the trees. Each tree houses 3 containers from one population. In order to identify females from each origin, the day before the test flies are painted with a dot of water based acrylic in their thorax. Colors are randomly assigned and permuted every day. Observations are performed as described previously. To prevent pheromone contamination, trees and cages are washed with pressurized tap water at the end of each test. The containers used for the artificial leks are also washed with hot water and dried in an oven at 150°C for one hour. To avoid females behaving differently due to adaptation of sensory organs and due to habituation in the brain, males and females should be kept in separate rooms so that females have no previous experience with the male pheromones. Data from the daily observations within each cage (i.e. replicate) are added to obtain an overall measure of the location of the females throughout the experiment. Statistical analysis is done by means of a paired t-test or the corresponding non-parametric Wilcoxon paired test as appropriate, in which the number of flies registered in the area, in the tree and at the artificial lek is compared between sectors. In the first step (i.e. field cages with empty control containers), significant differences indicate that females use chemical cues to find males. In the second step (i.e. comparing male types), significant differences indicate that females orient towards one male type, while non-significant differences indicate either lack of capacity to discriminate or no preference towards any male type.

Figure 1. Walk-in field cage set up to evaluate female response to male pheromone: **a** artificial lek hanging from the tree **b** *Anastrepha fraterculus* female over an artificial lek.

Table 3. Results from the Wilcoxon sign rank test to evaluate orientation *A. fraterculus* females from different populations to artificial leks (containers with sexually mature males).

			Area		Tree		Lek	
Lek combination	**Female**	**N**	**Z**	**p-level**	**Z**	**p-level**	**Z**	**p-level**
Control†	Piracicaba	5	0,73	0,4652	2,02	0,0431	2,02	0,0431
Control	Tucumán	8	1,12	0,2626	1,12	0,2626	2,20	0,0277
Tucumán – Piracicaba	Piracicaba	7	0,54	0,5896	1,35	0,1763	0,40	0,6858
Tucumán – Piracicaba	Tucumán	7	1,86	0,0630	0,51	0,6121	0,53	0,5930

†In the control cages one of the two trees had empty containers with no males inside.

this species, the numbers of males calling was correlated with the amount of pheromone released (Bachmann et al. 2015). Along with the monitoring of calling males, several mating cages can be operated in parallel, in which males and females are released at the same time of the pheromone attraction test to confirm female readiness to mate.

An alternative approach to solve differences in calling times and in number of calling males is the use of collected volatiles or artificial blends made of synthetics pheromone analogs in the right proportions. Such an approach has been evaluated by Milet-Pinheiro et al. (2014) with promising results. These authors found that females from a population from Coruripe, Brazil (Brazilian-3 morphotype) were attracted to antennally active pheromone component candidates either used in a synthetic mix or singly. Individual compounds were significantly less attractive in comparison with the blend. In addition, this synthetic blend was equally attractive to females when compared with the volatiles collected from the males, both in laboratory and in field cages. Although the aim of Milet-Pinheiro et al. (2014) research was to find potent female attractants, the same methodology can be applied to evaluate the role of pheromones in mate recognition of closely related species. Similar attractiveness of volatile extracts of calling males and live calling males was reported also by López-Guillén et al. (2011) who found that the number of *A. obliqua* females captured by traps with volatile extracts of calling males was not significantly different to that caught by live calling males. However, to substitute the natural blend trapped from calling males with synthetics, it is necessary to have access to synthetic compounds (which may not be commercially available and its synthesis may be quite expensive) and to assess technological problems associated with dispenser type(s) that guarantee the pheromone release in comparable concentration and compound ratios like naturally calling males.

Concluding remarks

Unlike small laboratory cage tests, walk-in field cage tests have shown to be reliable and powerful tools to measure the level of mating compatibility among different species and populations of a putative single species. This experimental arena under semi-natural con-

ditions can also provide good information on the types of pre-zygotic isolation barriers that contribute to reproductive incompatibility. The intermediate scale of field cages, i.e. between small laboratory cages and open field observations, allows this experimental approach to be implemented in several places and without the need of much infrastructure. It also permits the evaluation of the behavior of flies coming from different regions provided adequate bio-security measures are in place (Cayol et al. 2002, Vera et al. 2006, Rull et al. 2012, 2013, Schutze et al. 2013, Devescovi et al. 2014) and, if desired, under different environmental conditions. Minor modifications of the general protocol can contribute to assess the relative importance of different isolation mechanisms, particularly when subtle differences in the time of mating activity are observed. In order to unravel which signals are crucial to recognize mates from their own species and hence avoid cross-mating, other experimental procedures might be necessary.

Walk-in field cages have also shown their usefulness in determining the role of chemical signals in species recognition. The use of calling males ensures the timely release of the compounds at the right proportions while the use of volatile compounds, instead of male flies, allows evaluating populations in which flies are active at different times of the day. Field cage tests can be accompanied by laboratory tests to assess antennal responses in the females to the various compounds present in the male pheromone. In addition, the role of close distance chemical signals such as cuticular hydrocarbons and other non-chemical signals such as acoustic and visual stimuli remains to be further explored.

Within the context of SIT application, the use of walk-in field cages to asses mating compatibility has not been restricted to the Tephritidae. For tsetse flies see Mutika et al. (2001, 2013) and for an evaluation of mating compatibility among codling moth, *Cydia pomonella* (L) populations from different regions of the world see Taret et al. (2010).

Overall, the so far attempted evaluation of different testing conditions has shown to provide a better understanding of the pre-zygotic isolation barriers occurring when mating incompatibility is found. As a general recommendation, the most convenient approach in a novel situation is to start with the traditional protocol and only in those cases in which sexual isolation is associated with temporal isolation, adaptations to the standard protocol should be made to evaluate mating compatibility. The experimental approach reviewed here provides a good source of information to delimit reproductive species boundaries. However, in any case it is advisable to follow this methodology as part of an integrative taxonomy approach, including also molecular, physiological and morphological traits in the assessments in order to achieve robust species delimitation.

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Relevant genetic differentiation among Brazilian populations of *Anastrepha fraterculus* (Diptera, Tephritidae)

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Abstract

We used a population genetic approach to detect the presence of genetic diversity among six populations of *A. fraterculus* across Brazil. To this aim, we used Simple Sequence Repeat (SSR) markers, which may capture the presence of differentiative processes across the genome in distinct populations. Spatial analyses of molecular variance were used to identify groups of populations that are both genetically and geographically homogeneous while also being maximally differentiated from each other. The spatial analysis of genetic diversity indicates that the levels of diversity among the six populations vary significantly on an eco-geographical basis. Particularly, altitude seems to represent a differentiating adaptation, as the main genetic differentiation is detected between the two populations present at higher altitudes and the other four populations at sea level. The data, together with the outcomes from different cluster analyses, identify a genetic diversity pattern that overlaps with the distribution of the known morphotypes in the Brazilian area.

* Both authors contributed equally to this work

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Keywords
Anastrepha fraterculus, microsatellites, population genetic differentiation, morphotypes

Introduction

The South American fruit fly *Anastrepha fraterculus* Wiedemann (Diptera: Tephritidae) belongs to the *fraterculus* group, which comprises a total of 34 formally described species (Norrbom et al. 2012) that can be distinguished only by minor morphological characters. The major diagnostic character for species within this group is the aculeus apex, which shows a large degree of intraspecific variation (Araujo and Zucchi 2006) as a result of genetic and environmental factors (Aluja 1994, Smith-Caldas et al. 2001).

The nominal species *A. fraterculus* is widely distributed from the Rio Grande Valley in northern Mexico to central Argentina, infesting over 100 hosts (Norrbom 2004), thus being a species of major economic importance in Brazil and other countries in South America (Steck 1991, Steck 1999, Zucchi 2008). Its distribution in South America was thought to be in two broad and unconnected bands along the east coast and the western and northern edges of the continent with a hiatus comprising the Amazon basin (Steck 1999, Malavasi et al. 2000). Yet, recent collections have recorded this species in the Brazilian Amazon in the states of Amapá, Pará, Tocantins, and Maranhão infesting 10 different hosts (Zucchi et al. 2011).

A. fraterculus has long been reported to show extensive morphological variation along its geographic distribution (Lima 1934, Stone 1942, Steck 1991, 1999). In his taxonomic review of the genus *Anastrepha*, Stone (1942) stated "it is possible that it will eventually be found to represent a complex of species rather than a single one". Since then, a good deal of research has documented and concluded that the nominal species *A. fraterculus* actually comprises an unresolved complex of cryptic species. Evidence comes from studies on morphological variation (Steck 1999, Hernández-Ortíz et al. 2012, for review), multivariate morphometric analyses (Hernández-Ortíz et al. 2004, 2012), differences in host use (Steck 1999, for review), behaviour (Yamada and Selivon 2001, Selivon et al. 2005, Vera et al. 2006, Vaníčková et al. 2015), the presence and degree of reproductive isolation (Devescovi et al. 2014 and references therein), and genetic analyses (Silva and Barr 2008, for review). However, the actual number of species within the *A. fraterculus* complex and their distribution is yet to be elucidated.

Genetic studies performed on *A. fraterculus* populations so far have revealed the following putative biological entities based on geography: an Andean lineage (Steck 1991), a Mexican species (Steck 1991, Smith-Caldas et al. 2001, Barr et al. 2005 and the morphometric studies of Hernández-Ortíz et al. 2004, 2012), a Guatemalan lineage (Smith-Caldas et al. 2001), a second Venezuelan species (Smith-Caldas et al. 2001), a Peruvian lineage (Steck 1991), and three Brazilian species (Morgante et al. 1980, Smith-Caldas et al. 2001, Selivon et al. 2005, and Ruiz et al. 2007a, 2007b) (Silva and Barr 2008, for review). In addition, morphometric studies revealed that Mexican populations formed a single morphotype, which was distinct from South

American populations (Hernández-Ortíz et al. 2004). Hernández-Ortíz et al. (2004) also identified an Andean morphotype (one population from Colombia) and a Brazilian morphotype comprising two populations from Brazil (states of São Paulo and Santa Catarina) and a population from Argentina (Tucumán). A study using isozymes, karyotypes, morphometry, and crossings on populations of *A. fraterculus* from Brazil recognized two species, *Anastrepha* sp.1 *aff. fraterculus* and *Anastrepha* sp.2 *aff. fraterculus* (Selivon et al. 2005). More recently, a multivariate morphometric analysis comprising 32 *A. fraterculus* populations identified seven distinct morphotypes: a Mexican morphotype, a Venezuelan morphotype, an Andean morphotype, a Peruvian morphotype, and three Brazilian morphotypes (Brazilian 1, Brazilian 2, and Brazilian 3) (Hernández-Ortíz et al. 2012). Although Brazilian populations of the nominal species *A. fraterculus* most likely comprise at least three morphotypes, published studies are based mostly on samples from the south-eastern region, while very few populations from other regions of the country have been examined.

Previous genetic studies using DNA sequencing of mitochondrial genes from different populations suggested that both the *fraterculus* group and the *A. fraterculus* complex have a recent evolutionary history, and thus molecular markers with a higher power of resolution were required to help understand the specific/subspecific differentiation within and between populations of this group (McPheron et al. 1999, Smith-Caldas et al. 2001). Microsatellites are a class of highly polymorphic molecular markers widely distributed in the genome of eukaryotes that can be useful to clarify such patterns of gene flow and to identify the spatial locations of genetic discontinuities (population boundaries) in studies of species complexes (Chambers and MacAvoy 2000, Barbará et al. 2007, Aketarawong et al. 2014). Within the Tephritidae, microsatellites have been successfully developed and applied for several *Bactrocera* species (Shearman et al. 2006; Aketarawong et al. 2006, 2007, Augustinos et al., 2008, Virgilio et al. 2010; Drew et al. 2011), for *Rhagoletis cerasi* L. (Augustinos et al. 2011), for a few *Ceratitis* species (Bonizzoni et al. 2001, 2004, Meixner et al. 2002, Baliraine et al. 2003, 2004, Silva et al. 2003, Delatte et al. 2013, Virgilio et al. 2013), for *Anastrepha suspensa* (Loew) (Fritz and Schable 2004, Boykin et al. 2010), and for *Anastrepha obliqua* (Macquart) (Islam et al. 2011). Microsatellites have only recently been isolated in *A. fraterculus* (Lanzavecchia et al. 2014) and have proven useful for the analysis of population dynamics and differentiation across the distribution range of this polymorphic species.

From an applied perspective, the correct identification of populations and species is an important step in the implementation of biologically-based control methods such as the Sterile Insect Technique (SIT) against this fruit fly complex (Silva and Barr 2008), which represents a serious constraint for fruit production in South America and a hindrance to the export of fresh fruit from regions where it occurs. Currently, control measures for this pest species rely solely on the use of insecticide cover or bait sprays. Therefore, there is demand for the development of the SIT as it would benefit South American countries such as Argentina, Brazil, and Peru (Cladera et al. 2014). However, the application and efficiency of species-specific control methods such as the SIT are critically dependent on the correct identification of the target pest populations

and the understanding of the spatial distribution of the pest species, thus the correct delimitation of species within the *A. fraterculus* complex is paramount.

This paper is centred on the assessment of genetic diversity among *A. fraterculus* populations from distinct geographic regions across Brazil, most likely belonging to at least three distinct morphotypes (Silva et al. unpubl. data). For this purpose we used highly informative SSR markers, which may capture the presence of eventual differentiative processes across the genome in different populations.

Methods

Six populations from three regions across Brazil were sampled from 2007 to 2013 (Table 1, Figure 1). In the Northeastern region, the populations from Monte Alegre (State of Rio Grande do Norte) and from Una and Porto Seguro (State of Bahia) were sampled. In the Southeastern region, samples from São Mateus (State of Espírito Santo) and from Campos do Jordão (State of São Paulo) were examined. In the Southern region, the population from Vacaria (State of Rio Grande do Sul) was sampled. For each locality, flies emerging from fruits collected from different trees were considered. Adult females were identified as *A. fraterculus* by Dr. Elton L. Araujo (UFERSA), Dr. Keiko Uramoto (USP), Dr. Miguel Francisco Souza Filho (Instituto Biológico) and Dr. Roberto A. Zucchi (USP) using the aculeus shape following Zucchi (2000) (Table 1). Voucher specimens were deposited at the insect collection of the Escola Superior de Agricultura "Luiz de Queiroz", USP, Piracicaba, SP, and at the Universidade Estadual de Santa Cruz, Ilhéus, BA, Brazil. According to the classification of Hernández-Ortíz et al. (2012), the flies collected in Una (BA) can be classified as Brazilian morphotype 3, while those from Vacaria (RS) and Campos do Jordão (SP) as Brazilian 1 (Silva et al. unpublished data). For the samples from Monte Alegre, Porto Seguro and São Mateus, no clear-cut information is available to assign them to a specific morphotype.

Table 1. Field collected samples of *Anastrepha fraterculus* Brazilian populations.

States	Sample site	Morphotype*	Host	Coordinate	Elevation
Rio Grande do Norte (RN)	Monte Alegre	?	Guava	-6.0678W; -35.3322S	51.816m
Bahia (BA)	Una	3	Guava	-15.2933W; -39.0753S	27.737m
Bahia (BA)	Porto Seguro	?	Guava	-16.4497W; -39.0647S	48.768m
Espírito Santo (ES)	São Mateus	?	Araçá	-18.7161W; -39.8589S	35.966m
São Paulo (SP)	Campos do Jordão	1	Raspberry	-22.7394W; -45.5914S	1627.9m
Rio Grande do Sul (RS)	Vacaria	1	Guava	-28.5122W; -50.9339S	970.79m

* The Morphotype classification is based on Hernández-Ortiz et al. (2012)

Figure 1. Map of the collected samples.

Microsatellite analysis

A total of 171 *A. fraterculus* individuals collected from the above mentioned populations were assessed for their SSR variability. DNA was extracted from three legs of each single fly using the "DNeasy Blood & Tissue" kit (Qiagen, Valencia, CA) following the standard DNeasy protocol. DNA samples were screened using the following ten microsatellite loci: AfD4, AfD105, AfA7, AfA112, AfA115, AfA120, AfA122, AfA117, AfA10, and AfC103 (Lanzavecchia et al. 2014). Allele scoring was performed using an automated ABI PRISM 310 Genetic Analyser (Applied Biosystem) following Aketarawong et al. (2006).

Data analysis

The mean number of alleles (na) and mean null allele frequency (*A*n) (non-amplifying alleles due to changes in the primer binding regions), expected and observed heterozygosity were estimated using GENEPOP version 4.0.7 (Raymond and Rousset 1995) for each population. Deviation from the Hardy-Weinberg equilibrium and linkage disequilibrium, together with their critical levels after the sequential Bonferroni test (Rice 1989), were tested using GENEPOP version 4.0.7 (Raymond and Rousset 1995). The allelic Polymorphic Information Content (PIC) was derived using CERVUS (Kalinowski et al. 2007).

Microsatellite Analyzer (MSA) (Dieringer and Schlötterer 2003) was applied to estimate the pairwise *F*st values among populations (Weir and Cockerham 1984). The statistical significance of each *F*st value was assessed by comparing the observed values with the values obtained in 10,000 matrix permutations. Spatial analyses of molecular variance were investigated using SAMOVA 2.0 (Dupanloup et al. 2002). This approach identifies groups of populations that are genetically homogeneous and maximally differentiated from each other without the constraint of being geographically close. The method requires the *a priori* definition of the number of groups (*K*) of populations that exist and generates *F*-statistics (F_{SC}, F_{ST} and F_{CT}) using an AMOVA approach. Different numbers of groups (*K*) were tested, and a simulated annealing procedure permitted the identification of the composition of each of the *K* groups that maximizes the F_{CT} index (proportion of total genetic variance due to differences between groups). The program was run for two to five groups ($K = 2$ to $K = 5$) each time with the simulated annealing process repeated 100 times, starting each time with a different partition of the population samples into the *K* groups. The analysis of molecular variance (AMOVA) was carried out using ARLEQUIN software version 3.11 (Excoffier et al. 2006). Principal Coordinate Analysis (PCoA) in the program GenAIEx 6.5 (Peakall and Smouse 2012) was applied to identify the relationships among populations on the basis of their allele frequencies.

Results

SSR variability

The variability estimates describing the suitability of the ten SSR loci (AfD4, AfD105, AfA7, AfA112, AfA115, AfA120, AfA122, AfA117, AfA10, and AfC103) for detecting the presence of differentiation among the *A. fraterculus* populations are shown in Table 2. The number of alleles per locus across populations ranges from 7 to 18 with a mean of 12.9, and the mean frequency of null alleles across the loci is generally low (0.04–0.08). Moreover, the Polymorphic Information Content (PIC) estimate for each locus ranges from 0.44 (A10a) to 0.88 (A120a), and the across loci average is 0.74, suggesting that this set of loci is informative for population analyses.

Tests for Hardy-Weinberg equilibrium (HWE) using Fisher's exact test with the sequential Bonferroni correction (Rice 1989) revealed that the populations conformed to Hardy–Weinberg equilibrium (HWE) at most loci. The very few observed locus/populations combinations that were not in HWE were not concentrated at any locus or in any population. Significant linkage disequilibrium was not detected between genotypes at the ten loci. As no evidence of linkage disequilibrium between loci was assessed, these 10 loci can be considered to be independent.

Population variability and differentiation

An estimate of variability distribution in and among the six tested populations (AMOVA) indicates that 90% of the variation occurs within populations while only about 10% of

Table 2. Microsatellite variability detected across the six Brazilian *Anastrepha fraterculus* populations.

Locus	na	Min-Max	PIC
D4a	7	2–5	0.56
D105a	15	5–11	0.72
A7a	13	7–11	0.83
A112a	18	8–12	0.82
A115a	14	7–12	0.80
A120a	15	7–13	0.88
A122a	12	5–9	0.74
A117a	11	6–9	0.77
A10a	13	2–11	0.44
C103a	11	7–9	0.80
Mean	12.9	5.6–10.2	0.74

na, mean number of alleles; Min-Max, minimum and maximum number of alleles; PIC, polymorphic information content.

Table 3. Genetic variability of wild populations of *Anastrepha fraterculus* from different geographical regions in Brazil estimated using 10 SSRs.

	na	He	Ho	F_{IS}
Una-BA	7,7	0,63	0,54	0,14
Porto Seguro-BA	8,2	0,70	0,67	0,02
Monte Alegre-RN	7,5	0,68	0,57	0,20
São Mateus-ES	6,3	0,66	0,66	0,08
Campos do Jordão-SP	8,3	0,71	0,61	0,13
Vacaria-RS	8,5	0,72	0,62	0,12

na, mean number of alleles; He, expected heterozygosity; Ho, observed heterozygosity; F_{IS} fixation index.

Table 4. Spatial Analysis of Molecular Variance (SAMOVA) for different population partitions.

Number of groups (*K*)	F_{CT}	*P*	Population partition
2	0.195	0.062	(Una, Porto Seguro, Monte Alegre, São Mateus), (Campos do Jordão, Vacaria)
3	0.182	0.015	(Una, Porto Seguro, São Mateus), (Monte Alegre), (Campos do Jordão, Vacaria)
4	0.190	0.050	(Una, Porto Seguro, São Mateus), (Monte Alegre), (Campos do Jordão), (Vacaria)
5	0.196	0.068	(Una, Porto Seguro), (São Mateus), (Monte Alegre), (Campos do Jordão), (Vacaria)

total variation is detected among populations. Indeed as shown in Table 3, the intrapopulation genetic variability is similar across the six samples. As a second step, the simulated annealing approach based on the SAMOVA algorithm was applied to identify the presence of genetically homogeneous groups across the considered Brazilian populations. For this, the spatial analyses of molecular variance was performed without constraints for geographic composition of the groups. As observed in Table 4 no great differences in the F_{CT} values were observed when we increased the group number (K): four of the five simulated groupings (2, 4, and 5) produced non-significant F_{CT} values. This implies that the molecular variance due to differences between populations within each group is weak, confirming the AMOVA data. Only in one case did we observe an F_{CT} estimate which statistically maximized the differences between groups. This is the grouping configuration which splits the six populations into three groups: i) Una, Porto Seguro and São Mateus, ii) Monte Alegre, iii) Campos do Jordão and Vacaria, (F_{CT} = 0.182, P = 0.015). These data indicate that the mountain populations of Campos do Jordão (state of São Paulo) and Vacaria (state of Rio Grande do Sul) are genetically homogeneous and differentiated from the group of the three coastal populations (Una, Porto Seguro and São Mateus), and from the other more distant coastal population of Monte Alegre. The pairwise F_{ST} estimates (Table 5) confirm the presence of differentiation between the group of the two mountain populations and the coastal populations which in turn share a certain degree of genetic relatedness. Principal Coordinate Analysis (PCoA) was performed to better clarify the genetic relations among the six populations. The first two axes explain a relatively high amount of the genetic variation (88%). The first axis (77.91%) separates Campos do Jordão and Vacaria from the other

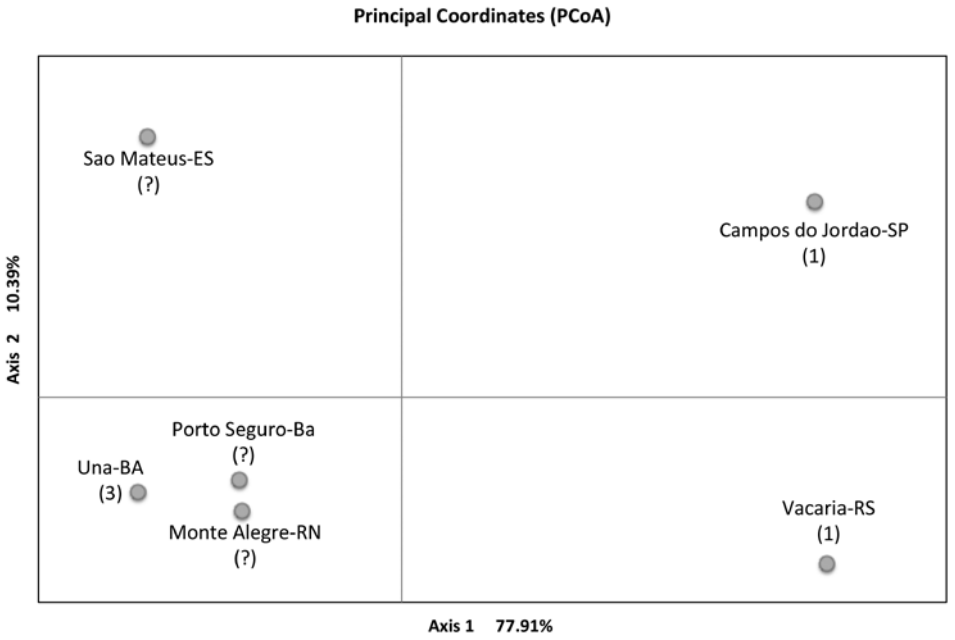

Figure 2. Two-dimensional plot of Principal Coordinate Analysis (PCoA) based on similarity matrix derived from *Anastrepha fraterculus* microsatellites data. The Morphotype classification (Hernández-Ortiz et al. 2012), relative to each sample is also reported.

Table 5. Pairwise-F_{ST} values among 6 population samples of *Anastrepha fraterculus* as derived from Microsatellite Analyser (Dieringer and Schlotterer 2003).

	Una-BA	Porto Seguro-BA	Monte Alegre-RN	São Mateus-ES	Campos do Jordão-SP	Vacaria-RS
Una-BA	-					
Porto Seguro-BA	0.015 ^{ns}	-				
Monte Alegre-RN	0.020	0.012 ^{ns}	-			
São Mateus-ES	0.031	0.031	0.042	-		
Campos do Jordão-SP	0.163	0.127	0.130	0.160	-	
Vacaria-RS	0.165	0.126	0.132	0.175	0.038	-

Some of the values are not significantly different from zero at P > 0.05 (ns)

populations. The second axis (10.39%) mainly differentiates São Mateus from the group of Una, Porto Seguro, and Monte Alegre, but also Campos do Jordão from Vacaria. It is interesting that there is a certain correspondence of the genetic grouping with the known morphotype classification (Hernández-Ortiz et al. 2012). The molecular variance represented by the first axis separates the populations on the basis of both geographical distance and altitude. On this basis, we attempted to disentangle the effect of geographical distance and altitude on the genetic differentiation of Campos de Jordão and Vacaria. The plots in

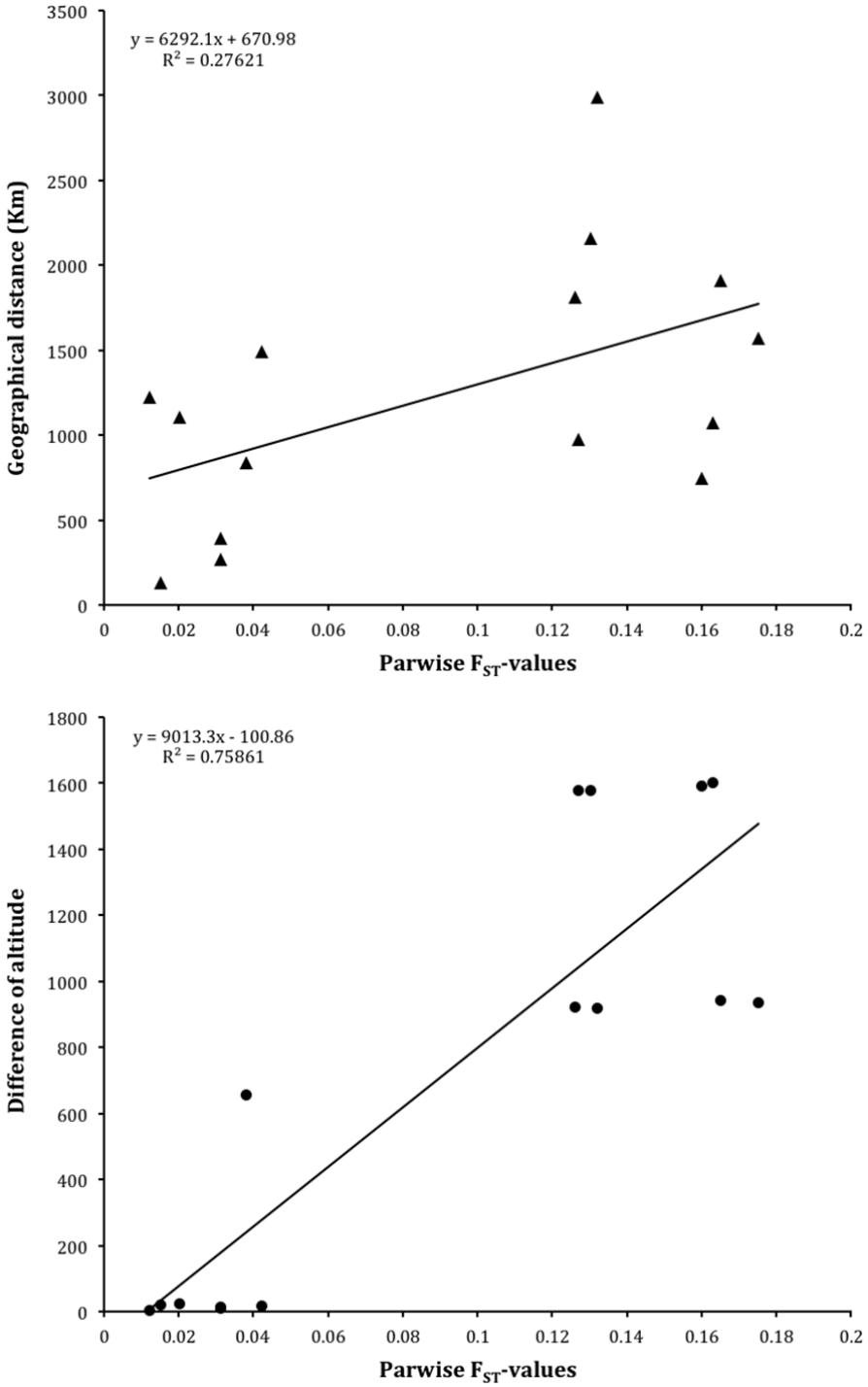

Figure 3. Correlation of *FST* values with the geographic distances (upper plot) and altitude differences (bottom plot) among the 6 Brazilian samples of *Anastrepha fraterculus.*

Figure 3 show the correlations between pairwise F_{ST} values with the differences in altitude (Figure 3A) and the geographic distance (Figure 3B), respectively. The correlation results clearly indicate that the difference in altitude has a greater impact (r^2= 0.76 vs r^2= 0.28) on genetic differentiation of mountain Vacaria and Campos do Jordão, than on the remaining samples belonging to the coastal plain populations.

Discussion

With this paper we initiated, in the polymorphic *A. frateculus* complex, an analysis of the underpinning genetic architecture and its interaction with correlated ecological, biological and morphological traits. In this context, we have used microsatellite markers to perform a genetic analysis of populations from a complex ecological area such as Brazil.

Although the chromosomal location of the considered SSR loci remains unknown, the assessed linkage equilibrium between them suggests they are statistically independent and that their variability patterns might reflect genome-wide patterns across populations. The six considered ecogeographic populations are here represented by highly polymorphic samples, which reflect a high degree of intrapopulation genetic diversity. Indeed only 10% of the total variability (AMOVA) is represented by the differences between the six geographic populations, while greater variability was found within populations.

The spatial analysis of genetic diversity indicates that the levels of diversity among the six populations vary significantly on an eco-geographical basis, as indicated by SAMOVA and PCoA data. More than by geographical distance, the genetic differentiation is influenced by altitude. The multivariate analysis of ten microsatellites depicts a structural pattern, which clearly separates populations on climatic distribution both on latitudinal and altitudinal basis. Particularly, altitude seems to represent a differentiating adaptation, as the main genetic differentiation is that detected between the populations present at higher altitudes (Campos de Jordão and Vacaria) and those populations from sea level. Genetic divergence between populations from low and high altitude areas has already been observed for populations within the *A. fraterculus* complex using isozymes (Morgante et al. 1980, Steck 1991, Selivon et al. 2005) and mtDNA (Steck and Sheppard 1993, Santos 1999, McPheron et al. 1999, Smith-Caldas et al. 2001, Barr et al. 2005). Steck (1991) concluded that the strong differences in allelic frequency between lowland and Andean populations of *A. fraterculus* in Venezuela was due to the fact that they actually represent two genetically distinct species albeit morphologically indistinguishable. These allopatric populations can be subject to divergent selection in response to ecological factors that change over large geographical scales such as altitude. Altitude may act as a barrier to gene flow as levels of life history divergence between high- and low-altitude populations can be correlated with levels of post-zygotic reproductive isolation (Orr and Smith 1998).

One interesting observation, which arises from our data, is that the observed structure of Brazilian populations is entangled with the presence of morphotypes. The actual number of these entities and their respective geographic range are questions that remain to be further elucidated. At the moment three different morphotypes are identified in Brazil (Hernández-Ortíz et al. 2012). As it appears clearly, the PCoA analysis depicts a genetic differentiative pattern that overlaps with the distribution of the known morphotypes. Now the open questions are: 1) is the observed population differentiation contributing to the underpinning genetic architecture of the morphotypes associated to these populations? and 2) do morphotypes track environmental variability? In retrospect, a further aim is to clarify the evolutionary relationships between populations, ecotypes, and morphotypes.

Conclusion

The population genetic approach, in addition to improving our knowledge of the underpinning genetic architecture of the *A. fraterculus* complex, is also important from an applied perspective. The overall level of genetic variability and the presence of differentiation that we detected among the Brazilian populations of *A. fraterculus* constitute an important contribution for any potential future application of SIT for the control of populations of this fruit fly pest in Brazil.

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Nuclear ribosomal internal transcribed spacer 1 (ITS1) variation in the *Anastrepha fraterculus* cryptic species complex (Diptera, Tephritidae) of the Andean region

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Abstract

The nuclear ribosomal internal transcribed spacer 1 (ITS1) was sequenced for *Anastrepha fraterculus* (Wiedemann, 1830) originating from 85 collections from the northern and central Andean countries of South America including Argentina (Tucumán), Bolivia, Perú, Ecuador, Colombia, and Venezuela. The

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ITS1 regions of additional specimens (17 collections) from Central America (México, Guatemala, Costa Rica, and Panamá), Brazil, Caribbean Colombia, and coastal Venezuela were sequenced and together with published sequences (Paraguay) provided context for interpretation. A total of six ITS1 sequence variants were recognized in the Andean region comprising four groups. Type I predominates in the southernmost range of *A. fraterculus*. Type II predominates in its northernmost range. In the central and northern Andes, the geographic distributions overlap and interdigitate with a strong elevational effect. A discussion of relationships between observed ITS1 types and morphometric types is included.

Keywords
molecular, fruit fly, Diptera

Introduction

The *Anastrepha* fauna of the central and northern Andes and Andean periphery is extremely rich, but incompletely known, and includes the poorly understood Andean populations of the serious economic pest species *A. fraterculus* (Wiedemann), which is interpreted as a cryptic species complex as recently reviewed by Hernández-Ortiz et al. (2012, 2015). The taxonomic structure of the *A. fraterculus* cryptic species complex remains inadequately resolved, even as to the number of species involved and their distributional patterns. As part of an ongoing project to improve the identification of *Anastrepha* species, an effort to better resolve and understand the taxonomic structure of this complex was undertaken utilizing sequence analysis of select mitochondrial and nuclear gene regions, with emphasis on the fauna of the Andean region.

Preliminary Sanger sequencing of potentially useful DNA regions for a subset of *A. fraterculus* specimens from two sites in the central Andes of Peru representing "low" and "high" elevation populations (Peru colony, Lima, (n=5) and Cusco, Calca, (n=5) respectively) included the mitochondrial cytochrome oxidase I (COI, in part, including the Folmer fragment) and ribosomal r16S (in part), and the nuclear ribosomal r18S (in part), internal transcribed spacer (ITS, complete), and r28S (in part, including the expansion domains D1-D8). It also included the nuclear protein coding genes elongation factor 1 (EF1∝, in part) and the carbamoyl phosphate synthetase domain (CAD, intron1-exon1, in part). These were also sequenced for other *Anastrepha fraterculus* species group taxa including *A. suspensa* (Loew), *A. distincta* Greene, *A. ludens* (Loew), and *A. obliqua* (Macquart).

This initial survey established that taxonomically informative and diagnostic sequence polymorphism was present in the ribosomal internal transcribed spacer 1 (ITS1). Further sequence analysis then concentrated on ITS1 and geographical coverage was expanded to include the entire project region. We here report ITS1 sequence polymorphism patterns, their geographical correlations, and taxonomic implications for Andean populations of *A. fraterculus*.

Methods

The majority of specimens in this study were adult females. Taxonomic determinations as *A. fraterculus* were based on adult morphological criteria following Norrbom et al. (2012 onwards). After removal of tissue for DNA analysis specimens were deposited in the FSCA as vouchers.

The geographical region chosen for the initial stage of this project included the central and northern Andes and Andean periphery of Bolivia, Perú, Ecuador, Colombia, Venezuela, and Argentina. For comparison, limited numbers of specimens were also available from outside of the core region including México, Guatemala, Costa Rica, Caribbean Colombia (Isla de San Andrés), southern and southeastern Brazil, and coastal Venezuela (Los Caracas), as well as ITS1 sequences (NCBI) derived from *A. fraterculus* originating from localities in Paraguay (Lopes 2010, Lopes et al. 2013).

Most specimens originated from McPhail-type fruit fly traps using yeast hydrolysate bait with, or without, borate preservative. Lesser numbers were collected using multi-lure traps (ML) or reared from host fruits. Trapped or reared specimens were stored in alcohol (70–100% isopropanol or ethanol); or in the case of some archival FSCA samples, stored frozen at -80°C from as early as 1988.
Acronyms for organizations that provided specimens or DNA sequences are as follows:

DSA Dirección de Sanidad Agroalimentaria, Gobierno Autónomo Departamental Santa Cruz, Bolivia.

FSCA Florida State Collection of Arthropods, Division of Plant Industry, Florida Department of Agriculture and Consumer Services, United States of America.

IAEA, IPCL International Atomic Energy Agency, Insect Pest Control Laboratory, Austria.

ICA Instituto Colombiano Agropecuario, Colombia.

NCBI National Center for Biotechnology Information, United States of America.

SENASA Servicio Nacional de Sanidad Agraria del Perú, Perú.

SENASAG Servicio Nacional de Sanidad Agropecuaria e Inocuidad Alimentaria, Bolivia.

USDA, ARS United States Department of Agriculture, Agricultural Research Service, United States of America.

ITS1 sequences were constructed for 299 *Anastrepha fraterculus* specimens representing 85 collections (Figure 1) from the central and northern Andes including Perú (n=119; SENASA, FSCA, IAEA-IPCL, this project), Bolivia (n=28; SENASAG, DSA, FSCA, this project), Colombia (n=125; ICA, IAEA-IPCL, this project), Ecuador (n=14; FSCA, this project), Venezuela (n=7; FSCA), and Argentina (n=6; IAEA-IPCL).

To help place geographical distributions and taxonomic identities in context, specimens of *A. fraterculus* from localities outside of the Andean region having identical

Figure 1. *Anastrepha fraterculus* collection localities.

ITS1 sequences to those recorded from the Andean localities were also included in this analysis. The latter represented 17 collections (n=44) from Mesoamerica, including México (n=3; FSCA, IAEA-IPCL), Guatemala (n=4; FSCA), and Costa Rica (n=2; FSCA), Brazil (n=26; FSCA, IAEA-IPCL, USDA-ARS), Colombia (n=8; ICA) and Venezuela (n=1; FSCA).

Paraguay was represented by ITS1 sequences deposited in the NCBI (HQ829865 to HQ829879) (Lopes 2010, Lopes et al. 2013).

Each collection listed below is described by country, collection number, locality, geographical coordinates (latitude and longitude) when provided, date, collector, other relevant label data, number of specimens, and sex (male=M, female=F, unknown=?): **ARGENTINA**: 1) **Tucumán**, colony 35 in Vienna, Austria, April 2004, IAEA-IPCL, 6 F. **BOLIVIA**: 2) **Chuquisaca**: Surema, 01-06-02-19, 15 June 2010, SENASAG, 2 F. 3) **Santa Cruz**: Refugio Los Volcanes, 63.60W 18.11S, 1040-1050m, reared ex fruit of *Pouteria glomerata* (Miq.) Radik, 20-24 October 2013, A.L. Norrbom & B.D. Sutton, 8 F; 4) Refugio Los Volcanes, 63.60W 18.11S, 1040-1050 m, reared ex fruit of *Myrciaria floribunda* (H. West ex Willd.) Berg., A.L. Norrbom & B.D. Sutton, 8 F; 5) Totaí, 62.2494W 17.20322S, 260 m, 19 August 2014, SENASAG, 6 F; 6) Totaí, 62.2494W 17.20322S, 260 m, 5 August 2014, SENASAG, 1 F; 7) Valle Grande, McPhail Trap, 13 September 2001, DSA, 3 F. **BRAZIL**: 8) **Paraná**: São João de Graciosa, Poço Preto, 48.86842W 25.39829S, 76m, reared ex fruit of *Psidium guajava* L., 28 February 2015,

M. Savaris & A.L. Norrbom, 4 F; 9) Palmas, Linha Algeria, Fazenda Cerro Chato, 51.67301W 26.50278S , 1208 m, butterfly trap, 2-4 March 2015, Sepulveda, 1 F. 10) **Rio Grande do Sul**: Vacaria, colony 36 in Vienna, Austria, May 2010, IAEA-IPCL, 2 larvae. 11) **São Paulo**: Piracicaba: colony 37 in Vienna, Austria, February 2011, IAEA-IPCL, 10 F; 12) Bertioga, sea level, reared ex *Terminalia catappa* L., 2 F; 13) Itaquera, reared ex guava, 25 July 1989, Malavasi & Morgante, 2 F. 14) **Santa Catarina**: Friburgo, reared ex Fejoa (*Acca sellowiana* (O. Berg) Burret), 2 F; 15) Joacaba, reared ex guava, 2 F; 16) P. N. Aparados da Serra, Linha Rio do Boi, 50.00469W 29.20229S, 208 m, butterfly trap, 6-9 March 2015, T. Sepulveda, 1 F. **COLOMBIA:** 17) **Antioquia**: Rionegro, Vereda Barro Blanco, 75.40494W 6.15378N, 2144 m, ML Trap, 11 July 2013, A. Florez (ICA), 8 F; 18) Rionegro, 75.4155W 6.1303N, 2151 m, ML Trap, 03 August 2010, E. Arévalo (ICA), 8 F; 19) Rionegro, Aeropuerto MC, El Platanal, 75.43533W 6.17496N, 2174 m, ML Trap, 30 May 2013, A. Florez (ICA), 8 F. 20) **Cauca**: Timbio, 76.63139W 2.42296N, 1752 m, ruta 415300007, 18 July 2014, Z. Vargas, 7 F. 21) **Cundinamarca**: Anolaima, Finca Villa Mariana, 74.47569W 4.80175N, 1558 m, ex *Coffea arabica* L., 16 September 2014, E.J. Rodriguez & P.A. Rodriguez, 2 larvae; 22) same location, multilure trap, 07 September 2014, E.J. Rodriguez & P.A. Rodriguez, 2 F. 23) Guaduas, campo de tejo, 74.55471W 5.03767N, 1668 m, 23 August 2014, P. Correa, 8 F. 24) **Huila**: Palestina, 76.1390W 1.7319N, 1531m, ruta 415300007, 21 February 2014, ICA, 2 F; 25) Palestina, 70.1726W 1.7033N, 1798m, ruta 415300007, 21 February 2014, ICA, 1 F; 26) Palestina, 76.1351W 1.7099N, 1769m, ruta 415300007, 20 February 2014, (ICA), 1 F; 27) Palestina, 76.1683W 1.7070N, 1790m, ruta 415300007, 21 February 2014, ICA, 1 F; 28) Palestina, 70.1174W 1.7175N, 1511m, ruta 415300007, 20 February 2014, ICA, 3 F; 29) **Magdalena**: Santa Martha, 73.9959W 11.28432N, 153 m, 09 May 2014, J. Amezquita, 1 F; 30) Santa Marta, 73.77654W 11.24910N, 46m, 12 August 2014, J. Amezquita, 1 F. 31) **Meta**: Lejanias, Predio el Vergel, 73.9073W 3.4556N, 482m, 15 June 2014, ICA, 1 F; 32) Lejanias, predio Buenos Aires, 73.9006W 3.4612N, 475m, 16 June 2014, ICA, 1 F. 33) **Nariño**: Tumaco, Kiosco Panorama, 78.73395W 1.82822N 8.7 m, ICA, 2 F; 34) Tumaco, Estrella del Mar, 78.75569W 1.81244N, 13.4 m, ICA, 8 F; 35) Arboleda, Los Balsos, 77.0964W 1.44708N, 1900 m, ICA, 9 F; 36) Buesaco, El Nispero, 77.16837W 1.36098N, 2007 m, ICA, 3 F; 37) La Union, Chaguarurco, 77.16488W 1.59419N, 1849 m, ICA, 4 F. 38) Pasto, Confrut El Eden, 77.27045W 1.19534N, 2562 m, ICA, 5 F. 39) **Putumayo**: Predio Rivera la Bomba, 76.91229W 0.33237N, 332 m, 15 March 2005, F. Cespedes, 8 F. 40) **Risaralda**: Apia, Via Apia Belen, 75.87456W 5.18857N, 1413 m, 12 August 2014, D. Garcia, 8 F; 41) Santuario, predio la Marina, 75.94676W 5.05153N, 1106 m, 04 June 2014, D. Gómez, 8 F; 42) Dos Quebradas, predio San Isidro, 75.66122W 4.84610N, 1497 m, 06 March 2014, D. Gomez, 8 F. 43) **San Andrés y Providencia**: Orange Hill, 814.7085W 12.5584N, 8m, 26 December 2013, ICA, 5 F; 44) Arlis Williams, 81.7086W 12.5584N, 0m, 29 November 2013, ICA, 3 F. 45) **Tolima**: Vereda de Gamboa, 4.40909W 75.33638N, 1635m, colony in Vienna**,** Austria, IAEA-IPCL, 2 F; 46) Ibagué/lavado, colony 40 in Vienna, Austria, July 2010, IAEA-IPCL, 5 F. **COSTA**

RICA: 47) Dominical, sea level, reared ex *Terminalia catappa*, March 1989, M. Condon, 2 F. **ECUADOR**: 48) **Azuay**: Gualaceo, ca. 10km s.e., reared ex peach, 10 March 1994, G.J. Steck & B. McPheron, 2 ♀. 49) **Guayas**: Taura, 100m, McPhail in mango, 28 January 2001, P. Ponce, 6 F. 50) **Pichincha**: Tubaco, Expt. Stn., ex toronja blanca, 24 February 1994, J. Vilatuna, 4 ♀. 51) **Tungurahua**: Patate, 2000 m, McPhail in *Psidium guajava*, 22 October 2000, P. Ponce, 2 F. **GUATEMALA**: 52) Chim., ex guava, November 1989, 2 F; 53) Chim., ex guava, November 1989, 2 F. **MEXICO**: 54) **Chiapas**: Tapachula, Metapa, ex guava, March 1989, 1 F. 55) **Veracruz**: Xalapa, colony 39 in Vienna, Austria, August 2010, IAEA-IPCL, 2 F. **PERÚ**: 56) **Amazonas**: Huayabamba, Huambo, Nuevo Horizonte, 17 August 2006, SENASA, 1 F; 57) Huayabamba, Longar, Michina, 21 September 2006, SENASA, 2 F; 58) Huayabamba, Huambo, Nuevo Horizonte, 21 September 2006, SENASA, 3 F. 59) **Ancash**: Casma, Urbano, Calles, 31 August 2006, SENASA, 9 F; 60) Casma, Urbano, Avenidas, 23 August 2006, SENASA, 3 F; 61) Casma, Urbano, Calles, 23 August 2006, SENASA, 8 F; 62) **Apurimac**: 23 June 2005, SENASA, 2 F. 63) **Ayacucho**: Huamanga, Huatatas, Huatatas, 06 December 2005, SENASA, 1 F; 64) Huamanga, Pongora-Chacco, Muyurina, 06 December 2005, SENASA, 1 F; 65) Huamanga, Yucfaes, Pucahuasi, 07 December 2005, SENASA, 2 F; 66) Huanta, Urbano, Huanta, 13 December 2005, SENASA, 3 F. 67) **Cajamarca**: Alto Jequetepeque, Alto Jequetepeque, 05 July 2006, SENASA, 1 F. 68) **Cusco:** colony in Vienna, Austria, IAEA–IPCL, 2 F. 69) Echarati, Chontachayoc, 19 January 2014, T. Guevara, 8 F. 70) Urubamba, Calca, Sillacancha, 29 September 2006, SENASA, 2 F. 71) Pilcopata, 71.41946W 12.89219S, 559m, 1 March-12 April 2013, M. Choque, 1 F. 72) **Huánuco**: Huallaga-Central, Aucayacu, Santa Lucia, 17 October 2006, SENASA, 3 F; 73) Huallaga-Central, Aucayacu, Pucayacu, 01 November 2006, SENASA, 3 F; 74) Huallaga-Central, Aucayacu, Pueblo Nuevo, 31 October 2006, SENASA, 1 F; 75) Huánuco: Huallaga-Central, Aucayacu, Pueblo Nuevo, 18 July 2006, SENASA, 5 F; 76) Huallaga-Central, Aucayacu, Pueblo Nuevo, SENASA, 07 March 2006, 2 F; 77) Huallaga-Central, Tingo Maria, Naranjillo, 06 March 2006, SENASA, 1 F. 78) **Ica**: Ica, Bajo, Santiago, 06 December 2006, SENASA, 1 F. 79) **Junín**: Chincana, 29 January 2007, SENASA, 9 F. 80) **Lambayeque**: 2000, SENASA, 2 F. 81) **Libertad**: Santa Catalina, Laredo, Galindo, 18 May 2006, SENASA, 2 F; 82) Viru, Viru, Chanquin, 29 May 2006, SENASA, 2 F; 83) Jequetepeque, Guadalupe, La Cinta, 15 May 2006, SENASA, 2 F; 84) Santa Catalina, Laredo, Galindo, 15 June 2006, SENASA, 1 F; 85) Libertad: Santa Catalina, Laredo, La Merced, 07 June 2006, SENASA, 1 F; 86) Jequetepeque, Chepén, Talambo, 06 June 2006, SENASA, 3 F. 87) **Lima**: Asia, El Platanal, TML-fg, 16 March 1994, G.J. Steck & B. McPheron, 2 F; 88) La Molina, La Molina, lab colony, 18 March 1994, G.J. Steck & B. McPheron, 2 F; 89) lab colony in Lima SENASA, 5 F. 90) colony 34 in Vienna, Austria, 2012, IAEA-IPCL, 7 F. 91) **Piura**: San Lorenzo, Malingas, Canal Malingas Huaraguaos, 29 August 2006, SENASA, 3 F. 92) **Tumbes**: Zarumilla, Zarumilla Alta, Papayal, 28 February 2006, SENASA, 1 F; 93) Zarumilla, Zarumilla Alta, Papayal, 07 March 2006, SENASA, 1 F; 94) Zarumilla, Zarumilla Baja, La Palma, 05 April 2006, SENASA, 1 F. 95) **VRAE Region**: San Miguel, Qanan, Qanan, 24 August 2005, SE-

NASA, 2 F; 96) San Miguel, Chaupin, Chaupin, 24 August 2005, SENASA, 3F; 97) 24 August 2005, SENASA, 5 F. **VENEZUELA**: 98) **Barinas**: Los Mesas, ex coffee, 1 M. 99) **Mérida**: Lagunilla, reared ex *Terminalia catappa*, 1989, A.L. Norrbom, 2 ?. 100) Mérida area, Finca San Antonio, >1600m, ex *Rubus glaucus* Benth., 31 May 1988, G.J. Steck & A.L. Norrbom, 1M 1?; 101) Merida, Sta. Rosa station, ca. 1600 m, ex coffee, 30 May 1988, G.J. Steck & A.L. Norrbom, 2 M. 102) **Vargas**: Los Caracas, Litoral central, sea level, reared ex *Terminalia cattapa*, 5 June 1988, C.J. Rosales, 1 M.

ITS1 in *Anastrepha* is highly base-biased with ~84–85% AT in the *fraterculus* species group. In addition, poly(A)/poly(T) subsequences of up to 20 or more bases are predominant. Initial PCR and Sanger sequencing of ITS1 resulted in what could be interpreted as substantial intra-individual polymorphism and/or PCR polymerase artifacts (slippage). Cloning of ITS1 PCR amplicons followed by comparative re-PCR/Sanger sequencing or direct Sanger sequencing of cloned DNA indicated that (1) PCR polymerase slippage-type artifacts were predominant (this does not rule out some level of intra-individual polymorphism) and (2) that the BigDye Terminator 3.1® (Thermo Fisher Scientific, Waltham, MA USA) sequence by synthesis (SBS) chemistry is highly resistant to polymerase slippage at this level of AT bias. In addition, it was found that less than perfect DNA quality can greatly increase polymerase artifacts.

A considerable effort was made to reduce PCR polymerase artifacts using high processivity polymerases, modified PCR conditions, and reaction adjuncts. The polymerases with the best performance included Phusion® (Thermo Fisher Scientific) under modified PCR conditions, KAPA® Hi Fi (KAPABiosystems, Wilmington, Massachusetts USA), and the KOD-based Platinum® Pfx (Thermo Fisher Scientific). No perfect solution was found; however, the overall level of PCR slippage in most non-degraded DNA samples was reduced to a point where confident analysis was possible. Homopolymer regions having greater than 14 or so A or T bases tended to exhibit PCR slippage even under optimal conditions, as also did sufficiently large repeats such as $(ATT)_n$ with n>5. Since polymerase artifacts can originate from either PCR or replication, or both, it is possible, if not probable, that homopolymeric or repeat regions are exhibiting some level of intra-individual polymorphism. These regions, fortunately, tended to be localized as single isolated homopolymers or repeats towards the 5' and/or 3' ends of ITS1 in the *A. fraterculus* cryptic species complex allowing bidirectional resolution.

In addition, individual DNA samples were found to often react differently to PCR optimizations, particularly in the all too common situation of trapped specimens having a significant probability of degraded DNA. The use of high-salt PCR buffers with relatively high annealing temperatures was generally useful in these cases; however, PCR reaction adjuncts such as TMAC (tetra ammonium carbonate) used to preferentially improve AT bonding while significantly reducing polymerase slippage and artifacts in some cases often increased polymerase artifacts in other samples having the same ITS1 sequence. This latter effect generally occurred in cases of poor DNA quality. Resource limitations precluded PCR optimization for individual samples except in special cases. As a general strategy, KAPA® HiFi became the standard polymerase for PCR.

Table 1. Primers used for PCR amplification and sequencing of ITS1 in *Anastrepha fraterculus*.

Primer	Primer Sequence 5'–3'
ADL 18sF	TAA CTC GCA TTG ATT AAG TCC C
ADL 5.8sR	GAT ATG CGT TCA AAT GTC GAT G
ADL ITS1 internal F	GAT TGA ATG ATA AGT TAA TTT GTT CAC
ADL ITS1 internal R	GTT GCG AAT GTC TTA GTT CAA C

Primers utilized for PCR and sequencing are given in Table 1: ADL 18sF was based upon the 18s sequences NCBI EU179519 (*A. ludens*) and AF187101 (*A. fraterculus*); ADL 5.8sR was modified from CAS5p8sB1d (Ji et al. 2003); ADL ITS1 internal F and R were constructed from complete ITS1 sequences of *A. fraterculus* generated by direct sequencing using the above PCR primers. The primers internal to ITS1 allowed improved sequence quality for regions subject to polymerase slippage artifacts.

Primers supplied in lyophilized form by Integrated DNA Technologies (IDT) were reconstituted in 1X pH 8.0 Tris-EDTA (TE) (Thermo Fisher Scientific) to a 100μM stock solution. Working solutions were diluted in HyClone® nuclease-free water (Thermo Fisher Scientific) to 10μM for PCR amplification and/or 2.5μM for Sanger reactions. Experimentally, modified primers were synthesized incorporating 3' phosphorothioate linkages to counter exonuclease degradation but this was not found to be helpful and was not continued.

Samples for DNA extraction generally consisted of 1 or more legs with attached muscle, or in some cases the complete thorax, frozen or in alcohol. The latter were air dried in a laminar flow hood to remove alcohol, then in both cases the samples were chopped, frozen in LN_2, and powdered with a Mini-Beadbeater-96 (Biospec Products, Inc., Bartlesville, Oklahoma USA) using a glass bead. DNA extraction and cleanup utilized the DNeasy Blood and Tissue Kit ® (QIAGEN, Venlo, Netherlands) following the manufacturer's protocol with overnight digestion by Proteinase K using the supplied ATL buffer. Final elution was in 50μL of AE buffer.

PCR amplifications were carried using a GeneAmp® PCR System 9700 thermal cycler (Thermo Fisher Scientific) with the temperature program recommended by KAPA Biosystems for the KAPA HiFi hotstart polymerase: initial denaturation 95°C/2min. followed by denaturation 98°C/20 sec., annealing at 65°C/15 sec., and extension at 72°C/15 sec. for 30 cycles with no final extension. PCR reactions also followed manufacturer's recommendations with a total volume of 20μL, 0.3μM final primer concentration, and 1U of polymerase. Mineral oil overlays (10μL) were used. Visualization of amplification products was by planar gel electrochromatography on a 2-2.5% agarose gel and TAE buffer. Gels were stained using SYBR Safe (Thermo Fisher Scientific). If the PCR amplicons were deemed acceptable, then the PCR reactions were cleaned up by spin column using the Roche High Pure PCR Product Purification Kit (Roche Diagnostics Corporation, Indianapolis, Indiana USA) following the manufacturer's recommended protocol and the DNA concentration quantified by a NanoDrop® (Thermo Fisher Scientific) μv spectrophotometer prior to Sanger reaction assembly.

The Sanger sequencing utilized the BigDye® Terminator™ 3.1 chemistry (Thermo Fisher Scientific) following the manufacturer's recommendations for a 0.25X reaction mix and thermocycler temperature program. Sanger reaction products were cleaned of unincorporated dye terminators using the BigDye® Xterminator™ Purification Kit (Thermo Fisher Scientific) with a modified protocol using PCR tubes rather than a 96-well plate. Separation of Sanger reaction products and visualization utilized an Applied Biosystems 3100-Avant Genetic Analyzer (Thermo Fisher Scientific) upgraded to 3130 specifications.

Sequence Scanner v1.0 (Thermo Fisher Scientific) was used for base calling of raw sequences with visual interpretation for final decisions. Base editing and manual alignment, and cluster analysis utilized MEGA6: Molecular Evolutionary Genetics Analysis software version 6.01 (Tamura et al. 2013). Overall similarity between sequences was inferred by UPGMA (unweighted pair group method with arithmetic mean) cluster analysis (Sneath 1973) with distances computed by the maximum composite likelihood method (Tamura et al. 2004) in number of base substitutions per site with gaps eliminated.

Given the high variability in DNA quality, even between individual specimens from the same collection, a triage-type sequencing strategy evolved in which individual samples were evaluated at multiple stages of the analysis and rejected if they failed to meet certain experimentally determined criteria. This included extracted DNA concentration as determined by μv spectroscopy, visual intensity of amplicons from high stringency PCR reaction conditions, and initial single primer reverse strand sequencing of the r5.8s–r18s region. Only approximately 1/2 of the DNA from trapped specimens resulted in acceptable ITS1 sequence quality.

A subset of, or all, samples from each locality having the best DNA quality were sequenced using the full set of four primers to increase coverage and to allow bi-directional coverage of the regions showing possible intra-individual polymorphism and/or PCR slippage artifacts. Generally in these regions a consistent dominant sequence was present and could be reconstructed with bi-directional coverage and/or manual deconvolution of the overlapping sequences. The accuracy of this approach was verified during method development by direct sequencing of cloned PCR products.

Results

The polymorphic ITS1 region of the Andean *A. fraterculus* cryptic species complex ranges from 534–563 nucleotides (nt) in length and is significantly AT-biased at approximately 84% AT (Figure 2, all base numbers with respect to the hypothetical alignment in this figure). In this group, ITS1 can be considered as a mix of A/T homopolymers of variable length with intervening base interruptions including G and/or C and bounded by 5' and 3' interrupted poly(A) subregions 52–89nt (bases 1–123) and 44–46nt (bases 588–637) in length, respectively, having individual A homopolymers up to 22 bases. At the 3' end of the 5' poly(A) region (bases ~125–150) is a small variable region.

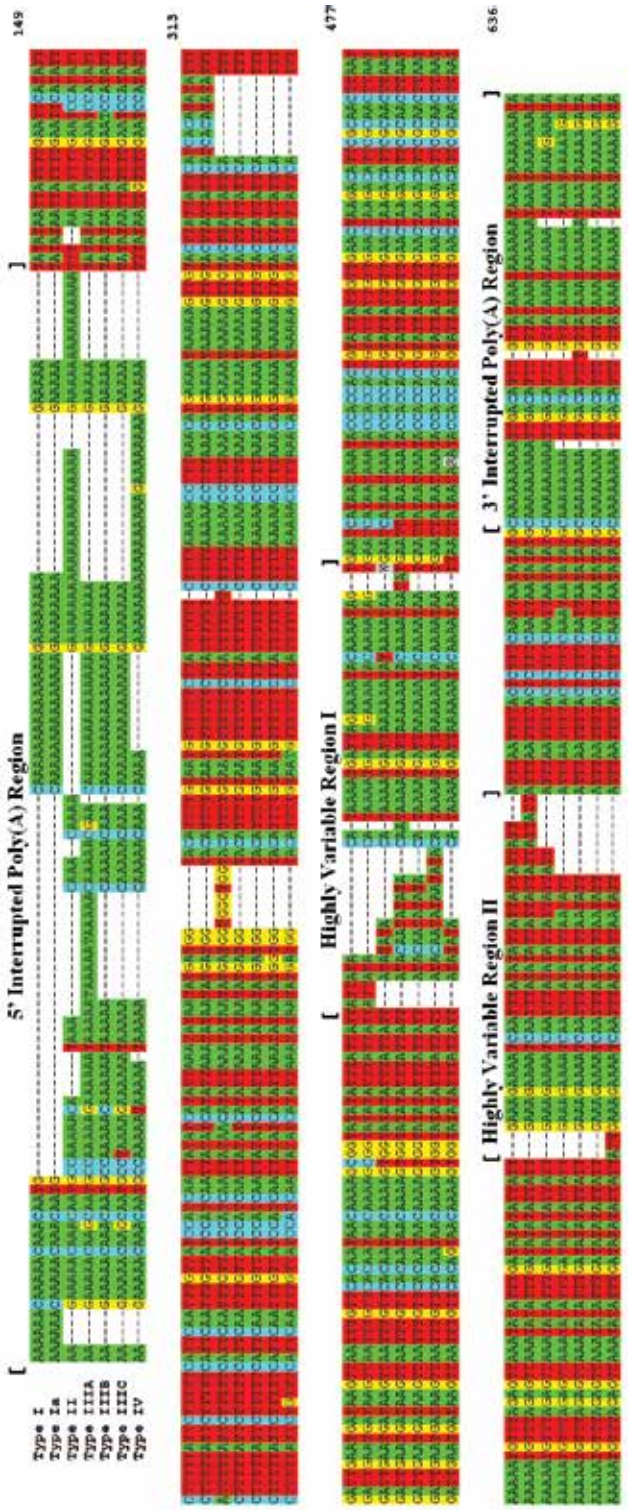

Figure 2. ITS1 polymorphic region sequences for Andean *Anastrepha fraterculus*; hypothetical alignment.

Table 2. Results: ITS1 sequence types by country and collection.

ITS1 Sequence Type	Country	Collection number	NCBI Accession
TI/TIa	Argentina	1 (TI, TIa)	
	Bolivia	2 (TI, TIa), 3 (TI, TIa), 4 (TI), 5 (TI), 6, (TI), 7 (TI, TIa)	KT893864
	Brazil	8–16 (all TI)	
	Peru	62 (TI, TIa), 63 (TIa), 64 (TI), 65 (TI), 66 (TI), 68 (TI), 70 (TI), 95 (TI, TIa), 96 (TIa) 97 (TI, TIa)	KT893865
	Colombia	29–32, 39, 43–44	
	Costa Rica	47	
TII	Guatemala	52–53	
	Mexico	54–55	
	Venezuela	102	
	Colombia	33–37	
TIIIA	Ecuador	49, 51	KT893866
	Peru	56–61, 67, 71–94	KT893867
	Colombia	21	
TIIIB	Ecuador	50	
	Venezuela	100–101	
	Colombia	45	KT893868
TIIIC	Ecuador	48	
	Venezuela	99, 100	
	Colombia	17–20, 22–28, 38, 40–42, 45–46	KT893869
TIV	Ecuador	50	KT893870
	Peru	69	
	Venezuela	98, 101	

The majority of sequence polymorphism in the intervening subregion between the bounding interrupted poly(A) regions is concentrated in 2 highly variable sequence regions, here designated HRV I (bases 369–418) and HRV II (bases 516–556) respectively (Figure 2). These regions are polymorphic in sequence within the *A. fraterculus* cryptic species complex with respect to single nucleotide polymorphisms (SNPs) as well as in the lengths of repeats and/or progressive repeats. Within the relatively conserved regions between HRV I, HRV II, and the bounding poly(A) regions are occasional scattered SNPs as well as 2 unique expansions or insertions.

The ITS1 sequences of the Andean *A. fraterculus* cryptic species complex can be placed into 4 groups, here designated as ITS1 sequence types TI, TII, TIII, and TIV, with a variant of TI designated TIa and TIII further subdivided into 3 subtypes designated TIIIA, TIIIB, and TIIIC (Table 2). Representative sequences have been deposited in the NCBI. The pattern of overall similarity among these sequence types is visualized by UPGMA clustering (Figure 3).

The ITS1 sequence type TI has a polymorphic region of length 534 or 537nt and is characterized by a unique CA to CATCACATATA expansion located approximately

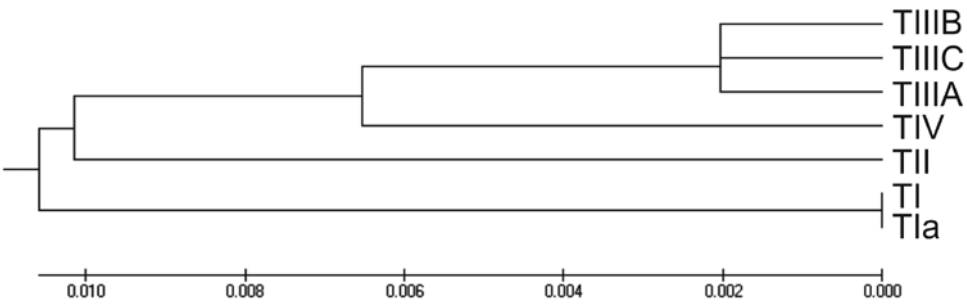

Figure 3. Overall similarity inferred by UPGMA (unweighted pair group method with arithmetic mean) cluster analysis (Sneath and Sokal (1973) of Andean *Anastrepha fraterculus* ITS1 sequence types (489nt). Distances were computed by the maximum composite likelihood method (Tamura et al. (2004) in number of base substitutions per site with gaps eliminated.

40nt from the 5' end of HVRI (bases 301 to 309), a 5' poly(A) region unique within this complex, a unique repeat expansion $(ATT)_2$ to $(ATT)_3$ starting at base 363, and unique SNPs at bases 142, 351, 401, and 415. Inter-individual polymorphism is present in an $(ATT)_n$ repeat with n=5 or 6 (the latter designated TIa in Figure 2) within HVRII starting at base 542 in the alignment.

The geographical distribution of TI in the central Andean region ranges from the Chiquitano forests into the eastern Andean dry valleys to at least 2000 m in Bolivia north to the higher elevation eastern dry valleys of the Cusco-Ayacucho region of Peru above *ca.* 2800 m. This ITS1 sequence type also extends south to Argentina (Tucumán), and east to Paraguay (Lopes 2010, Lopes et al. 2013) and at least southern and southeastern Brazil (Figure 4).

Geographical variation in the HVRII $(ATT)_n$ repeat may be present with the n=5 variant (TI) most common in SE Brazil and n=6 (TIa) predominant in the Peru dry valleys; however, sample sizes are insufficient to determine if this is a real trend.

The ITS1 sequence type TII polymorphic region is 554nt in overall length and characterized by a unique expansion of TGG to TGGTGGGTGG starting at base 214, located 86nt from the 3' end of the 5' poly(A) region. The 5' poly(A) region is unique in sequence within the *A. fraterculus* complex; however, the presence of consecutive A_{22} and A_{15} homopolymers (estimated lengths) increases the likelihood of PCR artifacts. The putative A deletions of TATATA to TTT at the 3' end of this region is consistent but must be considered hypothetical. Additional unique ITS1 sequence elements includes a possible $(T)_6$ to $(T)_7$ expansion starting at base 251, SNPs at bases 149,191, 408, and 630, and the $(ATT)_4$ repeat starting at base 539 in HVRII. An inter-individual polymorphism (A or T) was observed at base 418 and is indicated by the IUPAC ambiguity code W in the alignment.

The geographical distribution of TII in this study was restricted to the periphery of the northern Andes including Caribbean coastal sites of Colombia and Venezuela, and south along the eastern foothills of the Cordillera Oriental of Colombia to at least the Ecuador border (Figure 4). This sequence variant also characterizes the specimens from Mexico, Guatemala, Costa Rica, and the Isla de San Andrés (Colombia).

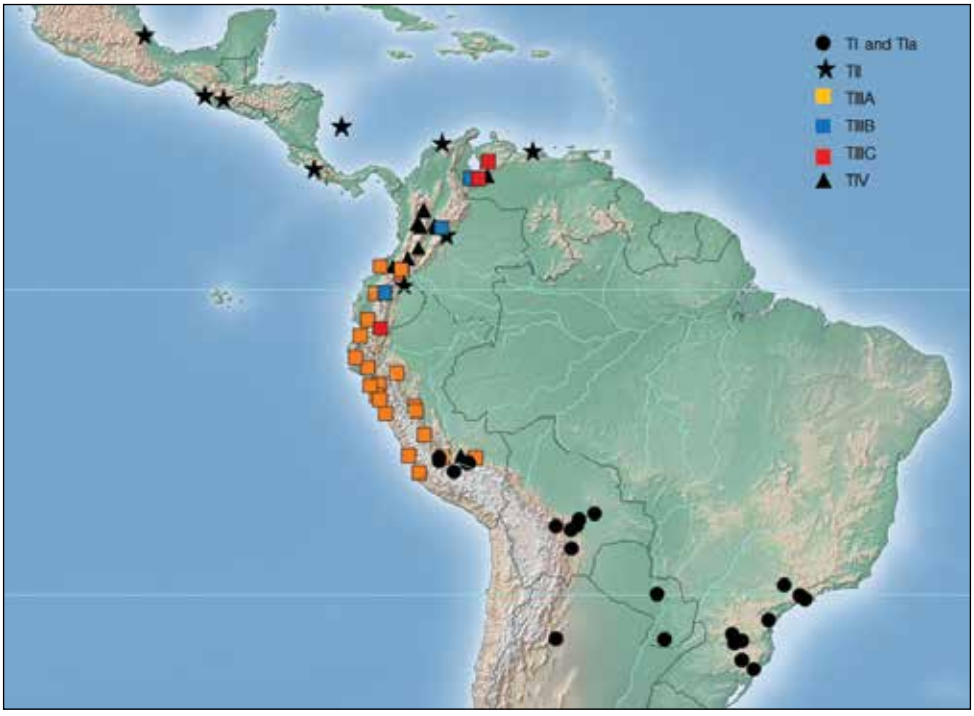

Figure 4. Geographical distribution of Andean *Anastrepha fraterculus* ITS1 sequence types.

The ITS1 sequence type TIII polymorphic region ranged from 550-563nt in length. The three sequence subvariants in this group are very close in overall ITS1 similarity (Figure 3) but polymorphic in HVRI with a common CAATATA sequence starting at base 375. The geographical distribution of TIII was restricted to the central and northern Andean region (Figure 4).

Subtype TIIIA with the ITS1 polymorphic region of length 563nt, is the most divergent of the three with a TAAAAA to $(TAAAAA)_3$ expansion starting at base 42 in the 5' poly(A) region, unique insertions in HVRI including an A (base 389) and TA (at bases 416 to 417), and a possible A expansion at base 577.

This sequence subtype characterizes *A. fraterculus* of the lower elevations of the Andean periphery in the Pacific plain and Andean foothills and valleys of Peru (generally less than ~1200 m in elevation), from Ica in the south (eradicated by SENASA south of Lima?) through western Ecuador to the north into at least SW Colombia. Specimens with this sequence were also seen from the eastern Andean valleys of Peru from the Kosñipata Valley of Cusco to at least Amazonas (Figure 4).

The ITS1 sequence subtypes IIIB (length of polymorphic region 550nt) and IIIC (length 551nt) are very similar differing by 2 SNPs in the 5' poly(A) region, an SNP at base 210, a complex expansion ATA to AATATA in HVRI starting at base 381, and putative homopolymer length polymorphisms towards the 3' end of the ITS1 polymorphic region (bases 606 and 621).

The geographical distributions of these subtypes overlap with scattered collection localities from the Cordillera de Mérida in Venezuela south to the Andes of Ecuador (Figure 4) at elevations above ~1200 m. Specimens having these sequences were collected as larvae; so far no trapped adults have been seen. Larvae having the subtype IIIB sequence were collected from coffee (*Coffea* L. sp., Colombia) and toronja blanca, or pomelo (*Citrus maxima* (Burm.) Merr., Ecuador), and together with IIIC from Andean blackberry, (*Rubus glaucus* Benth.) in Venezuela.

The ITS1 sequence type TIV polymorphic region length is 553nt. This type is rather more generalized in ITS1 sequence within the *A. fraterculus* cryptic species complex, perhaps closer to the *fraterculus* species group groundplan than the others. Type TIV is characterized by a unique G interruption at base 99 in the 5' poly(A) region, unique SNPs at bases 133, 160, 341, and 419, and by a unique repeat expansion $(ATT)_2$ to $(ATT)_3$ in HVRII starting at base 510.

The geographical distribution of TIV in the northern Andes extends from the Cordillera de Mérida of Venezuela, the Cordilleras Central and Oriental of Colombia, south to at least the northern Andes of Ecuador (Figure 4) generally above ~900 to 1000 m. In addition, specimens of *A. fraterculus* with this ITS1 sequence were collected in the vicinity of Echarate, in the southeastern Andean foothills of Peru at about 960 to 1400 m.

Discussion

The ITS1 sequence types were not randomly distributed and they present a geographical pattern that is generally consistent with previous concepts about the *A. fraterculus* cryptic species complex of the Andean region.

ITS1 sequence type TI is widespread in South America from at least SE Brazil west into northern Argentina and north in the eastern Andes to at least the Cusco region of Perú. Specimens having this ITS1 sequence type include those from the IAEA, IPCL colonies originating from Argentina (Tucumán, sampled 2014 and 2015) and Brazil (Vacaria, sampled 2010, and Piracicaba sampled 2014, 2015), as well as the published sequences of specimens from Paraguay (Lopes 2010, Lopes et al. 2013). Concurrently, this type seems identifiable with *Anastrepha* sp. 1 (Selivon et al. 2004) (=*Anastrepha* sp. 1 *aff. fraterculus* Yamada & Selivon 2001, Selivon et al. 2005) and morphotype 1 of Hernández-Ortiz et al. (2012).

Specimens of the *A. fraterculus* complex having ITS1 sequence type TII were restricted to Mesoamerica and northeastern South America and include specimens from the IAEA, IPCL, colony originating from México (Xalapa, sampled 2013). A specimen with this ITS1 sequence originated from the los Caracas, Venezuela collection (1988) on which the "Vz-Lowland" population of Steck (1991) was based. TII appears to correspond to the "Mexican" form of Hernández-Ortiz et al. (2012), and likely includes specimens from "lowland" Venezuela. The latter were apparently not compared with the Mesoamerican *A. fraterculus* by Hernández-Ortiz et al. (2012).

The identity of the group having the ITS1 sequence type TIII is more complicated. Subtype TIIIA appears to be well defined and includes specimens from the IAEA IPCL, Perú colony (sampled 2014) and can probably be identified with the *Anastrepha* sp. 4 *aff. fraterculus* of Selivon et al. (2004) and the Peruvian morphotype of Hernández-Ortiz et al. (2012). *Anastrepha fraterculus* having the TIIIB and TIIIC sequence subtypes, however, are of uncertain taxonomic status and identity. Specimens from the highland Venezuela *A. fraterculus* collections of Steck (1991) include those with TIIIB, TIIIC, and TIV ITS1 sequences; unfortunately, specimens analyzed in the earlier isozyme analysis were destroyed during the process preventing molecular analysis of the same individuals. In Colombia, both subtype IIIB and type IV ITS1 sequences were found in specimens reared from larvae collected from coffee near Bogotá (this project); specimens from an IAEA, IPCL colony from Tolima, Colombia (sampled 2015) include subtype TIIIC. Both subtypes and type IV are present in collections of *A. fraterculus* from the Andes of Ecuador as well. It is not clear if a polymorphic ITS1 species is present in the highlands of the northern Andes, or if multiple sympatric species exist. Only specimens having ITS1 sequence type TIV were seen in trap samples (ICA) from Colombia, as well as from the IAEA, IPCL colony from Colombia (Ibagué, sampled 2014), the latter now lost.

Specimens having the TIV ITS1 sequence pattern seem to fit the "Andean" form of Hernández-Ortiz et al. (2012), but it remains unclear how those with the TIIIB and TIIIC sequence types fit into this morphotypic scheme. Steck (1991) failed to find isozyme heterogenity in the same collections of *A. fraterculus* from the Cordillera de Mérida, Venezuela, having specimens with the TIIIB, TIIIC, and TIV ITS1 sequence types. This is consistent with ITS1 sequence polymorphism. Additional collections from this region will be required to clarify this.

These results indicate that ITS1 sequence patterns can help resolve taxonomic structure in the *Anastrepha fraterculus* cryptic species complex, at least at some level. It remains to determine the lower limits of this resolution, ie. if a finer level of taxonomic structure in *A. fraterculus* exists in the Andean region. In addition, the sampling here was not geographically exhaustive. Significant parts of the Andean region remain poorly sampled including, but not restricted to, Ecuador and Venezuela. Beyond the Andean region; moreover, the diversity of the *Anastrepha* fauna in general and *A. fraterculus* in particular, of Amazonia and Guayana remain poorly known.

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Characterisation of the chemical profiles of Brazilian and Andean morphotypes belonging to the *Anastrepha fraterculus* complex (Diptera, Tephritidae)

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Abstract

Fruit fly sexual behaviour is directly influenced by chemical and non-chemical cues that play important roles in reproductive isolation. The chemical profiles of pheromones and cuticular hydrocarbons (CHs) of eight fruit fly populations of the Andean, Brazilian-1 and Brazilian-3 morphotypes of the *Anastrepha fraterculus* cryptic species complex originating from Colombia (four populations) and Brazil (four populations) were analysed using two-dimensional gas chromatography with mass spectrometric detection. The resulting chemical diversity data were studied using principal component analyses. Andean morphotypes could be discriminated from the Brazilian-1 and Brazilian-3 morphotypes by means of male-borne pheromones and/or male and female CH profiles. The Brazilian-1 and Brazilian-3 morphotypes were found to be monophyletic. The use of chemical profiles as species- and sex-specific signatures for cryptic species separations is discussed.

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Keywords
Cryptic species, chemotaxonomy, GC×GC/MS, PCA

Introduction

Communication is a crucial process for both intra- and interspecific interactions (Kroiss 2008). Chemical signals are probably the oldest form of communication in living organisms, and insects constitute one group of animals that rely heavily on chemical communication signals (Levine and Millar 2009). Unique messages are created by varying the structure of the chemicals that comprise a message and by combining these chemicals in blends with different ratios (Jallon 1984, Millar 2000). Over time, the components of the messages have been tuned up by natural selection according to function within the context in which they evolved (Levine and Millar 2009, Vaníčková 2012).

The South American fruit fly, *Anastrepha fraterculus* (Wiedemann 1830) (Diptera: Tephritidae) constitutes a cryptic species complex with different degrees of pre- and postzygotic isolation (reviewed by Vaníčková et al. 2015a). Eight taxonomically distinct morphotypes have been recognized thus far; these are the Andean, Brazilian-1, Brazilian-2, Brazilian-3, Ecuadorian, Mexican, Peruvian and Venezuelan morphotypes (Hernández-Ortiz et al. 2004, 2012, 2015). Recent studies have shown that the Andean, Brazilian-1, Brazilian-3, Mexican, and Peruvian morphotypes have varying degrees of sexual incompatibility (Vera et al. 2006, Cáceres et al. 2009, Segura et al. 2011, Rull et al. 2013, Devescovi et al. 2014). *Anastrepha fraterculus* exhibits a complex mating system, which involves auditory, visual and chemical signals (Morgante et al. 1983, Aluja 1994). Males of this species form leks on host or non-host trees to release volatile compounds, which serve at first to attract other males and subsequently to attract conspecific females. These chemical mixtures therefore act as aggregation as well as sexual pheromones. Differences in the *A. fraterculus* male-borne volatiles produced by flies from geographically distinct populations from Argentina, Brazil and Peru were previously described (Lima et al. 2001, Cáceres et al. 2009, Břízová et al. 2013, Lima-Mendonça et al. 2014). Recently, chemical and electrophysiological analyses have shown that six compounds of the volatile mixture produced by males of *A. fraterculus* from northeastern Brazil are antennaly active in conspecific females (Milet-Pinheiro et al. 2015). The attractiveness of conspecific females in laboratory and semi-field bioassays for a synthetic mixture of the six components to conspecific females is similar to the attractiveness of male headspace samples (Milet-Pinheiro et al. 2015).

It is thought that tephritid fruit flies use contact pheromones in the last phase of courtship when a female briefly touches a male with its proboscis or front legs (Morgante et al. 1980, Vaníčková et al. 2014, Vaníčková et al. 2015b). Contact or short-range pheromones are relatively non-volatile, nonpolar compounds called cuticular hydrocarbons (CHs), which are synthesized from fatty acid precursors and deposited on an insect's cuticle. Essentially, they serve as protection against desiccation. They also

play a crucial role in communication and sex, species, or reproductive status recognition (Blomquist and Bagnères 2010, Kather and Martin 2012). Due to their species-specificity, CH profiles are widely used for cryptic species determination, as documented in diverse insect families such as Termitoidae (Haverty et al. 2000), Miridae (Gemeno et al. 2012), Tephritidae (Vaníčková et al. 2014, Vaníčková et al. in press), Drosophilidae (Oliveira et al. 2011, Jennings et al. 2014), and many others.

In *A. fraterculus* age- and sex-dependent CHs production has been investigated in one Argentinean population (Vaníčková 2012, Vaníčková et al. 2012b). Males of *A. fraterculus* from this population produce a set of unsaturated CHs, not present in female body washes, suggesting these compounds may function as contact pheromones in chemical communication. A recent study on intraspecific variation of male CH profiles from six populations of the *A. fraterculus* cryptic species complex, ranging from Argentina to Mexico, has shown that the chemical signatures are specific to the putative species and therefore may be used for identification of particular morphotypes within the complex (Vaníčková et al. in press). In the same study, female CH profiles of Brazilian-1, Peruvian and Mexican morphotypes revealed qualitative and quantitative differences in CH composition. Nevertheless, a detailed study using more populations of the same *A. fraterculus* morphotype from different regions is necessary for further evaluation of the male pheromone and CH profiles as potential chemotaxonomic markers in species differentiation. Furthermore, information on the variability of female CH profiles within putative species of the *A. fraterculus* complex needs to be evaluated.

The present work aims to (i) clarify differences in the composition of male-borne volatiles among eight different populations belonging to three morphotypes (Andean, Brazilian-1 and Brazilian-3) of the *A. fraterculus* cryptic species complex; (ii) evaluate the potential use of male CH profiles for *A. fraterculus* identification of the Andean, Brazilian-1 and 3 morphotypes; (iii) investigate divergence in female CH profiles of the Andean and Brazilian morphotypes.

Methods

Insects

Eight laboratory populations, previously analysed for the morphotype identification by Hernández-Ortiz et al. (2004, 2012, 2014 unpublished data), originated from unique collections (for more detail see Table 1). After eclosion, the insects were separated by sex and put into plastic chambers (30 × 20.5 × 16 cm). The flies were fed an artificial diet (Sobrinho et al. 2009). Brazilian morphotypes were reared in the Chemical Ecology Laboratory at the Universidade Federal de Alagoas (Maceio, Brazil). The temperature of the insectarium was 25 °C, relative humidity was 60%, and the photoperiod was 14:10 light:dark. The Andean morphotypes were kept in the entomological laboratories at the Universidad del Tolima (Ibague, Colombia). They were reared at 22 °C; relative humidity was 70%, and the photoperiod was 12:12 light:dark.

Table 1. Geographical location of the sampled populations of *Anastrepha fraterculus* in Brazil and Colombia and the analyses of their cuticular hydrocarbons (CH) and male-borne pheromones.

Country, State	Population	Code	Alt. [m]	Latitude	Longitude	Morphotype[†]	CH analyses	Pheromone analyses
Brazil, RS	Pelotas[‡]	PEL	7	29°28.19'S	50°37.03'W	Brazilian-1	+	+
Brazil, RS	Bento Gonçalves[‡]	BEN	690	29°17.08'S	51°51.89'W	Brazilian-1	+	+
Brazil, SC	São Joaquim[§]	SAO	1360	28°17.38'S	49°55.54'W	Brazilian-1	+	+
Brazil, AL	Alagoas[|]	AL	16	10°08.01'S	36°10.34'W	Brazilian-3	+	+
Colombia	Ibague[¶]	IBA	1580	04°26.20'N	75°13.55'W	Andean	n.a.	+
Colombia	Sibundoy[¶]	SIB	2136	01°20.33'N	76°91.92'W	Andean	+	+
Colombia	Duitama[¶]	DUI	2569	05°49.50'N	73°04.49'W	Andean	+	+
Colombia	Cachipay[¶]	CAC	1850	04°45.27'N	74°23.02'W	Andean	+	n.a.

[†]Morphotypes were identified by Dr. Hernández-Ortiz. Institutions and laboratories which provided the particular populations: [‡]EMBRAPA-Empresa Brasileira de Pesquisa Agropecuária, Brazil; [§]UFB- Universidade Federal de Bahia, Brazil; [|]UFAL-Laboratory of Chemical Ecology at Universidade Federal de Alagoas, Brazil; [¶]UT-Universidad del Tolima, Colombia. n.a. – samples were not avaliable for the analyses.

Male-borne volatile collection

For the male-volatile collection following populations were available: three Colombian (Duitama, Ibague, Sibundoy) and four Brazilian (Alagoas, Bento Gonçalves, Pelotas, São Joaquim) (Table 1). To obtain male-borne volatiles, procedures described by Břízová et al. (2013) and Milet-Pinheiro et al. (2015) were used. Groups of 20 sexually mature virgin males of *A. fraterculus* were placed in a glass desiccator (180 mm high; 200 mm diameter), and volatiles were collected using dynamic headspace methods. The inlet of the desiccator was modified by the addition of an inlet tube containing SuperQ® (100 mg; Chromapack) to adsorb the released volatiles. The volatiles were then collected using an air pump (Resun® AC 2600) coupled to a flow meter (Supelco®) and absorbed on the SuperQ® filter. The air flow through the filter was 500 mL min^{-1} for 24 h. The volatiles trapped in the filter were then eluted with 500 μL of redistilled trace analysis grade hexane (Sigma-Aldrich, Brazil). The samples were stored in 2 mL vials, which were kept in the freezer (-5 ºC) until chemical analyses. Ten replicate samples of volatiles were collected for each of the study populations.

Extraction of cuticular hydrocarbons

For the extraction of CHs following populations were available: three Colombian (Duitama, Cachipay, Sibundoy) and four Brazilian (Alagoas, Bento Gonçalves, Pelotas, São Joaquim) (Table 1). CHs of 20-days-old virgin males (N = 10) and females (N = 10) were extracted from the study populations following previously described methods (Vaníčková et al. 2012b, Vaníčková et al. 2014, Vaníčková et al. in press). 1-Bromdecane (Sigma-Aldrich, Czech Republic) was added as an internal standard for quantification (10 ng per 1 μL of the extract). Each extract was concentrated to approximately 100 μL under a constant flow of nitrogen and stored in a freezer (-5 °C) until analysis.

Chemical analyses

Two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC/MS) was used for the quantification and identification of male-borne volatiles and CH profiles. Identical conditions were used for the analyses of all samples and for chromatographic data evaluation, as described in previous studies on *A. fraterculus* male pheromones (Břízová et al. 2013, Milet-Pinheiro et al. 2015) and CHs (Vaníčková 2012, Vaníčková et al. 2012b, Vaníčková et al. in press). Standards of *n*-alkane (C_8–C_{38}; Sigma-Aldrich, Czech Republic) were co-injected with authentic samples to determine the retention indices (*RI*) of the analytes. Compounds were identified by comparison of their mass spectra fragmentation patterns, *RI* and authentic synthetic standards when available (Van Den Dool and Kratz 1963, Carlson and Yocom 1986, Vaníčková 2012, Vaníčková et al. 2012a, Břízová et al. 2013, Milet-Pinheiro et

al. 2015, Vaníčková et al. 2014, Vaníčková et al. in press). Detailed chemical identification of the CHs has been published previously (Vaníčková et al. 2012b, Vaníčková et al. in press).

Chemicals

Except for (*S,S*)-(-)-epianastrephin, which was provided by Prof. Jim Nation (University of Florida, Gainesville, USA), all chemicals were purchased either from Sigma-Aldrich, Brazil [α-pinene, limonene, (*Z*)-3-nonen-1-ol] or from Penta, USA [(*E,Z*)-3,6-nonadien-1-ol]. Chemicals were > 95% pure, based on the results from capillary gas chromatography.

Statistics

The relative peak areas of seven male-borne volatile compounds and forty-eight male and female CHs (as identified by the GC×GC/MS in the deconvoluted total-ion chromatogram mode) were calculated for each replicate of the study populations.

The differences in the chemical composition of the samples from study populations were analysed by principal component analysis (PCA). Prior to PCA, peak areas were subjected to logarithmic transformation; intraspecific scaling was performed by dividing each species score by its standard deviation; the data were centred by species' scores. In PCA analyses, hierarchical clustering based on Pearson correlation showed that populations with similar chemical profiles cluster together.

A heat map was used to visualize male-borne volatiles organized as matrices. The heat map performed two actions on a matrix. First, it reordered the rows and columns so that rows and columns with similar profiles were closer to one another, causing these profiles to be more visible to the eye. Second, each entry in the data matrix was displayed as a colour, making it possible to view the patterns graphically. Dendrograms were created using correlation-based distances and the Ward method of agglomeration was used in the present analysis (Key 2012). All computations were performed with R 3.1.2 (R Core Team 2014), and the R packages *FactoMineR* (Husson et al. 2015), and *gplots* (Warnes et al. 2015) were used.

Results

Male-borne volatiles

Significant quantitative differences ($P < 0.05$) were found in the male-borne volatiles, namely α-pinene, limonene, (*Z*)-3-nonen-1-ol, (*E,Z*)-3,6-nonadien-1-ol, (*Z,E*)-α-farnesene, (*E,E*)-α-farnesene and epianastrephin, among the investigated populations

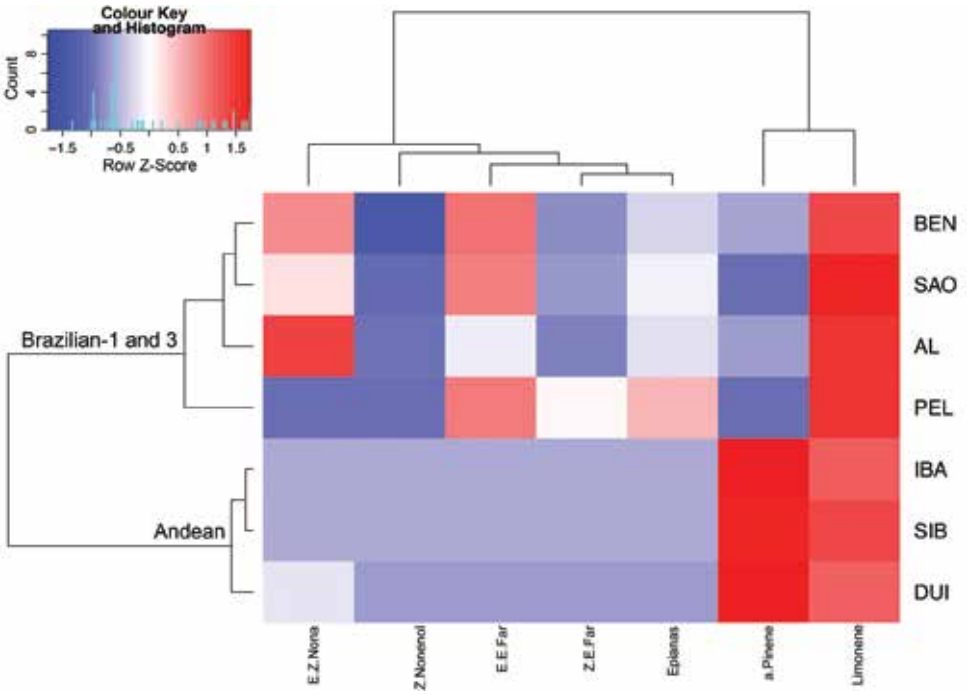

Figure 1. Heat map of seven male-borne volatiles (columns) identified by GC×GC/MS analyses in seven populations (rows) of the *Anastrepha fraterculus* cryptic species complex. The dendrograms were created using correlation-based distances and the Ward method of hierarchical clustering ($P < 0.05$). Key: AL - Alagoas, AL, Brazil; BEN - Bento Gonçalves RS Brazil; DUI - Duitama, Colombia; IBA - Ibague, Colombia; PEL - Pelotas, RS, Brazil; SAO - São Joaquim, SC, Brazil; SIB - Sibundoy, Colombia. Epianas – Epianastrephin; Z.E.Far - (*Z*,*E*)-α-farnesene; E.E.Far - (*E*,*E*)-α-farnesene; Z.Nonenol - (*Z*)-3-nonen-1-ol; E.Z.Nona - (*E*,*Z*)-3,6-nonadien-1-ol; a.Pinene - α-pinene.

of *A. fraterculus*. A heat map was constructed to visualize the relative proportions of the seven volatiles in each of the populations (Figure 1). Colombian populations (DUI, IBA, SIB) have similar proportions of the seven compounds, whereas the Brazilian populations (AL, BEN, PEL, SAO) have diverse volatile profiles. In the compound dendrogram, monoterpenes (α-pinene, limonene) formed one cluster, while sesquiterpenes [(*Z*,*E*)-, (*E*,*E*)-α-farnesenes], unsaturated alcohols [(*Z*)-3-nonen-1-ol, (*E*,*Z*)-3,6-nonadien-1-ol] and a lactone (epianastrephin) grouped in a second cluster. The most abundant volatile among all the populations was limonene. (*Z*)-3-Nonen-1-ol was the least abundant compound in the study strains. α-Pinene was more abundant in the Andean morphotype (DUI, IBA, SIB) than in the Brazilian morphotypes (AL, BEN, PEL, SAO). The relative proportion of (*E*,*Z*)-3,6-nonadien-1-ol varied the most between populations.

The PCA analyses of GC×GC/MS data showed the Andean morphotype formed one cluster while Brazilian-1 and Brazilian-3 morphotypes formed another cluster (Figure 2, Hierarchical clustering). The first two dimensions represented 85% of

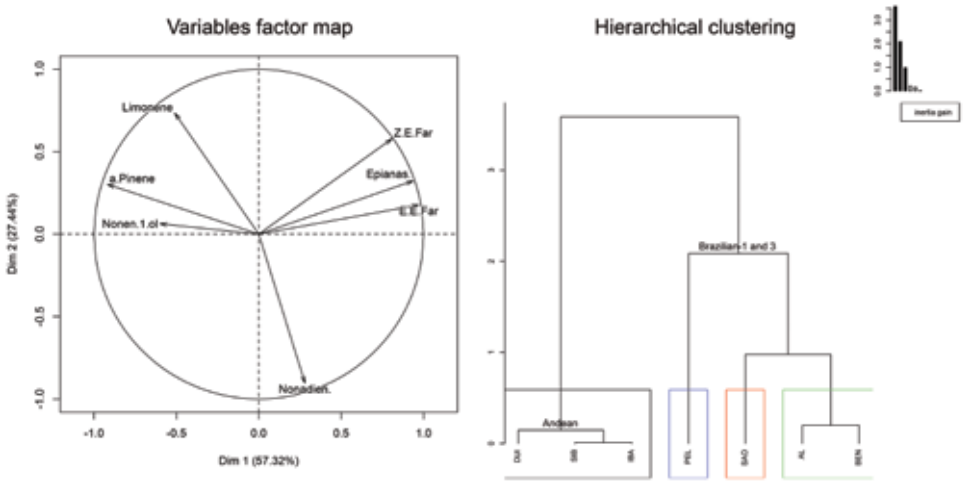

Figure 2. Principal component analyses (PCA) of transformed GC×GC/MS data of seven male-borne volatiles produced by groups of 20 sexually mature individuals from seven populations of the *Anastrepha fraterculus* cryptic species complex. Variables factor map represents projection of variables on the plane defined by the first two principal components. Hierarchical clustering is score plot describing the populations and their clustering. Key: AL - Alagoas, AL, Brazil; BEN - Bento Gonçalves RS Brazil; DUI - Duitama, Colombia; IBA - Ibague, Colombia; PEL - Pelotas, RS, Brazil; SAO - São Joaquim, SC, Brazil; SIB - Sibundoy, Colombia. Epianas. – Epianastrephin; Z.E.Far - (*Z*,*E*)-α-farnesene; E.E.Far - (*E*,*E*)-α-farnesene; Nonen.1.ol - (*Z*)-3-nonen-1-ol; Nonadien. - (*E*,*Z*)-3,6-nonadien-1-ol; a.Pinene - α-pinene. Colored boxes indicate particular clusters.

the total variance. The monoterpenes α-pinene and limonene and (*Z*)-3-nonen-1-ol were specific to the Andean morphotype, whereas sesquiterpenes (*Z*,*E*)- and (*E*,*E*)-α-farnesene, the lactone epianastrephin and (*E*,*Z*)-3,6-nonadien-1-ol were typical for the Brazilian morphotypes (Figure 2, Variables factor map). The three populations representing the Andean morphotype (DUI, IBA, SIB) grouped together forming the first cluster (Figure 2, Hierarchical clustering) whereas the second cluster was formed by the Brazilian populations representing the Brazilian-1 (BEN, PEL, SAO) and Brazilian-3 (AL) morphotypes.

Male CH profiles

Forty-eight male CHs, including 19 linear *n*-alkanes (A1-19), 11 methylbranched alkanes (B1-11), 11 alkenes (C1-11), and 7 alkadienes (D1-7), were evaluated by PCA for the possible use in the identification of Andean (CAC, DUI, SIB), Brazilian-1 (BEN, PEL, SAO) and Brazilian-3 (AL) morphotypes (Figure 3). The first two PCA dimensions accounted for 55% of the total variance. The populations clustered in two main groups, and each group was composed of two clusters. Clusters one and two were formed by Andean morphotype populations (DUI and CAC, SIB, respectively),

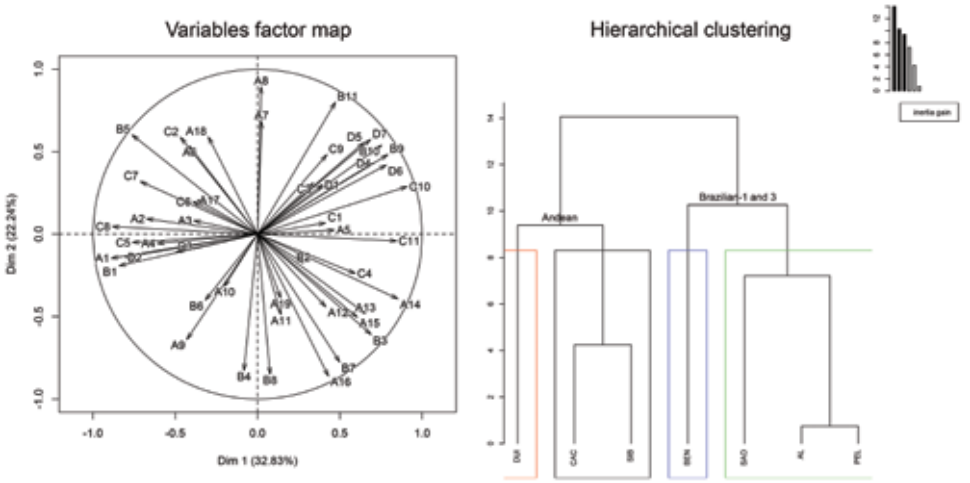

Figure 3. Principal component analyses (PCA) of transformed GC×GC/MS data of 48 male CHs from seven populations of the *Anastrepha fraterculus* cryptic species complex. Variables factor map represents projection of variables on the plane defined by the first two principal components. Hierarchical clustering is score plot describing the populations and their clustering. Key: AL - Alagoas, AL, Brazil; BEN - Bento Gonçalves RS Brazil; CAC - Cachipay, Colombia; DUI - Duitama, Colombia; PEL - Pelotas, RS, Brazil; SAO - São Joaquim, SC, Brazil; SIB - Sibundoy, Colombia. A1-19 - *n*-alkanes; B1-11 -methylbranched hydrocarbons; C1-11 - alkenes; D1-7 - alkadienes. Colored boxes indicate particular clusters.

whereas clusters three and four consisted of Brazilian morphotypes populations (BEN and AL, PEL, SAO; Figure 3, Hierarchical clustering). Linear *n*-alkanes (*n*-nonacosane A14, *n*-hentriacontane A16), methylbranched hydrocarbons (3-methylheptacosane B3, methylheptatriacontane B11) and 11-tritriacontene (C10) were responsible for the formation of the Brazilian morphotype clusters (AL, BEN, PEL, SAO), whereas *n*-dodecane (A1) and the mix of odd methylbranched hydrocarbons (9-/11-/13-methylnonacosane B5) were characteristic for the Andean group (Figure 3, Variables factor map, Suppl. material 1).

Female CH profiles

Female CH profiles from study populations consisted of 48 saturated and unsaturated compounds with chain lengths ranging from 12-38 carbons. In female body washes, unsaturated male-specific CHs were absent, namely 7-heneicosene, 7-docosene, 7-tricosene and 7-pentacosene. In the PCA analyses the populations segregated into two main groups (Figure 4, Hierarchical clustering). The three Colombian populations (CAC, DUI, SIB) grouped together forming one cluster. The second cluster was formed by the four Brazilian populations (AL, BEN, PEL, SAO) (Figure 4, Hierarchical clustering). The compounds responsible for this separation were *n*-docosane (A7), methylbranched hydrocarbons (2-methyloctacosane B4, 3-methylnonacosane B7) and

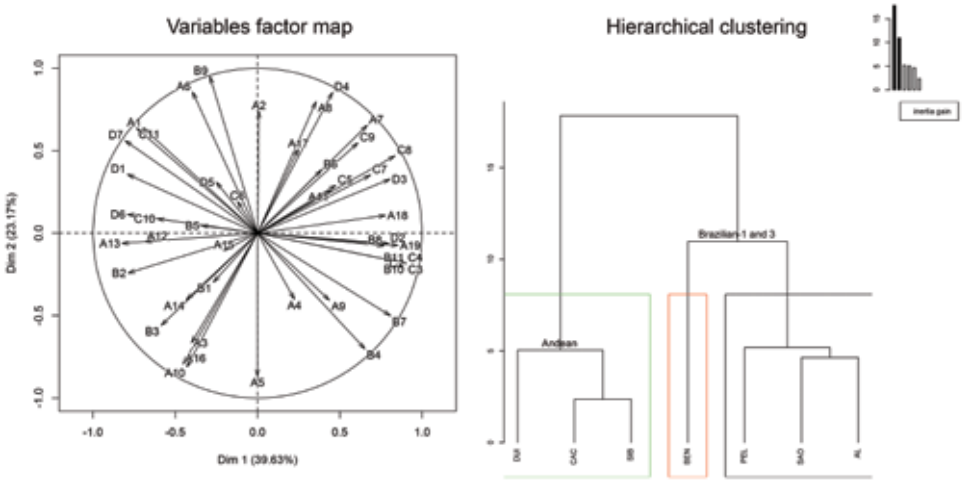

Figure 4. Principal component analyses (PCA) of transformed GC×GC/MS data of 48 female CHs from seven populations of the *Anastrepha fraterculus* cryptic species complex. (Variables factor map) projection of variables on the plane defined by the first two principal components. (Hierarchical clustering) score plot describing the populations and their clustering. Key: AL - Alagoas, AL, Brazil; BEN - Bento Gonçalves RS Brazil; CAC - Cachipay, Colombia; DUI - Duitama, Colombia; PEL - Pelotas, RS, Brazil; SAO - São Joaquim, SC, Brazil; SIB - Sibundoy, Colombia. A1-19 - *n*-alkanes; B1-11 -methylbranched hydrocarbons; C1-11 - alkenes; D1-7 - alkadienes. Colored boxes indicate particular clusters.

unsaturated CHs (hentriacontene C8, tetratriacontadiene D4), which were specific to the Brazilian morphotypes. The Andean morphotype (CAC, DUI, SIB) was defined by the presence of linear compounds (*n*-dodecane A1, *n*-pentacosane A10, *n*-hentriacontane A16), a mix of methylbranched CHs (9-/11-/13-methylhentriacontane B9) and heptatriacontadiene (D7) (Figure 4, Variables factor map, Suppl. material 1).

Discussion

Chemical profiles of *A. fraterculus* varied quantitatively among populations from diverse regions of South America. To some extent, the chemical profiles showed comparable patterns among populations belonging to the same morphotype. Comparison of the chemical profiles of the Andean and two Brazilian morphotypes showed that the more geographically distant the morphotypes are, the more diverse their pheromone and CH profiles are. Nevertheless, this trend was not observed between the Brazilian-1 and Brazilian-3 morphotypes. Within the *A. fraterculus* complex, the Andean morphotype is allopatric, while the Brazilian morphotypes (Brazilin-1, Brazilian-2 and Brazilian-3) are sympatric (Hernández-Ortiz et al. 2012, Selivon et al. 2005). The sympatry of the Brazilian entities may be one of the possible factors contributing to the similarity of their chemical profiles. Evidence of similarities of pheromone profiles within and between the species complexes of sympatric populations comes from an extensive evo-

lutionary study of Hawaiian Drosophilidae CHs (Alves et al. 2010). Such evolutionary studies are missing for the *A. fraterculus* complex and nothing is known about the role of pheromones (short- or long-range) in the speciation process of this species.

The Andean morphotype populations had very different pheromone profiles from that of the two Brazilian morphotypes. Břízová et al. (2013) and Cáceres et al. (2009) reported extensive qualitative and quantitative differences in the male pheromone composition of Brazilian, Argentinean and Peruvian populations of the *A. fraterculus* complex. Our study demonstrated that the common plant monoterpene, limonene, varies least among all populations. Two of the most variable volatiles from the male pheromone mixture were (*E*,*Z*)-3,6-nonadien-1-ol and (*E*,*E*)-α-farnesene. As reported by Milet-Pinheiro et al. (2015), (*E*,*Z*)-3,6-nonadien-1-ol, α-farnesene, and epianastrephin are highly attractive to conspecific females after approaching a mating site, while plant compounds such as α-pinene or limonene are used by female to find mating and brood sites (Robacker and Hart 1986). We speculate that the different ratios of (*E*,*Z*)-3,6-nonadien-1-ol, (*E*,*E*)-α-farnesene, and epianastrephin, together with the diverse CH profiles, result in the final rejection of a male by an heterospecific female. A recent study on reproductive compatibility demonstrated that the Andean morphotype is fully incompatible with the Brazilian-1 and Brazilian-3 morphotypes (Devescovi et al. 2014). The authors stated that prezygotic sexual incompatibility might be a result of the differences in the timing of mating activities between the morphotypes studied. Considering the mating of *A. fraterculus*, where chemical communication plays an important role in acceptance or rejection of males, we suggest that the differences in the chemical profiles identified in the present study may also contribute to sexual incompatibility. Nevertheless, further electrophysiological and behavioural studies involving different morphotypes of the *A. fraterculus* complex need to be performed in order to evaluate this hypothesis.

Male and female flies of the Andean morphotype and the Brazilian morphotypes can be separated using CH profiles (Vaníčková et al. in press). The Andean morphotype formes a separated group whereas the Brazilian-1 and Brazilian-3 morphotypes create a monophyletic cluster. Nonetheless, Brazilian populations belonging to the same geographical areas do not group together. Variation in the chemical composition of CH profiles identified here may be influenced by genetic variability within and between populations of the Brazilian-1 and 3 morphotypes. It is important to note that all of the Brazilian populations investigated here were created during several generations under identical laboratory conditions, which could possibly influence the results presented here. Houot et al. (2010) reported the effects of laboratory acclimation on the variation of male courtship, mating and the production of sex pheromone, in *D. melanogaster*. These authors concluded that the reproduction-related characters could diverge between neighboring *D. melanogaster* populations, and differently adapt to stable laboratory conditions. Nevertheless, in tephritidae these kinds of studies are missing.

Selivon et al. (2004) found conspicuous differences between sex chromosomes that separated the Brazilian-3 from the Brazilian-1 and Brazilian-2 morphotypes. The possibility for hybridization between distinct cryptic species within the *A. fraterculus* complex and meiotic recombination of chromosomal markers could form the genetic

basis by which CHs vary between related putative species. The evidence comes from experiments with *Drosophila* spp. that explained interspecific variation of CH profiles (Coyne et al. 1994, Coyne and Oyama 1995, Doi et al. 1996, Coyne and Charlesworth 1997). Future genetic analyses are necessary for evaluation of this hypothesis within the *A. fraterculus* complex.

CHs in insects serve primarily to prevent desiccation by reducing water loss (Blomquist et al. 1987, Blomquist and Bagnères 2010). Populations living in warmer and drier environments lose water less rapidly and usually have longer chain-length CHs than populations in humid habitats (Blomquist and Bagnères 2010). However, the relationship between CH structure and the capacity to resist desiccation is not so simple. In our previous work on CH profiles from geographically distinct populations of the *A. fraterculus* complex, we reported that the relative proportions of these compounds vary, depending on relative humidity, relative temperature and altitude (Vaníčková et al. in press). In the present study, CH profiles from the Andean morphotype formed one single cluster. These populations live naturally at high altitudes with lower relative temperatures, and therefore their specific CHs are long methylbranched compounds (e.g. methylheptatriacontane). However, the same conclusion is not possible to draw for the Brazilian-1 and Brazilian-3 morphotypes studied. The three Brazilian morphotypes may co-occur in the same localities infesting guava fruits (*Psidium guajava* L.) (Selivon et al. 2004). The Brazilian-3 morphotype seems to be restricted to the Atlantic coastal areas and may co-occur with the Brazilian-2 morphotype infesting guava and tropical almond (*Terminalia catappa* L.). The Brazilian-1 morphotype occurs from northern Argentina through southern and southeastern Brazil and may also co-occur together with the Brazilian-2 morphotype infesting guavas and oranges (*Citrus* sp.). In Brazil there are 70 different host plants for the *A. fraterculus* complex recorded (Zucchi 2007). Etges and Jackson (2001) reported that the variation of CH profiles between closely related species or between populations of these species of *D. mojavensis*, reflects the adaptation to different host plants. In these flies, the ratio of the principal CHs rapidly changed with laboratory acclimation, and influenced courtship and mating (Stennett and Etges 1997, Houot et al. 2010). These CH changes depend on enzymes whose level could represent a metabolic adaptation to host-plant chemicals (Higa and Fuyama 1993, Jones 2001, Houot et al. 2010). Varying the availability of different nutrients could also account for CH variation between strains raised in the laboratory (Stennett and Etges 1997). These factors, possibly influencing CH composition in the *A. fraterculus* complex, need to be carefully investigated in future studies.

Conclusion

The present study demonstrates that pheromone components and CH profiles diverge qualitatively between Andean and Brazilian-1 and Brazilian-3 morphotypes and may be used to some extent to delimit morphotypes in the *A. fraterculus* species complex. Comprehensive studies, which simultaneously examine environmental, behavioural,

genetic and chemical features are necessary to be performed aiming to understand which factors affect the geographical variation in the male-borne volatiles and CH profiles in the *A. fraterculus* complex.

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Supplementary material 1

Table S1. *Anastrepha fraterculus* male and female characteristic.

Authors: Lucie Vaníčková, Radka Břízová, Antonio Pompeiano, Luana Lima Ferreira, Nathaly Costa de Aquino, Raphael de Farias Tavares, Laura D. Rodriguez, Adriana de Lima Mendonça, Nelson Augusto Canal, Ruth Rufino do Nascimento

Data type: species data

Explanation note: *Anastrepha fraterculus* male (m) and female (f) characteristic cuticular hydrocarbons identified by principal component analyses.

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Current knowledge of the species complex *Anastrepha fraterculus* (Diptera, Tephritidae) in Brazil

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Abstract

The study of the species complex *Anastrepha fraterculus* (*Af* complex) in Brazil is especially important in a taxonomical, evolutionary and pest management context, because there are evidences that some of them may occur in sympatry. In this review, we analyzed the main results supporting evidences that three cryptic species occur in Brazil. The taxonomical and phylogenetic relationships based on eggshell morphology, adult morphometrics, as well as cytotaxonomy and genetic differentiations are discussed. We also review available information on sexual behavior including acoustic communication of males during courtship and sexual incompatibility; and chemical signals involved in the communication between sexes, with a special focus on sex pheromones. We examined the role of long- and short-range pheromones (male-produced volatiles and cuticular hydrocarbons, respectively), their implications in sexual isolation, and their possible use for chemotaxonomic differentiation of the putative species of the *Af* complex.

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Keywords
South American fruit fly, cryptic species, taxonomy, sexual behavior, chemical communication, acoustic communication

Introduction

The fruit fly *Anastrepha fraterculus* constitutes a complex of cryptic species (*Af* complex) currently composed of eight taxonomically recognized morphotypes (Hernández-Ortiz et al. 2012, 2015). Its geographical distribution ranges from Southern Texas through Eastern Mexico, Central and South America (Stone 1942, Hernández-Ortiz and Aluja 1993, Norrbom et al. 1999). In Brazil, *A. fraterculus* is one of the most important polyphagous pests infesting about 70 host plant species (Zucchi 2007, 2008). Several studies confirmed that natural Brazilian populations of *A. fraterculus* have morphological, biological, and genetic differences throughout their geographical distribution (Stone 1942, Malavasi and Morgante 1983, Steck 1991, Selivon and Perondini 1998, Selivon et al. 1997, 1999, 2004, 2005a,b, Silva and Barr 2008). Three entities of the *Af* complex, termed as *A*. sp.1 *aff. fraterculus*, *A*. sp.2 *aff. fraterculus*, and *A*. sp.3 *aff. fraterculus*, occur in Brazil (Yamada and Selivon 2001, Selivon et al. 2004, 2005a). Although, an extensive review of genetics and biology of *A. fraterculus* from Argentina has been published (Cladera et al. 2014), information about the ecology, taxonomy, and behavior of *A. fraterculus* putative species in Brazil is still insufficient and this imposes constrains to implementation of environmental friendly control methods, such as the Sterile Insect Technique (SIT) (Dyck et al. 2005). In order to apply SIT, insect strains are reared on a massive scale in facilities with the potential to produce millions of sterile insects per week (Hendrichs et al. 1995). Compatibility between wild and laboratory reared insects is critical for the success of this pest management method (Wong et al. 1982, Cayol 1999, Meza-Hernández and Díaz-Fleischer 2006, Benelli et al. 2014a).

Here, we present a revised synthesis on the current status of our knowledge of the *A. fraterculus* complex in Brazil, focusing on divergence among Brazilian populations by evaluating multiple aspects: (i) taxonomy and relationships, (ii) sexual behavior and reproductive incompatibility, and (iii) chemical communication between sexes.

Taxonomy and relationships

The first documented evidence of a cryptic species complex appeared in the comprehensive taxonomic revision of the genus *Anastrepha* by Stone (1942). He described extensive morphological variation among specimens from Mexico through South America and considered these samples to constitute geographical races. Stone stated: "As treated here it [*Anastrepha fraterculus*] extends from the Rio Grande valley in Texas

south to Argentina, and it is possible that it will eventually be found to represent a complex of species rather than a single one."

Since then, enough information has been gathered to affirm that the nominal species *Anastrepha fraterculus* in fact represents a cryptic species complex (*Af* complex). Some studies conducted in the 1990's correlated morphological traits and genetics of Brazilian samples (Selivon and Perondini 1998, Selivon et al. 1996, 1997, 1999), enabling the recognition of two different biological entities within the complex (referred in this paper as *A.* sp.1 or Brazilian-1 and *A.* sp.2 or Brazilian-2). Later Selivon et al. (2004, 2005a,b) used diverse sources and proposed the existence of a third Brazilian entity named *A.* sp.3 *aff. fraterculus* (abbreviated in this paper as *A.* sp.3 or Brazilian-3).

Adult morphology

The historical taxonomy of the genus *Anastrepha* is largely based on adult characters of the female aculeus, external morphology of the body, and the wing pattern (Stone 1942, Steyskal 1977) (Figure 1). Although males of many species currently cannot be distinguished at all, recent studies have found characters in the male genitalia useful for identification of some species groups and phylogenetic relationships (Norrbom et al. 1999, 2012).

Taxonomic studies of the nominal *A. fraterculus* (*sensu lato*) done by Lutz and Lima (1918), Lima (1934), Greene (1934) and later by Stone (1942), showed high variability among adult populations, so currently six synonyms are based on Brazilian specimens: *Tephritis mellea* Walker, 1837 (St. Paul's, Brazil); *Anastrepha fraterculus* var. *soluta* Bezzii, 1909 (São Paulo, Brazil); *Anastrepha braziliensis* Greene, 1934 (Viçosa, Minas Gerais, Brazil); *Anastrepha costarukmanii* Capoor, 1954 (Itajuba, Minas Gerais, Brazil); *Anastrepha scholae* Capoor, 1955 (Agua Preta, Bahia, Brazil); *Anastrepha pseudofraterculus* Capoor, 1955 (Itatiaia, Rio de Janeiro, Brazil).

Besides, morphometric assessment proved to be useful for the recognition of *Anastrepha* species. Araujo and Zucchi (2006) performed a discriminant function analysis on linear measures of the aculeus to separate Brazilian samples of *A. fraterculus* (*s.l.*) from *A. obliqua* (Macquart), *A. sororcula* Zucchi, *A. zenildae* Zucchi and *A. turpiniae* Stone. Recently geometric morphometrics of the wing has been also used for the recognition of species such as *A. fraterculus* (*s.l.*), *A. obliqua*, and *A. sororcula* (Perre et al. 2014). All species are related as they were classified within the "*fraterculus* species group" (*sensu* Norrbom et al. 2012). Comparative studies on morphometrics of the wing of males and females, and the aculeus tip was done with Brazilian *A.* sp.1 and *A.* sp.2. These results demonstrated that wing morphometrics could be used to distinguish these species efficiently, and wing sexual dimorphism was also recognized (Selivon et al. 2005a).

However, based on adult morphology, the first evidence of differences among Brazilian populations of the *Af* complex from other countries was made with compari-

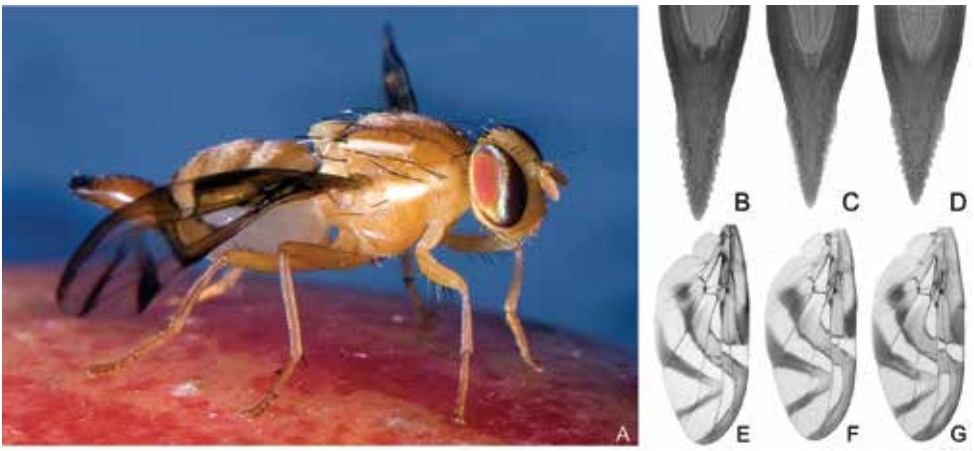

Figure 1. Adult female *Anastrepha fraterculus* (**A**) and typical forms of the aculeus tip and the wing pattern of morphotypes Brazilian-1 (**B, E**), Brazilian-2 (**C, F**), Brazilian-3 (**D, G**), respectively. (The photo of adult was made by Dr. Hoskovec, the images of aculeus and wings were modified from Hernández-Ortiz et al. 2012).

sons of seven samples from Mexico, two from Brazil (São Paulo and Piracicaba), and each one from Argentina (Tucumán) and Colombia (Tolima) (Hernández-Ortiz et al. 2004). They used measures of the aculeus, wing and mesonotum of females. These data clearly separated a Mexican morphotype, and the Brazilian and the Argentinean samples were clearly differentiated from the Colombian sample, resulting in the naming of these clusters as Brazilian and Andean morphotypes, respectively.

Further morphometric analyses using 32 populations from Mexico, Central America, and South America (including Venezuela, Colombia, Ecuador, Peru, Brazil and Argentina), confirmed previous findings and clearly added that within the *Af* complex seven morphotypes could be discerned throughout the Neotropical region (Hernández-Ortiz et al. 2012). The eight Brazilian populations examined belong to the biogeographical sub-regions Chacoan and Paranaense, distinguishing three discrete clusters nominated morphotypes; the Brazilian-1 morphotype comprised samples from the states of São Paulo, Santa Catarina, and Minas Gerais; the Brazilian-2 morphotype was represented by two samples from Ilha Bela and São Sebastião (state of São Paulo); and the Brazilian-3 morphotype was characterized by a single sample from Ubatuba (São Paulo).

Egg morphology

Differences in egg morphology discovered between Brazilian populations of the *Af* complex suggested the existence of two different taxonomic entities for the first time. Through scanning electron microscopy, Selivon and Perondini (1998) de-

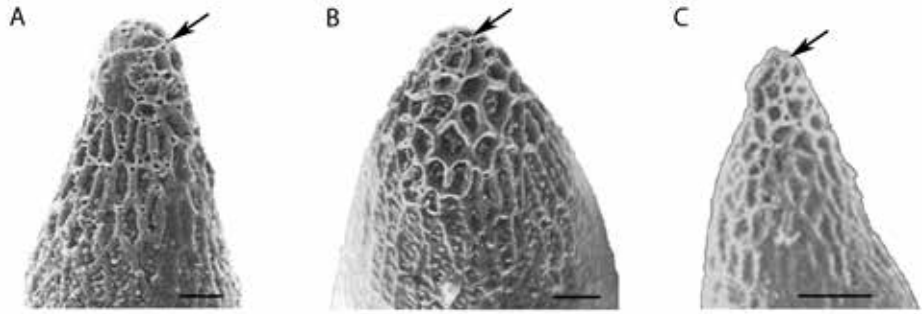

Figure 2. Scanning electron micrographs of the anterior tip (dorsal view) of eggs from Brazilian-1 (**A**), Brazilian-2 (**B**), Brazilian-3 (**C**) morphotype of *Anastrepha fraterculus*. The arrow shows aeropyles. Bars = 20 µm (**A, B**) and 50 µm (**C**). The images were modified from Selivon et al. 2004, and from Selivon and Perondini 1998, with permision.

scribed the external morphology of the chorion characterizing two distinct biological entities called *A. fraterculus* sp.1 and *A. fraterculus* sp.2, which were later named *A.* sp.1 *aff. fraterculus* and *A.* sp.2 *aff. fraterculus* (Yamada and Selivon 2001). Eggs of *A.* sp.1 are smaller than those of *A.* sp.2; in the former there is a papilla at the anterior pole which is absent in the *A.* sp.2; the micropyle is closer to the apex in *A.* sp.2; the anterior pole is ornamented by folds of the chorion forming irregular polygons in both, although in eggs of *A.* sp.2 these folds are arranged in a rosette around the micropyle. Aeropyles are found almost exclusively on the ventral side of the anterior pole, being more numerous in *A.* sp.1. Later Selivon et al. (2004) described the eggs of Brazilian *A.* sp.3, showing that it differs from *A.* sp.1 and *A.* sp.2 in terms of size, position of the micropyle, and the ornamentation of the chorion at the anterior pole (Figure 2).

Geographic distribution and host use

Concerning the species distribution and host use of the Brazilian members of the complex, there is very limited information and few inferences can be made. For example, two species of the Brazilian complex, *A.* sp.1 and *A.* sp.2 exhibited preferentially an allopatric distributional pattern. However, in most of 18 locations sampled from Brazilian Inland Plateau, they can be found together infesting guavas (*Psidium guajava*) and oranges (*Citrus* sp.), respectively, and only two locations in the Paraíba valley (Santa Isabel and Jambeiro, in the state of São Paulo) recorded the co-occurrence of the three Brazilian forms (Selivon et al. 2004, Selivon and Perondini 2007). Other species, *A.* sp.3 was very common in the Atlantic coastal region (in the states of Rio de Janeiro, São Paulo, and Santa Catarina), and no records were documented in most locations from Brazilian Inland Plateau (in the states of Santa Catarina, Paraná, São

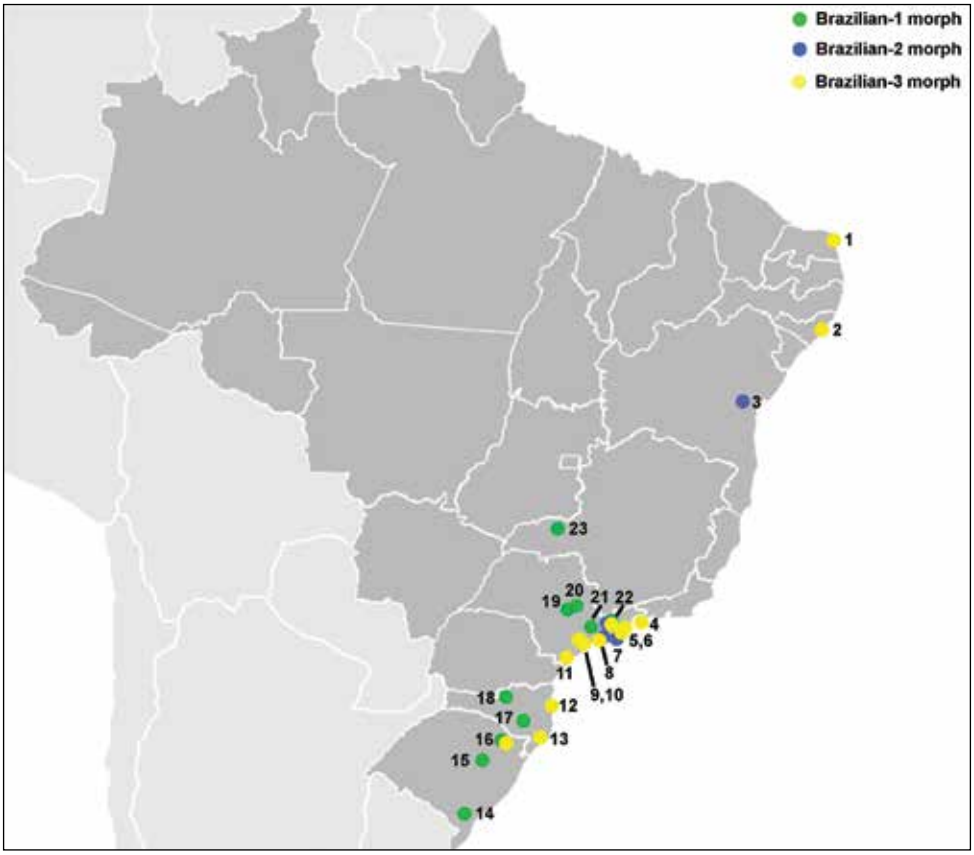

Figure 3. Map of Brazil indicating the geographic locations from which Brazilian-1 (green), Brazilian-2 (blue) and Brazilian-3 (yellow) morphotypes of *Anastrepha fraterculus* were collected. **1** Parnamirim, RN **2** Maceio, AL **3** Conceição de Almeida, BA **4** Parati, RJ **5** Ubatuba, SP **6** Caraguatatuba, SP **7** Ilhabela, SP **8** São Sebastião, SP (region of sympatry of Brazilian-2 and Brazilian-3 morphotypes, Selivon et al. 2004, Hernández-Ortiz et al. 2012) **9** Maresias, SP **10** Miracatu, SP **11** Morretes, SP **12** Porto Belo, SC **13** Criciúma, SC **14** Pelotas, RS **15** Bento Gonçalves, RS **16** Vacaria, RS (region of sympatry of Brazilian-1 and Brazilian-3 morphotypes, Selivon et al. 2004) **17** São Joaquim, SC **18** Caçador, SC **19** Botucatu, SP **20** Piracicaba, SP **21** São Paulo, SP **22** Santa Isabel and Jambeiro, SP (Paraíba valley - region of sympatry of the three morphotypes, Selivon et al. 2004) **23** Uberlandia, MG.

Paulo, Goiás, and Minas Gerais) (Figure 3). Therefore, *A*. sp.3 seems to be restricted to the coastal plain areas, where it co-occurs with *A*. sp.2 and can be found even infesting the same host fruits, guava or tropical almond (*Terminalia catappa*). Food preferences of the *Af* complex remain uncertain, since about 70 host plant species have been recorded in Brazil for the nominal *A. fraterculus* (*s.l.*) (Zucchi 2007, 2008). While a number of these records may be questionable due to possible misidentification of fruit flies, understanding the relationships of the Brazilian entities with their native hosts will be of great relevance to determine the pest status of each taxonomic entity within the species complex.

Cytotaxonomy

The first cytological evidence of differences between samples of the *Af* complex was reported by Bush (1962). He compared the karyotypes of a Mexican sample respective to the Brazilian population described by Mendes (1958). Bush suggested that this difference might represent a case of chromosomal polymorphism or, more likely, sibling species. This conclusion was supported based on morphological differences attributed to geographical variation and distinct host preferences because the Brazilian population is a pest of citrus, while the Mexican population does not infest citrus and therefore is not considered economically important in Mexico.

Later Solferini and Morgante (1987) studied the karyotype of eight *Anastrepha* species from Brazil, highlighting that all of them could be identified on the basis of chromosome morphology. Samples from six Brazilian localities were studied – Itaquera, Sorocaba, São Roque (from São Paulo state); Conceição do Almeida, Cruz das Almas, and Santo Antonio de Jesus (from Bahia state). Four distinguishable karyotypes were found; two of them from the state of São Paulo, and two others from the state of Bahia, suggesting that they represent sibling species. However, one karyotype described from Bahia actually corresponded to *Anastrepha sororcula* Zucchi (Morgante et al. 1993). In the nominal species *A. fraterculus*, similar acrocentric autosomes and chromosome numbers (2n=12) have been described from populations in Mexico, Brazil and Argentina (Mendes 1958, Bush 1962, Solferini and Morgante 1987, Selivon et al. 1996, Selivon et al. 1997, Basso et al. 2003). In the Brazilian species complex, conspicuous differences in the sex chromosomes were found. Chromosomes X and Y were larger in *A*. sp.2 respective to *A*. sp.1 and *A*. sp.3; in addition, to differences in the distribution and location of the blocks of constitutive heterochromatin (Selivon et al. 2004, 2005a).

More recently, Goday et al. (2006) performed a comparative analysis of heterochromatin organization in the sex chromosomes to determine the rDNA loci. They used sequential staining techniques with DAPI and chromomycin A3 fluorochromes, which have different affinities for DNA bases, followed by C-banding. A specific sex–chromosome banding pattern was obtained. This technique demonstrated structural differences on the Y chromosome between *A*. sp.1 and *A*. sp.3, allowing an accurate separation of these two species with this method.

Genetics

The first molecular study of intraspecific variation in the *Af* complex in Brazil was performed by Morgante et al. (1980) using an isozyme electrophoresis analysis. They studied 11 enzymatic loci for 16 populations of *A. fraterculus* (*s.l.*) coming from six localities of southern, southeastern and northeastern Brazil [Itaquera, Sorocaba, Sao Roque (São Paulo); Conceição do Almeida, Cruz das Almas, and Santo Antonio de Jesus (Bahia)]. They summarized the "*Af* complex" as consisting of four population subgroups, with the northeastern populations being more different from the others.

However, one member of the subgroup from northeastern Brazil (Bahia) was later determined to belong to *A. sororcula* Zucchi (Morgante et al. 1993), and corresponding to the karyotype 3 formerly described by Solferini and Morgante (1987).

A similar isozymic analysis conducted by Steck (1991) involved samples spanning a wide geographical range (Mexico, Costa Rica, Venezuela, Peru and Brazil). His results showed strong genetic differentiation within the nominal *A. fraterculus*. Extreme frequency and/or fixed allele differences were found among samples from Andean vs lowland Venezuela and also between Brazilian samples from the south (São Paulo) vs the northeast (Bahia). Separation among samples were far greater than any observed among populations of the reference species, suggesting that the *Af* complex as it now stands may even not be monophyletic (Steck 1999). Shortly thereafter, Steck and Sheppard (1993) corroborated the findings of isozyme data by using mitochondrial DNA restriction fragment length polymorphism (RFLP). This method separated populations from northeast and southeast Brazil. They also demonstrated that specimens from coastal Venezuela and from the Bahia region were highly differentiated, even though they originally seemed similar based on isozymic analyses.

Studies of mitochondrial DNA (Santos 1994) also reported strong evidence of large inter-population variability in Brazilian samples, recognizing the existence of two haplotypes within the nominal species *A. fraterculus* when compared with *A. obliqua* and *A. sororcula*. McPheron et al. (1999) investigated 16S rDNA data to analyze the relationships among *Anastrepha* species, however they only included two sequences of the *Af* complex. They found that a single specimen from Mérida (Venezuela) was distinct from a specimen collected in the state of São Paulo (Brazil). The phylogenetic relationships inferred from mtDNA sequences of COI by Smith-Caldas et al. (2001), further supported the presence of multiple gene pools within the nominal *A. fraterculus*. They suggested a cryptic species exists in the high elevations of the Andes and further corroborated the non-monophyly among samples of the *Af* complex.

Rocha and Selivon (2004) analyzed samples involving the three Brazilian entities, subjecting the total DNA to fragmentation by restriction endonucleases. The banding pattern showed specificity among species with *A.* sp.3 being very distinct from *A.* sp.1 and *A.* sp.2. Some bands were common to species of the *Af* complex and six other *Anastrepha* species as well. However, other bands were only observed in the three entities of the *Af* complex but in different arrangements within the genome (Selivon and Perondini 2007). Selivon et al. (2005a) conducted combined analyses of isozymes, karyotypes, morphometry, and crossings from 10 Brazilian populations. The isozymic study comprised a survey of 16 enzymatic systems of 19 loci, and results showed significant differences in the allele frequencies at four loci (FUM, ME, HEX, and LDH). Results showed the presence of two clearly distinct genetic clusters, which were related differentially with other species of the *fraterculus* species group, suggesting that *Af* complex would not be monophyletic. The most important finding of this work was that both clusters differed in the length of their sex chromosomes and the size and location of heterochromatic regions.

Barr et al. (2005) used the nuclear gene *period* to reconstruct the phylogeny of *Anastrepha*, but their tree included only seven specimens of *A. fraterculus*, four from Venezuela (Mérida and Caracas), two from México, and a single one from Brazil (São Paulo). The Brazilian specimen was distinct from the two other clades composed by Mexican and Venezuelan specimens, further suggesting that the nominal *A. fraterculus* is not monophyletic.

Vaníčková (2012) performed a comparative study of COII and ITS1 using two populations of *A. fraterculus* (Bento Gonçalves, Rio Grande do Sul; Tucumán, Argentina). The sequenced parts of the COII gene were not different. The sequencing of ITS1 gene resulted in AT-rich sequences (84%) and released tandem-repeats/poly-N stretches and poly-A-stretches. Nevertheless, the variability of the sequences was very low. These results confirmed that the studied populations belong to the same Brazilian-1 morphotype. Further studies including brother populations sampling were suggested in order to confirm COII and ITS1 as suitable genes for resolution of the three Brazilian forms inside the *Af* complex.

Recently, Silva et al. (2014) investigated the variability of COI among 200 specimens of *A. fraterculus* sampled from Brazil, Mexico, and Argentina. The COI genetic variation in *A. fraterculus* was high. Three haplotypes were exclusive to Brazilian collections, one to Argentina, and five to Mexico. For Brazil, the most common haplotype was seen among 73% of the samples. These authors concluded that based on the phylogenies and geography of samples, the COI gene has limited utility in recognizing cryptic species.

Unfortunately, available data as a whole do not permit correlating different karyotypes or genetic and molecular traits, with the morphology and distribution of the Brazilian sibling species, mainly because studies were carried out with flies from different locations. A synthesis of molecular datasets from the existing literature is precluded because the original authors applied different methodologies or genetic loci to analyse *A. fraterculus* samples (Silva and Barr 2008).

Sexual behavior and reproductive incompatibility

The lek polygyny mating system displayed by *A. fraterculus* was first described by Malavasi et al. (1983) through systematic field observation. Calling males aggregate in the top of host and non-host trees from the first hour after dawn until mid-morning, forming groups in which males fight to defend a small territory where they court females and mate (Segura et al. 2007, Benelli et al. 2014b). In *A. fraterculus* male aggressions are not frequent and/or outcomes are not crucial for mating success (Segura et al. 2007, Benelli 2015a, 2015b). The courtship behavior exhibited by *A. fraterculus* lekking males is complex and composed of visual, acoustical, and chemical displays (Mankin et al. 1996, Segura et al. 2007, Gomez Cendra et al. 2011). Differences in either time of mating or patterns of courtship behavior among species from the *Af* complex have the potential to affect mating recognition and ultimately lead to reproductive isolation (Morgante et

al. 1983, Selivon et al. 1999, Vera et al. 2006, Cáceres et al. 2009, Segura et al. 2011, Dias 2012, Rull et al. 2013, Devescovi et al. 2014). Herein, time of mating, patterns of courtship behavior, aspects of reproductive compatibility/incompatibility and acoustic communication will be reviewed regarding the Brazilian populations of the *Af* complex.

Courtship behavior

Overall, time of mating and the period in which *Af* complex males from the studied Brazilian populations display their courtship behavior in leks seem to occur mainly in the morning, shortly after the sunrise (Malavasi et al. 1983, Lima et al. 2001, Vera et al. 2006, Rull et al. 2012, Dias 2012). So far, differences in mate timing have not been reported yet for Brazilian entities of the *Af* complex.

Lek formation and courtship displays were compared among five Brazilian populations of the *Af* complex from South (Bento Gonçalves, Pelotas, and Vacaria - Rio Grande do Sul; São Joaquim - Santa Catarina) and Southeast regions of Brazil (Piracicaba - São Paulo) by Dias (2012). Males from both Brazilian regions seem to be attracted to the same leks because no differences were found in the male lek distribution in field cages; however, males from the same regions differed in the frequency of some courtship displays. According to Dias (2012), 12 behavioral units, defined as distinct steps of male courtship, characterize the sexual behavior displayed by *Af* complex males from South and Southeast Brazil and comparisons made on five behavioral unit frequencies associated with mating success revealed differences among them.

Reproductive incompatibility

Although Brazil is the South American country with the highest number of *Af* complex entities (Hernández-Ortiz et al. 2012), little is known about the sexual compatibility of its *A. fraterculus* populations. Partial postzygotic isolation between two Brazilian populations, determined as *A*. sp.1 and *A*. sp.2, was reported by Selivon et al. (1999). In this work, some degree of postzygotic reproductive isolation was found among F1 crosses between *A*. sp.1 males (Vacaria, Rio Grande do Sul) and *A*. sp.2 females (Conceição do Almeida, Bahia). Cytoplasmic incompatibility between different *Wolbachia* strains found in eggs of *A*. sp.1 and *A*. sp.2 has been suggested as one of the causes of their postzygotic reproductive isolation (Selivon et al. 1996; Selivon et al. 1999; Selivon et al. 2002). Later, *Wolbachia* strains were also found in adults from Piracicaba (Cáceres et al. 2009) as well as in *A*. sp. 1, *A*. sp.2, and *A*. sp. 3 from Southeastern Brazil (Marcon et al. 2011). Postzygotic reproductive isolation was reported by Selivon et al. (2005a) based on crosses between two population clusters of *Af* complex from Northeastern (Rio Grande do Norte, Bahia), Southeastern (Minas Gerais, São Paulo), and Southern (Rio Grande do Sul) Brazil, corroborating a previous study (Selivon et al. 1999).

Vera et al. (2006) found partial sexual isolation between *A. fraterculus* from Piracicaba (São Paulo) and Argentina (Tucumán), as well as high sexual isolation between them and two Peruvian (La Molina, Piura) *A. fraterculus* populations. Dias (2012) reported full sexual compatibility among *A. fraterculus* populations from the south region of Brazil (Bento Gonçalves, Pelotas, Vacaria, and São Joaquim), but partial sexual isolation between flies from the south and southeast (Piracicaba) regions. Rull et al. (2012) found prezygotic and postzygotic reproductive compatibility among three populations of Brazilian-1 morphotype from Pelotas, Vacaria, and Tucumán. In contrast, the same Brazilian populations from Pelotas and Vacaria, both characterized as Brazilian-1 morphotype, showed strong prezygotic isolation when compared to the Mexican morphotype (Xalapa, Veracruz (Rull et al. 2013)). In addition, prezygotic and postzygotic reproductive isolation were found among some populations from the Andean (Ibagué), Mexican (Xalapa), Peruvian (La Molina), Brazilian-1 (Tucumán, Argentina), and Brazilian-3 (Parnamirim, Brazil) morphotypes, patterns potentially due to the presence of *Wolbachia* (Devescovi et al. 2014).

Although some progress has been made toward our understanding about the reproductive isolation barriers among the Brazilian populations of the *Af* complex, this advance is still discrete given the high cryptic species diversity that could be potentially found in the north, northeast, south, and southeast of Brazil. Further studies need to be conducted in order to elucidate the mechanisms involved in the divergence among the cryptic species of the *Af* complex, which could help to predict their distribution.

Acoustic communication

Acoustic communication during reproductive behavior has been identified in twenty-four species of tephritid flies and characterized in ten species, including some *Anastrepha* spp. (Takata 2010). This type of communication is characteristic of polyphagous Tephritidae in which males form leks (Shelly 2001). In *Anastrepha* species two types of sound have been described. The "calling song" is characterized by rapid backward-forward wing movement that generates pulses trains with pulses variation between 0.1 to 0.5 s duration with interpulses of the same duration and dominant frequency of ca. 80 - 150 Hz. The "precopulatory" song is characterized by continuous wing vibration of about 170 Hz, this song is highly variable in duration lasting few seconds to some minutes (Webb 1983, Sivinski 1984, Mankin 1996, Briceño 2009, Takata 2010).

Intra- and interspecific variations in sound production may be important in the eventual reproductive isolation of species/populations and could contribute to speciation. Mankin et al. (1996) studied differences in song repertoires and characteristics of four species of *Anastrepha* [*A. fraterculus* (two populations), *A. obliqua*, *A. sororcula* and *A. grandis*] from different regions of Brazil and found that differences in calling sounds produce pre-copulatory reproductive isolation. Although sound is important, pheromone and behavioral differences may play a larger role in separating species (Sivinski and Webb 1985, Sivinski 1988). In the two populations of *A. fraterculus* [Itaquera

(São Paulo), Cruz das Almas (Bahia)] the calling song did not show differences that could contribute to reproductive isolation (Mankin et al. 1996). Similar results were reported by Joachim-Bravo et al. (2013) working with four populations of *A. fraterculus* from different regions of Brazil, namely Piracicaba (São Paulo), São Joaquim (Santa Catarina), Vacaria (Rio Grande do Sul) and Bento Gonçalves (Rio Grande do Sul). Nevertheless, a recent study that include some of the Brazilian species of the *Af* complex (*A.* sp.1, *A.* sp.2, *A.* sp.3), *A. obliqua* and *A. amita* showed the calling song could be a signal used in interspecific recognition contributing to reproductive isolation. The authors concluded that calling songs constitute a relevant factor maintaining the genetic integrity of the species (Takata 2010). Traits that determine reproductive isolation among species are subjected to the stochastic nature of evolutionary forces that might vary across taxa (Lenormand et al. 2009, Nosil et al. 2009). Hence, acoustic communication may be an important factor determining reproductive isolation among *Anastrepha*, but not among taxonomic entities of the *Af* complex. Overall, unless the difference in a particular trait is correlated to female mate preference, sexual isolation among species is questionable (Ritchie 1996, Ritchie et al. 1999).

Future work should test the role that acoustic communication plays on the reproductive behavior and also if the temporal (time components) and spectral (frequency and intensity components) characteristics could be used in species/populations recognition. In addition, vibratory substrate-borne components of acoustic signals could transmit information between individuals; this possibility has not yet been studied in tephritid flies.

Chemical communication

Sex pheromones

Sex pheromones play an important role in species/partner recognition and in the mating behavior of *Anastrepha* species (Sivinski 1989, Sugayama and Malavasi 2000). During 'calling' (one of the first courtship behaviors), males disseminate a volatile mixture of compounds to attract males and females (Nation 1989, Lima et al. 2001). The studies involving the chemicals which trigger communication in *Anastrepha* species have been conducted considering two distinct perspectives: 1) the way flies store and release their sex pheromones, and 2) how these infochemicals are used to attract females and influence mating (Landolt and Averill 1999). The volatile compounds released by *A. fraterculus* males were first isolated from salivary gland extracts of specimens from the south of Brazil (Pelotas, Rio Grande do Sul) (Lima et al. 2001). A lactone, (*E*,*E*)-suspensolide, two isomeric sesquiterpenes, (*E*,*Z*)-α-farnesene and (*Z*,*E*)-α-farnesene and four alkylpyrazines, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine, were found in those extracts as major compounds, and 3-butyl-2,5-dimethylpyrazine was detected as a minor component. The monoterpenes, (*Z*)-β-ocimene and limonene, were also identified in the mixture of volatiles released

by males, and the isomeric lactones, anastrephin and (*S*,*S*)-epianastrephin, were found in extracts derived from washing the aeration chamber where males were placed for volatile collection (Lima et al. 2001).

A subsequent study carried out by Santos (2003), using *A. fraterculus* males from northeast Brazilian population (Alagoas), identified 23 compounds in the mixture of volatiles released by the flies. One alcohol, (*E*,*Z*)-3,6-nonadien-1-ol, four sesquiterpenes, α-*trans*-caryophyllene, (*E*,*E*)-α-farnesene, α-*trans*-bergamotene and β-bisabolene and the isomeric lactones, suspensolide, anastrephin and (*S*,*S*)-epianastrephin were reported among the identified compounds.

Further studies on the chemical composition of sexual pheromone of *A. fraterculus* and its perception by conspecific females were conducted (Břízová et al. 2010, Břízová 2011, Vaníčková 2010, Zyková 2013). The laboratory population used in these studies was *A*. sp.1 (Tucumán, Argentina) and 5 compounds, which elicited antennal depolarization on conspecific female antenna, were detected from male emanations. These compounds were identified as (*Z*)-3-nonen-1-ol, (*Z*,*Z*)-3,6-nonadien-1-ol, (*E*,*E*)-α-farnesene, (*Z*,*E*)-α-farnesene and epianastrephin. Furthermore, the study on age-dependant changes in the production of male-borne volatiles has shown that quantitative production of volatiles was lower in younger flies (5 days old) and increased with age (15-20 days old) reaching its maximum on the 20th day after eclosion (Zyková 2013).

Břízová et al. (2013) conducted chemical and statistical analysis of the volatile pheromone components produced by males from seven populations of *A. fraterculus*. Six populations were from three distinct geographic regions of Brazil: Pelotas (Rio Grande do Sul), Vacaria (Rio Grande do Sul), Bento Gonçalves (Rio Grande do Sul) and São Joaquim (Santa Catarina) (south of Brazil); Piracicaba (São Paulo) (southeast of Brazil) and Alagoas (Alagoas) (northeast of Brazil), and the seventh population was from Argentina (Tucumán). In this study, 14 volatile compounds emitted by males including terpenoids, alcohols and aldehydes were identified (Table 1). Multivariate statistical analyses showed that the populations from Vacaria, Pelotas, Alagoas and Tucumán are most dissimilar from the remaining populations (São Joaquim, Bento Gonçalves and Piracicaba) in terms of volatiles produced (Figure 4). The authors hypothesized that there may also be other compounds in the male pheromone mixture that are responsible for the attraction and/or repulsion of conspecific and/or heterospecific females. The differences among the pheromone mixtures released by the males of different Brazilian and Argentinean populations of *A. fraterculus* might also be regulated by various genes, as previously reported for *Drosophila* spp. (Ferveur et al. 1997, Ferveur 2005). The variability in the male-borne volatile profiles may directly influence the responses of females from these populations and change the manner in which they respond to the pheromone mixtures released by homospecific and heterospecific males (Břízová et al. 2013).

A recent study on chemical and electrophysiological analyses and behavioural bioassays was performed using a population of *A. fraterculus* from Alagoas (*A*. sp.3, Alagoas, Brazil) revealing the presence of 29 compounds in headspace samples of *A. fraterculus* males (Milet-Pinheiro et al. 2015). However, only six compounds, i.e.

Table 1. Chemicals identified in the male sex pheromone mixture of *Anastrepha fraterculus* Brazilian morphotypes: *A*. sp.1 PEL – Pelotas (RS, BR), BEN – Bento Gonçalves (RS, BR), VAC – Vacaria (RS, BR), SAO - São Joaquim (SC, BR), TUC – Tucumán (AR); *A*. sp.3. AL – Alagoas (AL, BR) [modified after Břízová et al. (2013)].

No.	Compound	*RI*	*A*. sp.1 PEL	*A*. sp.1 BEN	*A*. sp.1 VAC	*A*. sp.1 SAO	*A*. sp.1 TUC	*A*. sp. PIR	*A*. sp.3 AL
1	*p*-Cymene	1022	++++	+	++	++	++	+	+++
2	2-Ethylhexan-1-ol	1029	+	+++	++++	+	++	+	tr
3	Limonene†	1041	++++	++	+++	++++	+++	+	++++
4	(*Z*)-β-Ocimene	1050	++	+	tr	++	+++	+++	-
5	Nonanal	1107	+	++	++++	++	tr	+	+
6	(*Z*)-3-Nonen-1-ol†	1159	tr	++	+	+	++	+	+
7	(*E*,*Z*)-3,6-Nonadien-1-ol†	1161	tr	++++	+	+++	+++	++++	++++
8	Decenal	1210	++	+	++	+	tr	+	+
9	(*Z*,*E*)-α-Farnesene†	1495	+	+	+	++	+	+	+
10	Germacrene D	1498	+	+	+	+	tr	+	+
11	(*E*,*E*)-α-Suspensolide	1506	++	++	+	++	++	++	++
12	(*E*,*E*)-α-Farnesene†	1512	++	++	+	++++	++++	++++	+
13	Anastrephin	1617	+	+	+	+	+	+	+
14	Epianastrephin†	1621	++	++	+	+++	+	++	+

tr ≤ 0.1 %; + ≤ 3 %; ++ ≤ 10 %; +++ ≤ 20 %; ++++ > 20 %; †Male-borne attractants identified by Milet-Pinheiro et al. (2015) in AL population.

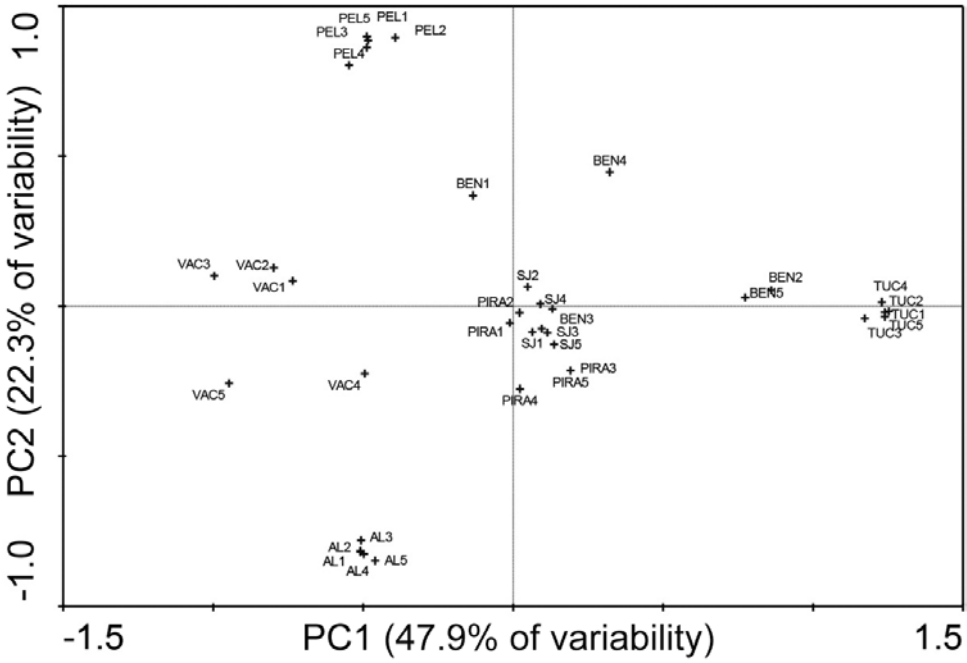

Figure 4. The results of the multivariate principal component analysis (PCA) of the sex pheromone of the males of *Anastrepha fraterculus* from 7 different populations representing two Brazilian morphotypes (A. sp.1, A. sp.3). A. sp.1. PEL – Pelotas (RS, BR), BEN – Bento Gonçalves (RS, BR), VAC – Vacaria (RS, BR), SAO - São Joaquim (SC, BR), TUC – Tucumán (AR); A. sp.3. AL – Alagoas (AL, BR); PIRA – Piracicaba (SP, BR) [modified after Břízová et al. (2013)].

α-pinene, limonene, (*Z*)-3-nonen-1-ol, (*E*,*Z*)-3,6-nonadien-1-ol, α-farnesene and (*S*,*S*)-(-)-epianastrephin, triggered antennal depolarization in conspecific females. Results from laboratory bioassays showed that synthetic compounds tested individually elicited more behavioral responses than a hexane control, but only the synthetic mixture composed of all EAD-active compounds triggered behavioral response in females similar to the headspace samples of conspecific males. In semi-field conditions, the synthetic mixture was more attractive to females than a hexane control and equally attractive to headspace extracts of males.

Further comparison of the male-borne chemical profiles of seven populations of *A*. sp.1 [Bento Gonçalves (Rio Grande do Sul), São Joaquim (Santa Catarina), Pelotas (Rio Grande do Sul)], *A*. sp.3 (Alagoas), and Andean (Duitama, Ibague, Sibundoy) morphotypes confirmed the previous findings on variability among male pheromone composition (Vaníčková et al. 2015). Male-specific compounds, which were proved by Milet-Pinheiro et al. (2015) to be attractive for *A. fraterculus* conspecific females, differed qualitatively among the seven studied populations (Vaníčková et al. 2015). The three Andean morphotype populations formed one separated cluster, whereas the one *A*. sp.3 together with three *A*. sp.1 populations grouped in a second cluster.

Cladera et al. (2014) stated that the key pheromone components of courtship and their roles as attractants to the two sexes have been overlooked in *A. fraterculus*. In contrary, here we present evidence proving that the pheromone and its role in the *A. fraterculus* chemical communication have been the main focus of studies performed by a few groups on the last 14 years. Together, the studies conducted on *A. fraterculus* pheromones suggest sexual specificity in the production and perception of individual pheromone components in this species. The semi-field bioassays conducted by Milet-Pinheiro et al. (2015) showed that the synthetic mixture of the *A. fraterculus* male-borne pheromone was effective in attraction of conspecific females, suggesting the use of this mixture for the control of this pest in infested orchards. Therefore, further experiments to evaluate the potential of traps baited with the pheromone mixture are necessary to confirm this hypothesis.

Cuticular hydrocarbons

Although long range attractants (sex pheromones) are essential for male and female flies to find each other, other substances, including cuticular hydrocarbons (CHs), may function as short range attractants and/or agents which trigger physiological changes, such as induction of receptivity in females and other behaviors associated with mating (Lockey 1991, Howard and Blomquist 2005). Although the primary role of CHs is to protect the insect body against desiccation; secondarily they play an important role in intra- and interspecific communication (Howard and Blomquist 2005, Blomquist and Bagnères 2010). In addition, the sex-specificity in CH profiles indicates the role of CHs in sexual communication (Blomquist and Bagnères 2010). CHs may also serve as species-specific fingerprints useful for delimitation of the putative species hidden within cryptic species complexes (Kather and Martin 2012).

Study of sex- and age-dependent differences in CHs production has been conducted for a laboratory population of *A. fraterculus* (*A.* sp.1, Tucumán, Argentina). It was found that sexually mature males had specific unsaturated hydrocarbons (7-monoenes) on their cuticles, which are absent in females (Vaníčková 2012, Vaníčková et al. 2012). The presence of the male specific CHs indicates that these compounds may play a role in the later phase of the mating, when the female touches the male with its front legs and proboscis (Vaníčková 2012, Vaníčková et al. 2012, 2014a, in press). Nevertheless, the exact role of those compounds in pheromone communication of *A. fraterculus* needs to be further explored.

Vaníčková et al. (2014b, in press) conducted a study including one south and one southeast Brazilian populations [Vacaria (Rio Grande do Sul), Piracicaba (São Paulo), respectively] and compared their chemical CH profiles with flies originated from Peru (La Molina), Mexico (Xalapa), Argentina (Tucumán) and Colombia (Ibague), representing the Peruvian, Mexican, *A.* sp.1 and Andean morphotypes of *Af* complex, respectively. The authors suggested eight potential taxonomic markers, specifically *n*-hexadecane, *n*-octadecane, *n*-eicosane, 4-methylhexacosane, 7-heneicosene, 7-tri-

cosene, 11-hentriacontene and 7,18-pentatriacontadiene, which could be used for the potential delimitation of males of the particular morphotypes within the *Af* complex. For example, Peruvian and *A*. sp.1 (Vacaria, Tucumán) morphotypes have unique CH profiles, suggesting CHs could be used to distinguish between these two subspecies.

Nevertheless, when compared the chemical profiles of males and females CHs from *A*. sp.1 [Bento Gonçalves (Rio Grande do Sul), Pelotas (Rio Grande do Sul), São Joaquim (Santa Catarina)], *A*. sp.3 (Alagoas) and Andean morphotype (Duitama, Cachipay, Sibundoy), CHs were found to have a limited use for distinguishing between *A*. sp.1 and *A*. sp.3 (Vaníčková et al. 2015). In all, the seven *A. fraterculus* populations analyzed formed two main clusters presenting Andean and Brazilian entities. The *A*. sp.1 and *A*. sp.3 populations created monophyletic cluster. These results point out that the relationships between the CH profiles and geographical isolation and/or influence of diet, host fruit, laboratory rearing and possible genetic variability are very complex and not yet understood among the *Af* complex. Therefore, it is necessary to conduct future studies, which will elucidate these complicated relationships of the CH chemical profiles and evaluate their use as specific taxonomic markers. The Brazilian-2 morphotype (*A*. sp.2) should be also examined for the CH composition and compared with the *A*. sp.1 and *A*. sp.3 morphotypes.

Remarks and conclusions

To date, three sibling species have been documented from the *Af* complex in Brazil. This complex work has relied on a number of analytical methods, such as differences in karyotypes (Mendes 1958, Bush 1962, Solferini and Morgante 1987, Selivon et al. 1996, 2004, 2005a,b), isozymic patterns (Morgante et al. 1980, Steck 1991, Selivon et al. 2005a), and DNA restriction patterns (Santos 1994, Steck and Sheppard 1993, Smith-Caldas et al. 2001, Rocha and Selivon 2004). Egg morphology (Selivon and Perondini 1998, Selivon et al. 2004, Selivon and Perondini 2007) and comparative morphometry of adults (Selivon et al. 2005a, Hernández-Ortiz et al. 2004, 2012, Araujo and Zucchi 2006) have also provided important clues to recognize cryptic species within the complex. Finally, factors involved in reproductive isolation among Brazilian populations, such as courtship behavior, pheromones, and CHs also play a role in distinguishing groups in the complex (Selivon et al. 1999, Santos et al. 2001, Břízová et al 2013, Vaníčková et al. 2014b, in press).

The three Brazilian species exhibit conspicuous differences in the sex chromosomes, both in terms of size and the amount and arrangement of heterochromatic blocks (Selivon and Perondini 2007). They may be also recognized by morphological analysis of the chorion of eggs (Selivon and Perondini 1998, Selivon et al. 2004) and by multivariate morphometrics based on certain linear measurements of the aculeus, wing and mesonotum (Hernández-Ortiz et al. 2012). Morphological data reported in this work show Brazilian morphs are more similar to each other than the other morphotypes described from the Neotropics. A crucial issue was that diverse samples tested by morphometrics corresponded to same populations identified as *A*. sp.1, *A*. sp.2 and

A. sp.3 by Selivon et al. (2004, 2005a,b). Therefore, the morphological divergence of these samples would be correlated with differences in egg-shell morphology, genetics or reproductive isolation. Further studies need to be conducted involving a wide range of populations of all three Brazilian morphotypes, in order to determine whether the different entities are consistent throughout their geographic and host range.

Out of the eight morphotypes currently recognized within the *Af* complex, there is compelling evidence that Brazilian morphs are the only ones to occur in sympatry in certain regions of the country. For instance, the presence of two karyotypes described from Itaquera (São Paulo) by Solferini and Morgante (1987), and the extreme allozyme variation found in samples of this particular locality by Steck (1991), suggests the coexistence of cryptic species. Nevertheless, a comprehensive study of the distribution of morphotypes throughout Brazil is required to improve the knowledge related to its entire distribution, sympatric areas, to understand the mechanisms of isolation and taxonomic relationships.

Studies on sexual compatibility, acoustic communication, chemical analyses of pheromones and cuticular hydrocarbons are, to some extent, complementary, as the Brazilian populations of the *A*. sp.1 morph used in these studies were the same. Specifically, *A*. sp.1 presented by Bento Gonçalves (Rio Grande do Sul), Vacaria (Rio Grande do Sul), São Joaquim (Santa Catarina) and Piracicaba (São Paulo) populations, revealed significant ($P < 0.01$) differences in the male pheromone composition (Břízová et al. 2013, Table 1), whereas CH profiles of Vacaria and Piracicaba (Vaníčková et al. 2014b, in press), and Bento Gonçalves with São Joaquim (Vaníčková el al. 2015) were comparably similar. Except for the Piracicaba population, all the three populations were sexually compatible between each other (Dias 2012, Joachim-Bravo et al. 2013) and non-significant differences were found between the sounds emitted by the males from these four populations. The chemical profiles and mating compatibility studies were also performed between populations of Pelotas (Rio Grande do Sul) Bento Gonçalves (Rio Grande do Sul), Vacaria (Rio Grande do Sul), and São Joaquim (Santa Catarina) (Dias 2012, Břízová et al. 2013, Joachim-Bravo et al. 2013, Vaníčková el al. 2015).

Together, all the evidence reviewed on three Brazilian entities regarding visual incompatibility, acoustic communication and chemical profiles suggest that the combination of all three types of signals will be necessary for the development of an effective pest monitoring and management program since these studies pointed out that (i) sexual specificity in the production and perception of individual pheromone components in this species might exist (ii) synthetic mixture of the *A. fraterculus* male-borne pheromone was effective in attraction of conspecific females, suggesting the use of this mixture for the control of this pest in infested orchards (Milet-Pinheiro et al. 2015), (iii) acoustic signals can be used as lures to attract tephritid flies (Webb et al. 1983, Mankin et al. 2004, Mizrach et al. 2005), and (iv) the CH profiles are species- and sex-specific, suggesting their use as possible chemotaxonomic markers for *Af* complex delimitation.

Future studies focused on electrophysiological and behavioral studies of the chemical communication of *Af* complex could help to understand the complex relationships between the three Brazilian entities. Research on mating behavioral sequences of the

three Brazilian entities of the *Af* complex as well as basic knowledge about *A. fraterculus* sexual communication could help to unravel mate assessment and mate choice dynamics, leading to the development of behavior-based control strategies and novel control tools for integrated pest management programs (Benelli et al. 2014a). Complementary molecular, genetics and morphological studies should be performed using the identical populations, in order to allow comparisons of all data obtained and subsequent implementation of effective control strategies of these pests.

From an applied perspective, area-wide integrated pest management programs based on the Sterile Insect Technique (SIT) cannot use only one *A. fraterculus* population to cover all Brazil. However, one population may be used in SIT programs covering wide areas that share the same morphotype, male courtship behavior and same time of the day when matings occur (Cladera et al. 2014). The use of lured semiochemicals (sex pheromones together with host fruit kairomones) combined with the SIT could improve the integrated pest management program by reducing dispersal, longevity and fecundity of *A. fraterculus* adults from the same morphotype. Despite an increasing number of studies demonstrating reproductive compatibility in the *A*. sp.1 morphotype and the partial incompatibility between *A*. sp.1 and *A*. sp.2, much remains to be learned about the *A*. sp.2, and *A*. sp.3 morphotypes.

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Gene flow and genetic structure of *Bactrocera carambolae* (Diptera, Tephritidae) among geographical differences and sister species, *B. dorsalis*, inferred from microsatellite DNA data

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Abstract

The Carambola fruit fly, *Bactrocera carambolae*, is an invasive pest in Southeast Asia. It has been introduced into areas in South America such as Suriname and Brazil. *Bactrocera carambolae* belongs to the *B. dorsalis* species complex, and seems to be separated from *B. dorsalis* based on morphological and multilocus phylogenetic studies. Even though the Carambola fruit fly is an important quarantine species and has an impact on international trade, knowledge of the molecular ecology of *B. carambolae*, concerning species status and pest management aspects, is lacking. Seven populations sampled from the known geographical areas of *B. carambolae* including Southeast Asia (i.e., Indonesia, Malaysia, Thailand) and South America (i.e., Suriname), were genotyped using eight microsatellite DNA markers. Genetic variation, genetic structure, and genetic network among populations illustrated that the Suriname samples were genetically differentiated from Southeast Asian populations. The genetic network revealed that samples from West Sumatra (Pekanbaru, PK) and Java (Jakarta, JK) were presumably the source populations of *B. carambolae* in Suriname, which was congruent with human migration records between the two continents. Additionally, three populations of *B. dorsalis* were included to better understand the species boundary. The genetic structure between the two species was significantly separated and approximately 11% of total individuals were detected as admixed ($0.100 \leq Q \leq 0.900$). The genetic network showed connections

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between *B. carambolae* and *B. dorsalis* groups throughout Depok (DP), JK, and Nakhon Sri Thammarat (NT) populations. These data supported the hypothesis that the reproductive isolation between the two species may be leaky. Although the morphology and monophyly of nuclear and mitochondrial DNA sequences in previous studies showed discrete entities, the hypothesis of semipermeable boundaries may not be rejected. Alleles at microsatellite loci could be introgressed rather than other nuclear and mitochondrial DNA. *Bactrocera carambolae* may be an incipient rather than a distinct species of *B. dorsalis*. Regarding the pest management aspect, the genetic sexing Salaya5 strain (SY5) was included for comparison with wild populations. The SY5 strain was genetically assigned to the *B. carambolae* cluster. Likewise, the genetic network showed that the strain shared greatest genetic similarity to JK, suggesting that SY5 did not divert away from its original genetic makeup. Under laboratory conditions, at least 12 generations apart, selection did not strongly affect genetic compatibility between the strain and wild populations. This knowledge further confirms the potential utilization of the Salaya5 strain in regional programs of area-wide integrated pest management using SIT.

Keywords
Carambola fruit fly, species complex, gene flow, incipient species, pest control, SIT, Salaya5 strain

Introduction

Bactrocera carambolae Drew & Hancock, the Carambola fruit fly, is a key insect pest belonging to the *B. dorsalis* species complex (Diptera, Tephritidae). Its native distribution covers the western part of the Indo-Australian Archipelago (determined by Wallace's and Huxley's lines), including the Thai/Malay Peninsula and Western Indonesia (White and Elson-Harris 1992, Clarke et al. 2005, EPPO 2014). Outside of Southeast Asia, the Carambola fruit fly was originally misidentified as *Dacus dorsalis* Hendel (Drew 1989), but later recognized as a separate species and named *B. carambolae*. The fly was firstly recorded to be a trans-continental pest in Paramaribo, Suriname, in 1975. Supposedly, tourists and trade by air between Indonesia and Suriname introduced the pest during the 1960s and 1970s. *Bactrocera carambolae* was also reported in other areas of South America: in 1986 in French Guyana (approximately 200 km from Paramaribo); in 1993 in Orealla, Guyana, at the border of Suriname (approximately 220 km from Paramaribo); and in 1996 in the Brazilian city of Oiapoque at the border with French Guyana (about 500 km from Paramaribo) (Malavasi et al. 2000, 2013). It can potentially spread to other countries in South America, Central America, and the Caribbean if effective control action is deficient (van Sauers-Mullers and Vokaty 1996). Currently, the Carambola fruit fly is in the process of being eradicated from the region of North Brazil (Malavasi et al. 2000, 2013). *Bactrocera carambolae* is regarded to be a polyphagous pest. It has a broad host range, including wild and cultivated fruits such as star fruit, mango, guava, and grapefruit (van Sauers-Mullers and Vokaty 1996, EPPO 2014). However, host plants for the fly were occasionally observed to be different between native and introduced areas as reported by van Sauers-Muller (2005). Despite being an important pest, knowledge of molecular ecology concerning species status and pest management is needed. There are no previous studies, except

multilocus phylogeny (Boykin et al. 2014), comparing it to closely related species such as *B. dorsalis* (Hendel) (Aketarawong et al. 2007, 2011, 2014a, 2014b, Khamis et al. 2009, Wan et al. 2011, Shi et al. 2012, Schutze et al. 2012, Krosch et al. 2013).

Within the *B. dorsalis* species complex, *B. carambolae* is still valid, even though a few members (i.e., *B. papayae* Drew & Hancock, *B. philippinensis* Drew & Hancock, and *B. invadens* Drew, Tsuruta & White) of the complex were recently synonymized with *B. dorsalis* (Schutze et al. 2012, 2013, and review in Schuzte et al. 2015). Based on a few species concepts, *B. carambolae* is distinct from *B. dorsalis*. For example, differences of morphology and morphometric data (Drew and Hancock 1994, Iwahashi 1999, Drew and Romig 2013) as well as monophyly confirmed by sequencing analyses of nuclear and mitochondrial DNA (Armstrong and Cameron 2000, Armstrong and Ball 2005, Boykin et al. 2014) were evidence to support morphological and phylogenetic species concepts, respectively. With regard to the biological species concept, pre- and post-reproductive isolation between the two species were also reported. Variations of reproductive morphologies (Iwahashi et al. 1999), host plants (Drew et al. 2008), and male pheromone components (Wee and Tan 2005b) and consumption doses (Wee and Tan 2005a) may reduce the number of sexual encounters between the two species. Furthermore, differences of mating times (McInnis et al. 1999, Schutze et al. 2013) and behavior (Schutze et al. 2013) account for reducing mating success. Nevertheless, inadequate reproductive isolation through hybridization was sometimes observed; viable *F*1 and further generations were produced under semi-natural conditions (Isasawin et al. 2014). Intermixing of pheromone components was consistently observed in semi-natural (Isasawin et al. 2014) and natural conditions (Wee and Tan 2005b). Slightly different genomes between the two species were observed in samples from inbreeding experiments using cytogenetic analysis (Augustinos et al. 2014). As such, the study of species status of *B. carambolae* and *B. dorsalis* is still an interesting issue. This information has implications not only for research but also for pest management and quarantine policies (Schutze et al. 2015).

In order to manage fruit fly pests, a method such as the Sterile Insect Technique (SIT) is commonly used to prevent, suppress, eradicate, or contain these pests (Klassen and Curtis 2005). In principle, male individuals are mass-sterilized and released into the target area. They competitively seek and mate with target fertile females. This leads to the production of nonviable offspring and subsequently suppresses the population. SIT is thus considered to be a target-specific, environmentally clean, and suitable birth control method. However, to enhance the effectiveness of SIT programs, the desired insect strains for irradiation and release are male-only strains (known as Genetic Sexing Strains or GSSs). For GSSs, male individuals can be sex-sorted before irradiation and release steps. An available GSS for *B. carambolae* has been successfully developed and evaluated, named Salaya5 (Isasawin et al. 2014). This strain was proven to competitively mate with two wild populations from Jakarta and North Sumatra, Indonesia, in field cage conditions. Nonetheless, for long-term pest control, a genetic compatibility between the mass-rearing colony (mother colony of released sterile insects) and wild population needs to be routinely monitored (Krafsur 2005, Cerritos et al. 2012). The genetic compatibility may drive mate choice and fertilization, especially in polyan-

drous pests wherein females have a post-mating opportunity to choose sperm from several males (review in Tregenza and Wedell 2000). Many invasive fruit fly pests are polyandrous such as *B. dorsalis*, *B. tryoni* (Froggatt), or *Ceratitis capitata* (Wiedemann) (Shelly and Edu 2008). Mating incompatibility between wild and released populations could result in an ineffective SIT program such as was the case for the New World screwworm in Jamaica (McDonagh et al. 2009).

Microsatellite DNA markers are a useful tool for population genetic and molecular ecological studies as well as pest management. The sequences of microsatellites are short tandem repeats that are widely distributed throughout the entire eukaryotic genome. Microsatellite loci selected for population genetics are Mendelian inherited, neutral, and polymorphic. Such markers generally provide a more contemporary estimate of diversity/structure because they mutate quicker and present a co-dominant feature, unlike mitochondrial DNA or other nuclear DNA markers (Hoshino et al. 2012). Likewise, using the genetic cluster approach based on microsatellite data can resolve intra- and interspecific relationships. Several microsatellite markers for invasive tephritid fruit flies were therefore established for studying population genetics in different geographical regions to infer colonization process (e.g., *C. capitata* (Bonizzoni et al. 2001, 2004, Meixer et al. 2002), *B. dorsalis* (Aketarawong et al. 2007, 2014a, Khamis et al. 2009, Wan et al. 2011, Shi et al. 2012), *Z. cucurbitae* (Coquillett) (Virgilio et al. 2010, Wu et al. 2011), *B. oleae* (Gmelin) (Nardi et al. 2005, Zygoridis et al. 2009, Dogaç et al. 2013)). In addition, established markers were used for solving species status in members of species complexes. For example, *B. dorsalis* and its synonym *B. papayae* were identified to have a single genetic cluster (Krosch et al. 2013) or weak population's genetic structure with no specific alleles (Aketarawong et al. 2014b), suggesting a single entity. However, members of the *Ceratitis* FAR complex were genetically divided, belonging to their species' genetic clusters, and some individuals were identified to be hybrid individuals, supporting a lack of reproductive isolation (Virgilio et al. 2013). Although isolated and developed for one species, the microsatellite primer sets can sometimes be used on related species (review in Barbara et al. 2007, Hoshino et al. 2012). Because of the conservation of microsatellite DNA sequences' flanking region across related species, cross-amplification is possibly an alternative approach for species whose data regarding microsatellite markers are unavailable.

The aim of this research, therefore, is to study the population genetics of *B. carambolae*, using modified cross-species amplification of microsatellite DNA markers derived from *B. dorsalis* and the junior synonym, *B. papayae*, with regard to three aspects of species status and pest management. Intra-specific variation was analyzed among seven populations, consisting of native and trans-continentally introduced populations, for inference of colonization processes. Moreover, samples of *B. dorsalis* were included to examine the population genetic structure and as an attempt to better understand the species boundary between *B. carambolae* and *B. dorsalis*. Lastly, concerning pest management aspect, we validated the potential for use the genetic sexing Salaya5 strain in regional SIT programs. The Salaya5 were genotyped and genetically compared to other wild *B. carambolae* populations, in order to evaluate genetic compatibility between them.

Methods

Sample collections

Nine wild fruit fly populations were collected from four geographical areas: Indonesia (6), Malaysia (1), Thailand (1), and Suriname (1) (Table 1 and Figure 1). These populations were from hosts and locations within the known ranges of *Bactrocera carambolae* (http://www.cabi.org/isc/datasheet/8700). In Indonesia, six populations were collected from three main islands; two populations (i.e., North Sumatra-NS and Pekanbaru-PK) were sampled from Sumatra; three populations (i.e., Depok-DP, Jakarta-JK, and Bandung-BD) were sampled from Java; and another (West Kalimantan-WK) was sampled from Borneo. In Thailand, one population was collected from the southern region (Nakhon Sri Thammarat-NT). All fruit fly samples were firstly characterized as *B. carambolae* based on Drew and Hancock (1994). In addition, the male pheromone profile (Wee and Tan 2005b) and/or ITS1 marker (Armstrong and Cameron 2000, Boykin et al. 2014) were also used to confirm the characterization (Table 1). Only populations BD and WK showed conflicting identifications and were classified as unidentified populations.

Three other populations of *B. dorsalis* were included in this study as outgroup samples for the investigation of genetic relationship between two cryptic species. These populations were collected from the known distributions of *B. dorsalis* (http://www.cabi.org/isc/datasheet /17685) and characterized as *B. dorsalis* using the same methods described before (Table 1). One population is from Ratchaburi, Thailand (RB), which is a representative source of *B. dorsalis* in Southeast Asia (Aketarawong et al. 2007, 2014a). Another is from the northern part of Thailand, Chang Mai (CM). The other was collected from Kaohsiung, Taiwan (KS). This sample is supposed to have been introduced from mainland China and has become an isolated population (Aketarawong et al. 2007, 2014a).

To record the genetic relationship between the genetic sexing Salaya5 strain (Isasawin et al. 2014) and wild populations, a sample of the Salaya5 colony (SY5) was included. The Salaya5 strain was created by hybridization and introgression of the genetic sexing *B. dorsalis* strain, named Salaya1 (Isasawin et al. 2012), and *B. carambolae* from Jakarta, Indonesia. This strain has a genetic background close to *B. carambolae* (99.9%) and a part of the Y-pseudo linked autosome carrying a dominant allele of white pupae alleles of Salaya1 strain (Isasawin et al. 2014). The strain was confirmed to be *B. carambolae* described by Isasawin et al. (2014). The Salaya5 colony has been maintained in the conditions presented in Isasawin et al. (2014).

All samples were preserved in 95% ethanol and kept at -20°C until use. The genomic DNA of each fly was extracted using the method of Baruffi et al. (1995).

Development of modified cross-species amplification of microsatellite DNA markers

Twelve microsatellite loci (Bd1, Bd9, Bd15, Bd19, Bd39, Bd42, and Bd85B derived from *B. dorsalis* s.s. (Aketarawong et al. 2006), and Bp58, Bp73, Bp125, Bp173, and

Table 1. Sample collection used in this study

Sample name	Type	Population characterization						
		Morphology[1]		Location[2]	Host plant [3]	Male pheromone[4]		ITS1[5]
		Bcar	*Bdor*			*Bcar*	*Bdor*	*Bcar*	*Bdor*
NS	Alive	x		North Sumatra, Indonesia (01°47'N; 099°02'E)	*Averrhoa carambola*	x		x	
PK	Dead	x		Pekanbaru, Riau, Indonesia (00°32'N; 101°27'E)	n/a			x	
DP	Dead	x		Depok, West Java, Indonesia (06°23'S; 106°49'E)	*Averrhoa carambola*			x	
JK	Alive	x		Jakarta, Indonesia (06°14'S; 106°49'E)	*Averrhoa carambola*	x		x	
BD	Dead	x		Bandung, West Java, Indonesia (06°54'S; 107°36'E)	n/a				x
WK	Dead	x		West Kalimantan, Indonesia (00°02'S; 109°19'E)	n/a				x
DK	Dead	x		Dengkil, Selangor, Malaysia (03°20'N; 101°30'E)	n/a			x	
NT	Alive	x		Nakhon Sri Thammarat, Thailand (08°19'N; 099°57'E)	n/a	x		x	
PR	Dead	x		Paramaribo, Suriname (05°52'N; 055°10'W)	n/a			x	
RB	Alive		x	Ratchaburi, Thailand (13°52'N; 099°48'E)	*Mangifera indica*		x		x
CM	Dead		x	Chang Mai, Thailand (18°47'N; 098°59'E)	n/a				x
KS	Dead		x	Kaohsiung, Taiwan (23°02'N; 120°35'E)	*Mangifera indica*				x
SY5	Alive	x		Thailand (Lab) (Introgression strain)	-	x		x	

1 followed the method of Drew and Hancock (1994)

2 covered known distribution of *B. carambolae* and *B. dorsalis* (http://www.cabi.org/isc/)

3 observed in the collection area

4 followed the method of Wee and Tan (2005b)

5 followed the method of Armstrong and Cameron (2000) and Boykin et al. (2014)

n/a no available data

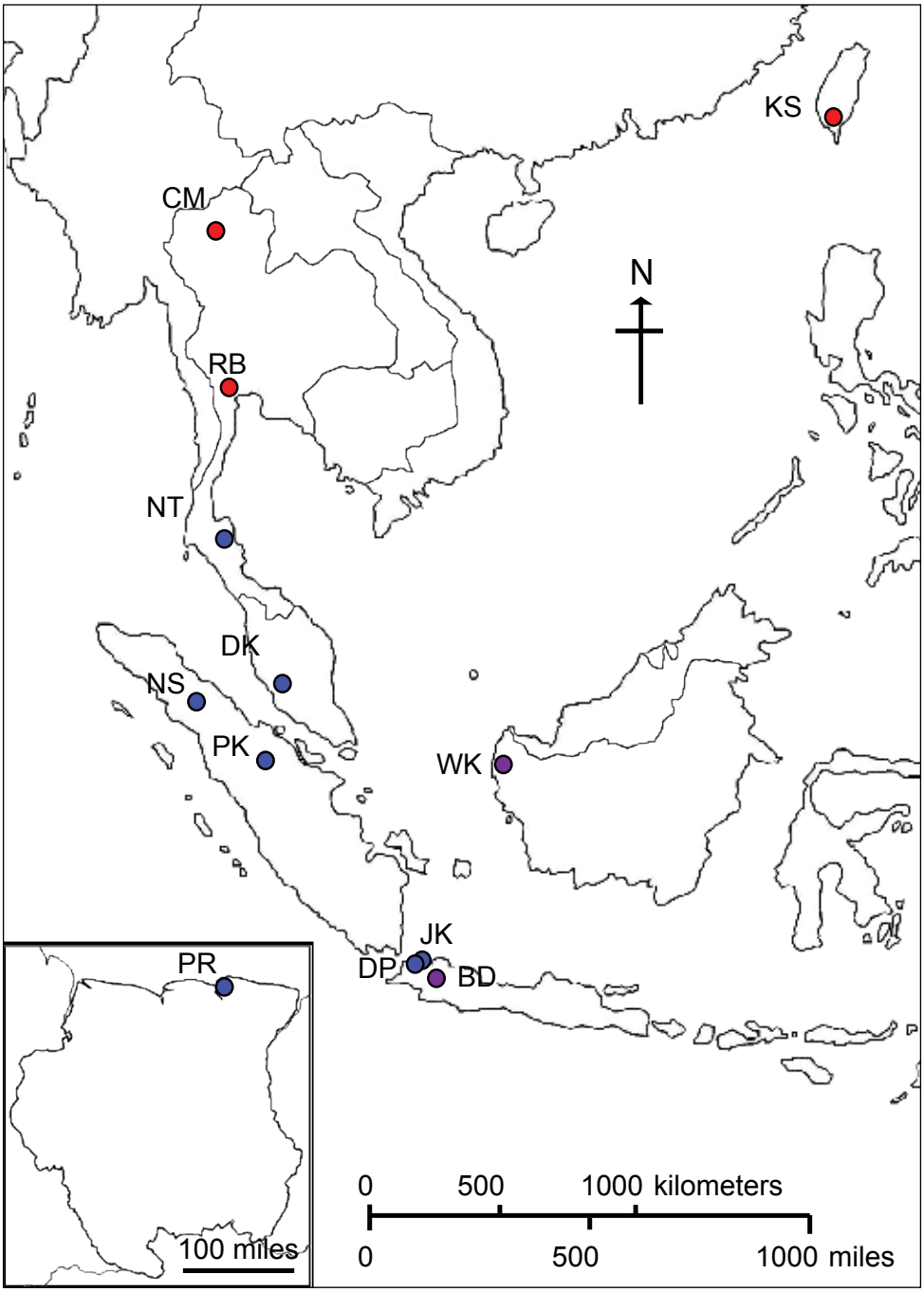

Figure 1. Sampling collections of *Bactrocera carambolae* and *B. dorsalis* in this study. Seven populations of *B. carambolae* (blue dots) were collected from Southeast Asia and Suriname. Three populations of *B. dorsalis* (red dots) were sampled from East and Southeast Asia. Two other unidentified populations (purple dots) were included. Information for each population is described in Table 1.

Bp181 derived from *B. papayae* (Shearman et al. 2006)), previously established for study of members in the same complex (Aketarawong et al. 2014b), were analyzed in *B. carambolae*. Amplifications were set up in a 15- µl volume reaction containing 1 × buffer, 2.5 mM $MgCl_2$, 25 µM dNTPs, 0.5 U *Taq* polymerase (Vivantis), 5 µM of each primer, and 100 ng of genomic DNA. PCRs were performed using the thermal cycler Flexcycler (AnalytikJena, Germany) using the conditions described by Aketarawong et al. (2014b). Amplicons of sizes consistent with fragments of *B. dorsalis* were cloned and sequenced using an ABI PRISM 310 genetic analyser (Macrogen, Korea). All sequences, except Bp173, showed homology to the original sequences in Genbank (Table 2).

To improve null allele problems due to mutations on primer-binding sites and/or unsuitable PCR conditions, a new set of primers were designed and renamed for the 11 loci using OLIGO version 4.0-s (Rychlik and Rhoads 1989). Moreover, the new annealing temperature for each primer pair is shown in Table 2.

Fifteen flies from Jakarta and North Sumatra, Indonesia were initially screened with the 11 sets of new primers using the PCR conditions mentioned above. Electrophoresis and allele scoring were determined as in Aketarawong et al. (2014b). Only eight loci were selected based on sharpness, specificity, and polymorphism of amplified products for further testing in all samples (Table 2).

Genetic variations

The descriptive parameters of population genetics were estimated using GENALEX v.6.5 (Peakall and Smouse 2012). These parameters include the mean number of alleles (n_a), mean effective number of alleles (n_e), mean number and frequency of private alleles (n_p and A_p, respectively), and mean observed and expected heterozygosity (H_O and H_E, respectively). Null allele frequency (A_n) was estimated following Brookfield (1996). Departure from Hardy-Weinberg equilibrium and the effect of linkage disequilibrium were tested using GENEPOP v.4 (Rosset 2008), with their critical levels set according to the sequential Bonferroni test (Rice 1989).

Population structure

Genetic differentiation (F_{ST}) among 13 populations was measured using MICROSATELLITE ANALYSER (MSA) (Dieringer and Schlötterrer 2003). In addition, genetically distinct groups (or clusters) were determined using the Bayesian approach implemented in STRUCTURE v.2.3.1 (Pritchard et al. 2000, Falush et al. 2003). The admixture model (the *F* model), assuming correlated allele frequencies, was run. The program computed the number of possible clusters (*K*) from one to 13, with the condition of the burn-in period being 100,000 steps, followed by 500,000 MCMC repetitions. For each *K* value, five iterations were performed. The other parameters were set at default values: a standard deviation of 0.05, prior F_{ST} mean of 0.01, and different values

Table 2. Microsatellite loci motif and primers used in this study

Locus	Repeat motif [GenBank Accession no.]	Original motif in *B. dorsalis*[1] and *B. papayae*[2] [Genbank Accession no.]	Primer (5'-3')	T_a (°C)	n_a	Size range in bp (n_a)		
						*Bcar**	*Bdor*	SY5
Bcar1	$CT(CA)_4CGCA$	$CT(CA)_4CG(CA)_2$	F: TGCTTAACAGTAATTGCTCCTT	62	11	96–112	96–108	100–108
	[KT355774]	[DQ482030][1]	R: AAGCAGTAAACAATAAAGTTCCAA			(9)	(7)	(4)
Bcar9	$(GT)_2AA(GT)_6GA$	$GA(GT)_7GA$	F: GCTGATATGTGTGCGTCTTATTTGTGA	69	16	156–182	140–172	168–186
	[KT355775]	[DQ482033][1]	R: ATCTCGTATTGTGGTTGCTTAAATATG			(12)	(8)	(6)
Bcar15	$(CA)_3CC(CG)_2CAA$ $(CA)_6CGTG(TACA)_3$	$(CA)_8CGCAA$ $(CA)_4CGTG(TACA)_3$	F: TGCCTTGTGCTATTTAATCTTTATCAA	63	12	183–199	155–195	191–195
	[KT355776]	[DQ482034][1]	R: AAATAAACAAAACAAAATGCAAATACA			(9)	(7)	(2)
Bcar19	$(CA)_2CT(CA)_6(TA)_2CA$	$(CA)_2CT(CA)_6(TA)_2(CATA)_2$	F: TAGATGGAGATGGGTGCGTGTACATG	71	13	149–171	155–175	167
	[KT355777]	[DQ482035][1]	R: GCGTGTTCACAAGGACTAATCGAA			(11)	(9)	(1)
Bcar39	$(GT)_8$	$(GT)_8$	F: GGTCAAACAAATCACTCAGTAAC	63	14	68–92	78–104	84–90
	[KT355778]	[DQ482037][1]	R: CCGTTATATCAGGCAAATCTATA			(8)	(11)	(4)
Bcar42	$CAAA(CA)_2AA(CA)_3(TA)_4$	$(CA)_7(TA)_7TG(TA)_2GC(CA)_3TA$	F: GCACAGTGAGCGTTACAAG	64	13	150–190	172–186	180–186
	[KT355779]	[DQ482038][1]	R: TGTTTTTACAGTTATACACTTCCCT			(10)	(8)	(4)
Bcar73	$(GT)_9$	$(GT)_5$	F: AGCGAAAACCAACTACTACCG	67	7	107–119	109–115	113–115
	[KT355780]	[AY847272][2]	R: CCACTACTTCATCTTGTTCCTGCAG			(7)	(4)	(2)
Bcar181	$(AC)_5ATAC$	$(AC)_8$	F: GTGCATGCCTTCGTGTAGCCTAACTCA	67	5	101–109	103 –109	103–105
	[KT355781]	[AY847280][2]	R: AATCTGCGAAGGATATCAACCATTCAC			(5)	(4)	(2)

[1]Aketarawong et al. (2006)

[2]Shearman et al. (2006)

*These data are calculated using seven *B. carambolae* populations

of F_{ST} for different subpopulations. The optimal number of hypothetical clusters was indicated by the Delta *K* method (Evanno et al. 2005). To identify the potential admixed individuals between *B. carambolae* and *B. dorsalis*, an individual-based genetic cluster was plotted. STRUCTURE analysis under different assumptions was also run to verify the consistency. The repeated analyses considering (1) uncorrelated allele frequency and (2) missing data as recessive homozygotes for the null alleles were set to avoid the shared descent of samples and to verify possible bias from null alleles, respectively.

Principle Coordinate Analysis (PCoA) was used to display genetic divergence among fruit fly populations in multidimensional space. This analysis was based on allele frequency data and performed on genetic distance using GENALEX v.6.5 (Peakall and Smouse 2012). The subprogram MOD3D in NTSYS-pc v.2.1 (Rohlf 2005) was subsequently used for plotting the first three principal coordinates.

To test genetic homogeneity in different hierarchical population structures, Analysis of Molecular Variance (AMOVA) in ARLEQUIN v.3.1.1 was used (Excoffier and Lischer 2010). After 1,000 permutations, populations were grouped according to nine criteria described in Table 5.

Isolation by distance (IBD)

The correlation analysis between genetic and geographic distance was performed using the subprogram ISOLDE in the GENEPOP package (Rousset 2008).

Genetic network analyses

Analyses of the genetic networks were performed using EDENetworks (Kivelä et al. 2015). This program is advantageous in that it can provide graphical representations of the structure and dynamics of a system of interaction between populations in multidimensional space, without *a priori* assumptions of the clustering of populations and some of the constraints (e.g., binary branching) compulsory in phylogenetic trees. Data types of 'genotype matrix, diploid, sampling site based' were used as input. The genetic distance metrics or network files were calculated using the F_{ST}-based distance of Reynolds (Reynolds et al. 1983); networks were constructed.

Networks consist of nodes (or vertices), corresponding to populations, connected by links (or edges), representing their relationships or interactions. Connectivity degree (or Degree) is the number of edges connected to a node summarizing how strongly a population associated with the other populations in the system and whether or not it is a source population. Betweenness-centrality (*BC*) determines the relative importance of a node within the network as an intermediary in the flow of information. Each network was weighted demonstrating genetic similarity associated with each link.

The network can be analyzed at various meaningful thresholds (*thr*). *thr* is the maximum distance considered as generating a connection in the network. One mean-

ingful distance is the one corresponding to the percolation threshold (D_p), edges with weights below the threshold were removed from the weighted network, and only the most important links were retained. Above the D_p level, there is a giant component containing almost all the nodes in the networks while below the D_p level, the network is fragmented into small disconnected components and the system therefore loses its ability to transport information across the whole system. Therefore, scanning at different thresholds was performed to analyze possible sub-structured systems to observe the sequential forms of clusters (Kivelä et al. 2015). The D_p and *thr* levels were determined by automatic and manual thresholding options, respectively.

Results

Genetic variability

All eight microsatellite loci tested within 13 populations have different levels of polymorphism in terms of number of alleles (ranging from moderately polymorphic, at five (Bcar181) to highly polymorphic at 16 (Bcar9)) and allele size range, as presented in Table 2. At locus Bcar73, allele 113 appeared to be fixed in male individuals of the SY5 strain, as reported by Isasawin et al. (2014). Linkage disequilibrium (LD) and Hardy-Weinberg equilibrium tests (HWE) were therefore performed for seven microsatellite loci for all populations. No significant evidence of LD among all loci was detected. After Bonferroni correction (Rice 1989), 28 out of 91 comparisons of loci and populations significantly deviated from the HWE results.

Overall genetic variations detected in each population is summarized in Table 3. *Bactrocera carambolae* samples collected from Southeast Asia showed relatively higher genetic variation than the introduced population (PR) for all parameters (i.e., n_a, n_e, n_r, A_r, n_p, A_p, H_O and H_E). In addition, the values of genetic variation of three *B. dorsalis* populations, two unidentified populations, and the SY5 strain were in the same range as *B. carambolae*. The n_p values were observed in all populations, except for the NS, DK and PR populations, varying from 0.125 (DP, JK, KS and SY5) to 0.625 (RB) per locus. Likewise, rare alleles (allele frequency less than 0.05) were also detected in all populations (n_r: ranging from 0.375 to 1.875 per locus), except KS. The average H_E values varied from 0.185 (PR) to 0.668 (RB). However, a deficiency in the average H_O values was found in all populations. Inbreeding (F_{IS}) was detected in all populations, ranging from 0.095 to 0.628. The average for null alleles was 0.12, varying from low (0.01) to high frequency (0.25), which may contribute to the deficiency of heterozygosity observed in the given populations.

Population structure

Genetic differentiation among 13 populations was measured by the fixation index (F_{ST}), as shown in Table 4. Among seven populations, the pairwise F_{ST} values range

Table 3. Genetic variation among thirteen populations.

Sample	n_a	n_e	n_p	A_p	n_r	A_r	H_O	H_E	F_{IS}
NS	3.375	1.980	0.000	0.000	0.625	0.032	0.202	0.410	0.532
PK	5.250	3.318	0.250	0.024	1.375	0.030	0.436	0.589	0.232
DP	5.000	3.257	0.125	0.059	0.625	0.030	0.381	0.648	0.386
JK	5.250	3.530	0.125	0.019	1.125	0.024	0.375	0.613	0.388
DK	4.625	2.937	0.000	0.000	0.875	0.024	0.347	0.528	0.258
NT	5.625	3.384	0.250	0.018	1.750	0.024	0.337	0.653	0.461
PR	2.000	1.324	0.000	0.000	0.375	0.015	0.152	0.185	0.095
BD	5.500	2.816	0.250	0.037	1.625	0.021	0.380	0.622	0.376
WK	5.750	3.257	0.375	0.092	1.875	0.026	0.406	0.660	0.393
RB	5.375	3.302	0.625	0.031	0.875	0.030	0.259	0.668	0.628
CM	4.250	2.466	0.250	0.074	0.750	0.037	0.387	0.572	0.315
KS	3.375	2.163	0.125	0.111	0.000	0.000	0.385	0.459	0.119
SY5*	3.286	1.952	0.125	0.016	1.000	0.032	0.317	0.433	0.274

*This data is calculated by using seven loci because locus Bcar73 is Y-pseudo linked.

n_a, mean number of alleles; n_e, mean effective number of alleles, $1/(1-H_E)$; n_p, mean number of private alleles; A_p, mean frequency of private alleles; n_r, mean number of rare alleles (allele frequency < 0.05); A_r, mean frequency of rare alleles; H_O, mean observed heterozygosity; H_E, mean expected heterozygosity; F_{IS}, mean inbreeding coefficient

from 0.134 (between PK and JK) to 0.631 (between DK and PR). Genetic differentiation was relatively high between native and introduced populations, ranging from 0.444 to 0.631. The introduced population (PR) was most similar to population PK (F_{ST} = 0.444) and JK (F_{ST} = 0.448). Within native areas of *B. carambolae*, the pairwise F_{ST} values ranged from 0.134 (PK and JK) to 0.344 (PK and DK). On the other hand, the pairwise F_{ST} values among samples of *B. dorsalis* varied from 0.210 (RB and CM) to 0.357 (CM and KS). Without PR, the degree of genetic differentiation between *B. carambolae* and *B. dorsalis* ranged from 0.181 (DP and RB) to 0.524 (NS and KS). The SY5 strain was revealed to be genetically closest to JK (F_{ST} = 0.278). The degree of pairwise F_{ST} among pairs of the SY5 strain and others varied from 0.372 (SY5 and DP) to 0.507 (SY5 and PR) (Table 4).

STRUCTURE analysis demonstrated the proportion of co-ancestry (Q) distributed in hypothetical clusters (K) whereas PCoA illustrated the genetic divergence of fruit fly populations in multidimensional space, as shown in Figure 2. Among seven *B. carambolae* populations, the Delta K value (Evanno et al. 2005) was determined to be K equals two (K = 2) as the optimal number. At K = 2, genetic clusters were divided into two groups: native and introduced *B. carambolae*. Cluster 1 contained all native populations (NS (Q = 0.979), PK (Q = 0.969), DP (Q = 0.984), JK (Q = 0.953), DK (Q = 0.991), and NT (Q = 0.995)) whereas the introduce population (PR) was distinguished, forming its own cluster (Q = 0.988) (Figure 2A). This subdivision corresponded to the first axis (44% of total variation) of the principal coordinate.

Table 4. Significant pairwise F_{ST} among 13 populations.

Population	NS	PK	DP	JK	DK	NT	PR	BD	WK	RB	CM	KS	SY5
NS													
PK	0.296												
DP	0.274	0.162											
JK	0.217	0.134	0.169										
DK	0.288	0.344	0.287	0.300									
NT	0.329	0.248	0.188	0.241	0.206								
PR	0.596	0.444	0.495	0.448	0.631	0.564							
BD	0.334	0.240	0.228	0.176	0.368	0.264	0.491						
WK	0.345	0.216	0.163	0.197	0.336	0.273	0.486	0.194					
RB	0.404	0.260	0.181	0.234	0.339	0.204	0.582	0.202	0.162				
CM	0.395	0.321	0.254	0.290	0.401	0.249	0.636	0.259	0.200	0.210			
KS	0.524	0.402	0.322	0.347	0.368	0.168	0.738	0.352	0.317	0.256	0.357		
SY5	0.468	0.389	0.372	0.278	0.381	0.343	0.507	0.348	0.321	0.354	0.409	0.414	

Table 5. Analysis of molecular variance (AMOVA).

Scenario*	Among groups			Among populations within groups			Within populations		
	V_a	Percentage	*P*	V_b	Percentage	*P*	V_c	Percentage	*P*
1	0.2533	11.8	0.1877	0.5952	27.72	<0.0001	1.2990	60.49	<0.0001
2	0.5817	23.21	0.1953	0.6256	24.96	<0.0001	1.2990	51.83	<0.0001
3	0.1833	7.98	0.0479	0.7805	33.98	<0.0001	1.3335	58.04	<0.0001
4	0.1450	6.26	0.0156	0.7282	31.46	<0.0001	1.4416	62.28	<0.0001
5	0.1377	6.15	0.2483	0.8262	36.88	<0.0001	1.2767	56.98	<0.0001
6	0.1744	7.69	0.0694	0.7810	34.47	<0.0001	1.3105	57.84	<0.0001
7	0.1607	6.98	0.0342	0.7288	31.67	<0.0001	1.4121	61.35	<0.0001
8	0.3298	13.96	0.0010	0.5910	25.02	<0.0001	1.4416	61.02	<0.0001
9	0.139	6.08	0.0039	0.7498	32.57	<0.0001	1.4121	61.35	<0.0001

*Scenario 1: Sumatra, Indonesia (NS and PK); Java, Indonesia (DP and JK); Malaysia (DK); Thailand (NT); and Suriname (PR)

Scenario 2: Southeast Asia (NS, PK, DP, JK, DK, and NT) and South America (PR)

Scenario 3: *B. carambolae* (NS, PK, DP, JK, DK, and NT) and *B. dorsalis* (RB, CM, and KS)

Scenario 4: *B. carambolae* (NS, PK, DP, JK, DK, and NT) and group of *B. dorsalis* (RB, CM, and KS), and BD and WK

Scenario 5: *B. carambolae* (NS, PK, DP, JK, DK, NT, and PR) and the SY5 strain

Scenario 6: *B. carambolae* (NS, PK, DP, JK, DK, NT, and PR) and *B. dorsalis* (RB, CM, and KS) and the SY5 strain

Scenario 7: *B. carambolae* (NS, PK, DP, JK, DK, NT, and PR); group of *B. dorsalis* (RB, CM, and KS), BD, and WK; and the SY5 strain

Scenario 8: STRUCTURE analysis (Figure 2B): cluster 1 (RB, CM, KS, BD, and WK), cluster 2 (NS, PK, DP, JK, DK, and NT), and cluster 3 (PR)

Scenario 9: STRUCTURE analysis (Figure 2C): cluster 1 (NS, PK, JK, DK, PR, and SY5) and cluster 2 (RB, CM, KS, BD, WK, DP, and NT)

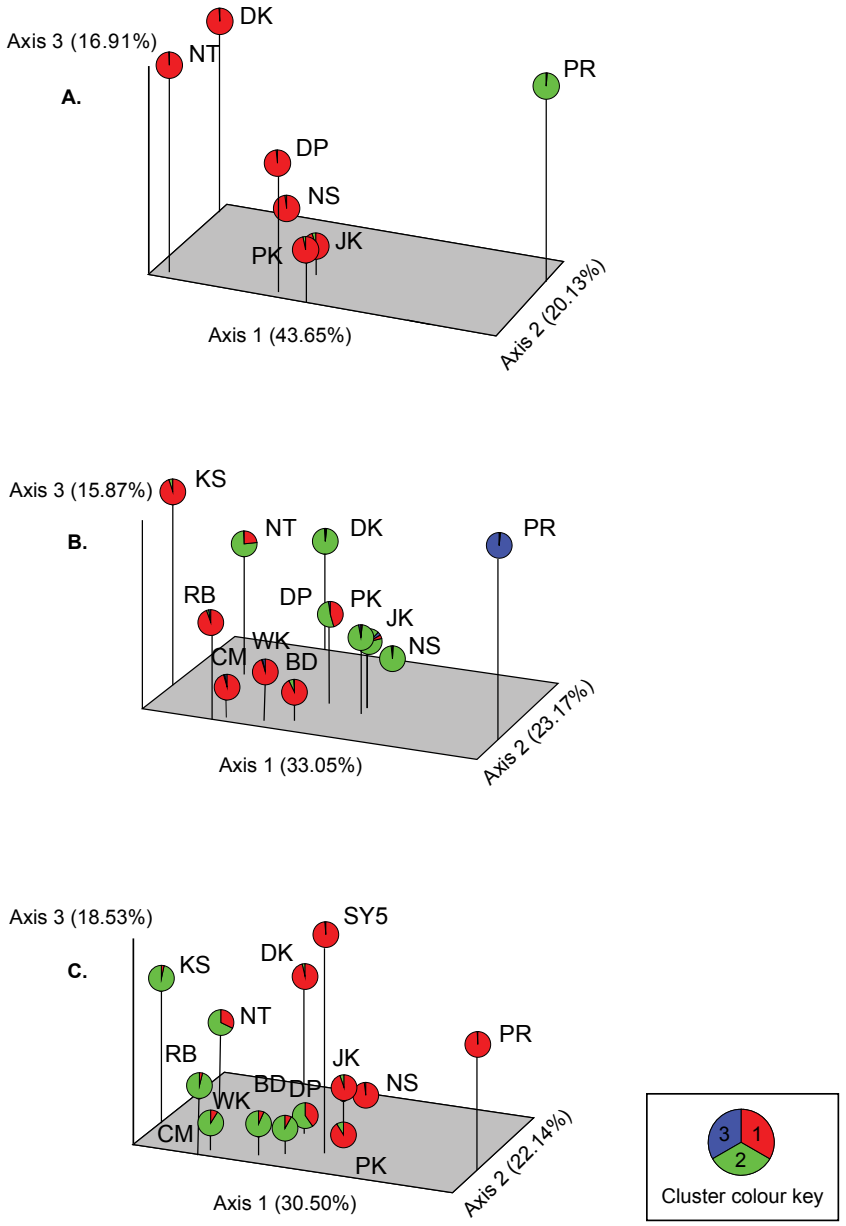

Figure 2. Three-dimensional plot of Principal Coordinate Analysis (PCoA) and STRUCTURE analysis. **A** the planes of the first three principal coordinates explain 43.65%, 20.13%, and 16.91% of total genetic variation, respectively, for seven *B. carambolae* populations using eight SSRs **B** the planes of the first three principal coordinates explain 33.05%, 23.17%, and 15.87%, respectively, for *B. carambolae* and *B. dorsalis* groups using eight SSRs **C** the planes of the first three principal coordinates explain 30.50%, 22.14%, and 18.53%, respectively, for the SY5 strain and wild populations using seven SSRs. Pie graphs, consisting of different colored sections, represent co-ancestor distribution of 185, 289, and 321 individuals in **A** two, **B** three, and **C** two hypothetical clusters, respectively.

When three additional populations of *B. dorsalis* (RB, CM and KS) and two unidentified populations (BD and WK) were included in the STRUCTURE analysis, the optimal number for *K* was three. Genetic clusters were separated into two groups: *B. dorsalis* belonged to cluster 1 while *B. carambolae* belonged to clusters 2 and 3 (Figure 2B). Six native populations of *B. carambolae* shared genetic memberships in cluster 2 (NS (Q = 0.976), PK (Q = 0.954), DP (Q = 0.519), JK (Q = 0.910), DK (Q = 0.970), and NT (Q = 0.758)). However, PR formed its own cluster, cluster 3 (Q = 0.986). Three *B. dorsalis* samples (RB (Q = 0.927), CM (Q = 0.955), and KS (Q = 0.952)) were grouped into the same genetic memberships (cluster 1) with two unidentified populations BD (Q = 0.927) and WK (Q = 0.953). Co-ancestor distribution between the *B. carambolae* and *B. dorsalis* clusters ($Q \geq 0.001$) was observed in populations DP (Q = 0.457) and NT (Q = 0.236). Using PCoA, the first plane (33% of total variation) of multidimensional space also separated PR from the rest of the populations. Although the other two axes (23% and 16%, respectively) did not clearly divide samples into two groups in accordance with the STRUCTURE results, the group of *B. dorsalis* appeared to be plotted separately from the *B. carambolae* group.

The individual-admixture plot for $K = 3$ is presented in Figure 3. Individuals contained in the proportion of genetic cluster (Q) between 0.100 to 0.900 ($0.100 \leq Q \leq 0.900$) were identified as admixed individuals (or potential hybrids). Among the *B. carambolae* group, 18 of 185 individuals (9.73%) were admixed individuals. On the other hand, 13 of 104 individuals (12.5%) of the *B. dorsalis* group were admixed individuals. Pure individuals ($Q > 0.900$) were identified between the two groups. Eight individuals from the *B. carambolae* group were labelled as *B. dorsalis* whereas one individual from the *B. dorsalis* group was indicated to be *B. carambolae*.

Adding the SY5 strain to the genetic cluster analysis, two was the optimal number for *K*. At $K = 2$, genetic clusters formed two groups likely corresponding to *B. carambolae* and *B. dorsalis* groups. Six populations of *B. carambolae* (NS (Q = 0.981), PK (Q = 0.913), JK (Q = 0.948), DK (Q = 0.964), and PR (Q = 0.993) belonged to cluster 1 (Figure 2C). The SY5 strain was also a member of the *B. carambolae* cluster (Q = 0.988). Cluster 2 consisted of the *B. dorsalis* group (CM (Q = 0.909), RB (Q = 0.960), KS (Q = 0.964), BD (Q = 0.918), and WK (Q = 0.933)). However, populations DP (Q = 0.596) and NT (Q = 0.677) also shared membership in this cluster and became an admixture structure. The principal coordinates did not clearly delineate the two clusters using the three-dimensional plot. The PR population was still isolated from the others while the *B. dorsalis* group seemed to form a peripheral group.

Analysis of Molecular Variance (AMOVA) was used to study the hierarchical structure of populations for different scenarios (Table 5). Overall, up to 23% of variation was attributed to the differences among groups whereas approximately 52% to 62% of variation was attributable to differences within populations. In scenarios 1 and 2, populations of *B. carambolae* were divided into subpopulations according to micro- and macro- geographies. At the micro-geographical level, seven populations were divided into five groups: Sumatra, Indonesia (NS and PK); Java, Indonesia (DP and JK); Malaysia (DK); Thailand (NT); and Suriname (PR). Non- significant differ-

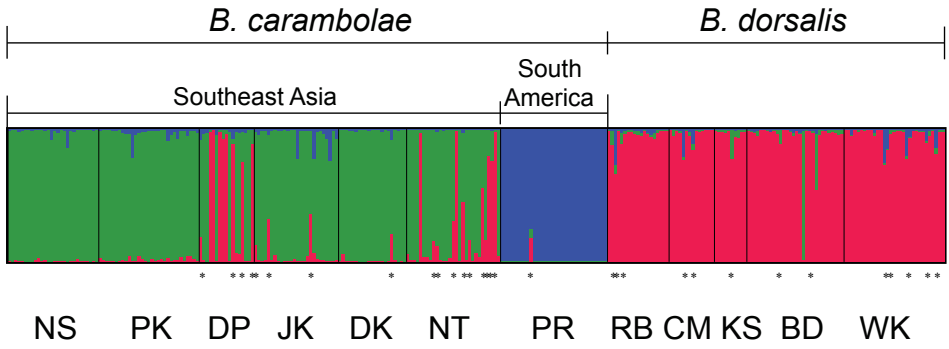

Figure 3. The individual admixture plot for $K = 3$. Each bar reveals a single individual. Each color of bars represents each genetic cluster. Samples of *B. carambolae* belong to clusters 2 and 3 (green and blue, respectively) while samples of *B. dorsalis* belong to cluster 1 (red). Potential hybrids have a proportion of genetic cluster (Q) between 0.100 to 0.900 ($0.100 \leq Q \leq 0.900$) as identified with asterisk (*).

ences among those groups were detected (scenario 1: $P = 0.1877$). Likewise, when all seven populations were divided based on macro-geography (Southeast Asia vs. South America), a non-significant difference between groups was still observed (scenario 2: $P = 0.1953$). A test of genetic homogeneity between *B. carambolae* and *B. dorsalis* is presented in scenario 3, illustrating a significant difference ($P = 0.0479$). Even though BD and WK were included in the *B. dorsalis* group, a significant difference was still detected (scenario 4: $P = 0.0156$). In scenarios 5 to 7, the SY5 strain was included to compare the genetic variation among other samples. The results revealed a non-significant difference between *B. carambolae* and the SY5 strain (scenario 5: $P = 0.2483$) and among *B. carambolae*, *B. dorsalis* and the SY5 strain (scenario 6: $P = 0.0694$). However, a significant difference was indicated when BD and WK were included in the *B. dorsalis* group (scenario 7: $P = 0.0342$). The last two scenarios was set following the STRUCTURE results (Figures 2B and 2C, respectively). Significant differences were detected in both scenarios ($P = 0.0010$ and $P = 0.0039$, respectively).

Isolation by distance (IBD)

The correlation between genetic and geographic distance was analyzed using only wild samples consisting of *B. carambolae* and *B. dorsalis* populations. The correlation between genetic and geographical distance became non-significant [$R^2 = 0.394$, $P = 0.106$, $F_{ST}/(1\text{-}F_{ST}) = 0.146$ Ln (geographical distance) - 0.572] when *B. carambolae* samples were analysed. This fact indicates that there is no limitation of gene flow among *B. carambolae* across the region. Among *B. carambolae* and *B. dorsalis* populations, analysis showed significant correlation between genetic and geographical distances [$R^2 = 0.449$, $P = 0.002$, $F_{ST}/(1\text{-}F_{ST}) = 0.180$ Ln (geographical distance) + (0.868)], even though only the PR sample was excluded ($R^2 = 0.119$, $P = 0.021$).

Genetic connectivity

Simplified networks were constructed for three different scenarios: (1) among seven *B. carambolae* populations, (2) among 12 populations belonging to *B. carambolae* and *B. dorsalis* clusters, and (3) among 13 populations, including the SY5 strain (Figures 4 to 6, respectively). Genetic distance metrics for the first and second data sets were estimated using eight microsatellite loci, but the third data set was analyzed using seven loci because the omitted locus is Y-pseudo linked in the SY5 strain (Isasawin et al. 2014). The networks were scanned for decreasing thresholds from the fully connected network to the percolation threshold (D_p) and lower threshold chosen (*thr*) to reveal the sequential substructure at decreasing thresholds. The first scenario was constructed based on data among seven *B. carambolae* populations (Figure 4). The percolation threshold D_p = 0.52 showed the emergence of a connection between native and introduced populations (Figure 4B). The node of JK had a larger degree (Degree = 6.0) and betweenness-centrality (*BC* = 5.0) than others and played a role connecting between native and introduced populations (Suppl. material 1: Table 1). The scanning at decreasing thresholds illustrated sub-cluster of native and introduced populations (*thr* = 0.40; Figure 4C). Moreover, JK and PK populations were the first to be jointed in the network (*thr* = 0.15; Figure 4D).

For the second scenario, five more populations belonging to the *B. dorsalis* cluster were included in the network analyses (Figure 5). At the percolation threshold D_p = 0.20, non-substructured system was observed (Figure 5B). Genetic connections between the *B. carambolae* and *B. dorsalis* groups were identified through the nodes of DP (Degree = 4; *BC* = 11.00), JK (Degree = 3; *BC* = 5.00) and NT (Degree = 2; *BC* = 5.00) (Suppl. material 2: Table 2). The network scanned below D_p demonstrated a serial disconnection of the system (Figure 5B). The first relationship of the network

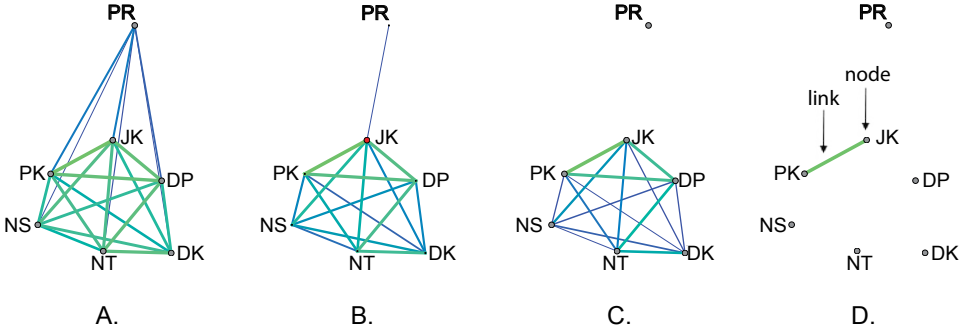

Figure 4. Simplified network of seven *Bactrocera carambolae* populations, and the sequential forms of cluster. The network was constructed using eight SSRs. Scanning was done for decreasing thresholds **A** is the fully connected network **B** is the percolation threshold (D_p = 0.52, with all links corresponding to distances superior to D_p excluded). JK plays an important role connecting between native and introduced populations **C**–**D** are the lower thresholds chosen (*thr* = 0.40 and 0.15, respectively) to reveal sub-structured network.

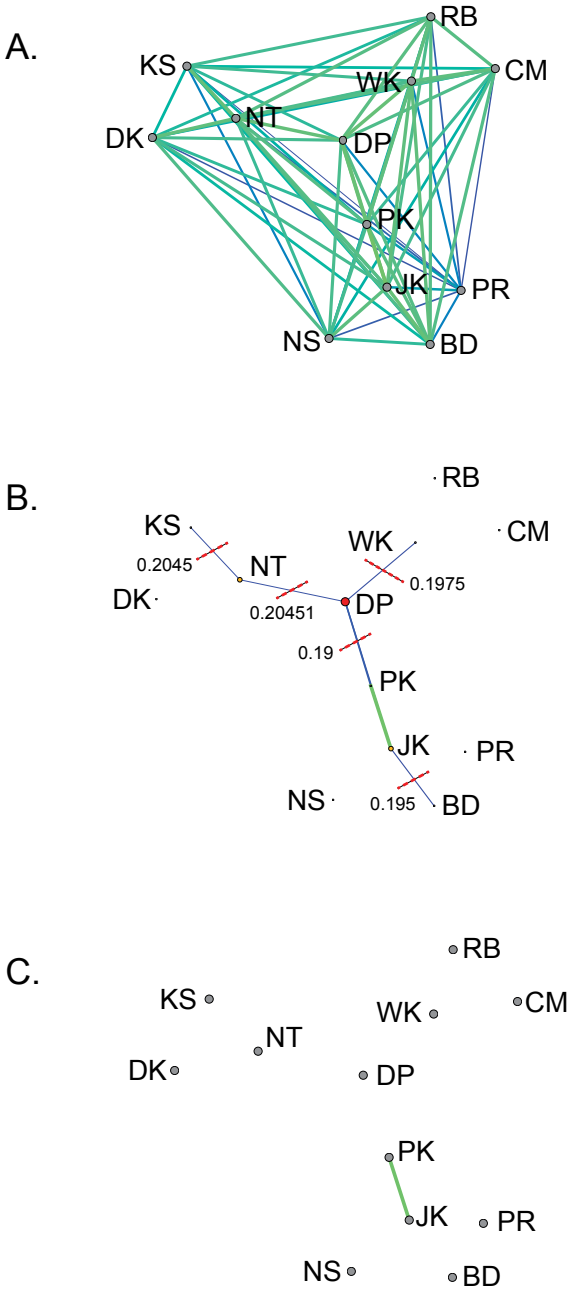

Figure 5. Simplified network of *Bactrocera carambolae* and *B. dorsalis* groups, and the sequential disconnection of the network. The network was constructed using eight SSRs. Scanning was done for decreasing thresholds **A** is the fully connected network **B** is the percolation threshold (D_p = 0.20, with all links corresponding to distances superior to D_p excluded). DP, JK, and NT are connecting between *B. carambolae* and *B. dorsalis* groups. Red dashed lines with number are corresponded to the threshold values, revealing serial disconnection of the network **C** is the lowest threshold (*thr* = 0.15).

was detected between JK and PK populations (*thr* = 0.15; Figure 5C). To the contrary, the genetic links between native (JK and PK) and introduced (PR) populations of *B. carambolae* were revealed when increasing the threshold to *thr* = 0.55 (Degree = 11 and *BC* = 4.61) (Suppl. material 2: Table 2).

For the third scenario, the SY5 strain was added into the network to evaluate the genetic similarity between the SY5 strain and wild populations (Figure 6). The links between the SY5 strain and wild populations were detected above the percolation threshold (*thr* = 0.34 to 0.75) (Figure 6A). The SY5 strain shared greatest genetic similarity to JK (*thr* = 0.34). At the percolation threshold D_p = 0.23, non-substructured network was recognized (Figure 6B). Scanning below D_p showed disconnection of the system (Suppl. material 3: Table 3). Again, the first relationship of the network was detected between JK and PK populations (*thr* = 0.15; Figure 6C).

Discussion

Native vs introduced populations of *B. carambolae*

At the macro-geographical level, comparing among seven populations of *B. carambolae* from Southeast Asia and South America, the populations across the species' native range possessed higher genetic variation than the introduced population, which is generally expected for invasive species. The first genetic connections between native and introduced populations were identified as Jakarta (JK) and Pekanbaru (PK), Indonesia. However, the genetic structure of the Suriname population (PR) (based on F_{ST}, STRUCTURE, PCoA, and genetic network analysis) was differentiated from the Carambola fruit fly in Southeast Asia. These results were congruent with multilocus phylogenetic analysis established by Boykin et al. (2014). They deduced that factors and processes shaping the genetic variation and population structure of *B. carambolae* in the introduced area potentially include genetic drift during the colonization process and local adaptation. Based on the genetic data in this study, JK and PK are possible sources of the PR population. *Bactrocera carambolae* accidentally invaded South America, likely by tourists and trade from Indonesia to Suriname. Even though this species was detected in 1975, it took up to 21 years (1975–1996) to establish its new populations in other areas, expanding 500 km eastward and westward from the original point of introduction in Suriname (Malavasi et al. 2000, 2013). In the meantime, according to the report of van Sauers-Muller (2005), host plants of *B. carambolae* (e.g., guava, Malay apple, carambola, West Indian cherry, and mango) in Suriname were limited to backyards; agricultural production had not yet developed. Hosts for the fly were occasionally recorded to be different between native and introduced areas (van Sauers-Muller 2005), as shown in Table 6. Regarding that evidence, the conditions of habitats, including sufficient time for genetic drift to take effect, may be natural factors causing high genetic differentiation between native and introduced populations. Likewise, the same factors, including limitations of human activity, among four countries

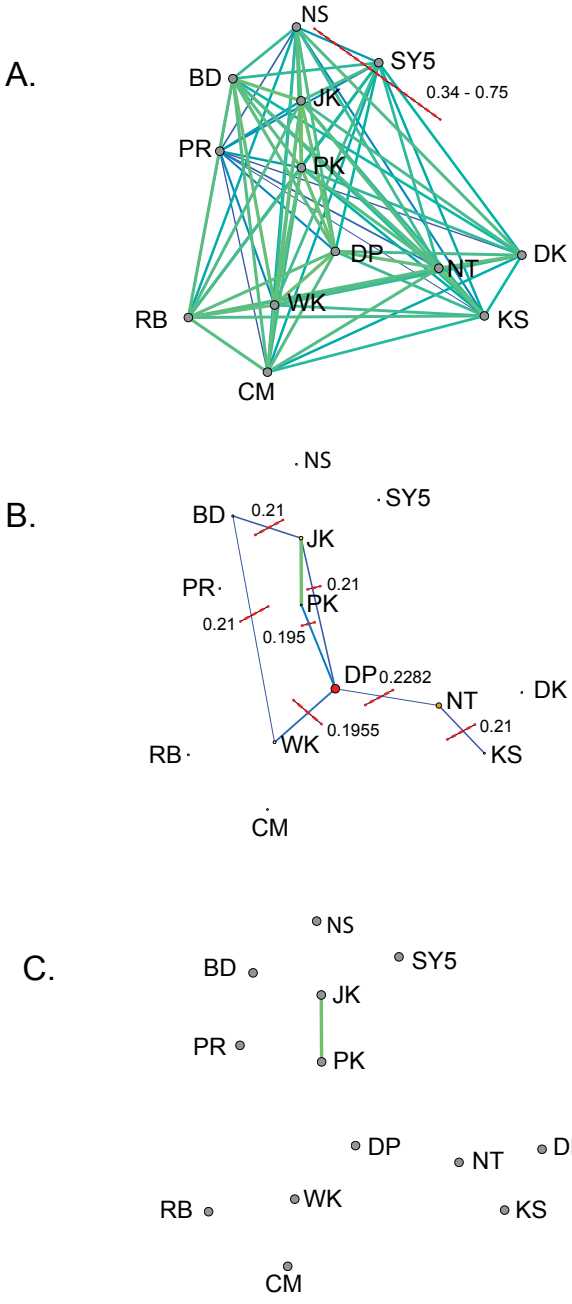

Figure 6. Simplified network of the SY5 strain and wild populations, and the sequential disconnection of the network. The network was constructed using seven SSRs. Scanning was done for decreasing thresholds **A** is the fully connected network **B** is the percolation threshold (D_p = 0.23, with all links corresponding to distances superior to D_p excluded). DP, JK, and NT are connecting between *B. carambolae* and *B. dorsalis* groups **C** is the lowest threshold (*thr* = 0.15). Red dashed lines with number are corresponded to the threshold values, revealing serial disconnection of the network.

Table 6. Record of different host plants in Southeast Asia and Suriname for *Bactrocera carambolae* (edited from van Sauers-Muller 2005).

Hosts found in Southeast Asia only	Hosts found in Suriname only
Annona montana Macf.	*Anacardium occidentale* L.
Annona muricata L	*Spondias cytherea* Sonn.
Thevetia peruviana (Pers.) K. Schum	*Spondias mombin* L.
Persea americana Mill.	*Garcinia dulcis* (Roxb.) Kurz
Artocarpus altilis (communis) (Park.) Fosberg	*Malpighia punicifolia* L.
Artocarpus heterophyllus Lam.	*Eugenia cf. patrisii* Vahl
Averrhoa bilimbi L.	*Citrus sinensis* (L.) Osbeck
Punica granatum L.	
Capsicum annuum L.	
Lycopersicon esculentum Mill.	

near Suriname may slow down species distribution in South America. This pattern is similar to the case of the related species *B. dorsalis* in several introduced areas such as Hawaii, Myanmar, and Bangladesh, in that their genetic variation and population structure were shaped by genetic drift and different local adaptations (Aketarawong et al. 2007). However, the current study included only one population from South America. Research using more samples, if available, from these regions should provide a better understanding of the demographic dynamics of the Carambola fruit fly within South America as well as between the two continents.

Within Southeast Asia, *B. carambolae* demonstrates high genetic variation and homogeneous population structure. West Java, in particular JK, is also a potential source of *B. carambolae* populations in Southeast Asia. JK showed relatively higher genetic variation and greater values of Degree and betweenness-centrality than other populations in the genetic network. Moreover, this area is important for the cultivation of star fruit and is a center for transportation to other cities and countries. The homogeneous genetic pattern of *B. carambolae* in native areas is similar to *B. dorsalis* collected from neighboring countries, including Thailand, Laos, and Cambodia (Aketarawong et al. 2007, 2014a, Schutze et al. 2012). Both not having limitations on human migration and the intensive cultivation of similar host plants could enhance gene flow, shaping genetic homogeneity among flies from nearby countries. Although the geography of Indonesia, Malaysia and Thailand is not entirely contiguous, increasing trade can promote the migration of insect pests within the country, as well as among other countries (Suputa et al. 2010). Therefore, effective quarantine measures are important to reduce the pest's distribution in Southeast Asia.

Pairwise F_{ST} between native and introduced populations was significantly higher than zero. Approximately 13.4% to 32.9% and 44.4% to 63.1% of genetic diversity were results of genetic difference among populations within native areas and among populations between native and introduced areas, respectively. From the comparison of the genetic diversity of other closely related species using eight similar microsatellite loci with different primer sets as the current study, lower levels of genetic diversity (ap-

proximately 2% to 18%) were estimated from *B. dorsalis* (Aketarawong et al. 2014b). The most likely explanation of the situation involves a high level of gene flow and/or recent population divergence. In case of *B. carambolae*, it implies that the level of colonization of this invasive fly may not be as high as *B. dorsalis*. Alternatively, it can be deduced that populations, in particular between native and introduced areas, became diverted, congruent with studies of multilocus phylogeny using sequence analyses (Boykin et al. 2014). However, the F_{ST} value can be influenced by the geographical difference of sampling locations (Neigel 2002). Samples of *B. dorsalis* were collected at no more than 200 km intervals whereas in this study, *B. carambolae* populations were collected from locations varying from 20 (Depok) to 18,022 km (Paramaribo) from Jakarta, Indonesia. Research using more samples from different locations on a fine scale and/or more genetic markers may provide more details.

Departures from HWE were quite high and null alleles might be responsible for the departures. The average null allele frequency was moderate (0.12), varying from low (0.01) to high frequencies (0.25). The high departure from HWE was also observed in other study using a single set of microsatellite markers for different species such as the *Ceratitis* FAR complex (52.4%, Virgilio et al. 2013). We verified possible bias produced by the presence of null alleles in our data set. The STRUCTURE analysis was also repeated considering missing data as recessive homozygotes for the null alleles. We found that the effect of null alleles was negligible (Suppl. material 4: Figure 1).

Species boundaries of *B. carambolae* and *B. dorsalis*

We found 44 alleles shared between *B. carambolae* and *B. dorsalis* while 14 and 27 alleles were detected in only *B. carambolae* and *B. dorsalis*, respectively. Coincidence of similar/different allele profiles between them at microsatellite loci may be due to several phenomena including retention of ancestral alleles in both sister species; substantial gene flow between the two species; size homoplasy (Estoup et al. 2002). For the latter case, homoplasy is possibly ruled out. It does not represent a significant problem for many types of population genetics (i.e., only 1–2% underestimation of genetic differentiation) considering only when microsatellite with high mutation rate and large population size together with strong allele size constraints were involved. The choice of more realistic mutation models than a common strict-Stepwise Mutation Model (SMM) should alleviate the homoplasy effect (review in Estoup et al. 2002, review in Selkoe and Toonen 2006).

Using the genetic cluster approach, assuming correlated allele frequencies in different clusters were likely to be similar due to migration or shared ancestral, species' genetic structures were determined. We found admixed individuals (potential hybrids) in both clusters with relatively similar ratio (9.73% in *B. carambolae* cluster and 12.5% in *B. dorsalis* cluster). To avoid the shared descent, a stricter model using uncorrelated allele frequencies was tested. The results still presented species' genetic cluster and admixed in-

dividuals (Suppl. material 4: Figure 1). Genetic connectivity also revealed that populations DP, JK, and NT in the *B. carambolae* cluster and population WK in the *B. dorsalis* cluster consisted of several admixed individuals, playing the role of linker between *B. carambolae* and *B. dorsalis* in the genetic network. Comparing to *B. dorsalis* and the junior synonym, *B. papayae*, they share better genetic profiles (Schutze et al. 2012, Krosch et al. 2013, Aketarawong et al. 2014b). Weak and no genetic structure were presented using both similar (but different primer sets) (Aketarawong et al. 2014b) and different microsatellite loci (Krosch et al. 2013). It was observed that a single cluster dominated the ancestral of all samples, when uncorrected allele frequencies were assumed in the analysis (Krosch et al. 2013). Using a different assumption of allowing similar allele frequencies between populations, the population's genetic structure was observed rather than species and admixed individuals were found among population clusters (Aketarawong et al. 2014b).

The current study therefore provided additional evidence to support an incomplete reproductive isolation between *B. carambolae* and *B. dorsalis* (Wee and Tan 2005b, Augustinos et al. 2014, Isasawin et al. 2014). Boundaries between *B. carambolae* and *B. dorsalis* may be semipermeable, varied as a function of genome region. Alleles at microsatellite loci used in this study could be introgressed between two species rather than other nuclear and mitochondrial DNA sequences (Boykin et al. 2014). To achieve a clearer picture of species boundary, genome-wide comparisons (ranging from modest number of microsatellite loci to full genome sequences, transcriptome, or SNPs analysis) between recently diverged forms or species should be performed. This may not only offer patterns of differentiation across the genome, but also define the dynamics of species boundary (Harrison and Larson 2014).

Implication of pest control using SIT for *B. carambolae*

Isasawin et al. (2014) reported a proof of concept to characterize and use the new genetic sexing Salaya5 strain (SY5) for controlling *B. carambolae*. At that time, two wild populations of *B. carambolae* were then included in the experiment. However, this study was focusing on how it would be possible to use the SY5 strain for pest control on a broader level, not only locally. Therefore, more samples of *B. carambolae* from native and introduced areas (i.e., Indonesia, Malaysia, Thailand, and Suriname) were included. More analyses on genetic variation, population structure and genetic network were performed between the SY5 strain and wild populations. We found that the SY5 strain had genetic variation, population structure, and genetic similarity comparable to *B. carambolae,* rather than *B. dorsalis*, in Southeast Asia. The results strongly confirmed that the Salaya5 strain had not diverted away from its original genetic makeup. Under laboratory condition, at least 12 generations apart, selection did not strongly affect genetic compatibility between the strain and wild populations. Therefore, the SY5 strain could be included in the pest control programs using male-only SIT for control *B. carambolae* at local and regional levels. However, an actual mating test is still required between the strain and samples from introduced populations.

SIT is a species-specific control method that can deliver environmental benefit. However, it may be restricted where at least two major target pests coexist and need to be controlled. Releasing sterile males of only one target may not ensure a reduction of all problems (Alphey et al. 2010). *Bactrocera carambolae* and *B. dorsalis* were reported to be sympatric species in some areas (e.g., Kalimantan). Although several lines of evidence suggested that both species showed mating compatibility to some degree (McInnis et al. 1999, Schutze et al. 2013, Isasawin et al. 2014), release of either sterile genetic sexing Salaya1 or Salaya5 strain may be not enough to control both target pests. The release both of the sterile genetic sexing Salaya5 and of Salaya1 strains for controlling *B. carambolae* and *B. dorsalis*, respectively, should maximize the success of SIT programs. A simulation of mating competitiveness tests and field operation, when releasing two species together, will be further required to gain knowledge for confirmation of the proposed idea.

In order to identify the fruit fly samples, although microsatellite data showed significantly different population structure of the two species, eight of 185 individuals (4.32%) and one of 104 individuals (0.96%) belonging to the *B. carambolae* and *B. dorsalis* clusters were identified as opposite to their original assumed identity. At the individual level, microsatellite data in this study may not provide definitive data for studying systematic questions of incipient species. However, at the population level, microsatellite data can be used to distinguish species. This is similar to the case of the *Ceratitis* FAR complex in that genetic clustering can solve three species' statuses whereas other data (i.e., morphology, phylogenetics based on DNA sequence analyses, and niche) cannot (Virgilio et al. 2013). In this study, the two unidentified populations should be good examples to support this particular advantage of using microsatellite markers. Therefore, a combination of microsatellite data with other approaches should be better than a stand-alone approach to define and confirm individual and/or population.

Conclusion

Pattern of connectivity and population structure, based on microsatellite DNA markers, showed that *B. carambolae* from an introduced population genetically differs from populations from the native range. Genetic drift during colonization process and local adaptation may be factors shaping its genetic diversity and population structure. However, only sampling from South America might not be sufficient to trace back the process of colonization within and between continents. West Sumatra (Pekanbaru-PK) and Java (Jakarta-JK) were identified as sources of the Suriname population, congruent with historical record of human migration between the two continents. A different pattern of population structure was observed in *B. carambolae* within native range, where free human movement and trading can promote genetic homogeneity. Between *B. carambolae* and *B. dorsalis* groups, potential hybrids provide evidence through individual-based admixture plots. This was additional supportive data suggesting that reproductive isolation between *B. carambolae* and *B. dorsalis* is somewhat leaky. Although morpho-

logical characterization and several nuclear and mitochondrial markers revealed distinct species, the hypothesis of semipermeable species boundaries between them cannot be rejected. Alleles at microsatellite loci could be introgressed rather than other nuclear and mitochondrial sequences. Regarding the final conclusion on pest management aspect, the genetic sexing Salaya5 strain for *B. carambolae* had not diverted away from its original genetic makeup (JK) and other neighbor populations. Under laboratory condition, at least 12 generations apart, selection did not strongly affect genetic compatibility between the strain and wild populations. Therefore, the Salaya5 strain could be possible to include in the pest control programs using male-only SIT in local and regional levels, although an actual mating test is still required between the strain and samples from introduced populations.

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Supplementary material 1

Component data at the five successive thresholds used to illustrate Figure 4

Authors: Nidchaya Aketarawong, Siriwan Isasawin, Punchapat Sojikul, Sujinda Thanaphum

Data type: measurement

Explanation note: Component data are used to illustrate the structure of the subset of *B. carambolae* populations. The Highest Betweenness-centrality is highlighted in blue.

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Supplementary material 2

Component data at the four successive thresholds used to illustrate Figure 5

Authors: Nidchaya Aketarawong, Siriwan Isasawin, Punchapat Sojikul, Sujinda Thanaphum

Data type: measurement

Explanation note: Component data are used to illustrate the structure of the subset of *B. carambolae* and *B. dorsalis* populations. The highest Betweenness-centrality is highlighted in blue.

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Supplementary material 3

Component data at the four successive thresholds used to illustrate Figure 6

Authors: Nidchaya Aketarawong, Siriwan Isasawin, Punchapat Sojikul, Sujinda Thanaphum

Data type: measurement

Explanation note: Component data are used to illustrate the structure of the subset of the Salaya5 strain and wild populations. The highest Betweenness-centrality is highlighted in blue.

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Supplementary material 4

Comparisons among three different the individual admixture plots

Authors: Nidchaya Aketarawong, Siriwan Isasawin, Punchapat Sojikul, Sujinda Thanaphum

Data type: measurement

Explanation note: Comparisons among the individual admixture plots of 289 individuals, for K = 3, considering correlated allele frequency, uncorrelated allele frequency, and missing data as recessive homozygotes for the null alleles, respectively.

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Cytogenetic and symbiont analysis of five members of the *B. dorsalis* complex (Diptera, Tephritidae): no evidence of chromosomal or symbiont-based speciation events

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Abstract

The *Bactrocera dorsalis* species complex, currently comprising about 90 entities has received much attention. During the last decades, considerable effort has been devoted to delimiting the species of the complex. This information is of great importance for agriculture and world trade, since the complex harbours several pest species of major economic importance and other species that could evolve into global threats. Speciation in Diptera is usually accompanied by chromosomal rearrangements, particularly inversions that are assumed to reduce/eliminate gene flow. Other candidates currently receiving much attention regarding their possible involvement in speciation are reproductive symbionts, such as *Wolbachia, Spiroplasma, Arsenophonus, Rickettsia* and *Cardinium*. Such symbionts tend to spread quickly through natural populations and can cause a variety of phenotypes that promote pre-mating and/or post-mating isolation and, in addition, can affect the biology, physiology, ecology and evolution of their insect hosts in

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various ways. Considering all these aspects, we present: (a) a summary of the recently gained knowledge on the cytogenetics of five members of the *B. dorsalis* complex, namely *B. dorsalis s.s.*, *B. invadens*, *B. philippinensis*, *B. papayae* and *B. carambolae*, supplemented by additional data from a *B dorsalis s.s.* colony from China, as well as by a cytogenetic comparison between the *dorsalis* complex and the genetically close species, *B. tryoni,* and, (b) a reproductive symbiont screening of 18 different colonized populations of these five taxa. Our analysis did not reveal any chromosomal rearrangements that could differentiate among them. Moreover, screening for reproductive symbionts was negative for all colonies derived from different geographic origins and/or hosts. There are many different factors that can lead to speciation, and our data do not support chromosomal and/or symbiotic-based speciation phenomena in the taxa under study.

Keywords
Tephritidae, *Wolbachia*, inversions, polytene chromosomes

Introduction

The *Bactrocera dorsalis* species complex currently consists of approximately 90 entities, whose limits are not fully resolved (Drew and Hancock 1994, Drew and Romig 2013, Krosch et al. 2013, Boykin et al. 2014, Schutze et al. 2015). However, species delimitation is of paramount importance when dealing with economic important species, since it can influence world trade through implementation of quarantine policies and/or facilitate the application of species specific, environmental friendly control methods, such as the Sterile Insect Technique (SIT). Driven by these considerations, much effort has been invested in the last decades to clarify the species status within the complex. Among the most recent advances in this area, Drew and Romig (2013) synonymised *B. papayae* and *B. philippinensis* under *B. papayae*, while Schutze and colleagues (Scutze et al. 2015) have proposed the further synonymization of these two taxa and *B. invadens* with *B. dorsalis s.s.,* under *B. dorsalis s.s.*

Recent studies have shown that efforts to resolve complex species status require multidisciplinary approaches (De Queiroz 2007, Schlick-Steiner et al. 2010), well-characterized material and extended sampling (Schutze et al. 2012, Krosch et al. 2013, Boykin et al. 2014). Such approaches have been also followed in other Tephritidae genera where species delimitation of species complexes is also an important concern, such as in *Anastrepha* (Selivon et al. 2005, Vera et al. 2006, Cáceres et al. 2009). This is due to the fact that speciation can be driven by a variety of forces, resulting in different speciation paths. The data basis can be complicated when speciation is ongoing (incipient). Therefore, in collaboration and through independent analysis, different research groups around the world, through the Coordinated Research Program: 'Resolution of Cryptic Species Complexes of Tephritid Pests to Enhance SIT Application and Facilitate International Trade' have accumulated a multitude of data that have contributed to the better understanding of the Tephritidae species complexes. One of the main targets was the resolution among five economic important taxa with unclear limits within the *B. dorsalis* complex. These were *B. dorsalis s.s., B. papayae, B. philippinensis, B. invadens* and *B. carambolae*.

A key pathway of speciation in Diptera is through chromosomal rearrangements (CRs), mainly inversions. More than fifty years of research on polytene chromosomes of *Drosophila* and mosquito species have shown that speciation is almost universally accompanied with inversions (Sturtevant and Dobzhansky 1936, Ashburner et al. 1982, Krimbas and Powell 1992, Noor et al. 2001, Rieseberg 2001, Kirkpatrick and Barton 2006, Bhutkar et al. 2008, Kulathinal et al. 2009, Stevison et al. 2011, Lee et al. 2013). The recent advances in whole genome sequencing and the availability of a number of genomes of *Drosophila* and mosquito species have verified the nuclear DNA rearrangements described in earlier cytogenetic studies (Kirkpatrick and Barton 2006, Ranz et al. 2007, Bhutkar et al. 2008, Schaeffer et al. 2008, Kulathinal et al. 2009, McGaugh and Noor 2012, Lee et al. 2013). Different models have been proposed to explain how CRs enhance speciation, recently focusing mainly on the restriction of recombination within and near inverted regions as the causal factor of restriction in gene flow (Noor et al. 2001, Rieseberg 2001, Kirkpatrick and Barton 2006, Faria and Navarro 2010).

However, sequencing of entire genomes cannot yet be easily applied to species with bigger genomes and a high proportion of repetitive DNA sequences. Shotgun sequencing approaches are relatively quick and cheap, but cannot provide insight into higher chromosomal organization of species lacking of a complete sequenced reference genome, at least up to now. Regarding the *B. dorsalis* complex, the draft genome of *B. dorsalis s.s.* currently consists of more than 86,000 contigs (http://www.ncbi.nlm.nih.gov/assembly/GCF_000789215.1). Even though the construction of several genome databases of Tephritidae species is ongoing, this methodology is so far (a) too slow and expensive to screen a large number of different populations and (b) it is not guaranteed to reveal structural chromosomal changes between species, unless coupled with molecular and genetic approaches, such as Sanger sequencing, cloning and *in situ* hybridization. Direct observation and comparison of chromosomes is still a very powerful approach to shed light on the higher organization and structure of chromosomes. Although mitotic chromosomes can also provide some information, polytene chromosomes are an excellent tool for resolution of CRs.

In Tephritids, there is a number of studies presenting and discussing mitotic karyotypes, especially for *Bactrocera* (Hunwattanakul and Baimai 1994, Baimai et al. 1995, 1999, 2000, Baimai 1998, 2000), *Anastrepha* (Cevallos and Nation 2004, Selivon et al. 2005, Goday et al. 2006, Selivon et al. 2007) and *Rhagoletis* species (Bush and Boller 1977). However, useful polytene chromosome maps, so far available for five genera, represent only 11 species: one of *Anastrepha* (*A. ludens*) (Garcia-Martinez et al. 2009), one of *Ceratitis* (*C. capitata*) (Zacharopoulou 1990), one of *Dacus* (*D. ciliatus*) (Drosopoulou et al. 2011b) and three of *Rhagoletis*, namely *R. cerasi* (Kounatidis et al. 2008), *R. cingulata* (Drosopoulou et al. 2011a) and *R. completa* (Drosopoulou et al. 2010). The genus *Bactrocera* can be regarded as the best studied so far, including four species of three different subgenera. These are *B. oleae* (subgenus *Daculus*) (Mavragani-Tsipidou et al. 1992), *B. cucurbitae* (subgenus *Zeugodacus*) (Zacharopoulou et al. 2011b) and *B. dorsalis s.s.* (Zacharopoulou et al. 2011a) plus *B. tryoni* (subgenus *Bactrocera*) (Zhao et al. 1998).

Cytogenetic studies have been used to distinguish between different members of the *B. dorsalis* complex in the past, based on mitotic chromosomes. Hunwattanakul and Baimai (1994) presented the typical karyotype of *B. dorsalis*, which is being referred to as form A. The mitotic karyotype of the complex is 2n = 12, consisting of five pairs of autosomes and a heterogametic XX/XY sex chromosome pair. In the following years, Baimai and colleagues presented numerous species within the complex with distinct mitotic karyotypes (Baimai et al. 1995, 2000, Baimai 1998). Although these studies are of great importance and reveal the power of cytogenetics for the resolution of species limits within species complexes, they suffered from limitations that could not be addressed or even predicted in the previous years. These include (a) the ongoing debate on species limits and taxonomy of the complex, (b) utilization of material from the field that cannot be evaluated with other approaches, since it was not colonized and, (c) lack of robust diagnostic tools within this complex. All these indicate that older taxonomic conclusions should be used with care and seen in the light of recent advances in the field.

To overcome such constraints, recent cytogenetic studies have used laboratory colonies from the Joint FAO/IAEA Insect Pest Control Laboratory (IPCL). These colonies are also material in a variety of research programs, are always available for further analyses and their status is routinely verified by expert taxonomists. Zacharopoulou and colleagues analysed colonized material of *B. dorsalis s.s.*, derived from Thailand and from a Genetic Sexing Strain (GSS) constructed in Hawaii (Zacharopoulou et al. 2011a). In this study, the form A mitotic karyotype was verified for *B. dorsalis s.s.*, and polytene chromosome map for this species was constructed, which includes 10 polytene arms. These arms correspond to the autosomes, which is consistent with the already described non-polytenization of the sex chromosomes in Tephritidae (Zacharopoulou 1990, Mavragani-Tsipidou et al. 1992, Zhao et al. 1998, Garcia-Martinez et al. 2009, Drosopoulou et al. 2010, Zacharopoulou et al. 2011a, Zacharopoulou et al. 2011b, Drosopoulou et al. 2011a, Drosopoulou et al. 2011b, Drosopoulou et al. 2012). Recently, a more extended cytogenetic analysis was performed (Augustinos et al. 2014b), shedding more light on the resolution of the species limits of the five taxa described before. Six laboratory colonies, representing *B. dorsalis s.s.* (two colonies), *B. papayae, B. philippinensis, B. invadens* and *B. carambolae,* were examined (Table 1) and all exhibited the form A mitotic karyotype. This was quite a surprise, since it is not in agreement with previous studies, where a distinct karyotype with a quite large X chromosome, carrying an 'elongated' arm with a secondary constriction, was described for *B. carambolae* from Thailand (Baimai et al. 1999). In addition, polytene chromosomes did not reveal any fixed CRs among these five taxa that could be used as diagnostic markers (Augustinos et al. 2014b) .

A second factor that should not be overlooked in studies addressing speciation phenomena is the presence of specific symbiotic bacteria, especially those referred to as 'reproductive parasites'. These are symbiotic bacteria mainly found in reproductive tissues and are best known to interfere with host reproduction, inducing a variety of phenotypes such as male killing, parthenogenesis, feminization and Cytoplasmic

Table 1. Material used in the present study.

No	Species	Origin	Reproductive symbiont screening*		Cytogenetically analyzed
			M	F	
1	*B. dorsalis*	Saraburi, Thailand	10	10	Zacharopoulou et al. 2011a Augustinos et al. 2014b
2	*B. dorsalis*	Nakhon Sri Thammarat, Thailand	10	10	Augustinos et al. 2014b
3	*B. dorsalis G17*	Bangkok, Thailand	10	10	
4	*B. dorsalis GSS*	Hawaii	10	10	Zacharopoulou et al. 2011a; Zacharopoulou and Franz 2013
5	*B. dorsalis (White body)*	OAP, Bangkok, Thailand	10	10	
6	*B. dorsalis*	Yunnan, China	10	10	
7	*B. dorsalis*	Fujian, china	10	10	
8	*B. dorsalis*	Pakistan	10	10	
9	*B. dorsalis*	Myanmar	10	10	
10	*B. dorsalis*	India	10	10	
11	*B. dorsalis*	Wuhan, China (colony 1)	10	10	Present study
12	*B. dorsalis*	Wuhan, China (colony 2)	10	10	
13	*B. carambolae*	Paramaribo, Suriname	10	10	Augustinos et al. 2014b
14	*B. carambolae*	Serdang, Malaysia	10	10	
15	*B. philippinensis*	Guimaras Island, Philippines	10	10	
16	*B. philippinensis*	Philippines	10	10	Augustinos et al. 2014b
17	*B. papayae*	Serdang, Malaysia	10	10	Augustinos et al. 2014b
18	*B. invadens*	Kenya	10	10	Augustinos et al. 2014b
19	*B. tryoni*	Australia	10	10	Present study

*Twenty flies were screened for the presence of the five reproductive symbionts listed in Table 2. None was positive for none of the symbionts.

Incompatibility (CI). Among them, *Cardinium, Arsenophonus, Spiroplasma, Rickettsia* and *Wolbachia* are commonly found in different arthropods (Bourtzis and Miller 2003, 2006, 2009, Perlman et al. 2006, Duron et al. 2008a, Werren et al. 2008, Saridaki and Bourtzis 2010, Zchori-Fein and Bourtzis 2011).

Wolbachia is probably the most ubiquitous bacterial symbiont in insects (Hilgenboecker et al. 2008, Zug and Hammerstein 2012) and is regarded as a putative 'speciation agent', since it can restrict gene flow through (CI) and lead to the selection and fixation of specific genotypes in a population. *Wolbachia*-induced CIs can co-exist with local selection on alleles involved in incompatibilities and, therefore, increase the migration rates that genetic variability can experience without getting lost. The combined act of the two aforementioned forces of incompatibility can lead to maintenance of the divergence among populations and enhance speciation (Flor et al. 2007, Telschow

et al. 2007, 2014). Besides theoretical and model predictions, the implication of *Wolbachia* in pre- and / or post-mating isolation phenomena has been experimentally supported in different insect systems including the parasitic wasps of the genus *Nasonia* (Bordenstein et al. 2001, Bordenstein and Werren 2007) and *Drosophila* (Jaenike et al. 2006, Koukou et al. 2006, Miller et al. 2010).

In tephritids, most studies have so far focused on the detection and characterization of *Wolbachia* infections. Although screening is far from complete, well-established infections have been found in some species. The best characterized species is *R. cerasi,* since all natural populations studied so far are 100% infected, usually with multiple-strain infections (Riegler and Stauffer 2002, Kounatidis et al. 2008, Arthofer et al. 2009, Augustinos et al. 2014a, Karimi and Darsouei 2014). More importantly, it is a well-documented example of the implication of *Wolbachia* in restriction in gene flow and enhancement of incompatibility between natural populations of the species (Riegler and Stauffer 2002). Other *Rhagoletis* species that seem to have persistent and multiple strain infections (although less populations are studied) are *R. pomonella* (Schuler et al. 2011) and *R. cingulata* (Drosopoulou et al. 2011a, Schuler et al. 2013), along with some *Rhagoletis* species of Japan (Coats et al. 2013). Outside *Rhagoletis*, the only species demonstrating persistent *Wolbachia* infections is *A. fraterculus,* (Selivon et al. 2002, Cáceres et al. 2009, Coscrato et al. 2009, Marcon et al. 2011, Martinez et al. 2012). All other tephritid species are so far considered as *Wolbachia*-free or only exhibiting low prevalence infections. Among them, *C. capitata* is also considered as *Wolbachia*-free (Bourtzis et al. 1994, Zabalou et al. 2004); however, there are two reports from a research group in Latin America discussing the presence of *Wolbachia* in local populations of the species (Rocha et al. 2005, Coscrato et al. 2009). The recent study on the *Wolbachia* presence in Australian fruit flies (Morrow et al. 2015) has extended our knowledge on the *Wolbachia* status of Tephritidae in a relatively unexplored area. In accordance with previous studies, few species were found infected and only a relatively small (although varying) percentage of individuals. However, this study demonstrated the presence of different *Wolbachia* strains, shared among natural populations of different species, raising the possibility of recent horizontal transmission events through shared parasitoids. Regarding the other four symbionts, there are up to now no reports of infected populations, at least to our knowledge. Especially for the *B. dorsalis* complex, there are only three reports of *Wolbachia* infections in natural populations. In all these cases, infections were found at a very low prevalence in nature (Kittayapong et al. 2000, Jamnongluk et al. 2002, Sun et al. 2007).

The purpose of this study was to (a) summarize gained knowledge and (b) provide new evidence regarding the cytogenetic and symbiotic status of the *B. dorsalis* complex, with the aim to identify factors possibly involved in speciation. Focus has been given on five taxa of economic importance and unclear species limits, namely *B. dorsalis s.s., B. papayae, B. philippinensis, B. invadens* and *B. carambolae.* Only material colonized at the Joint FAO/IAEA IPCL was analysed, that was also used in other Joint FAO/IAEA IPCL research programs (Wee et al. 2002, Krosch et al. 2013, Boykin et al. 2014, Schutze et al. 2013, Tan et al. 2013, Bo et al. 2014). More specifically, our cytogenetic

analysis was extended to (a) a *B. dorsalis s.s.* population derived from China, a cytogenetically unexplored area of great interest for the complex, (b) a new Australian colony of *B. tryoni,* a species that is genetically discrete though not phylogenetically distant from the *B. dorsalis* complex and (c) F1 bidirectional hybrids of *B. dorsalis s.s.* and *B. tryoni*. In addition, an extensive PCR screening was performed aiming at the detection of the five aforementioned reproductive symbionts in 18 different colonies available for the *dorsalis* complex and the colony representing *B. tryoni* (Table 1).

Methods

Material used

Nineteen colonies currently kept at the Joint FAO/IAEA IPCL were screened for the presence of different reproductive symbionts (Table 1). Eighteen of them represent the five members of the complex under discussion (*B. dorsalis s.s., B. papayae, B. philippinensis, B. invadens* and *B. carambolae*), while one colony represents *B. tryoni* from Australia that was included as a closely related outgroup. Two colonies were cytogenetically analysed (*B. dorsalis s.s.* from China-Wuhan and *B. tryoni* from Australia) and were added to the seven colonies previously analysed (see Table 1 and references therein). The F_1 bidirectional hybrids of *B. dorsalis s.s.* x *B. tryoni* were also analysed.

Mitotic chromosome preparations

Chromosome preparations were made as described in Zacharopoulou (1990) and Mavragani-Tsipidou et al. (2014). Brain tissue was dissected in 0.7 % NaCl, transferred to 1 % sodium citrate on a well slide for at least 15 min and fixed in fresh fixation solution (methanol/acetic acid 3:1) for 3 min. Fixation solution was removed and a drop of acetic acid (60 %) was added. Tissue was dispersed using a micropipette and the cell suspension was dried on a clean slide placed on a hotplate (40-45°C). Chromosomes were stained with Giemsa (5 % Giemsa in 10 mM phosphate buffer, pH 6.8). Chromosome slides were analysed at 100 x magnification, using a phase contrast microscope (Leica DMR), and photographs were taken using a CCD camera (ProgRes CFcool; Jenoptik Jena Optical Systems, Jena, Germany). At least 15 good quality preparations per sample and at least 10 well-spread nuclei per preparation were analysed.

Polytene chromosome preparations

Polytene chromosome preparations were made from 3rd instar larvae, as described in Zacharopoulou (1990), Mavragani-Tsipidou et al. (2014). Larvae were dissected in acetic acid (45 %), and salivary glands were transferred to HCl (3 N) for 1 min, fixed

in 3:2:1 fixation solution (3 parts acetic acid: 2 parts water: 1 part lactic acid) for ~5 min (until transparent) and stained in 2% lacto-aceto- orcein for 5-7 min. Glands were washed with 3:2:1 solution to remove excess stain and squashed. Chromosome slides were analysed at 100 x magnification using a phase contrast microscope (Leica DMR) and photographs were taken using a CD camera (ProgRes CFcool; Jenoptik Jena Optical Systems, Jena, Germany). At least 15 good quality preparations per sample and at least 10 well spread nuclei per preparation were analysed.

DNA extraction and PCR screening for reproductive symbionts

DNA was extracted from single flies, using the CTAB protocol (Doyle and Doyle 1990). To verify DNA quality, PCRs were performed for randomly selected samples with the universal primer pair 12SCFR/12SCRR that amplifies 420 bp of the insect mitochondrial 12S rRNA gene (Hanner and Fugate 1997). In total, 380 samples were screened for the presence of *Wolbachia, Spiroplasma*, *Arsenophonus*, *Rickettsia* and *Cardinium*. Screening was performed using bacterial species-specific 16S *rRNA* gene-based PCR. Depending on the set of primers used, the amplified DNA fragment varied in size from 200 bp to 611 bp. The amplification was performed in 20 μl reactions, each containing 2 μl of 10x KAPA *Taq* Polymerase Buffer A (with 1.5 mM of $MgCl_2$ at 1x), 0.1 μl of dNTPs (25 mM), 0.5 μl of the forward primer (25 μM), 0.5 μl of the reverse primer (25 μM), 0.1 μl of KAPA *Taq* DNA Polymerase (5 U/μl), 15.8 μl of sterile double distilled water and 1 μl of DNA. The PCR protocol included an initial 5 minute denaturation at 95 °C, followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at the optimum melting temperature for each pair of specific primers, 1 minute at 72 °C and a final extension step of 10 minutes at 72 °C, with the exception of *Wolbachia,* where 30 cycles were used. The products were electrophoresed on a 1.5 % agarose gel in order to determine the presence and size of the fragments. Primer pairs and PCR conditions are summarized in Table 2.

Results and discussion

As already stated in the Introduction, material colonized in IPCL was used in the present study. This is in the frame of utilizing multi-disciplinary approaches, using the same samples if possible, to contribute to the species resolution in the *dorsalis* complex (Schutze et al. 2015). For such approaches utilization of colonized, well-characterized material is essential. This is even more evident for cytogenetics, since live material is needed. On the other hand, results obtained from laboratory colonies must be verified in larger samples of different origin before elevating to species level. As it has been shown by different studies (Gilchrist et al. 2012; Parreño et al. 2014, Zygouridis et al. 2014), lab colonization is accompanied by an adaptation process including severe bottlenecks, hitch-hiking effects and extended inbreeding. This can affect the genetic

Table 2. PCR screening for five reproductive symbionts.

Genus	Primer 5'-3'	Tm°C	Product Size	Reference
Wolbachia	wspecF YATACCTATTCGAAGGGATAG	55°C	438 bp	Werren and Windsor 2000
	wspecR AGCTTCGAGTGAAACCAATTC			
Spiroplasma	63F_CG	60°C	450 bp	Mateos et al. 2006 Fukatsu and Nikoh 2000
	GCCTAATACATGCAAGTCGAACGG			
	TKSSspR			
	TAGCCGTGGCTTTCTGGTAA			
Arsenophonus	ArsF	56°C	611 bp	Duron et al. 2008b
	GGGTTGTAAAGTACTTTCAGTCGT			
	ArsR5			
	CCCTAAGGCACGYYTYTATCTCTAA			
Rickettsia	16SA1	55°C	200 bp	Fukatsu and Nikoh 2000
	AGAGTTTGATCTGGCTCAG			
	Rick16SR			
	CATCCATCAGCGATAAATCTTTC			
Cardinium	CLO-f1	56°C	466 bp	Gotoh et al. 2007
	GGAACCTTACCTGGGCTAGAATGTATT			
	CLO-r1			
	GCCACTGTCTTCAAGCTCTACCAAC			

structure of the populations and, possibly, their symbiotic communities. Therefore, results derived from colonized material should be 'interpreted' wisely and in combination with the analysis of natural collections.

Mitotic karyotypes – agreements and inconsistencies with older studies

The *B. dorsalis s.s.* colony from China showed the *B. dorsalis s.s.* mitotic karyotype known as form A. This is the typical and probably ancestral karyotype of the *dorsalis* complex. The above, together with previous results, show that the Joint FAO/IAEA IPCL colonies, representing the five investigated taxa, possess the same mitotic karyotype (Zacharopoulou et al. 2011a, Zacharopoulou and Franz 2013, Augustinos et al. 2014b)**.** Older studies (Baimai et al. 1999) describe a different karyotype for *B. carambolae* from Thailand. Although the *B. carambolae* colony analysed recently, available at the Joint FAO/IAEA IPCL (Augustinos et al. 2014b), was derived from a Suriname population, it is highly unlikely that the different origin is the explanation for this difference. Incorrect species identification due to the limitations discussed in the Intro-

duction Section is the most probable explanation. This is further supported by the fact that an independent study on the mitotic karyotypes of *B. carambolae* from Malaysia also found the typical form A karyotype for this taxon (Yesmin and Clyde 2012).

The examination of new material representing *B. tryoni* from Australia was in accordance with the previously published mitotic karyotype for this species (Zhao et al. 1998). This karyotype has five pairs of autosomes and a heterogametic XX/XY sex chromosome pair. The three larger autosome pairs are metacentric to submetacentric, while the two shorter autosome pairs are submetacentric to acrocentric. Y is the smallest of the set, while X is large and probably larger than or comparable to the largest autosomes.

Polytene chromosome comparisons and species resolution

Polytene chromosome nuclei of *B. dorsalis s.s.* from China are shown in Figure 1. Its polytene chromosomes show the same banding pattern with the published maps of *B. dorsalis s.s.* (Zacharopoulou et al. 2011a), and therefore can be regarded as homosequential with all other colonies analysed so far (Augustinos et al. 2014b). The characteristic asynapsis at regions 73-74 of arm 5R previously observed in all colonies (Zacharopoulou et al. 2011a, Augustinos et al. 2014b) was also found here at a relatively high frequency (Figure 2). Its polymorphic presence in all colonies analysed so far points to the close genetic proximity of these five taxa.

Another interesting finding from the analysis of the China colony is the high presence of an asynapsis at the telomeric region of 3L (Figure 3). Although previously observed in other colonies (Zacharopoulou et al. 2011a, Augustinos et al. 2014b), its frequency in the specific colony is much higher than in the colonies analysed before. Again, this can be considered as an inter species, intra population variation.

In the recent proposed revisions that synonymize four out of the five *Dorsalis* taxa under study (Drew and Romig 2013, Schutze et al. 2015), *B. carambolae* is maintained as a distinct species within the complex, but closely related to *B. dorsalis s.s.* The recent cytogenetic analysis on these five taxa failed to find any fixed diagnostic CRs among *B. dorsalis s.s.* and *B. carambolae* (Augustinos et al. 2014b). However, as discussed in that paper, the high frequency of small asynapses observed in the *B. dorsalis s.s.* x *B. carambolae* F_1 hybrids, in comparison to the *B. dorsalis s.s.* x *B. invadens* F_1 hybrids could be an indication of the presence of small CRs between the *B. dorsalis s.s.* and *B. carambolae* genomes, undetected with microscopy.

To explore the limitations of cytogenetic analysis in species resolution, we performed a polytene chromosome comparison between the *dorsalis* complex and *B. tryoni*, a species also belonging to the subgenus *Bactrocera* and routinely used as a closely related outgroup in different studies (Krosch et al. 2012; Boykin et al. 2014; Virgilio et al. 2015). To do so, polytene chromosome squashes from an IPCL laboratory colony were prepared and directly compared with the published *B. dorsalis s.s.* map (Zacharopoulou et al. 2011a), the already published *B. tryoni* map (Zhao et al. 1998) and photos from polytene chromosomes of the five taxa of the *dorsalis* complex. This analysis

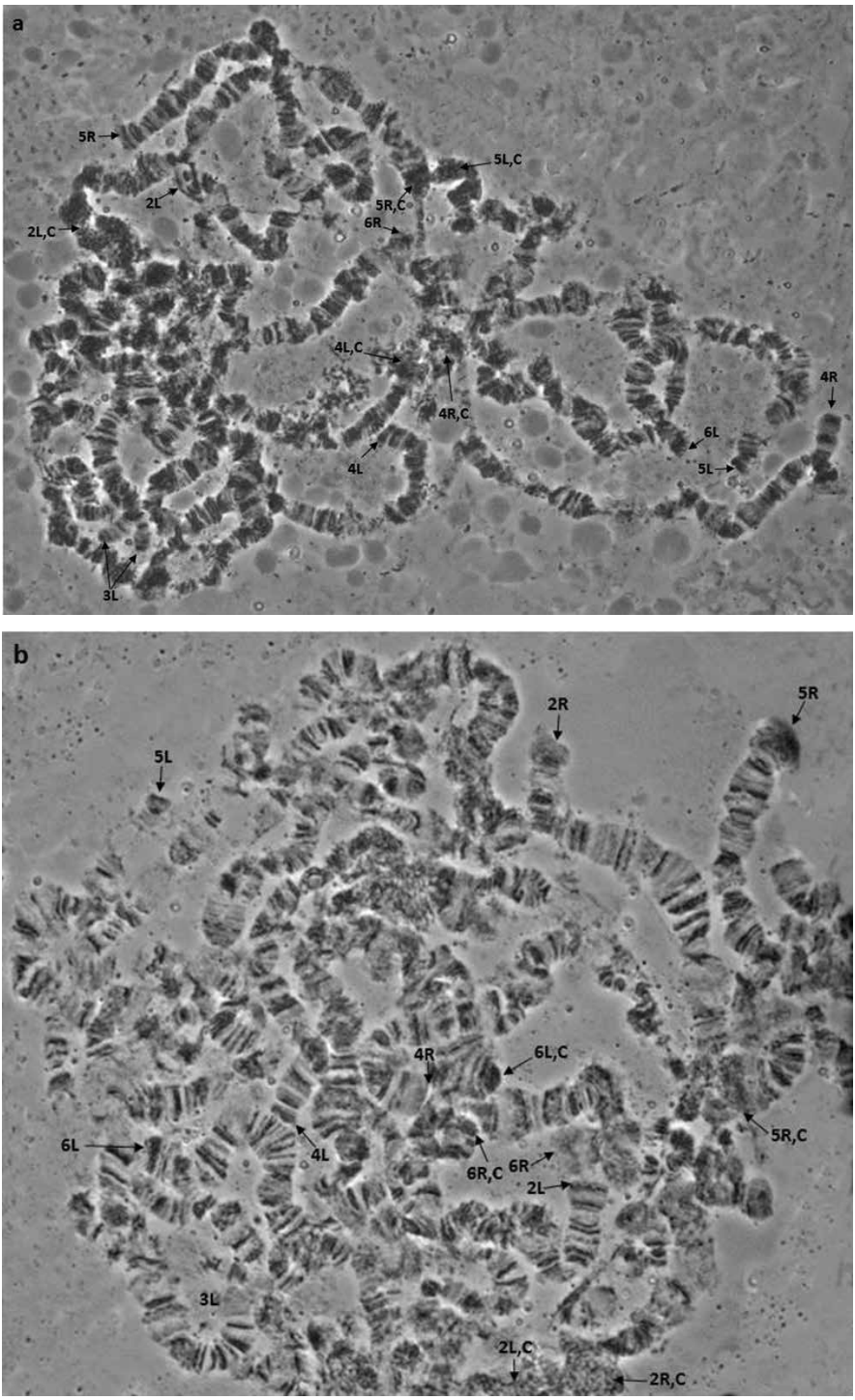

Figure 1. a, b Polytene nuclei of *B. dorsalis s.s.* from China. Chromosome arms are shown. Tips are marked with arrows and centromeres are indicated with 'C'.

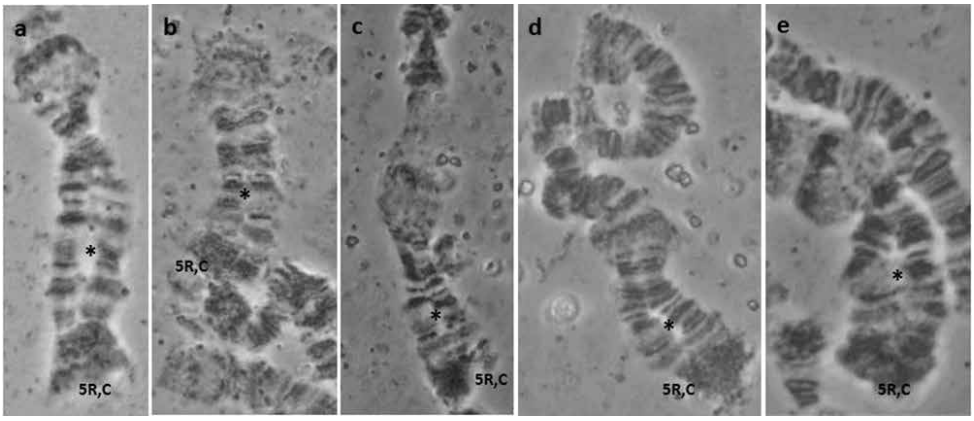

Figure 2. a–e Characteristic asynapsis in 5R chromosome arm, close to the centromere (regions 73-74), observed in the *B. dorsalis s.s.* colony derived from China. Asterisks (*) mark the asynaptic region, while 'C' marks the 5R centromere.

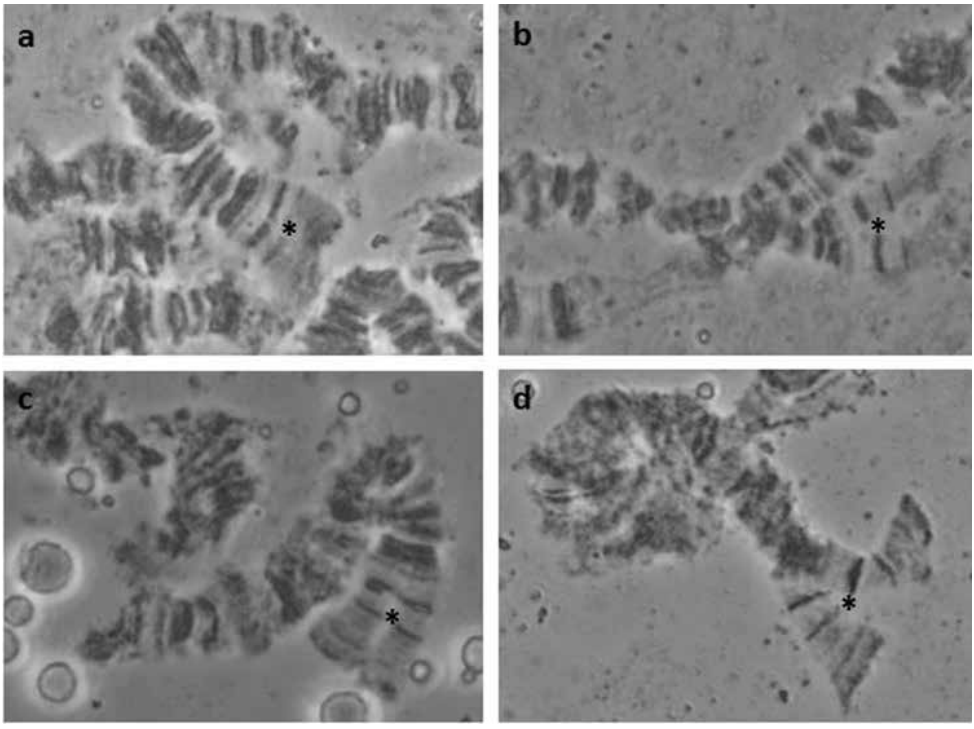

Figure 3. Characteristic asynapsis in the 3L, close to the tip region, observed in *B. dorsalis* colony derived from China. **a** almost completely synapsed region **b–d** asynapses of the same region; asterisks (*) indicate the specific region.

showed that this colony is homosequential with the previously published map of *B. tryoni*. A comparison between *B. tryoni* and the five *dorsalis* taxa showed that nine of the ten polytene arms can be regarded as highly homosequential, verifying the genetic proximity between them (Figures 4–6). However, a fixed chromosomal inversion that was previously described (Augustinos et al. 2014b), based on a comparison of polytene chromosome maps of the two species (*B. dorsalis s.s.* and *B. tryoni*), was verified in the new polytene chromosome squashes of the IPCL colony (Figure 7). This CR is quite extended, covering a large region of arm 2R.

To further verify the proposed syntenies, a cytogenetic analysis of F_1 bidirectional hybrids of *B. dorsalis s.s.* and *B. tryoni* was performed. Consistently with the aforementioned conclusions good synapsis can be seen in 9/10 polytene arms, while asynaptic regions are also present, as expected for hybrids of well-differentiated species (Figure 8). The inversion covering a large part of the 2R chromosome arm (Figure 8b) can also be observed, although its extension usually leads to chromosome breaks that make the mapping of breakpoints rather difficult (Figure 9).

As discussed in the Introduction, CRs are regarded as key players in Diptera speciation. In Tephritidae, all species analysed so far are differentiated by CRs, mainly inversions and transpositions. Focusing on the better studied Tephritidae species (*C. capitata*) and species of two genera that are phylogenetically close to each other (*Bactrocera* and *Dacus*), polytene chromosome comparisons performed either in older studies or in the present study have revealed specific CRs that are diagnostic in genus, subgenus and species level. Comparative analysis of the published polytene chromosome maps shows that the pericentric inversion in chromosome 5, firstly described by Zhao et al. (1998), also differentiates *C. capitata* from the other four *Dacus/Bactrocera* species studied so far (Zacharopoulou 1990, Mavragani-Tsipidou et al. 1992, Zacharopoulou et al. 2011a, 2011b, Drosopoulou et al. 2011b). Within the *Dacus/Bactrocera* clade, polytene chromosomes provide evidence for the genetic proximity of *Bactrocera Zeugodacus* and *Bactrocera Daculus* (to a lesser extend) with *Dacus.* More specifically, there are certain CRs shared between *B. cucurbitae (Zeugodacus*), *B. oleae* (*Daculus*) and *D. ciliatus* in contrast to the two species of the *Bactrocera* subgenus (*B. dorsalis s.s.* and *B. tryoni*). A characteristic example is a pericentric inversion in chromosome 6 that changes the length ratio of the two arms, clearly evident when comparing the maps of these species (Mavragani-Tsipidou et al. 1992, Zhao et al. 1998, Zacharopoulou et al. 2011a, 2011b, Drosopoulou et al. 2011b). On the other hand, *B. oleae* shares also some characteristic CRs with the typical *Bactrocera* (Mavragani-Tsipidou et al. 1992, Zhao et al. 1998, Zacharopoulou et al. 2011a, 2011b, Drosopoulou et al. 2011b). Informative is also chromosome 2, since its right arm is considered as highly polymorphic among the different Tephritidae species. The region involved in the 2R inversion described before does not only differentiate *B. tryoni* from the *B. dorsalis* taxa analysed so far. This region has a unique banding pattern and/or position among the five *Bactrocera/Dacus* species analysed so far (Mavragani-Tsipidou et al. 1992, Zhao et al. 1998, Zacharopoulou et al. 2011a, 2011b, Drosopoulou et al. 2011b). All the above findings are in accordance with recent studies discussing either the genetic proximity

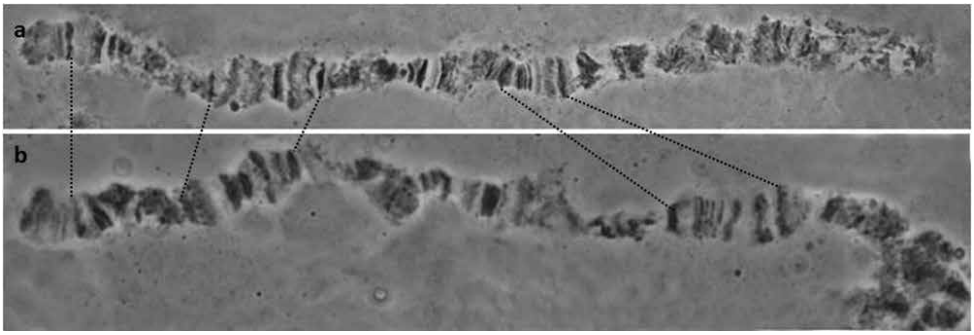

Figure 4. Comparison of the 3L polytene chromosome arm of **a** *B. tryoni* and **b** *B. dorsalis s.s.*. Dot lines connect characteristic landmarks of the two chromosomes.

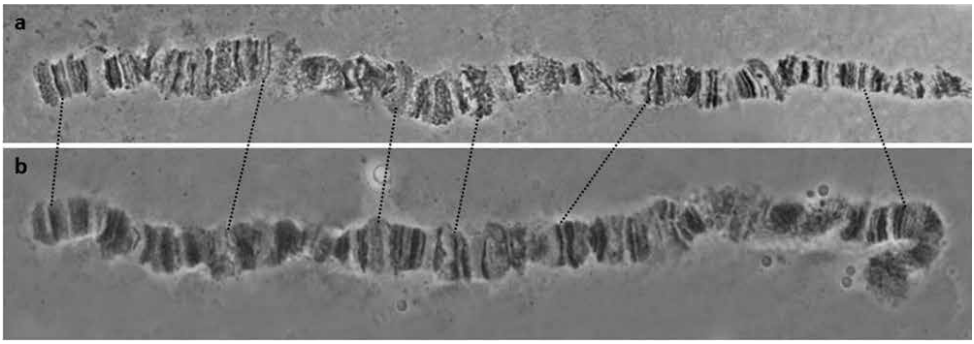

Figure 5. Comparison of the 4L polytene chromosome arms of **a** *B. tryoni* and **b** *B. dorsalis s.s.*. Dot lines connect characteristic landmarks of the two chromosomes.

of specific *Bactrocera* subgenera with *Dacus* or the actual status of specific subgenera, especially the *Zeugodacus* subgenus (Virgilio et al. 2009, Krosch et al. 2012, Virgilio et al. 2015).

Taking together that (a) all different Tephritidae species analysed so far exhibit characteristic CRs and (b) no diagnostic CRs could be observed in the five taxa of the *B. dorsalis* complex analysed here, it is clear that polytene chromosome analysis does so far not support a CR-mediated speciation event in the taxa under study.

Reproductive symbiont screening – lack of evidence for symbiotic involvement in speciation events

The PCR screening for *Arsenophonus, Cardinium, Spiroplasma, Rickettsia* and *Wolbachia* did not reveal any signs of infection in the 19 colonies tested (Table 1). However, since this analysis was performed on populations colonized for many generations, this does not necessarily represent the 'actual' symbiotic status of these species in the

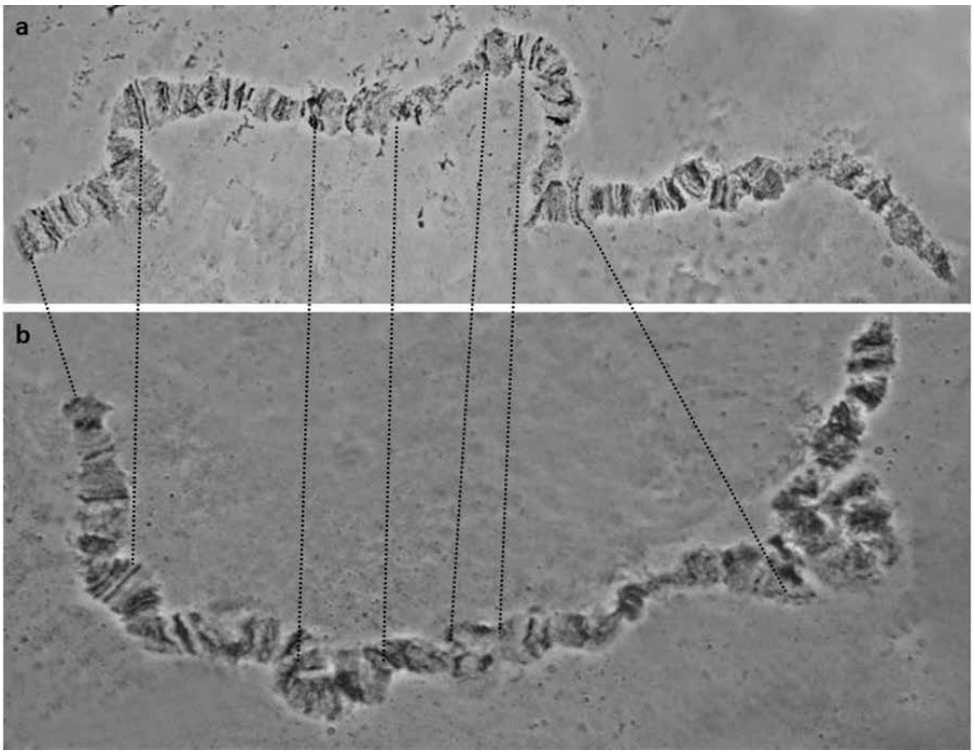

Figure 6. Comparison of the 5L polytene chromosome arms of **a** *B. tryoni* and **b** *B. dorsalis s.s.*. Dot lines connect characteristic landmarks of the two chromosomes.

wild. Colonization might have drastically affected the symbiotic communities of the respective populations. Although there is no evidence for the implication of reproductive symbionts on speciation events between the investigated taxa, symbiotic analysis of wild populations is thus crucial to fully resolve the symbiotic status of these taxa and the *dorsalis* complex in general.

In Tephritidae, only *Wolbachia* has so far been found in a limited number of species, while there are no reports of the presence of the other four symbionts. This can partly be attributed to a lack of comprehensive surveys. Regarding the *B. dorsalis* complex, there are reports for the presence of *Wolbachia* in natural populations (Kittayapong et al. 2000, Jamnongluk et al. 2002, Sun et al. 2007), however only a few populations and at very low frequencies. The first of them (Kittayapong et al. 2000) reports a *Wolbachia* PCR screening of fruit flies of Thailand, collected in the years 1995-1998. Screening was based on the *ftsZ* gene and supergroup-typing on *wsp* sequences. Only 2/222 of the mitotic form A samples and one out of two of the mitotic form K samples were infected. The infection was reported as belonging to supergroup B. Later on, the same research group, using the same samples, suggested the presence of multiple *Wolbachia* infections (Jamnongluk et al. 2002). More recently, a study performed on Chinese populations of *B. dorsalis s.s.* (Sun et al. 2007) revealed very low levels of

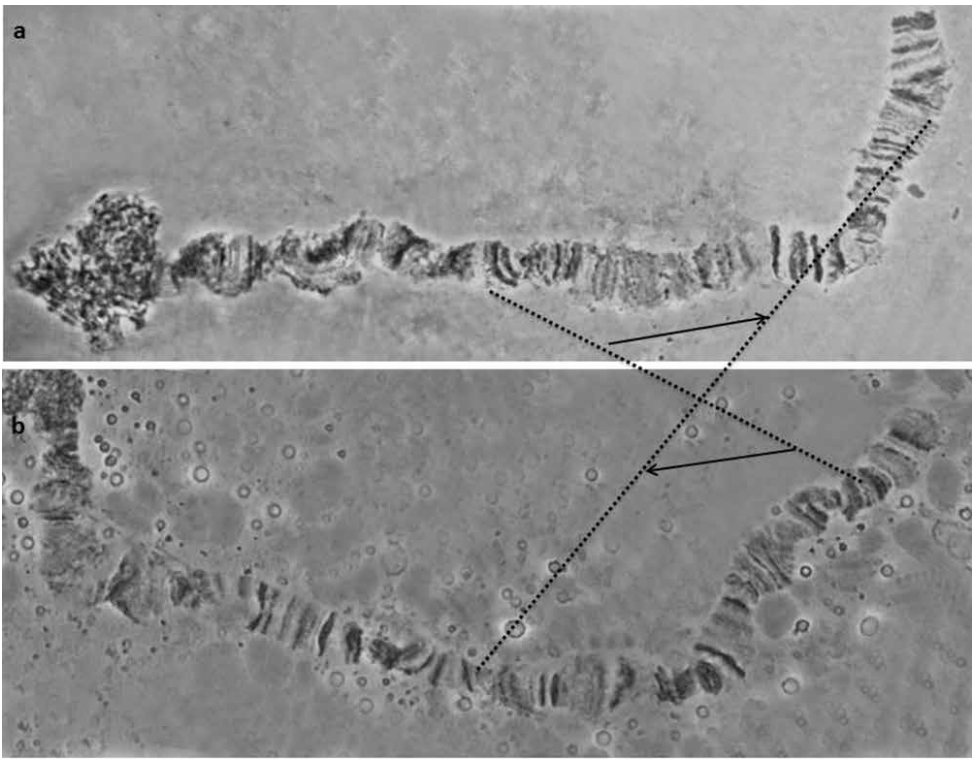

Figure 7. The inverted region on the 2R polytene arm that differentiates *B. tryoni* from the five members of the *B. dorsalis* complex: **a** *B. tryoni* **b** *B. dorsalis s.s.* Dotted lines mark the chromosomal region involved in the inversion while arrows indicate the orientation.

Wolbachia infections (19 positive samples of 1500), belonging either to supergroup A or B, based on *wsp* sequencing. Given the available knowledge at the time of these screens, the specimens tested might have not been properly identified at the species level. In any case, it is highly unlikely that at such low frequencies *Wolbachia* infection could trigger or support a speciation event.

Conclusion

CRs are a well-known indicator of speciation in Diptera, while symbionts obtain only during the last years more recognition as putative speciation factors. Analysing possible paths of speciation with multidisciplinary approaches (integrative taxonomy) is now acknowledged as the best way to provide robust results in species delimitation (De Queiroz 2007, Schlick-Steiner et al. 2010). Our analysis, focused on five economically important members of the *B. dorsalis* complex currently colonized at the Joint FAO/IAEA IPCL, failed to identify any fixed CRs or specific reproductive symbionts that could have partici-

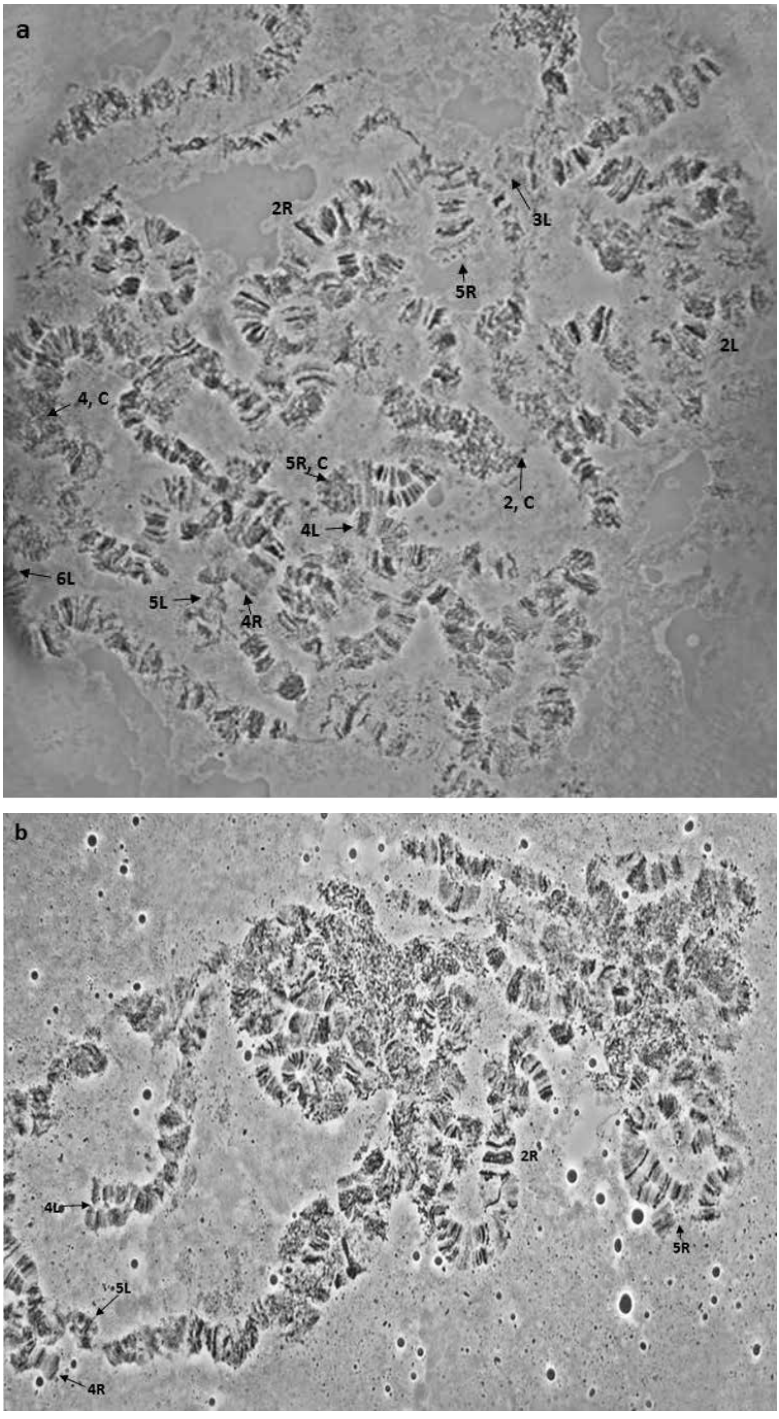

Figure 8. a, b Polytene nuclei derived from the F_1 *B. dorsalis s.s.* x *B. tryoni* hybrids. Chromosome arms are indicated. Tips are marked with arrows and centromeres are indicated with 'C'. Note the overall banding pattern homosequentiallity and the presence of limited asynapses.

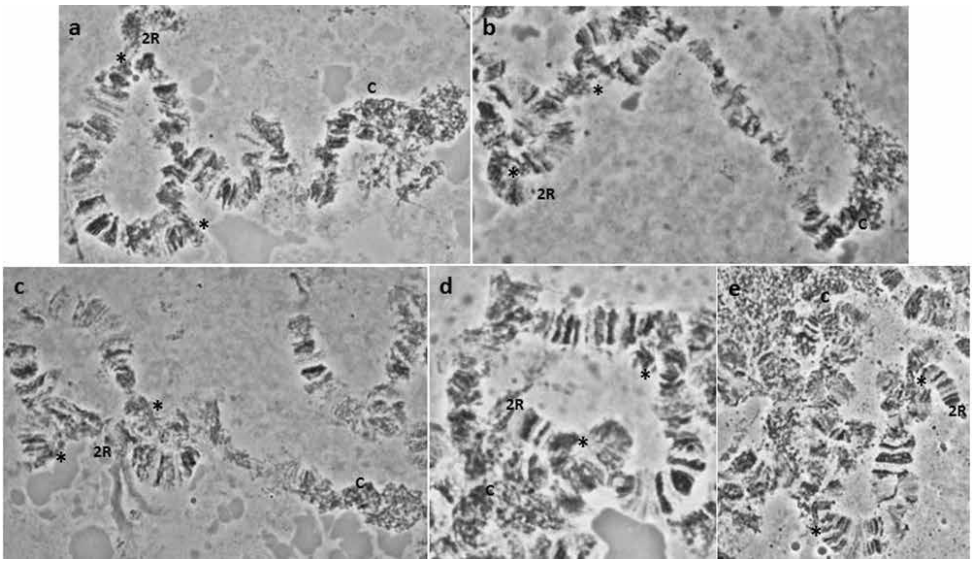

Figure 9. a–e Part of the 2R chromosome arm including the fixed inversion. Photos derived from different polytene chromosome preparations. Asterisks (*) indicate the inversion breakpoints. 'C' indicates the 2R centromere.

pated in the speciation process in the complex. These results are in line with recent data that question the 'actual' number of species within the *B. dorsalis* complex (Krosch et al. 2013, Schutze et al. 2013, San Jose et al. 2013, Boykin et al. 2014) and have led to the recent synonymization proposed by Schutze and colleagues (Schutze et al. 2015). Analysis of species within the complex that are more clearly differentiated from the five taxa under study could shed more light on the speciation process within the *B. dorsalis* complex.

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RESEARCH ARTICLE

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Inter-regional mating compatibility among *Bactrocera dorsalis* populations in Thailand (Diptera,Tephritidae)

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Abstract

Mating compatibility among recently colonized (wildish) populations of *Bactrocera dorsalis* (Hendel) from different geographic origins in Thailand was assessed through inter-regional mating tests. Outdoor octagonal nylon screen field cages containing single potted mango trees (*Mangifera indica* L.) were used. Sexual compatibility was determined using the index of sexual isolation (ISI), the male relative performance index (MRPI), and the female relative performance index (FRPI). The ISI values indicated that the northern population of *B. dorsalis* from Chiang Mai province was sexually compatible with the southern population of *B. dorsalis* (previously *B. papayae*) from Nakhon Si Thammarat province. The MRPI values showed that the northern males had a slightly higher tendency to mate than southern males, while the FRPI data reflected that females of both origins participated equally in matings. In all combinations there were no differences between homotypic and heterotypic couples in mating latency. Southern males tended to mate first with southern females, followed by northern males mating with northern females, while the latest matings involved heterotypic couples, in particular northern males mating with southern females. Overall, more couples were collected from higher parts of the field cage and the upper tree canopy, while there were no differences between the origins of flies in terms of elevation of couples within the cage.

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Laboratory assessments of fecundity showed no differences in the average number of eggs resulting from inter-regional crosses. Development of immature stages was also equal in the two hybrid crosses, with no differences found in the number of pupae produced, percentage pupal recovery, and percent adult emergence. The practical implication of this study is that colony of *B. dorsalis* derived from any northern or southern region of Thailand can potentially be used in sterile insect technique programs against this pest.

Keywords
Populations, different geographic origins, mating compatibility, field cages, sterile insect technique

Introduction

Polyphagous fruit fly species (Diptera: Tephritidae) are considered major threats to many countries as a result of their pest status, widespread distribution, invasive ability and potential impact on market access (Stephens et al. 2007). These flies infest a broad range of host plants including fruits and vegetables wherever they occur. In south-east Asia, most pest fruit flies belong to the genus *Bactrocera* Macquart, a very large genus of well over 500 species. Several of the most serious *Bactrocera* pest species are indigenous to Thailand and peninsular Malaysia, and amongst these the most important is *Bactrocera dorsalis* (Hendel) (Clarke et al. 2001, 2005).

Following the taxonomic revision of Drew and Hancock (1994), *Bactrocera dorsalis* was considered to occur in a broad swath across much of Asia, from the Indian subcontinent and Andaman Islands to southern China, Taiwan, and southeast Asia, extending southwards to central/southern Thailand as far south as the Isthmus of Kra on the Thai/Malay Peninsula, which Drew and Hancock considered its southern limit. *Bactrocera papayae* (Drew & Hancock) was considered a new species in 1994 separate from *B. dorsalis s.s.* based on subtle morphological characters and identification relied heavily on their respective geographical distributions to discriminate among them (Drew and Hancock 1994). The distribution of *B. papayae* began at the Isthmus of Kra and extended south to southern Thailand, Malaysia, Singapore, Kalimantan and eastward into the Indonesian archipelago, the large island groups of Sumatra, Java, and Borneo (Drew and Hancock 1994, CABI/EPPO 1998, Clarke et al. 2005, Steck 2007, David and Ramani 2011, Plant Health). The geographical ranges of the two taxa were thought to abut or overlap on or around the Isthmus of Kra, a recognized biogeographic barrier located on the narrowest portion of the Thai peninsula (Krosch et al. 2013). However, due to the recent synonymization of *B. invadens, B. papayae,* and *B. philippinensis* with *B. dorsalis* by Schutze et al. (2015a), the distribution of *B. dorsalis* now extends throughout much of sub-Saharan Africa, across the Indian subcontinent to the southeast Asian Indo/Malay Archipelago, and as far east as New Guinea and the Philippines.

In Thailand, *B. dorsalis,* as defined by Drew and Hancock (1994), was trapped in northern and central parts of the country. It was most abundant in the far north with a unimodal population peak, building up from the start of the monsoon season and

peaking around June, while September through January was a distinct low period. *Bactrocera papayae* was restricted to southern Thailand, again with a unimodal population peak, with the peak late in the monsoon season (August/September) and dropping off during the dry season (Clarke et al. 2001). *Bactrocera papayae* was considered the most abundant species in different agro-forested locations and in guava, *Psidium guajava* L., orchards in southern Thailand (Danjuma et al. 2013). Recently, fruit fly records in Thailand have been changed following the synonymization of *B. papayae* with *B. dorsalis* (Schutze et al. 2015a): it is now recognized that *B. dorsalis* occurs in all parts of Thailand.

While Schutze et al. (2015a) have synonymized *B. papayae* with *B. dorsalis*, as Thai based agricultural researchers we considered it important to carry out local work on diversity in *B. dorsalis* populations so as to inform local research and policy decisions. Mating compatibility studies among populations of *B. dorsalis* from northern Thailand and populations from southern Thailand (previously *B. papayae*) were needed to assess the sexual compatibility of flies from these different origins. Confirming the compatibility of flies from localities 1,500 km apart would endorse the recent synonymization and allow expansion of pilot SIT campaigns, which are currently applied as part of an integrated area-wide approach, to a wide range of environmental and geographical conditions to suppress this pest in Thailand.

Materials and Methods

Source of flies

Naturally infested fruits from northern Thailand (Chiang Mai province, 19°27'48.5"N; 98°57'50.3"E and 19°23'13.9"N; 98°57'58.9"E) and southern Thailand (Nakhon Si Thammarat province, 8°18'25.8"N; 99°37'50.3"E) were collected from host plants and brought to the fruit fly mass-rearing facility of the Department of Agricultural Extension (DOAE) in Pathumthani province. There they were placed on sawdust in containers to let the larvae mature and pupate. Larvae and pupae were kept at 25±2°C, 80-90% RH and a 12: 12 (L: D) photoperiod. Emerged adult flies were provided a standard diet consisting of enzymatic yeast hydrolysate and sugar (1: 3) with water supplied *ad libitum*. Wild flies at least 14 days old were identified at the laboratory of the Department of Agriculture, Bangkok, using external morphological characters to confirm their identity in accordance with their taxonomic descriptions (Drew and Hancock 1994). The *B. dorsalis* colony from Chiang Mai was obtained from the following fruits: mango (*Mangifera indica* L.), rose apple (*Eugenia javanica* Lam.) and star fruit (*Averrhoa carambola* L.), while the *B. dorsalis* colony from Nakhon Si Thammarat was obtained from Kluai Leb Mu Nang banana (*Musa sapientum* L.) and guava (*Psidium guajava* L.). The identity of flies was further confirmed by using the additional diagnostic tools of pheromone and genetic analyses.

Pheromone analysis

Five individual rectal (= pheromone) glands of individually methyl-eugenol fed and non-fed sexually mature wild males from southern Thailand, identified as *B. dorsalis* using morphological characters, were dissected out and stored in 95% alcohol. Samples were sent to the Laboratory of Entomology and Chemical Ecology, Department of Biology, Faculty of Science, Universiti Putra Malaysia for pheromone analysis. Samples were prepared using sample homogenization and solvent concentration under nitrogen before individual glands were transferred to conical glass vials for GC-MS analyses. The methyl-eugenol metabolites: 2-allyl-4, 5-dimethoxyphenol (DMP) and (*E*)-coniferyl alcohol (CF), were detected in all samples that were fed with methyl-eugenol. No samples were found to have the endogenous compounds present in *B. carambolae* males such as (3-methylbutyl) acetamide, ethyl benzoate, benzamide, 6-oxo-1-nonanol and 1, 6-nonanediol. These results confirmed that the southern colony of *B. dorsalis* (previously *B. papayae*) was not contaminated with *B. carambolae,* which is restricted to southern Thailand (Clarke et al. 2001).

Genetic analysis

The rest of the bodies of the *B. dorsalis* males from southern Thailand from which the rectal glands were removed for pheromone analysis, together with sexually mature complete females, were sent for genetic analysis in groups of five preserved in propylene glycol to the Diagnostics for Biosecurity laboratory at Lincoln University, Christchurch, New Zealand. DNA was extracted using PrepGem, and PCR amplified and sequenced for ITS1 using the PCR primers reported in Boykin et al. (2013). DNA sequences were aligned using Sequencher and a neighbor joining sequence-similarity representation developed with MEGA. Sequences of species-verified reference specimens from both Boykin et al. (2013) (specifically *B. dorsalis s.s.* from Taiwan, the Philippines and Malaysia, and *B. carambolae* from Suriname) and other in-house samples (specifically *B. dorsalis* [ex *B. papayae*] from Malaysia and *B. carambolae* from Suriname and Indonesia, (Mataram, Lombok)) were included in the alignment. The results further confirmed that the colony from the southern population was consistent with *B. dorsalis s.s.*, as per Boykin et al. (2013).

Flies maintenance protocol

Sexually mature *B. dorsalis* from each region were exposed to fresh mature Kluai Nam Wa banana (*Musa sapientum* L.) as an oviposition substrate and larval rearing medium. Bananas with eggs were removed from the rearing cages and placed on sawdust in a ventilated container. Pupae were collected daily and held in 20–25°C room for maturation. This allowed synchronization of development for the sexual compatibility tests.

Eight day-old pupae were manually sifted and transferred to standard quality control Plexiglas cages (30 × 40 × 30 cm) (FAO/IAEA/USDA 2003) with screen mesh windows on two sides and one window on the top for water supply. Standard sugar-yeast diet was provided under low-stress conditions after fly emergence.

Mating compatibility tests

Wildish *B. dorsalis* flies (2nd and 3rd generations) from northern (Chiang Mai) and southern (Nakhon Si Thammarat) Thailand were used for mating compatibility studies. Adult flies were sorted by sex within five days of emergence and virgin flies, once sexually mature at 21 and 23 days of age, were selected for the field cage tests. Based on preliminary studies to assure the sexual maturation of wild flies (Orankanok et al. 2013), flies were provided for mating compatibility test at 23 days of age for fertile wild males and at 21 days of age for fertile wild females. Flies were marked by immobilizing them and placing a small dot of acrylic color on each fly's scutum for both males and females in the early morning of each test day. Colors used were alternated between the two populations. All marked flies for the different replicates were maintained in cylindrical plastic containers, 12.5 cm diameter × 15 cm height, with a triangular mesh on the lid where water-agar and sugar-protein diet was supplied.

Mating compatibility tests between populations from the two regions were performed near the DOAE fruit fly mass-rearing facility. Outdoor octagonal field cages (size 120 cm each side and 220 cm height made from 32 mesh nylon screen), with each cage containing a single potted mango tree (*Mangifera indica* L.) ca. 180 cm in height. These were used since mate choice experiments in large, walk-in field cages containing a host plant have proven useful tools in discriminating among closely related sibling species (Cayol et al. 1999, Petit-Marty et al. 2004, Vera et al. 2006, Orozco et al. 2007, Cáceres et al. 2009, Schutze et al. 2013, Bo et al. 2014) and as reviewed by Juárez et al. (2015); protocols for such trials are now well established and widely applied.

Six replicates of control tests (NxN and SxS) and inter-regional (NxS) mating compatibility test were carried out during December 2012, with the six replicates of the two control combinations completed in one day, and the six replicates of the inter-regional combination completed on another. General procedures followed those outlined in the FAO/IAEA/USDA (2003) manual. As *B. dorsalis* mates at dusk (Arakaki et al. 1984), for each replicate, 20 males from each of the two populations under study were released into the field cage 1 to 2 hours before sunset (16.00-16.30 hrs) and 30 minutes before the females, to give the males enough time to establish territories and form leks (Prokopy and Hendrichs 1979, Shelly and Kaneshiro 1991), followed by the release of 20 females from each of the same two populations (16.30-17.00 hrs). Only healthy marked flies were released; non-active or dead flies were replaced. Temperature, relative humidity and light intensity were recorded immediately after females were released and then every half hour. The formation of copulating pairs was observed continuously, and five minutes after initiation of a mating, the

mating pairs were collected into small vials. For each mating couple, the following data were recorded: copulation start time, copulation location in cage, temperature, relative humidity, light intensity, male and female color. For position within cage, high elevation was defined as the top of the canopy of the tree or the ceiling of the field cage; low elevation was defined as the mid-lower canopy or the side-lower cage wall. The mated flies were not replaced or released back into the cage after separation (Cayol et al. 1999). Experiments concluded when flies became inactive, which occurred after sunset when light intensity dropped to 0-10 lux (Schutze et al. 2015b).

Data analysis

Sexual compatibility was measured using several indices. The Proportion of Flies Mating (PM) measures the suitability of the flies and the environment for mating and represents the overall mating propensity of the flies: Data are discarded if the proportion of flies mating is less than 20% (FAO/IAEA/USDA 2003). The Index of Sexual Isolation (ISI) takes into account the difference existing between homotypic and heterotypic matings; it ranges from -1 (complete negative assortative mating, that is, all matings are with members of the opposite population) to 0 (complete random mating or equal proportion of the possibilities of mating) to +1 (complete positive assortative mating or total sexual isolation, that is total mating isolation of the two populations and males and females only mated with their respective populations). The Male Relative Performance Index (MRPI) highlights any relative difference between males of both populations in terms of overall mating performance; it ranges from -1 (only males of the reciprocal population mated) to 0 (equal mating performance between males of both populations or males of both populations participated equally in mating) to +1 (only males of one population mated). The Female Relative Performance Index (FRPI) highlights any relative difference between females of both populations in terms of overall mating performance. The range of FRPI is similar to MRPI, but applied to females. The combined application of ISI, MRPI, and FRPI provides a comprehensive measure of mating compatibility, as it demonstrates the degree of isolation between populations or species and the relative participation of the sexes of each population or species (Cayol et al. 1999).

F1 hybrid fitness

Six replications of ten pairs representing both combinations of crosses of flies between *B. dorsalis* from northern (N) and southern (S) Thailand were individually assessed for the number of eggs, pupae and F1 adults produced. Eggs of each cross were collected and observed from day 7 until 90 day-old parents. All daily collected eggs of each cross were counted, introduced into semi-ripe bananas and laid on fine sawdust in separately ventilated containers for larval maturation. Pupae were separated and transferred into the cages. Adults of each cross were examined for successful emergence.

Results

Mating compatibility tests

Six replicates of field cage mating compatibility studies involving northern *B. dorsalis* and southern *B. dorsalis* (ex-*B. papayae*) were completed. The propensity for mating (PM) values was larger than 0.20 in all replicates, indicating that the conditions under which the tests were run were satisfactory. The mean proportion of flies mating in control tests of NN and SS were 0.57 and 0.33, respectively, and 0.38 for the inter-regional tests (NS).

The ISI value of 0.07 illustrates random mating between northern and southern *B. dorsalis* populations. Northern males showed slightly higher effectiveness at obtaining mates than males of southern population (MRPI = 0.21), while females of both origins participated equally in mating (FRPI = 0.05) (Figure 1).

Total numbers of mated pairs for the control populations of *B. dorsalis* were lower for the southern population (SS = 80 pairs) and higher for the northern population (NN = 136 pairs). Total pairs across all combinations and comparisons for either homotypic or heterotypic couples of the inter-regional study were not different (SS = 21; NN = 28; SN = 17; NS = 21).

Couples were found mating between 2,000 lux and 35 lux and at temperatures between 28 - 31°C. There was no difference in mating latency for couple formation in the two control studies (SS = 67.77 minutes; NN = 70.18 minutes). Averaged across all combinations and comparing homotypic or heterotypic couples of the inter-regional study, there were no differences in mating latency (SS = 69.58 minutes, NN = 70.61 minutes, SN = 71.60 minutes, NS = 72.47 minutes).

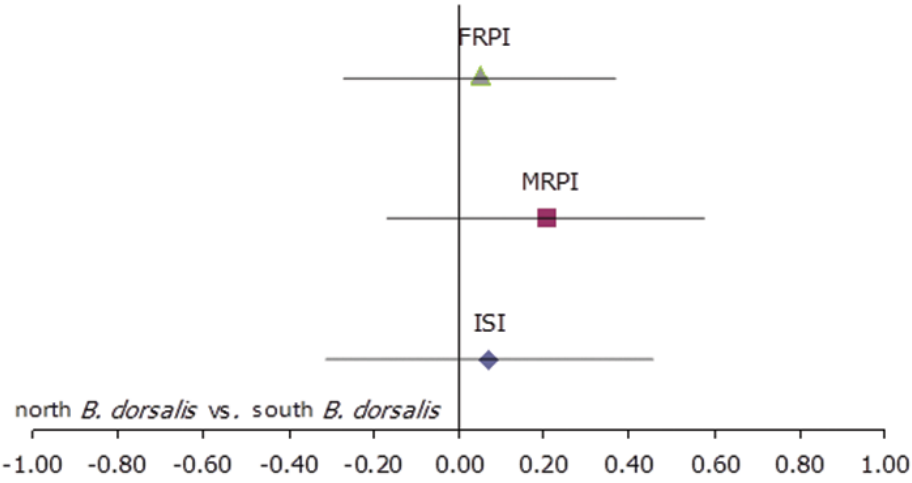

Figure 1. Index of Sexual Isolation (ISI) and Relative Performance Indices for Males (MRPI) and Females (FRPI) for the field cage mating compatibility tests between the two *B. dorsalis* populations from northern and southern Thailand.

Table 1. Mean percentages of pairs of all mating combinations which were collected at two heights within the field cage during mating compatibility tests between populations of *B. dorsalis* from northern and southern Thailand. High height is defined as the upper canopy of the tree or the ceiling of the field cage; low height is defined as the mid-lower canopy or the mid-lower cage wall.

Crosses		Height	
Male	**Female**	**High (%)**	**Low (%)**
Northern			
B. dorsalis	*B. dorsalis*	100.00	0.00
Southern			
B. dorsalis	*B. dorsalis*	97.62	2.38
Northern vs Southern			
(N) *B. dorsalis*	(N) *B. dorsalis*	86.57	13.43
(N) *B. dorsalis*	(S) *B. dorsalis*	86.11	13.89
(S) *B. dorsalis*	(N) *B. dorsalis*	70.83	29.17
(S) *B. dorsalis*	(S) *B. dorsalis*	100.00	0.00

Table 2. Average number of eggs, pupae and adults per female of reciprocal crosses within *B. dorsalis* populations from northern and southern Thailand.

Crosses		Average number of eggs	Average number of pupae	Average percent of pupae recovery	Average percent emergence of adults	
male	**female**				**Complete**	**Abnormal**
(N) *B. dorsalis*	(S) *B. dorsalis*	548.33	119.60	26.33	95.68	4.32
(S) *B. dorsalis*	(N) *B. dorsalis*	435.90	144.40	27.44	95.34	4.66

More couples in controls and inter-regional mating tests involving the two *B. dorsalis* populations were collected from high in the field cage (either from the ceiling of the cage or the upper canopy) relative to lower locations; there were no differences between the two population origins in terms of height of the couples inside the field cage (Table 1).

F1 hybrid fitness

Six replicates of ten pairs for each of the two crosses among *B. dorsalis* from northern and southern Thailand were completed. No differences were found in the mean number of eggs produced, the mean number of pupae produced, percentage pupal recovery (i.e. egg to pupation percentage), and mean percent adult emergence (Table 2).

Discussion

There was no evidence of any pre- or post-mating incompatibility between the *B. dorsalis* populations from northern (Chiang Mai) and southern (Nakhon Si Thammarat) (ex-*B. papayae*) Thailand, despite the populations originating from locations approximately 1,500 km apart. The combined data, using the different indices (ISI, MRPI,

FRPI), provided a complete and reliable picture of sexual compatibility among northern and southern *B. dorsalis* populations. The ISI demonstrated good sexual compatibility between northern and southern populations, indicating that individuals from the northern *B. dorsalis* population mate satisfactorily with those from the southern population. The MRPI indicated a general tendency of wild *B. dorsalis* males from northern Thailand to succeed in mating in slightly greater proportion with northern or southern females compared to southern males. The FRPI showed that northern and southern females are equally receptive in mating with northern or southern males.

Under similar experimental conditions using field cage mating trials, Schutze et al. 2013 also demonstrated random mating among all pair-wise combinations involving *B. dorsalis* and *B. papayae*. At the same time, similar field-cage tests with other fruit flies were able to detect sexual incompatibility among populations of different geographic origin (Vera et al. 2006). All these studies confirm the effectiveness of field cages with trees under semi-natural conditions to evaluate mating compatibility or sexual isolation in cryptic species complexes (Juárez et al. 2015, FAO/IAEA/USDA 2003).

Bactrocera dorsalis mates at dusk and the slightly higher proportion of northern males participating in copulations may be a climatic factor at the time of testing in December, when lower temperatures occur at dusk in central Thailand. A potential causal factor for the earlier time at which the southern populations tended to start mating may be the shorter period of optimum light intensity after sunset. Sunsets occurred approximately 14 and 11 minutes of later in Nakhon Si Thammarat relative to Chiang Mai and Bangkok, respectively, and sunset is three minutes earlier in Chiang Mai relative to Bangkok (based on 2012 sunset data; www.sunrise-and-sunset.com). This *ca*. ten minute difference may have affected the mating latency of flies from the extremes of geographical location compared to central Thailand, so that northern flies were delayed while southern flies were enhanced in their mating activity. According to Schutze et al. (2015b) the slight delay in time of sunset at Nakhon Si Thammarat relative to Bangkok may be sufficient to influence mating latency in flies of early-generation laboratory colonies causing earlier mating compared to flies from northern Thailand. Differences in the onset of mating behavior can be readily manipulated by changes in daily light patterns in other tephritid species (Miyatake et al. 2002).

Our inter-regional sexual compatibility results between *B. dorsalis* and *B. papayae* (that in the meantime has been synonymized with *B. dorsalis*) confirmed the high levels of inter-specific mating compatibility among *B. dorsalis* and *B. papayae* found in different countries (McInnis et al. 1999, Tan 2000, Wee and Tan 2000, Schutze et al. 2013). Also the laboratory assessments of the viability of the offspring of reciprocal crosses confirmed that *B. dorsalis* from southern Thailand (ex-*B. papayae*) and *B. dorsalis* from northern Thailand represent the same biological species. The capability of females of both populations to produce viable eggs with good pupal recovery after the inter-regional crosses means that there are no post-zygotic barriers to hybrid offspring viability. These results confirmed previous findings by Tan (2003), who demonstrated that *B. dorsalis* and *B. papayae* interbreed and produce viable offspring under laboratory conditions. Furthermore, compared to the *B. dorsalis* mass-reared in our production facility, the level of pupal recovery from all crosses was similar and acceptable.

Conclusion

The level of sexual compatibility detected in our study confirms the recent synonymization of *B. papayae* with *B. dorsalis*. It also opens the possibility of using *B. dorsalis* flies from either northern or southern populations in Thailand to initiate colonies for mass-rearing facilities. This will allow expanding pilot SIT campaigns, which are currently applied as part of an integrated area-wide approach, to a wider range of environmental and geographical conditions to suppress diverse populations of this pest in Thailand. Also, the mass-reared *B. dorsalis* flies currently being produced for the ongoing SIT program in Thailand can be used to suppress wild populations of this pest in either northern or southern regions of the country.

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Comparative sensitivity to methyl eugenol of four putative *Bactrocera dorsalis* complex sibling species – further evidence that they belong to one and the same species *B. dorsalis*

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Abstract

Males of certain species belonging to the *Bactrocera dorsalis* complex are strongly attracted to, and readily feed on methyl eugenol (ME), a plant secondary compound that is found in over 480 plant species worldwide. Amongst those species is one of the world's most severe fruit pests the Oriental fruit fly, *B. dorsalis s.s.*, and the former taxonomic species *B. invadens*, *B. papayae* and *B. philippinensis*. The latter species have been recently synonymised with *B. dorsalis* based on their very similar morphology, mating compatibility, molecular genetics and identical sex pheromones following consumption of ME. Previous studies have shown that male fruit fly responsiveness to lures is a unique phenomenon that is dose species-specific, besides showing a close correlation to sexual maturity attainment. This led us to use ME sensitivity as a behavioural parameter to test if *B. dorsalis* and the three former taxonomic species had similar sensitivity towards odours of ME. Using Probit analysis, we estimated the median dose of ME required to elicit species' positive response in 50% of each population tested (ED_{50}). ED_{50} values were compared between *B. dorsalis* and the former species. Our results showed no significant differences between *B. dorsalis s.s.*, and the former *B. invadens*, *B. papayae* and *B. philippinensis* in their response to ME. We consider that the

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Bactrocera males' sensitivity to ME may be a useful behavioural parameter for species delimitation and, in addition to other integrative taxonomic tools used, provides further supportive evidence that the four taxa belong to one and the same biological species, *B. dorsalis*.

Keywords
Bactrocera dorsalis, *B. invadens*, *B. papayae*, *B. philippinensis*, methyl eugenol, male response, lure sensitivity

Introduction

A number of fruit fly species in the *Bactrocera dorsalis* complex are pests of economic importance. The most notorious is the Oriental fruit fly, *B. dorsalis* (Hendel), a widely distributed and invasive species which includes the recently synonymised *B. invadens* Drew, Tsuruta & White, *B. papayae* Drew & Hancock and *B. philippinensis* Drew & Hancock. The presence of this species in the tropics and subtropics has caused significant damage by rendering infested fruits inedible and prohibiting fruit exports due to strict quarantine restrictions. Direct and indirect losses attributed to pestiferous tephritids are believed to be over US$2 billion annually (Malavasi 2014). As a case in point, the 2014 ban on the entry of the popular mango (*Mangifera indica*) cv. 'Alphonso' from India to the EU countries (European Commission 2014) due to detection of Oriental fruit fly, made headlines globally and created an uproar in India, which is the largest mango producer in the world. Although the ban was lifted in January 2015 (European Commission 2015), the duration of the ban had adversely affected the lucrative mango export and livelihood of growers.

Further adding to the global fruit fly problem was the incursion into Africa of suspected Oriental fruit fly in 2003 (Lux et al. 2003), subsequently described as a new species, *B. invadens*, two years later (Drew et al. 2005). The *B. invadens*' African invasion was so serious that within three years of first incursion into Kenya, it had already been found to attack over 50 types of fruits in over 20 countries (Ekesi et al. 2006). Whilst *B. invadens*, *B. dorsalis*, *B. papayae* and *B. philippinensis* were known to appear similar morphologically and genetically, could interbreed and produce viable offspring as well as having identical male sex pheromone components (Tan et al. 2011, 2013, Schutze et al. 2013), it was only recently that all four species were synonymized as *B. dorsalis* (Schutze et al. 2015).

Previous studies have shown that male fruit fly responsiveness to the male lure methyl eugenol (ME) is a unique phenomenon that is dose species-specific, besides showing a close correlation to sexual maturity attainment (Wong et al. 1989, 1991, Wee and Tan 2000a). For example, *B. papayae* and *B. dorsalis* demonstrate similar ranges of sensitivity to ME at nanogramme levels, while *B. carambolae* has been shown to be at least 10 times less sensitive to ME compared to the former two species (Wee et al 2002). This led us to hypothesize that if *B. dorsalis*, *B. invadens*, *B. philippinensis* and *B. papayae* are different names for the one biological species, then their sensitivity to ME could be used as a behavioural parameter to confirm, or refute that assumption,

i.e. we evaluated if the sensitivity of those putative species to ME were significantly different (as might be expected if different biological species), or were the same (as might be expected if they are populations of the same biological species). In this paper, the minimum dose of ME needed to elicit species' positive response in 50% of the population tested (ED_{50}) was determined using Probit analysis and compared between the putative species as a species' delimiting tool.

Methods

Insects

Colonies of adult *B. dorsalis* and the former *B. invadens*, *B. philippinensis* and *B. papayae* were maintained in UPM insectary under strict quarantine. *Bactrocera papayae* were raised from locally collected infested starfruits, *Averrhoa carambola* L.; while *B. dorsalis*, *B. invadens*, *B. philippinensis* were obtained as pupae from the FAO/IAEA Insect Pest Control Laboratory in Seibersdorf, Austria in 2010. Pupae were imported into Malaysia using permits issued by the Director-General of Department of Agriculture Malaysia to AKW Hee. The origins of the Seibersdorf cultures are as follows: *B. dorsalis*, Saraburi, Thailand; *B. invadens,* Kenya; and *B. philippinensis*, Guimaras, the Philippines. All adult flies were provided with water and a mixture of sugar and hydrolysed protein (3:1 w/w) *ad libitum*. The flies were bred under conditions of 25-29°C with 83–90% relative humidity, and a 12 L: 12 D photoperiod. Male flies were separated within three days of emergence (DAE) to prevent mating and were maintained in separate cages (30 cm × 30 cm × 30 cm) until required for bioassay at 19 DAE.

Chemical preparation

Different concentrations of ME (50, 100, 300, 500, 700 and 1000 ng per 5 µl of absolute ethanol, respectively) were prepared by serial dilution from pure ME (>99.8% purity; Merck-Schuchardt, Germany) following preliminary dose-response tests that showed attraction of male flies to ME of between 15–85%, which is in the linear portion of the population response curve (Heong et al. 2013). The use of six dilutions exceeds the minimum of five suggested for studies of this type by Robertson et al. (2007).

Probit regression of male flies' attraction to methyl eugenol

Laboratory bioassays with sexually mature and virgin male flies for their attraction to ME were conducted with slight modifications from the protocol of Wee et al. (2002). Males (30 males per replicate per cage [40 cm × 40 cm × 40 cm]) were acclimatized for 24 h before experimentation, in an isolated indoor area to prevent exposure of ME to

other colonies of flies that were yet to be assayed for ME response. Starting with 50 ng, the lowest concentration, 5 µl of diluted ME was dispensed using a Hamilton® 5 µl syringe onto a filter paper disc (3 cm × 3cm; Whatman® No.1) placed in a disposable Petri dish. During the bioassay, we promptly removed any male that approached the ME spot and attempted to feed. After 5 min, the total number of flies that responded positively was recorded. The same procedure was conducted for new batches of males at higher ME concentrations. There were between 5-9 replicates performed for each dosage and putative species using flies from different cohorts on different days. Absolute ethanol (>99%) was used as a control. All used filter papers and petri dishes were securely disposed in airtight containers to remove traces of ME from the laboratory environment.

The data obtained were pooled and subjected to Probit analyses using the PoloPlus software (LeOra Software 2002) based on Finney (1971). Probit regressions along with ED_{50} (effective median dose – that dose which elicits a response in 50% of the population tested) values were generated. Regression lines fitted to the dose response curves were subjected to a parallelism test using PoloPlus. This is necessary as relative potency, a measure of the species' responses to ME (based on ratios of ED_{50}), can only be valid when those ME dose-response regression lines are found to be parallel (Robertson et al. 2007; Heong et al. 2013).

Results

The male flies displayed typical behaviour in response to ME i.e. immediate zig-zag flying in locating the source of ME upon sensing the lure, followed by compulsive feeding on ME. When offered to the flies, ME attracted all four putative species at all of the tested doses (Figure 1). Approximately 25% of *B. dorsalis* responded to 50 ng of ME, which was the lowest concentration of ME tested and their responses were observed to gradually increase when subjected to higher dosages (Figure 1). The ED_{50} response to ME for *B. dorsalis* males was 268 ng (Table 1). Similar behavioural responses were also observed in the males of the other three putative species. At the lowest dose of 50 ng, ME attracted 18%, 22% and 16% of *B. invadens*, *B. philippinensis* and *B. papayae* males, respectively (Figure 1), with their corresponding ED_{50} values being 222 ng, 256 ng and 247 ng (Table 1). The validity of the ED_{50} values was confirmed with heterogeneity factors of each species' response to ME showing values < 1 (*B. dorsalis*, 0.484; *B. invadens*, 0.714; *B. philippinensis*, 0.502 and *B. papayae*, 0.859), indicating the data fits well with the model of standardized residuals when plotted against the predicted values. The likelihood ratio test of parallelism revealed that the slopes of the regression lines for *B. dorsalis*, *B. invadens*, *B. philippinensis* and *B. papayae* were not significantly different (Figure 2) (p >0.05; λ^2 = 7.27; df = 3). This allowed us to compare the relative potencies of the four species to ME. The attraction of *B. dorsalis*, *B. invadens*, *B. philippinensis* and *B. papayae* males to ME were found to be not significantly different based on their relative potency values which ranged between 0.9 and 1.1(Table 1). In all controls, no males were attracted to filter paper containing absolute ethanol.

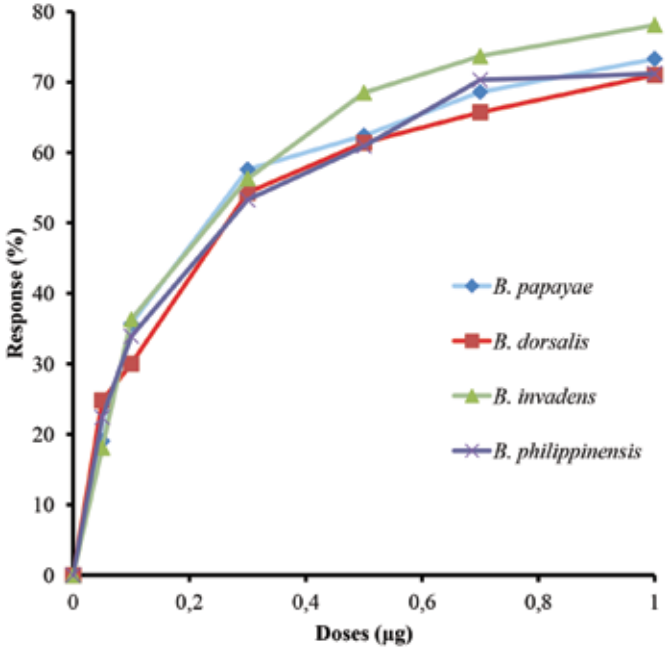

Figure 1. Dose-sensitivity response curves of *B. dorsalis* and the former taxa *B. papayae*, *B. invadens* and *B. philippinensis* to methyl eugenol at different doses.

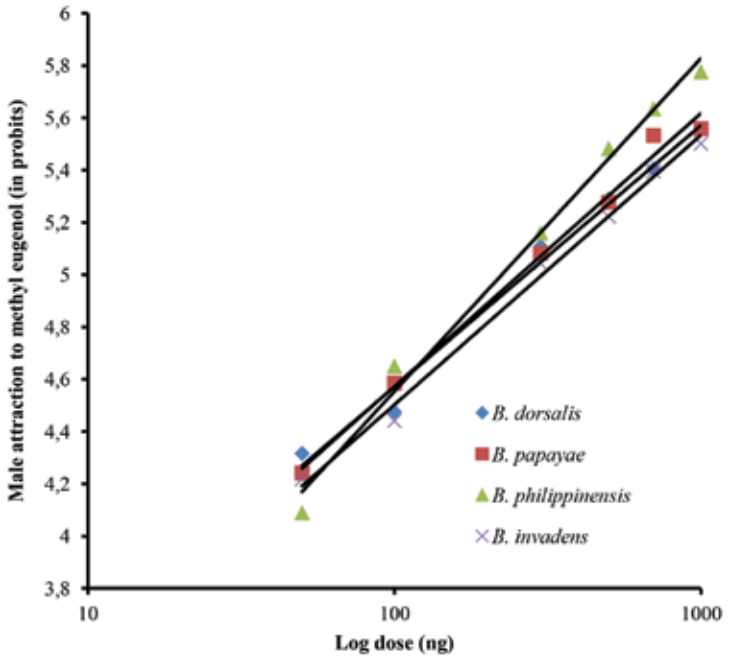

Figure 2. Probit lines of *B. dorsalis* and the former taxa *B. papayae*, *B. philippinensis* and *B. invadens* attraction to methyl eugenol.

Table 1. Probit analysis of male attractancy to methyl eugenol for *Bactrocera dorsalis* and the former taxa *B. papayae*, *B. philippinensis* and *B. invadens*.

Species	*n*[a]	Regression equation	χ^2	*df*	ED_{50}[b] (ng)	95% fiducial limits (ng)		Relative potency
						Lower	Upper	
B. papayae	210	y=1.108x-2.651	3.435	4	247	211	287	1.0
B. philippinensis	330	y=1.043x-2.511	2.008	4	256	224	290	1.0
B. invadens	270	y=1.266x-2.970	2.857	4	222	195	250	0.9
B. dorsalis	210	y=1.002x-2.433	1.936	4	268	226	316	1.1

[a] *n* - number of insects tested per dose

[b] ED_{50} (effective median dose) is the dose required to elicit a positive response in 50% of the fruit fly population tested.

Discussion

Bactrocera dorsalis and the three former species *B. invadens*, *B. philippinensis* and *B. papayae* all showed similar sensitivity to the male lure, ME, with non-significant differences in ED_{50} and potency values. Only marginal difference in the ED_{50} between *B. papayae* and *B. dorsalis* in the current study is in contrast to our earlier work that showed approximately twice the level of ED_{50} of *B. papayae* over *B. dorsalis* (Wee et al. 2002). We believe that the discrepancy in those ratios is attributed to the different geographical strains of *B. dorsalis* used, as the strain that was used by Wee et al. originated from Taichung, Taiwan, whilst the current strain used in this study originated from Saraburi, Thailand. Further, it must be noted that there was also no significant difference in the consumption of ME between *B. dorsalis* and *B. papayae* (Wee et al. 2002). The absence of parallelism test in Wee et al. was due to the fact that the inclusion of *B. carambolae* in that study elicited a very high ED_{50} value (17 and 9 times higher than that of *B. dorsalis* and *B. papayae*) that resulted in the unsuitability of parallelism test of the regression lines for that study.

ME is found naturally in over 480 plant species (Tan and Nishida 2012) and is a pheromone precursor/booster for ME-sensitive *Bactrocera* species (Tan and Nishida 1996, Tan et al. 2014). Synthetic ME is widely used to control pestiferous *Bactrocera* species such as *B. dorsalis* in male annihilation programmes (Steiner et al. 1965, Vargas et al. 2014). However, it was not until the discovery that the responses of male fruit fly species such as *B. dorsalis* and *B. carambolae* to ME was quantifiable (Wee et al 2002), that we have used male ME response as a species' delimitation tool. As an example, the fact that *B. carambolae* is able to interbreed with *B. papayae* and produce viable offspring both in the laboratory and in the wild (Wee and Tan 2000b, 2005, Tan 2003; but see Schutze et al. 2013) suggested that the former might be a sub-species belonging to a single species. However, that male *B. carambolae* possess significantly lower sensitivity to ME compared with that of *B. dorsalis* in cage assays (Wee et al. 2002) further prompted efforts to evaluate the status of *B. carambolae* leading to the recognition that

B. carambolae is currently a distinct, perhaps an incipient, species from that of *B. dorsalis* (Schutze et al. 2015).

An important applied finding of this study, separate to the species delimitation issue, involves the use of ME in the field as a pest control. When ME is applied in the field, the different levels of male attraction to ME between species may impact on chances of male annihilation success. In the case of *B. dorsalis* and the former taxonomic species *B. invadens*, *B. papayae* and *B. philippinensis*, application of male annihilation technique against those flies is not expected to affect the success of the programme given that all four species (now synonymised as *B. dorsalis*) have been proven to have similar sensitivities to ME.

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REVIEW ARTICLE

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Historical perspective on the synonymization of the four major pest species belonging to the *Bactrocera dorsalis* species complex (Diptera, Tephritidae)

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Abstract

An FAO/IAEA-sponsored coordinated research project on integrative taxonomy, involving close to 50 researchers from at least 20 countries, culminated in a significant breakthrough in the recognition that four major pest species, *Bactrocera dorsalis*, *B. philippinensis*, *B. papayae* and *B. invadens,* belong to the same biological species, *B. dorsalis.* The successful conclusion of this initiative is expected to significantly facilitate global agricultural trade, primarily through the lifting of quarantine restrictions that have long affected many countries, especially those in regions such as Asia and Africa that have large potential for fresh fruit and vegetable commodity exports. This work stems from two taxonomic studies: a revision in 1994 that significantly increased the number of described species in the *Bactrocera dorsalis* species complex; and the description in 2005 of *B. invadens,* then newly incursive in Africa. While taxonomically valid species, many biologists considered that these were different names for one biological species. Many disagreements confounded attempts to develop a solution for resolving this taxonomic issue, before the FAO/IAEA project commenced. Crucial to understanding the success of that initiative is an accounting of the historical events and perspectives leading up to the international, multidisciplinary collaborative efforts that successfully achieved the final synonymization. This review highlights the 21 year journey taken to achieve this outcome.

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Keywords
Bactrocera dorsalis species complex, *B. philippinensis*, *B. papayae*, *B. invadens*, synonymization, integrative taxonomy

Introduction

The genus *Bactrocera* Macquart of true fruit flies belongs to the Dacinae - a subfamily of Tephritidae (Drew 1989). Over 500 species have been described as belonging to this genus, making it one of the largest genera within the Tephritidae (Drew 1989). It has been estimated that total damage caused by tephritid fruit flies affecting production, harvesting, packing, and marketing of fruits globally amounts to over US$2 billion annually (Shelly et al. 2014).

Within *Bactrocera*, the *B. dorsalis* species complex contains almost 100 species that are morphologically similar and occur in the tropics and subtropics where fruit growing areas are extensive (Drew and Romig 2013). A number of species in this complex are of economic importance and highly invasive, the best known of which is the Oriental fruit fly, *Bactrocera dorsalis* (Hendel). Closely linked in pest status with *B. dorsalis* are the now synonymized species *B. papayae* Drew & Hancock, *B. philippinensis* Drew & Hancock and *Bactrocera invadens* Drew, Tsuruta & White. *Bactrocera dorsalis* is known to cause devastating losses in fruit commodities, especially in the Asia-Pacific and Africa regions (Kawasaki et al. 1991, Ye 2001, Verghese et al. 2004, Clarke et al 2005, De Meyer et al. 2010, Khamis et al. 2012, Li et al. 2012).

The existence of a complex of closely related, morphologically similar species to *B. dorsalis*, had been recognized for over 40 years (Hardy and Adachi 1954) before a major taxonomic revision of the group was undertaken by Drew and Hancock (1994). This revision described over 50 new species in the complex and, while the biological validity of most described species has not been questioned, this has not been the case for all taxa, particularly some of the very important pest species such as *B. papayae*. For these pest species, serious questions have been raised as to the validity over their status as separate species. This issue was accentuated when Drew et al. (2005) described *B. invadens* from Africa, a new species which again could not be consistently and readily separated from *B. dorsalis*.

The extensive similarities between *B. dorsalis s.s* and the three putative species established, *B. invadens, B. papayae* and *B. philippinensi*s, has led to much debate on the delimitation of these species, particularly in terms of seeking congruence between the biological and taxonomic status of these entities (Tan 2003, Clarke et al. 2005, Drew et al. 2008). Numerous studies over the past two decades have contributed to our understanding of the pest species in the *B. dorsalis* complex particularly with respect to their morphological and biological attributes. Nonetheless, these studies had not reduced the complexities of the species' status, as evident from a rise in the number of species in the *B. dorsalis* complex to almost 100 in the years since the 1994 revision in (Drew and Romig 2013).

To address and resolve the longstanding issue of species delimitation in the key pest species of the *B. dorsalis* complex, a multidisciplinary approach was adopted by an international team of more than fifty researchers from over twenty different countries. Under the auspices of the Joint FAO/IAEA Division on Nuclear Techniques in Food and Agriculture, a 5-year Coordinated Research Project (CRP) on '*Resolution of Cryptic Species Complexes of Tephritid Pests to Overcome Constraints to SIT Application and International Trade'* was established in 2010. The aim of the project was to define the species limits of pest species complexes within the Tephritidae, with the *B. dorsalis* complex identified as a priority. Studies that had been independently developed in the past, including morphometric, cytogenetic, molecular, behavioural and chemoecological datasets were re-examined, and gaps crucial for answering questions of how taxonomic species could be reconciled as biological species were filled. This project has led to the synonymization of *B. papayae*, *B. philippinensis* and *B. invadens* with *B. dorsalis,* based on the conclusion that there is insufficient evidence to maintain the former three taxa as biological species distinct from *B. dorsalis* (Schutze et al. 2015a).

It is not the aim of this paper to again provide the evidence for the synonymization of the four major pest species, as this has already been provided (Schutze et al. 2015a). Rather, it is our intent here to ensure that the long and arduous journey taken to achieve this outcome is understood. We feel that it is vitally important that the younger generation of fruit fly workers, who though armed with advanced scientific skills and techniques, will appreciate the background and good science conducted from the beginning in resolving this issue of significant transboundary importance for international agricultural trade. Thus, this paper aims to provide a historical account of the events leading to the FAO/IAEA-sponsored international efforts in resolving this prickly issue.

Taxonomic history of *B. dorsalis* complex

Before describing some of the biological insights which led to the questioning of taxonomic validity of these species, this section details the taxonomic history of the taxa of concern.

Bactrocera papayae, *B. carambolae* and *B. philippinensis*

Prior to the taxonomic revision of the *B. dorsalis* complex by Drew and Hancock (1994), the taxa endemic to the southeast Asian region of Malaysia, Indonesia and Thailand were identified as a single species, viz. *Bactrocera* (= *Dacus*) *dorsalis* (Hendel) (Hardy and Adachi 1954, Tan and Lee 1982). A second taxon was subsequently recognized, being referred to before description as Malaysian B (Drew 1991) and 'sp. near *B. dorsalis* (B)' (White and Elson-Harris 1992), before being formally described as *B. papayae* Drew and Hancock (1994). This taxon was given the common name of Asian

papaya fly (Drew 1997), although studies in Malaysia had shown that papaya was not the preferred host of this species, which prefers instead starfruit and banana (Tan and Nishida 1996). The detection of *B. papayae* in northern Queensland, Australia, in 1995 resulted in a successful eradication programme costing over US$32.5 million (Fay et al. 1997, Cantrell et al. 2002).

The concern over the destructive potential of *B. papayae* also underscored the importance of another closely related species, *B. carambolae* Drew & Hancock, which itself had formerly been referred to as Malaysian A (Drew 1991) and sp. near *B. dorsalis* (A) (White and Elson-Harris 1992) and was found together with *B. papayae* in Peninsular Malaysia and southern Thailand (Clarke et al. 2001). Together, *B. papayae* and *B. carambolae* accounted for the most damage to fresh fruits in Malaysia. These species were already known to be morphologically similar and able to interbreed resulting in viable laboratory offspring with hybrid rectal pheromonal compounds, even up to the F_3 generation (Wee 2000). Natural hybrids of both *B. carambolae* and *B. papayae* possessing similar rectal pheromonal compounds to those of laboratory hybrids had also been detected from the field (Wee and Tan 2005).

Additional to 'species near *B. dorsalis* (A) and (B), was a third taxon, designated as 'sp. near *B. dorsalis* (C) (White and Elson-Harris 1992). This population was only known from the Philippines and was subsequently described by Drew and Hancock (1994) as *B. philippinensis*.

Bactrocera invadens

As for the South-east Asian pest species of the *B. dorsalis* complex, confusion also existed for *B. invadens* in Africa, a devastating pest species now widespread in Africa, which has largely displaced other long established pests such as Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Hill and Treblanche 2014). Between its first detection in Kenya, 2003 and subsequent description as a new species (Drew et al. 2005), it was believed to originate from the *B. dorsalis* group, and was most likely *B. dorsalis* s.s. (Lux et al. 2003). Drew et al. (2005) and Drew et al. (2008) treated *B. invadens* as part of the *B. dorsalis* complex, but it was subsequently removed from the complex (Drew and Romig 2013).

Accumulating evidence for synonymisation – 1994 to 2010

Biological insights from chemical ecology

Efforts of fruit fly workers over two decades to resolve the biological species status of *B. papayae*, *B. philippinensis*, *B. invadens* and *B. dorsalis* started with the question from one of us (Keng-Hong Tan) on why males of certain fly species, such as *B. dorsalis,* are so strongly attracted to methyl eugenol (ME). This research effort was partly in

response to a challenge posed by N. Tanaka of the USDA Hawaiian Fruit Flies Investigations Laboratory in 1980 to figure out the role of ME in the biology of male tephritid flies. Whilst ME was first discovered a century ago as attracting male fruit flies (Howlett 1915), it was not until 60 years later that it was shown to be a highly potent attractant for *B. dorsalis* (Metcalf et al. 1975). While known to respond to ME, the question of why male flies are uniquely attracted to ME has been considered as a great mystery of tephritid biology (Cunningham 1989). ME is found as a common phenylpropanoid in numerous species of plants (>450 species in 80 families of plants) (Tan and Nishida 2012); and synthetic ME has been successfully used for monitoring and control programmes, including area-wide and border surveillance, quarantine and male annihilation programmes worldwide against many ME-responding tephritid pests of major economic importance (see reviews in Vargas et al. 2010; Shelly et al. 2014, Tan et al. 2014).

While Tan and colleagues searched for the reason as to why male *Bactrocera* respond to ME, significant insights were also gained as to the likely biological relationships between *B. dorsalis* and *B. papayae* (especially). This section describes the history of that process.

In the early 1980s work was undertaken using ME for field ecological surveys and population dynamics of *Dacus* species. This work demonstrated for the first time that temporary habituation of the male flies to ME is possible (Tan and Lee 1982; Tan 1984, 1985; Tan and Jaal 1986; Tan et al. 1987; Tan and Serit 1988). Following that and concomitant with continued ecological work involving ME, there was also interest in understanding the fate of consumed ME in relation to fruit fly physiology. Initial work by Keng-Hong Tan showed that, based on thin layer chromatography (TLC) analyses of haemolymph and digestive tissues of ME-fed and ME-deprived male flies, consumed ME was detected in the crop organ only. Furthermore, two saliva spots (with different relative mobilities from ME), that resulted from ME feeding by live sexually mature males (without prior exposure to ME), were detected on the developed-TLC plate only for extracts of ME-fed male rectal gland, crop and haemolymph (least visualized). This suggested the possibility that the rectal (pheromone) gland contained some compounds derived from the crop upon feeding of ME that were carried to the gland by the haemolymph. The transport of the ME-derived compounds to the rectal gland was subsequently confirmed (Hee and Tan 2004, 2005, 2006).

In 1986, the research collaboration of Keng-Hong Tan and Ritsuo Nishida of Kyoto University on the ecological significance of male fruit fly attractants took off after the introduction of Nishida by Professor S. Takahashi also of Kyoto University to Tan. This research collaboration resulted in the detection of the phenylpropanoids, (*E*)-coniferyl alcohol (CF) and 2-allyl-4,5-dimethoxyphenol (DMP), as sex pheromone components of male *B. dorsalis* (samples from Malaysia later known as 'Malaysian B' and then *B. papayae* - see next paragraph) following ME consumption (Nishida et al 1988a, 1988b).

During that time, *B. dorsalis* in Malaysia had been taxonomically split into two sibling species, Malaysian A and Malaysian B in 1991, and subsequently in 1994 de-

scribed as *B. carambolae* and *B. papayae,* respectively. These sibling species, together with *B. dorsalis s.s.,* were morphologically very similar to each other, particularly for male flies, and much work was concentrated on attempts to differentiate these species. In 1994, results from a primary chemotaxonomic diagnostic tool were presented in the 4th International Symposium of Fruit Flies of Economic Importance held in Tampa, Florida, demonstrating that CF and DMP were detected in both *B. dorsalis* (ex-Hawaii) and *B. papayae,* and with improved male mating competitiveness, following males' consumption of ME (Tan and Nishida 1996). Both the compounds were also shown to be male attractants, although CF was more attractive than DMP to conspecific females (Khoo et al. 2000). At the point source of highest concentration of these chemicals, females were observed to extrude their ovipositor, a sign of mating acceptance, confirming their role in mating behavior (Tan and Nishida 1996, Hee and Tan 1998). This information revealed the role of ME as a precursor to the ME-derived male sex pheromone components, which might be the main reason for the high mating percentages observed between the two sibling species in an outdoor cage (Tan and Nishida 1996).

The discovery that both *B. dorsalis* and *B. papayae,* having very similar morphological characters, also possessed identical pheromone components and mated readily under semi-natural conditions and producing viable offspring over generations provided the first strong evidence that the two species deserved to be considered as a single biological species. These are the basic criteria for species delimitation of Mayr's 'Biological Species Concept' (Mayr 1957).

Biochemical and genetic analyses

Following the revision of the *B. dorsalis* complex in 1994, differences in male genitalia length was used as the sole morphological basis of separating *B. papayae* from *B. dorsalis* (Drew and Hancock 1994; but see also Iwahashi 2001). Isozyme and other genetic evaluation of the two species were also conducted in an effort to provide evidence for species discrimination. However, while a discrimination was deemed possible, both species were shown to possess close genetic affinity (Yong 1994), including the lack of species-specific alleles and loci (Yong 1995). In general isozymes are now known to not be suitable markers to differentiate species due to factors such as hidden genetic variation (Behura 2006).

The development of DNA-based markers meant that a broader range of molecular tools were available to researchers in clarifying the status of species. Using ribosomal DNA markers, for example, had shown in tephritids that it was possible to differentiate distinct species within and between genera of flies, but not within a complex of closely related species (Armstrong and Cameron 2000). For example within the *B. dorsalis* complex, no ribosomal markers in this region could be found to distinguish between *B. dorsalis, B. papayae* and *B. philippinensis*. In addition, another

investigation that included an additional sympatric sibling species, *B. carambolae*, showed that this species also could not be discriminated by phylogenetic analyses using mitochondrial sequences (Muraji and Nakahara 2001). In this paper, the authors noted "*Although the monophylies of most of the species were supported by both topologies and bootstrap analyses, the two individuals of B. papayae did not form a clade for a species. B. papayae and its allied insects were formerly considered as a single species, B. dorsalis (Hendel)*". The authors further stated "*Thus, B. papayae samples used in this study may be hybrids or interbreeding descendants, which might explain why the nucleotide sequences did not match the species identification based on morphological characteristics*". In addition, Smith et al. (2003) also showed that of the four sibling species in the *B. dorsalis* complex that included *B. carambolae* and *B. caryeae*, both *B. dorsalis* and *B. papayae* belonged to the same clade within the 24 *Bactrocera* species analysed. Based on a phylogenetic species theory, this suggested that *B. dorsalis* and *B. papayae* were not distinct species.

Additional molecular work on species in the *B. dorsalis* complex began with the analysis of the actin gene family in *B. dorsalis* (He and Haymer 1994). Specifically, intron sequences from these genes were used for the analysis of genetic variation in populations of *B. dorsalis* and its sibling species in the *B. dorsalis* complex (He and Haymer 1997, 2003). Introns were used primarily because, as noncoding sequences, they were typically known to be much more variable than conserved coding sequences. Because of this, it was presumed that intron sequences could serve to identify genetic markers useful for differentiating species and populations. The variable intron markers identified were also used to construct oligonucleotide arrays for rapid screening of genetic variation in populations of *B. dorsalis*, *B. papayae* and *B. carambolae* (Naeole and Haymer 2003). In this work, Naeole and Haymer showed that one of three actin alleles were identical in DNA sequence and therefore shared between *B. dorsalis* and *B. papayae*. They speculated that this result was more consistent with these taxa representing populations of the same species, rather than being distinct species. Further discussions on this possibility, as reported by Tan (2003), allowed for the beginnings of a comprehensive picture to emerge incorporating support for this idea from a wide range of datasets.

Following this, samples of *Bactrocera* flies were sent to Alfred Handler (USDA, Gainesville, Florida) from Keng-Hong Tan. The analysis of insertions of the *piggyBac* transposable element in these specimens revealed that the gene was inserted at identical loci in both *B. dorsalis* and *B. papayae*, but at different loci in other clearly distinct species (Handler et al. 2008). During the 9th Exotic Fruit Fly Symposium in Fresno, California in 2007, Handler confirmed to Tan (personal communication) that this result would be expected to occur only with extremely closely related taxa, such as between different strains of the same species that can easily interbreed and produce viable offspring, as opposed to entities representing distinct species. As a matter-of-fact, using this same approach, Zimowska and Handler (2005) had earlier found no significant difference between that of *B. dorsalis* and *B. papayae*.

Absence of post-zygotic reproductive isolation between *B. dorsalis* and *B. papayae*

That *B. dorsalis* and *B. papayae* were not distinct species was consistent with earlier observations that interbreeding between *B. papayae*, *B. carambolae* and *B. dorsalis* results in viable offspring/hybrids (unpublished data c/f Tan 2000). Further, studies in Malaysia showed that mating compatibility between *B. dorsalis*, *B. papayae* and *B. carambolae* increased following males' consumption of ME (Wee and Tan 2000; Tan 2003). In these cases, the production of an identical phenylpropanoid, CF, functioning as a male sex pheromone component in males of those species, was suggested to be a factor in explaining the enhanced interbreeding seen in males that had consumed ME (Tan and Nishida 1996, Tan 2000). Furthermore no post-zygotic isolation was observed in matings between *B. dorsalis* (ex-Hawaii) and *B. papayae*, resulted even in F_3 hybrid offspring in the laboratory (Tan 2003).

While some authors have refuted this line of argument supporting the view that *B. dorsalis* and *B. papayae* are the same species, noting that hybridization between *Bactrocera* species is easy to achieve in laboratory cages even when using species from different subgenera (Cruickshank et al. 2001; Drew et al. 2008), interspecific matings between sibling species of *B. dorsalis*, *B. papayae* and *B. carambolae* have been reported to occur in field (McInnis et al. 1999; Wee and Tan 2000). Furthermore, in field studies, using modified ME-baited clear traps without a toxicant, the occurrence of natural hybridization between the sympatric sibling species, *B. papayae* and *B. carambolae* has been supported by captures of wild hybrids and chemotaxonomy (Wee and Tan 2005).

Persistence in maintaining the validity of *B. dorsalis* and *B. papayae* as distinct species

The failure to discover robust diagnostics markers to separate the species within the complex, and indeed the accumulating evidence that supported the idea that at least *B. dorsalis* and *B. papayae* were populations of the same species, prompted further studies by Drew et al. (2008) using additional morphometric measurements in examining the taxonomic status of a number of species in the *B. dorsalis* complex including *B. dorsalis* and *B. papayae*. In that paper, the authors argued that the accumulated evidence, including data based on morphometrics, pheromones, allozyme and nucleotide studies still supported maintaining these taxa as distinct species, even in light of the information presented in the preceding paragraphs that strongly suggested that at least *B. dorsalis* and *B. papayae* should be considered a single species. Further, whilst a review by Clarke et al. (2005) highlighted the difficulties in using morphological criteria to distinguish between those species in the *B. dorsalis* complex, particularly that of *B. dorsalis* and *B. papayae*, the use of certain genetic diagnostic tools in the discrimination of those two species was then generally accepted. For the evidence at that time, as to why Clarke et al. and Drew et al. argued the species were valid, the reader is directed to those papers. In retrospect, and despite the strengths and weaknesses of the many individual studies and their interpretations, it seems that at the time a fully integrative

taxonomic study might have contributed to resolving this issue by asking whether species that can be separated only morphologically can, in fact, be said to represent valid biological species (Clarke and Schutze 2014).

As soon as the paper by Drew et al. (2008) was published, Tan was alerted by Jorge Hendrichs of the FAO/IAEA of the authors' arguments in support of the idea that *B. dorsalis* and *B. papayae* were in fact distinct biological species. This paper included the statement that "*Although genetic similarities between allopatric populations of B. dorsalis and B. papayae have been documented (Tan, 2003), this does not cast doubt on their species status*". It is believed that the game changer to this issue was the sharp response of Keng-Hong Tan (in a form of a 4-page rebuttal letter sent via email to the journal editor) in relation to this article. In his letter, Tan pointed out that regrettably, the authors of the paper chose to ignore the increasing evidence clearly showing that the two taxa deserved recognition as one biological species. Instead, as the core focus of the article, they insisted that they were valid species based largely on morphological evaluation. The rebuttal letter was also informally sent to fruit fly researchers and members of FAO/IAEA and the International Plant Protection Convention Fruit Fly Technical Panel. Taking cognizance of the facts pointing to both *B. papayae* and *B. dorsalis* as indistinct species, we (Keng-Hong Tan, Suk-Ling Wee and Alvin Kah-Wei Hee) have already been referring to both taxa as that of *B. dorsalis* as early as 2005 in our papers (Hee and Tan 2005, 2006, Wee and Tan 2007), which were several years earlier than the implementation of the FAO/IAEA-sponsored CRP.

Furthermore, it must also be pointed out that at the same time of this fierce debate, evidence was being accumulated showing the devastation caused by another species, *B. invadens* as it invaded and spread rapidly across the continent of Africa in the early 2000s, together with the establishment of quarantine barriers by countries that did not harbour *B. invadens*, even though they harboured *B. dorsalis*.

Bactrocera invadens **as the final impetus for coordinated international action**

The detection of an unknown pest fruit fly in Kenya, 2003, first reported as an unknown species suspected to be of Asian origin and related to the Oriental fruit fly (Lux et al. 2003), struck fear in the hearts of quarantine and trade authorities. Given much was known about the destructive losses caused by the *B. dorsalis* in many countries, the incursion of a species closely related to *B. dorsalis* was of great concern as it could potentially devastate the agricultural and fruit industries that many African countries in the tropics and subtropics depend upon. The incursion was in fact swift and destructive, with over 40 species of fruits of economic importance infested in 30 African countries (Khamis et al. 2012). This had the international fruit fly scientific community, particularly workers in Africa and Europe, scrambling resources to seek more information on the fly's basic ecology, biology and control. Described as a new species, *B. invadens*, in 2005, over 80 refereed papers have been published on this fly since (for a brief overview, see Schutze et al. 2015b).

One major reason for the need to resolve the taxonomic status of this new pest was to confirm or reject the validity of the quarantine barriers which were established following the *B. invadens* incursion between importing countries (for example in Asia) where *B. dorsalis* was endemic and exporting African nations. In addition, an effective fruit fly management programme requires correct identification of the target pest species. In the case of *B. invadens*, while copious amounts of work have been done in understanding its ecology and biology, its true taxonomic status remained confused despite the availability of an array of modern analytical tools to aid in the identification of the species. For example, using *piggyBac* gene insertions, Zimowska and Handler (2005) found that *B. invadens* was indistinguishable from *B. dorsalis s.s.* and *B. papayae*, but this workshop abstract was not taken on to the peer reviewed literature. It must be noted that the results of Zimowska and Handler (2005) were based on the use of fly samples obtained from Tim Holler of USDA-APHIS, just prior to the formal description of these as *B. invadens*. These samples had been collected from two different populations in Tanzania (location in and around Dar Es Salaam) in December 2003 (Handler, personal communication). At the time, much of work focused on morphological evaluation, resulting in much continuing controversy on the status of this taxon as a distinct species. As an example, one of the diagnostic distinguishing characters was the appearance of varying shades of scutum colour from pale- to red-brown and almost black in *B. invadens* (Drew et al. 2005; Drew et al. 2008). The uncertainty over the species' status based on morphological features alone raised fears among some fruit fly workers that deliberation over the identity of *B. invadens* would set the stage for another grueling scientific argument, similar to that which occurred over the status of *B. dorsalis* and *B. papayae*.

The Coordinated Research Project

In conjunction with the alarming spread of *B. invadens* in Africa and doubts cast over its taxonomic status, as well the ongoing failure to resolve the species status of the Asian species, a clear need and opportunity arose for the international fruit fly research community to address the question of species boundaries in the *B. dorsalis* complex, involving not only *B. invadens* but also *B. dorsalis*, *B. papayae*, *B. philippinensis* and *B. carambolae*, all of which rank among the world's most destructive and highly invasive alien pest species. At the request from member states, the Joint FAO/IAEA Division took on an instrumental role in establishing in 2010 a six-year Coordinated Research Project (CRP) on '*Resolution of Cryptic Species Complexes of Tephritid Flies to Overcome Constraints to SIT Application and International Trade*'. While the first meeting of researchers to coordinate the research programme of this CRP took place in Vienna in 2010, it must be noted that it was during the FAO/IAEA consultants meeting in 2009 (that included Jorge Hendrichs and Keng-Hong Tan) that the CRP was designed.

The objectives of the CRP were not only to resolve the species issues within the *B. dorsalis* complex but also of other tephritid genera where close species relationships were

an issue, including *Anastrepha* and *Ceratitis* pest populations or species. Research coordination meetings were held in Vienna, Austria (2010), Brisbane, Australia, (2012), Tucuman, Argentina (2013), and La Reunion, France (2015), where research progress was critically evaluated and follow-up research work-plans developed. A multidisciplinary consensus was finally reached that while there was sufficient evidence confirming the separate species status for *B. carambolae,* the four species *B. dorsalis*, *B. invadens*, *B. papayae* and *B. philippinensis* constituted only a single biological species. This culminated in the synonymization of the later three species with *B. dorsalis* (Schutze et al. 2015a). The large authorship of the paper (49 authors from 15 countries) attests to the commitment, dedication and involvement of the international community to resolving this issue. As this outcome is celebrated, it must be remembered that doing good science through perseverance is not the only prerequisite for success, but strong scientific leadership in adopting the philosophy that asking and pursuing answers to the right questions is of paramount importance. Finally, we acknowledge that more research needs to be conducted to fill gaps in the biological, ecological and evolutionary understanding of other sibling species within the *B. dorsalis* complex, particularly incipient species in the Asia-Pacific region.

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RESEARCH ARTICLE

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A phylogenetic assessment of the polyphyletic nature and intraspecific color polymorphism in the *Bactrocera dorsalis* complex (Diptera, Tephritidae)

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Abstract

The *Bactrocera dorsalis* complex (Tephritidae) comprises 85 species of fruit flies, including five highly destructive polyphagous fruit pests. Despite significant work on a few key pest species within the complex, little has been published on the majority of non-economic species in the complex, other than basic descriptions and illustrations of single specimens regarded as typical representatives. To elucidate the species relationships within the *B. dorsalis* complex, we used 159 sequences from one mitochondrial (*COI*) and two nuclear (*elongation factor-1a* and *period*) genes to construct a phylogeny containing 20 described species from within the complex, four additional species that may be new to science, and 26 other species from *Bactrocera* and its sister genus *Dacus*. The resulting concatenated phylogeny revealed that most of the species placed in the complex appear to be unrelated, emerging across numerous clades. This suggests that they were placed in the *B. dorsalis* complex based on the similarity of convergent characters, which does not appear to be diagnostic. Variations in scutum and abdomen color patterns within each of the non-economic species are presented and demonstrate that distantly-related, cryptic species overlap greatly in traditional morphological color patterns used to separate them in keys. Some of these species may not be distinguishable with confidence by means other than DNA data.

Keywords

Bactrocera, *dorsalis*, intraspecific variation, phylogenetics

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Introduction

Most of the Dacine fruit flies (Tephritidae: Dacini) are in the genera *Bactrocera* (651 described species) and *Dacus* (270 species), with many species (73 *Bactrocera* and 11 *Dacus*) bred from commercial/edible fruit and fleshy vegetables (Vargas et al. 2015). Species of *Bactrocera* thrive in the endemic rainforest habitats of South-East Asia and Australasia, with a high degree of host specialization and a large number of cryptic species (Drew and Hancock 2000, Drew 2004).

Among the pest species, *Bactrocera dorsalis* (Hendel) (= the Oriental fruit fly) is the most destructive and polyphagous species (Vargas et al. 2015), belonging to a large complex of similar-looking species: the *Bactrocera dorsalis* complex (hereafter referred to as the OFF complex). The first reference to the OFF complex was by Hardy (1969), who recognized and provided a key to *B. dorsalis* and 15 other non-economic species. Subsequently, Drew and Hancock (1994) revised the group from South-East Asia, describing 40 new species and splitting *B. dorsalis* into four distinct species, resulting in a total of 52 species, plus 16 species in Australasia (Drew 1989). Among the combination of character states defining the complex, they included a mostly black scutum and abdomen terga III–V with a medial longitudinal band forming a "T-shaped" pattern with the transverse band at base of tergum III, and with variable dark patterns on lateral margins of terga III–V. Currently, 85 species are recognized, taking into account the recent revision (Drew and Romig 2013) and synonymization (Schutze et al. 2014). Six of the species (*B. carambolae* Drew & Hancock, *B. caryeae* (Kapoor), *B. dorsalis*, *B. kandiensis* Drew & Hancock, *B. occipitalis* (Bezzi), and *B. trivialis* (Drew)) in the complex are significant pests of cultivated fruit (Vargas et al. 2015).

While literature abounds on the taxonomy, genetic diversity, biology and management of the economic species (Clarke et al. 2005, Schutze et al. 2014a), very little is known about most of the other species in the OFF complex, other than basic taxonomic descriptions. Identification to species level is challenging for many species, due to uniform appearance and extensive intraspecific morphological variation. Morphological diagnostic tools were developed for the economic species, based of wing morphometrics (Schutze et al. 2012) and ovipositor and aedeagus lengths (Iwaizumi et al. 1997, Drew et al. 2008, Krosch et al. 2013, White 2000). Some of the species, especially *B. dorsalis*, display a broad range of color patterns and length of aedeagus and ovipositor, that have resulted in the description of geographic variants as new species, which were subsequently argued to be conspecific (Schutze et al. 2012, 2014b), and synonymized (Schutze et al. 2014a). The range of color variation in the scutum and abdomen was characterized to some extent for *B. dorsalis* and *B. carambolae* (e.g. Nishida and Vargas 1992, Iwahashi 1999, Drew et al. 2005, Leblanc et al. 2013), but no information has been published for the other 83 species.

Species descriptions and illustrations in published monographs (Drew 1989, Drew and Hancock 1994, Drew and Romig 2013) are based on the most commonly encountered morphological variants, and little information is presented on intraspecific variation. The dichotomous key in Drew and Hancock (1994) is based on these most

common variants, hence difficult to use to identify more atypical specimens. An attempt to account for variation in an interactive CD-ROM key (Lawson et al. 2003) yielded limited success (Clarke et al. 2005). In addition to the described species, there may likely exist cryptic species, hard to distinguish by morphological means, which can be separated with the help of genetic sequencing (e.g. Carew et al. 2011, Dujardin and Kitthawee 2013).

Clarke et al. (2005), when reviewing the data available at the time, stated that phylogenetic studies using limited taxa and genes may not demonstrate the monophyly of the complex. However, recent molecular phylogenies which include the OFF complex have found that most species form a well-defined monophyletic clade (Krosch et al. 2012, Virgilio et al. 2015). However, these studies only included methyl eugenol-attracted species and were limited to six economic species and six, mainly Australian, non-pest species such as *B. cacuminata* (Hering) and *B. opiliae* (Drew and Hardy). An alternate, polyphyletic complex was indicated by a phylogeny based on one mitochondrial and two nuclear genes by San Jose et al. (2013), but sampling was limited.

Our goal was to examine the *B. dorsalis* species complex more broadly than the few frequently targeted pest species. This is accomplished by reporting and analyzing novel molecular and morphological data on 22 non-pest species in the complex, in the context of the main pest species and selected outgroups. These data are used to: (i) determine through phylogenetic analysis if the complex is monophyletic or polyphyletic; (ii) provide diagnostic molecular data for over 25 species for which such data is currently lacking; and (iii) determine the utility of thoracic and abdominal color/pattern variation as species level diagnostic characters.

Materials and methods

Taxa sampling

The molecular phylogenies presented here are based on DNA sequences of 53 specimens collected in Asia, Australia, Oceania, the United States and Africa. These specimens include 47 species of *Bactrocera* belonging to five subgenera (including 24 species from the OFF complex), three species of *Dacus*, and *Ceratitis capitata* (Wiedemann) as the outgroup, (Table 1). In addition, we examined the morphology of thousands of specimens of the economic species and over 1,600 specimens of 22 non-economic species in the OFF complex. Two hundred and thirty seven representatives of these, selected to cover a broad range of color variants, were sequenced for the *COI* gene, as detailed below, to confirm morphological identifications and document intraspecific variation in morphological characters. In addition to examining the color pattern of individual specimens, photographs of the scutum and abdomen were taken, for all the sequenced specimens, and used to compile the variation plates (Figures 2–15). The number of specimens examined and sequenced for individual species are included in the figure captions.

Table 1. Species, lure response, collecting locality and voucher code and GenBank accession number for sequences for the species used in this study.

Species	Lure	Locality	Voucher	GenBank Accessions		
***Bactrocera* (*Bactrocera*)**				*COI*	*EF-1a*	*Period*
Species in *B. dorsalis* complex						
B. bivittata Li & Wang	Methyl eugenol	Laos: Luang Namtha	ms1305	KT594878	KT594827	KT594785
B. cacuminata (Hering)	Methyl eugenol	Australia: NSW, Valery	ms1997	KT594887	KT594822	KT594787
B. carambolae Drew & Hancock	Methyl eugenol	Malaysia: Penang, Teluk Bahang	ms1439	KF184076	KF184222	KF184149
B. dongnaiae Drew and Romig	Cue-lure	Cambodia: Koh Kong	ms1109	KT594897	KT594830	KT594789
B. dorsalis (Hendel) (sensu stricto)	Methyl eugenol	Hawaii: Oahu, Makiki	ms0853	KF184084	KF184230	KF184157
B. dorsalis (Hendel) (*B. invadens*)	Methyl eugenol	Sénégal: Ziguinchor	ms0898	KF184092	KF184238	KF184165
B. dorsalis (Hendel) (*B. papayae*)	Methyl eugenol	Malaysia: Penang, Teluk Bahang	ms1428	KF184067	KF184213	KF184140
B. fuscitibia Drew & Hancock	Cue-lure	Cambodia: Koh Kong	ms1175	KT594899	KT594831	KT594790
B. kanchanaburi Drew & Hancock	Methyl eugenol	Cambodia: Koh Kong	ms1300	KT594905	KT594833	KT594792
B. kohkongiae Leblanc	Cue-lure	Cambodia: Koh Kong	ms1139	KT591145	KT591136	KT591129
B. laithieuiae Drew & Romig	Cue-lure	Cambodia: Koh Kong	ms3762	KT594916	KT594823	KT594793
B. latilineola Drew & Hancock	Methyl eugenol	Cambodia: Koh Kong	ms1114	KT594917	KT594834	KT594794
B. lombokensis Drew & Hancock	Cue-lure	Laos: Luang Namtha	ms1548	KT594922	KT594836	
B. melastomatos Drew & Hancock	Cue-lure	Malaysia: Kedah, Mount Jerai	ms1411	KT594924	KT594837	KT594796
B. occipitalis (Bezzi)	Methyl eugenol	Philippines: Los Baños	ms1985	KT594931	KT594824	KT594798
B. osbeckiae Drew & Hancock	Cue-lure	Cambodia: Koh Kong	ms1163	KT594938	KT594841	KT594801
B. paraarecae Drew & Romig	Methyl eugenol	Laos: Luang Namtha	ms1110	KF184040	KF184186	KF184113
B. propinqua (Hardy & Adachi)	Cue-lure	Laos: Luang Namtha	ms1167	KF184053	KF184199	KF184126
B. quasiinfulata Drew & Romig	Cue-lure	Laos: Luang Namtha	ms1546	KT594970	KT594843	KT594803
B. raiensis Drew & Hancock	Methyl eugenol	Laos: Luang Namtha	ms1331	KT594972	KT594844	KT594804
B. thailandica Drew & Hancock	Cue-lure	Thailand: Chiang Mai	ms1047	KT594985	KT594852	KT594812
B. usitata Drew & Hancock	Cue-lure	Cambodia: Koh Kong	ms1173	KT594999	KT594854	KT594814
B. species 54	Cue-lure	Thailand: Chiang Mai	ms1182	KT594976	KT594847	KT594807
B. species 55	Cue-lure	Laos: Luang Namtha	ms1181	KT594979	KT594848	KT594808
B. species 59	Cue-lure	Laos: Luang Namtha	ms1164	KT594981	KT594849	KT594809

Species	Lure	Locality	Voucher	GenBank Accessions		
B. species 60	Methyl eugenol	China: Jinghong	ms3633	KT594982	KT594850	KT594810
Other species						
B. aethriobasis (Hardy)	Methyl eugenol	Cambodia: Koh Kong	ms1557	KT594862	KT594825	KT594783
B. albistrigata deMeijere	Cue-lure	Malaysia: Penang, Teluk Bahang	ms1395	KT594863	KT594826	KT594784
B. bhutaniae Drew & Romig	Cue-lure	Laos: Luang Namtha	ms1166	KF184052	KF184198	KF184125
B. bryoniae (Tryon)	Cue-lure	Australia: Bundaberg	ms1515	KT594886	KT594828	KT594786
B. correcta (Bezzi)	Methyl eugenol	Cambodia: Koh Kong	ms1093	KT594896	KT594829	KT594788
B. kirki (Froggatt)	Cue-lure	French Polynesia: Tahiti	ms0894	KF184090	KF184236	KF184163
B. latifrons (Hendel)	Latilure/cade oil	Hawaii: Oahu	ms0882	KF184085	KF184231	KF184158
B. limbifera (Bezzi)	Cue-lure	Cambodia: Koh Kong	ms1108	KT594921	KT594835	KT594795
B. nigrotibialis (Perkins)	Cue-lure	Cambodia: Koh Kong	ms1033	KT594930	KT594838	KT594797
B. ochrosiae (Malloch)	Cue-lure	Mariana Islands: Saipan	ms1485	KT594932	KT594839	KT594799
B. paradiospyri Chen, Zhou & Li	Methyl eugenol	Thailand: Chiang Mai	ms1470	KT594956	KT594842	KT594802
B. rubigina (Wang & Zhao)	Cue-lure	China: Jinghong	ms3544	KT594974	KT594845	KT594805
B. tryoni (Froggatt)	Cue-lure	French Polynesia: Tahiti	ms0892	KF184088	KF184234	KF184161
B. tuberculata (Bezzi)	Methyl eugenol	Thailand: Chiang Mai	ms1083	KT594998	KT594853	KT594813
B. umbrosa (Fabricius)	Methyl eugenol	Cambodia: Koh Kong	ms1002	KF184032	KF184178	KF184105
B. wuzhishana Li & Wang	Methyl eugenol	Thailand: Chiang Mai	ms1070	KT595000	KT594855	KT594815
B. zonata (Saunders)	Methyl eugenol	Thailand: Chiang Mai	ms1559	KT595002	KT594857	KT594817
***Bactrocera* (*Daculus*)**						
B. oleae (Gmelin)	No lure	USA: California	ms1387	KT594933	KT594840	KT594800
***Bactrocera* (*Notodacus*)**						
B. xanthodes (Broun)	Methyl Eugenol	French Polynesia: Rurutu	ms0896	KT595001	KT594856	KT594816
***Bactrocera* (*Sinodacus*)**						
B. hochii (Zia)	Cue-lure	Laos: Luang Namtha	ms1369	KT594904	KT594832	KT594791
***Bactrocera* (*Zeugodacus*)**						
B. cucurbitae (Coquillett)	Cue-lure	Cambodia: Koh Kong	ms0987	KF184104	KF184250	KF184177
B. scutellaris (Bezzi)	Cue-lure	Thailand: Chiang Mai	ms1030	KT594975	KT594846	KT594806
B. tau (Walker)	Cue-lure	Laos: Luang Namtha	ms1006	KT594984	KT594851	KT594811

Species	Lure	Locality	Voucher	GenBank Accessions		
Genus *Dacus*						
D. (*Didacus*) *ciliatus* Loew	None	South Africa: Stellenbosch	ms1576	KT595004	KT594859	KT594819
D. (Psilodacus) *pullescens* Munro	None	South Africa: Calitzdorp	ms1578	KT595005	KT594860	KT594820
D. (*Mellesis*) *sinensis* Wang	Cue-lure	Laos: Luang Namtha	ms1372	KT595006	KT594861	KT594821
Genus *Ceratitis*						
C. capitata (Wiedemann)	Trimedlure	Hawaii: Oahu	ms0865	KT595003	KT594858	KT594818

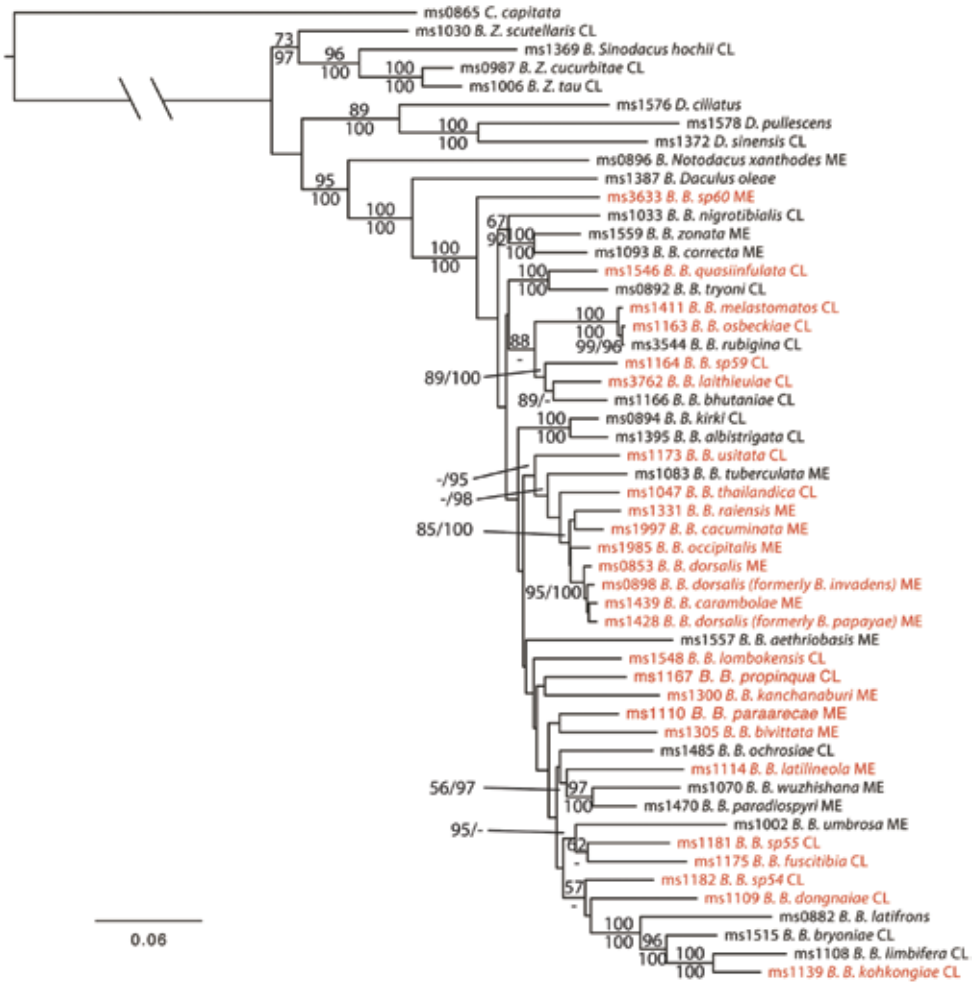

Figure 1. Maximum likelihood tree, concatenated, based three gene (*COI, period, EF-1a*) dataset. Support values above branches are Maximum Likelihood Bootstrap values / Bayesian Posterior Probabilities. Scale bar indicates the number of substitutions per site. Species in the Oriental fruit fly complex are outlined in red.

Validation of identification

Our specimens in the OFF complex were initially tentatively identified to species using available resources (Drew and Hancock 1994, Lawson et al. 2003, Drew and Romig 2013). These determinations were then confirmed by comparing pinned representatives and photographic plates of color variation to the large series of specimens used to produce the above publications, deposited in the Queensland Department of Agriculture and Fisheries (QDAF) insect collection (Ecosciences Precinct, Brisbane). The identifications were also confirmed by R.A.I. Drew, an expert on *Bactrocera* morphol-

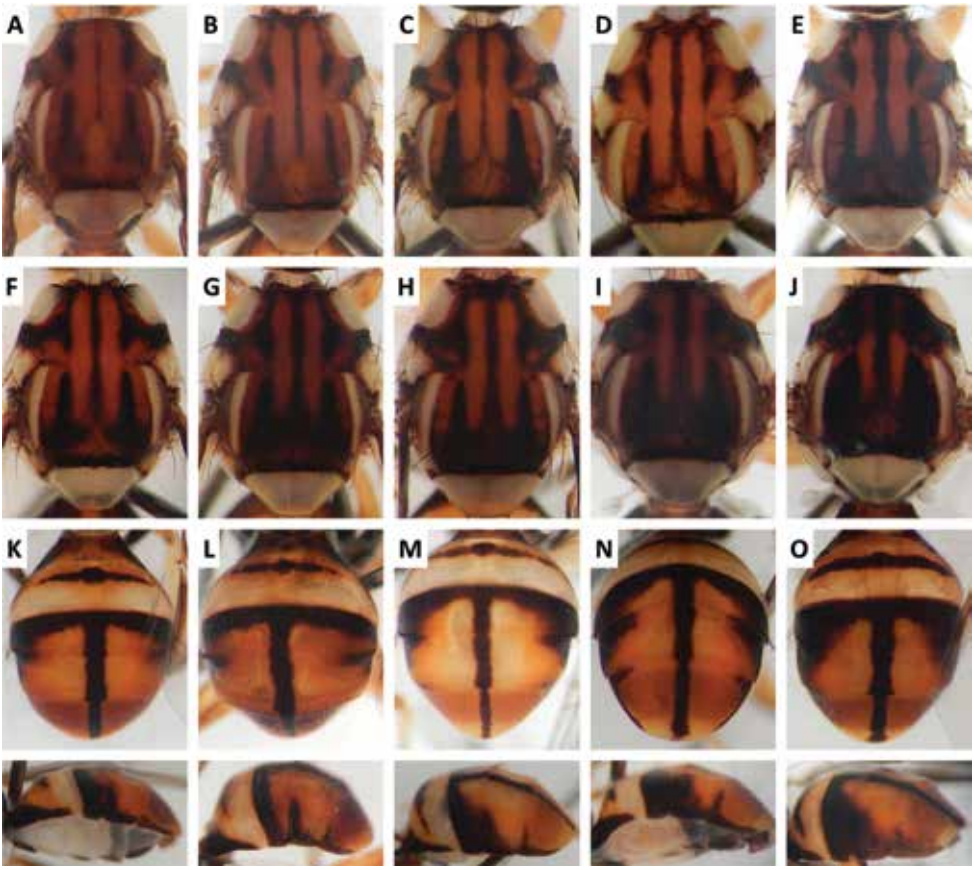

Figure 2. Variation in color pattern of scutum and abdomen in *Bactrocera bhutaniae* Drew and Romig (321 specimens examined and 36 sequenced). Voucher codes are: **A** ms3593 **B** ms3531 **C** ms3533 **D** ms4321 **E** ms2034 **F** ms1166 **G** ms2031 **H** ms3527 **I** ms3580 **J** ms1168 **K** ms2030 **L** ms3578 **M** ms4329 **N** ms3527 **O** ms1168.

ogy. Species referred to by numbers in previous publications (San Jose et al. 2013, Leblanc et al. 2013, 2014) and included in this study were identified as *B. osbeckiae* Drew and Hancock (species 22), *B. bhutaniae* Drew and Romig (species 25), *B. paraarecae* (species 26), and *B. propinqua* (Hardy and Adachi) (species 45).

DNA extraction, amplification, and sequencing

For each specimen, one to three legs were used for total genomic DNA extraction. The remainder of the specimen was deposited as a voucher in the University of Hawaii Insect Museum (UHIM) for preservation and morphological studies (Table 1). Genomic DNA was extracted using the DNeasy animal blood and tissue extraction kit

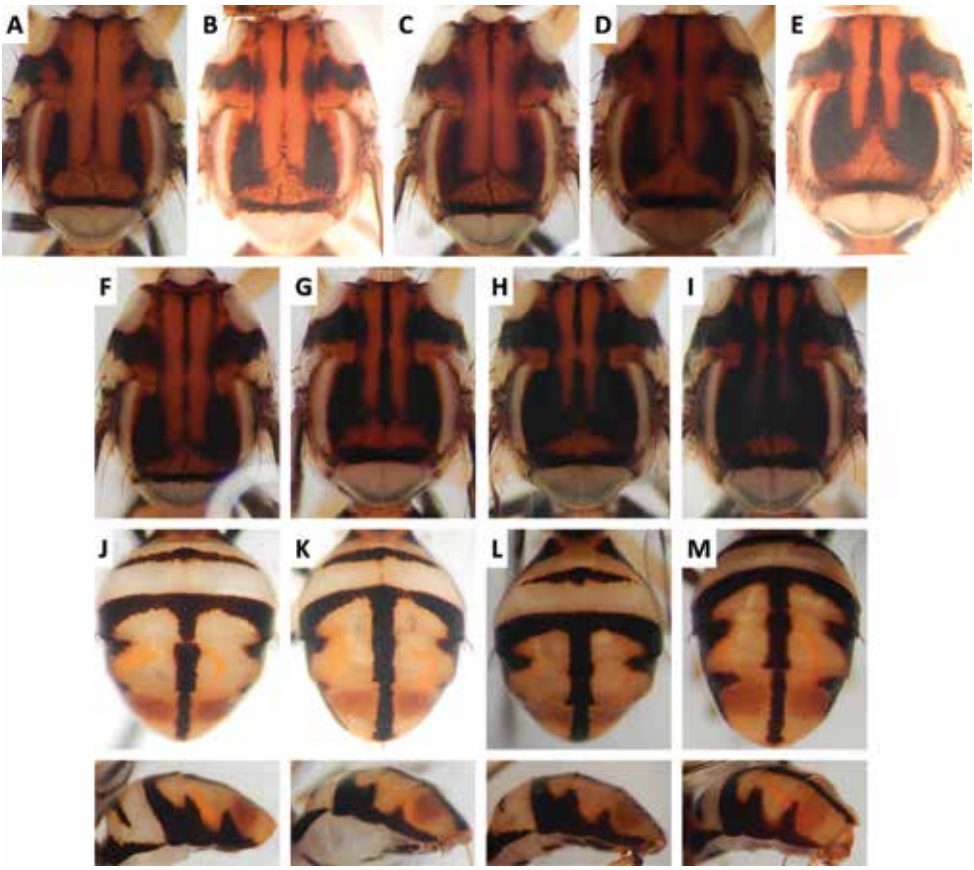

Figure 3. Variation in color pattern of scutum and abdomen in *Bactrocera bivittata* Li and Wang (47 specimens examined and 10 sequenced). Voucher codes are: **A** ms3606 **B** ms1305 **C** ms1304 **D** ms3607 **E** ms3605 **F** ms3604 **G** ms3609 **H** ms3608 **I** ms1790 **J** ms3605 **K** ms3606 **L** ms3609 **M** ms3604.

following manufacturer's protocol (Qiagen, Inc., Valencia, CA). Three different gene regions were amplified: the mitochondrial gene *cytochrome c oxidase I* (*COI*, 780 bp) and the nuclear genes, *elongation factor-1a* (*EF-1a*, 759 bp) and *period* (*PER*, 450 bp). These three genes were selected because each has been demonstrated to be informative in distinguishing populations, species complexes, species, or genera in Diptera (Folmer et al. 1994, Simon et al. 1994, Cho et al. 1995, Bauzer et al. 2002, Moulton and Wiegmann 2004, Barr et al. 2005, Foley et al. 2007, Virgilio et al. 2009, Gibson et al. 2011, San Jose et al. 2013). Gene amplification followed San Jose et al. (2013). All polymerase chain reaction (PCR) products were visualized on 1% agarose gel and purified using QIAquick spin columns (Qiagen, Inc.) according to the manufacturer's protocol. Bidirectional DNA sequencing was performed at the Advanced Studies of Genomics, Proteomics and Bioinformatics (ASGPB) sequencing facility of the University of Hawaii at Manoa (http://asgpb.mhpcc.hawaii.edu/).

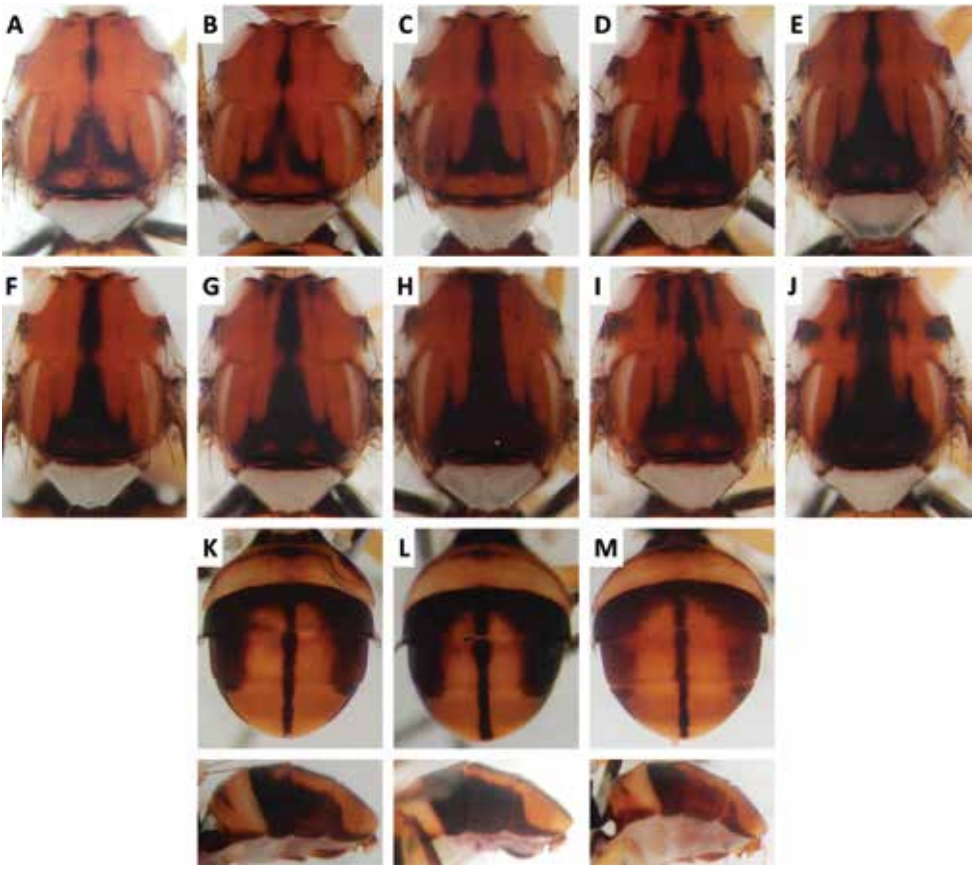

Figure 4. Variation in color pattern of scutum and abdomen in *Bactrocera cacuminata* (Hering) (> 300 specimens examined and 12 sequenced). Voucher codes are: **A** ms2003 **B** ms2005 **C** ms1998 **D** ms2008 **E** ms1999 **F** ms1997 **G** ms2010 **H** ms2004 **I** ms2009 **J** ms2002 **K** ms2005 **L** ms2008 **M** ms2009.

Sequence alignment, nucleotide composition, and phylogenetic analysis

Sequence alignments were performed with the software package Geneious 7.1.7 (Biomatters ltd.). Heterozygosity in the nuclear genes was present in most samples. Ambiguity codes (i.e., notation according to International Union of Pure and Applied Chemistry (IUPAC)) were used to denote heterozygous base pairs, and these codes were used in the subsequent analysis. Sequence alignment for each gene was conducted in Geneious using the Muscle option with default settings (Edgar 2004). We used jModeltest and the Akaike information criterion (Darriba et al. 2012) to determine the most appropriate evolutionary model for each gene in our analysis. Phylogenetic analyses were performed with both Maximum Likelihood and Bayesian Inference. MrBayes 3.2.1 (Ronquist et al. 2012) was used for Bayesian analyses and RaxML (Stamatakis et al. 2008) was used for maximum likelihood (ML). We used jModeltest (Darriba et al. 2012) to determine the most appropriate model for each partition. We concatenated our datasets by gene and used a GTR+ Γ

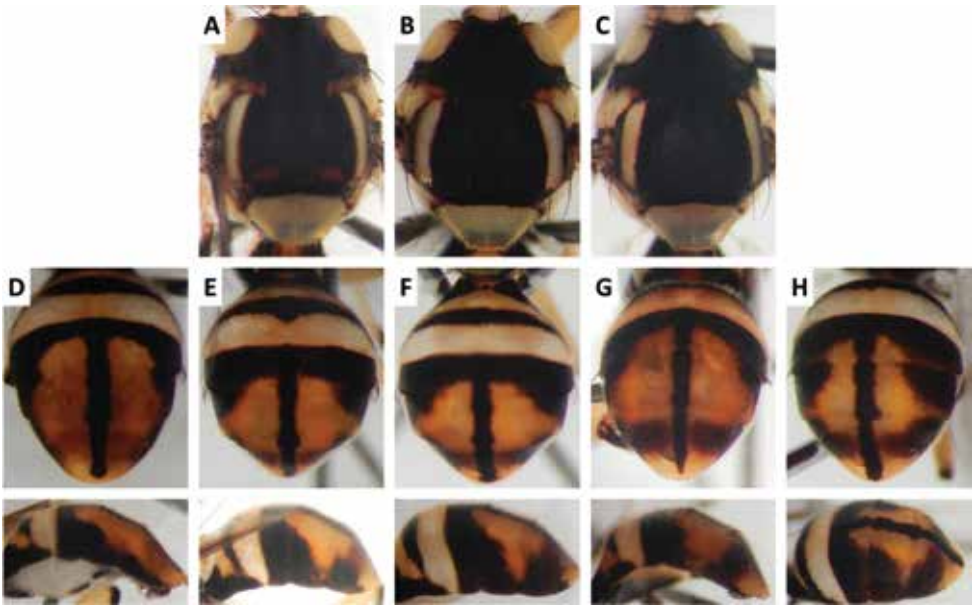

Figure 5. Variation in color pattern of scutum and abdomen in *Bactrocera fuscitibia* (Drew and Hancock) (33 specimens examined and 6 sequenced). Voucher codes are: **A** ms1178 **B** ms1177 **C** ms1297 **D** ms1175 **E** ms1176 **F** ms1177 **G** ms1178 **H** ms1297.

model for each gene in the Bayesian analysis general time reversible model (Tavaré 1986) with gamma distribution of rates (GTRGAMMA) for each gene in our likelihood analysis. We first analyzed each gene separately and subsequently concatenated them into a single dataset partitioned, by gene, using Maximum Likelihood and Bayesian inference. For each individual gene analysis (*COI, period,* and *EF-1α*) we ran four independent Bayesian runs in MrBayes 3.2.1 using the default settings. Each run started from a random tree using default priors sampling every one thousand generation for 10 million generations with a relative burn-in of 25%. We used the program Tracer 1.5 (Rambaut and Drummond 2009) to assess convergence of standard deviation in variance for Bayesian analyses. For RaxML analyses, each dataset included 10 ML tree searches with default settings, using a random starting tree to find the tree with the best likelihood score. One thousand Maximum Likelihood bootstrap replicates were conducted in Raxml to assess support for inferred relationships. For the concatenated dataset, we partitioned the data by gene and ran MrBayes using the same settings as the individual gene analyses except the parameters statefreq, revmat, shape, and pinvar were unlinked between partitions. For the Maximum Likelihood analysis of the partitioned concatenated dataset, we ran RaxML using the same settings and analyses for each partition as when genes were analyzed individually. Trees were visualized using FigTree v1.4.0 (Rambaut 2012) and rooted with *Ceratitis capitata*. *COI* sequences for all non-economic species in the *B. dorsalis* complex for which at least four sequences were available were analyzed using the program DNAsp to provide basic population genetic variability summary statistics (*Hn, h π, S*).

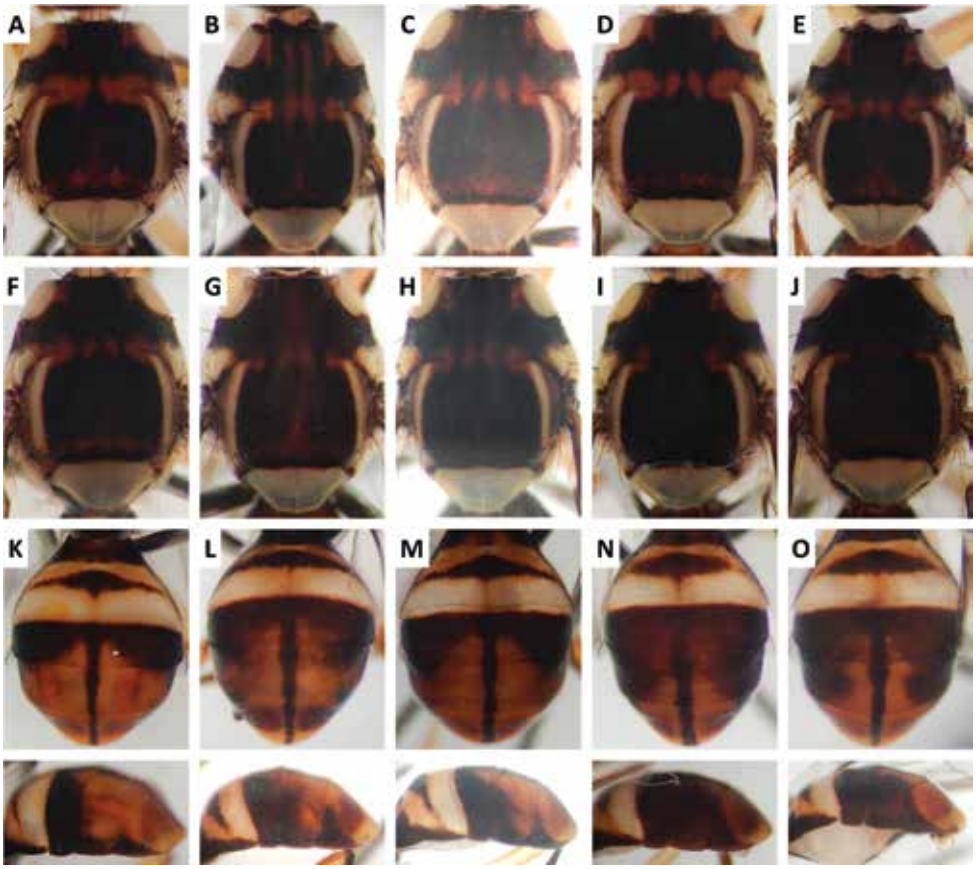

Figure 6. Variation in color pattern of scutum and abdomen in *Bactrocera kanchanaburi* Drew and Hancock (47 specimens examined and 16 sequenced). Voucher codes are: **A** ms3599 **B** ms1300 **C** ms3598 **D** ms1303 **E** ms3725 **F** ms1302 **G** ms3597 **H** ms3596 **I** ms3728 **J** ms3603 **K** ms3599 **L** ms3728 **M** ms1300 **N** ms1301 **O** ms3729.

Data Resources

Sequences listed on Table 1, as well as COI sequences for all specimens included on all figure plates, were deposited into GenBank KT591129 to KT591164 and KT594783 to KT595006.

Results

Topological differences between the individual gene trees were not supported with high bootstrap values and posterior probabilities (<50% BS <0.9 PP) and overall individual gene trees were poorly resolved, with *COI* providing more signal for the more recent divergences (Suppl. material 1) and the nuclear genes providing signal for deep-

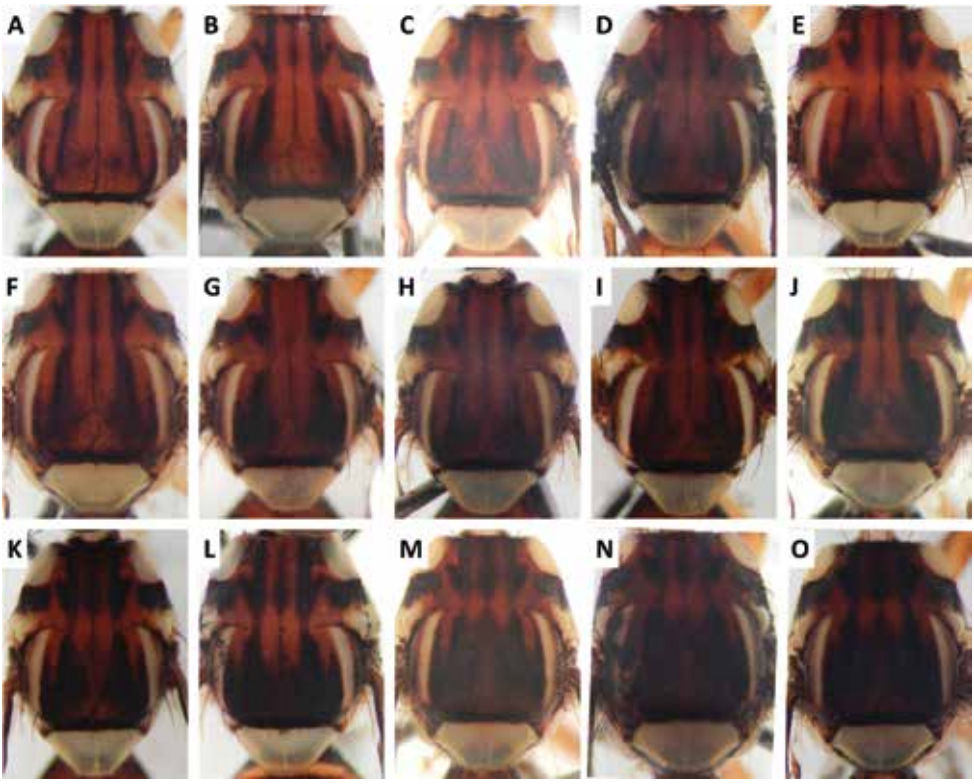

Figure 7. Variation in color pattern of scutum in *Bactrocera kohkongiae* Leblanc (210 specimens examined and 22 sequenced). Voucher codes are: **A** ms1149 **B** ms1144 **C** ms1142 **D** ms1780 **E** ms1148 **F** ms1145 **G** ms1307 **H** ms1143 **I** ms1141 **J** ms1146 **K** ms1151 **L** ms1785 **M** ms1140 **N** ms1781 **O** ms1150.

er relationships (Suppl. material 2, 3). However the concatenated analysis produced a well-resolved tree (Figure 1) which is consistent with previous studies (Krosch et al. 2012, San Jose et al. 2013, Virgilio et al. 2015). In the concatenated phylogeny, the *Zeugodacus* group of subgenera (as defined by Drew and Hancock 2000) is sister to *Dacus* and the *Bactrocera*+*Notodacus*+*Daculus* clades, which themselves are sister taxa. This renders *Bactrocera* paraphyletic with respect to *Dacus*, as suggested previously (White 2006, Krosch et al. 2012, Virgilio et al. 2015). However the relationship is not strongly supported in the tree and additional genes and taxa are necessary to fully resolve this relationship. The subgenus *Bactrocera* is monophyletic in the concatenated phylogeny (100 BS, 1.0 PP). The inclusion of many non-economic OFF complex species in our study shows with high support that despite a similar appearance, the complex is a highly polyphyletic group. Multiple, well-supported, clades (75–100% BS values) in the subgenus *Bactrocera* contain a mix of species previously thought to belong to the OFF complex and non-OFF complex species. One clear example is the inclusion of non-OFF complex *B. bryoniae*, *B. latifrons*, *B. limbifera*, with *B. kohkon-*

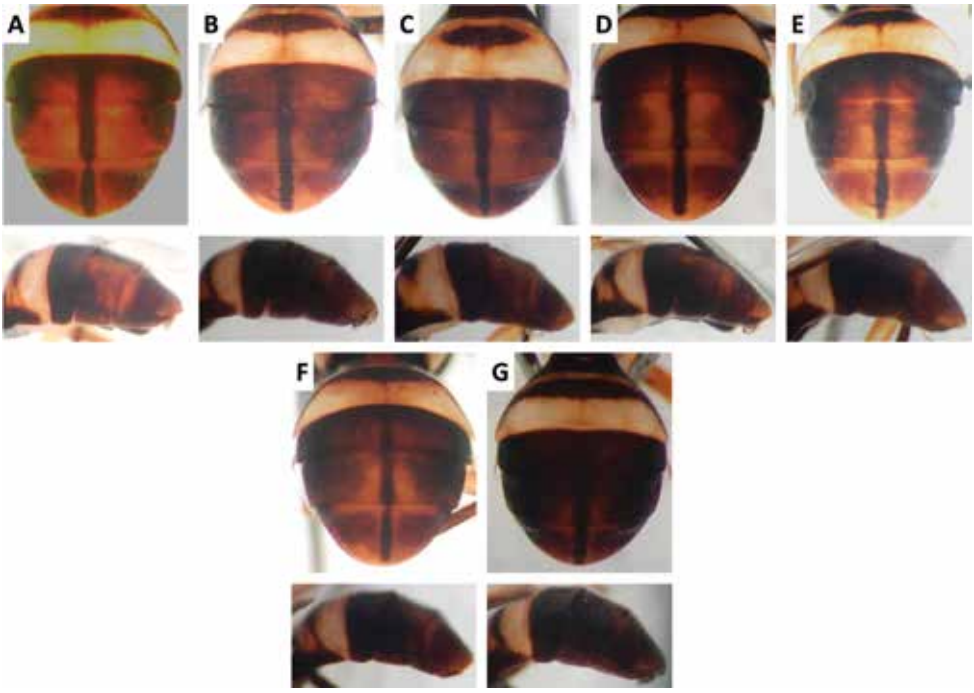

Figure 8. Variation in color pattern of abdomen in *Bactrocera kohkongiae* Leblanc. Voucher codes are: **A** ms1149 **B** ms1147 **C** ms1145 **D** ms1785 **E** ms1146 **F** ms1139 **G** ms1137.

giae, which fits in the OFF complex (Figure 15 A–C) in a strongly supported (100%, 100% PP value) clade (Figure 1). This indicates that, despite low support for the backbone topology in the subgenus *Bactrocera,* the polyphyletic nature of the OFF complex is still well supported. The main pest species in the complex (*B. carambolae* and *B. dorsalis*, now including *B. papayae* and *B. invadens*, see Schutze et al. 2014a) form a monophyletic unit with very little genetic differentiation (<1.3% in *COI*) between them, and rest within a well defined clade that includes several other species attracted to methyl eugenol (*B. occipitalis*, *B. cacuminata*, *B. raiensis*). Three species, *B. melastomatos* (Figure 9F–O), *B. osbeckiae* (Figure 10) and *B. rubigina* (Figure 14F), were genetically indistinguishable using *COI* (0.1% pair-wise difference) in the phylogeny, appearing together in a single lineage, despite having very distinctive color patterns. Interestingly, they were slightly more distinct in the nuclear genes (1.1% *EF-1a* and 1% *period* pair-wise difference), which was not the case for most species. Population genetic statistics, based on *COI* sequences, showed high levels of haplotype diversity for most of the non-economic species in the *B. dorsalis* complex (Table 2).

Color patterns of scutum and/or abdomen (Figures 2–15) varied extensively within some of the species (Figures 2A–J, 3A–I, 4A–J, 5D–H, 6K–O, 7, 8, 9F–O, 10, 11B–E, 12A–J, 13F–O), and were relatively uniform in others (Figures 2K–O, 3J–M, 4K–M, 5A–C, 6A–J, 9A–E, 11A, 12K–O, 13A–E). Scutum color pattern was highly

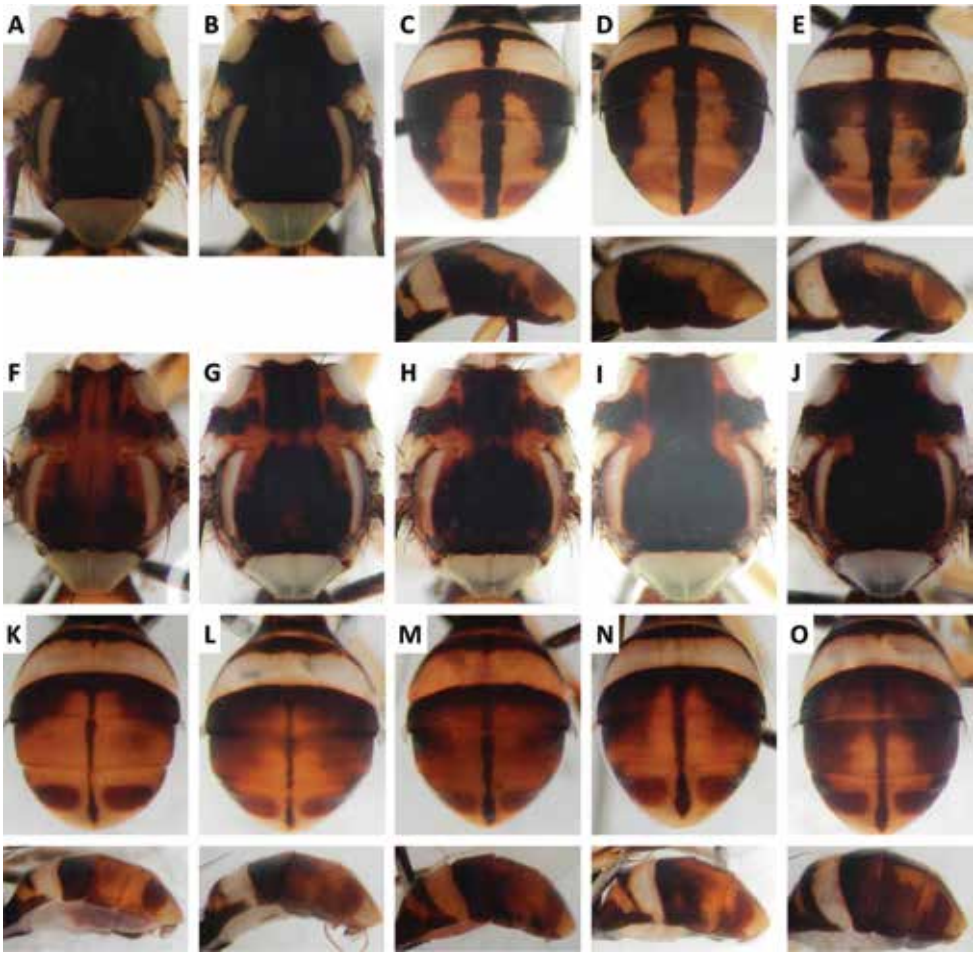

Figure 9. Variation in color pattern of scutum and abdomen in *Bactrocera latilineola* Drew and Hancock (**A–E**) (11 specimens examined and 4 sequenced) and *B. melastomatos* Drew and Hancock (**F–O**) (46 specimens examined and 8 sequenced). Voucher codes are: **A** ms1114 **B** ms2025 **C** ms2025 **D** ms2024 **E** ms1299 **F** ms1415 **G** ms1416 **H** ms1410 **I** ms1412 **J** ms1411 **K** ms1416 **L** ms1417 **M** ms1413 **N** ms1410 **O** ms1411.

polymorphic in *B. bhutaniae* (Figure 2), *B. bivittata* (Figure 3), *B. kohkongiae* (Figure 7), *B. melastomatos* (Figure 9F–O), *B. osbeckiae* (Figure 10), and *B. propinqua* (Figure 12). Abdomen pattern was confusingly polymorphic, yet scutum remained uniform in *B. thailandica* (Figure 13).

Scutum color and variation followed three basic patterns among species for which series of specimens were examined. In *B. bhutaniae* (Figure 2), *B. bivittata* (Figure 3), *B. kohkongiae* (Figure 7), and *B. osbeckiae* (Figure 10), scutum was predominantly red–brown with a highly variable dark lanceolate pattern. The pattern was composed of a medial and two lateral bands, generally interrupted at the level of the transverse suture,

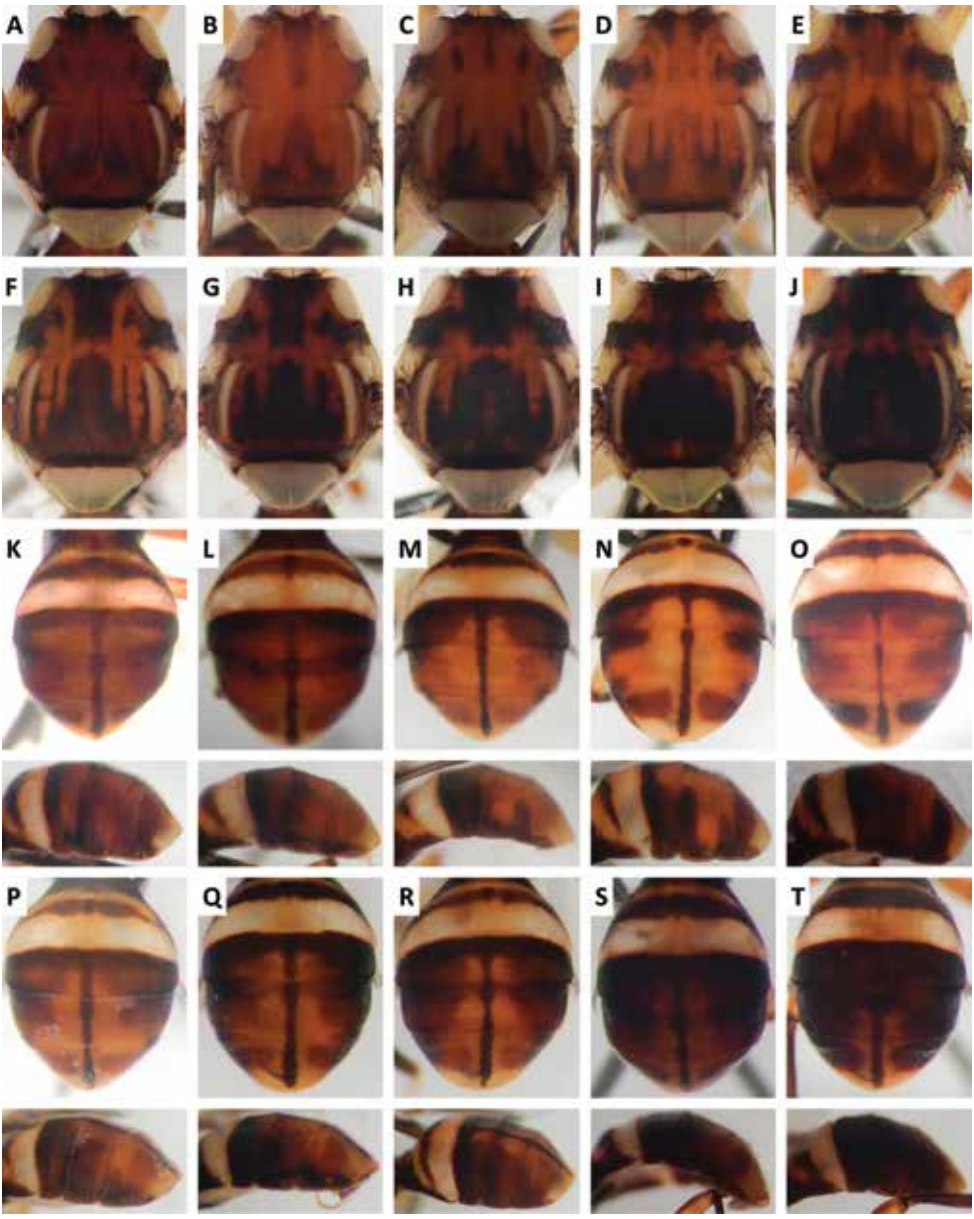

Figure 10. Variation in color pattern of scutum and abdomen in *Bactrocera osbeckiae* Drew and Romig (100 specimens examined and 39 sequenced). Voucher codes are: **A** ms1161 **B** ms3559 **C** ms3558 **D** ms3553 **E** ms3555 **F** ms3561 **G** ms1163 **H** ms3785 **I** ms3764 **J** ms3768 **K** ms1153 **L** ms3758 **M** ms3554 **N** ms1180 **O** ms1138 **P** ms3555 **Q** ms3784 **R** ms3560 **S** ms1154 **T** ms3768.

in *B. bhutaniae* and *B. bivittata* (medial band usually narrower and lateral bands very broad). The lanceolate pattern was highly variable in *B. kohkongiae*, from extensively pale with a narrow medial band to almost entirely dark with light markings restricted

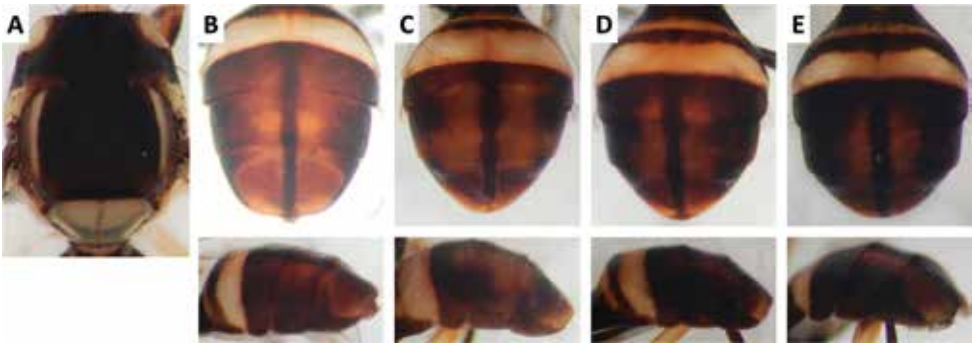

Figure 11. Variation in color pattern of scutum and abdomen in *Bactrocera paraarecae* Drew and Romig (10 specimens examined and 5 sequenced). Voucher codes are: **A** ms1295 **B** ms1296 **C** ms2040 **D** ms1294 **E** ms1110.

Table 2. Summary statistics of genetic variability, based on *COI* gene sequences, for non-economic species in the *B. dorsalis* complex.

Species	Sample size	Haplotypes (Nh)	Haplotype diversity (h)	Nucleotide diversity (pi)	Segregating sites (s)
B. bhutaniae	33	25	0.966	0.02557	86
B. bivittata	10	9	0.978	0.00320	10
B. cacuminata	11	4	0.491	0.00161	5
B. fuscitibia	6	5	0.933	0.00676	14
B. kanchanaburi	15	13	0.981	0.00768	31
B. kohkongiae	22	17	0.952	0.00472	23
B. latilineola	4	3	0.833	0.00320	5
B. melastomatos	8	4	0.643	0.00127	4
B. osbeckiae	35	13	0.704	0.00717	18
B. paraarecae	5	5	1.000	0.01536	29
B. propinqua	24	23	0.996	0.01047	40
B. thailandica	56	13	0.386	0.00145	24
B. usitata	5	5	1.000	0.01076	17

to the transverse suture, and *B. osbeckiae*, from mostly dark fuscous, with red-brown markings at level of postpronotal lobes and along transverse suture, to extensive lanceolate red–brown pattern with a broad medial longitudinal band, which can be faint or absent. In *B. cacuminata* (Figure 4), scutum was red–brown with a single medial dark band widened at apex of scutum and anteriorly narrowed to a point, and with two short lateral bands pointed anteriorly. A similar pattern was frequently observed in *B. propinqua* (Figure 12), in which the scutum varied from *B. cacuminata*-like to uniformly dark with light markings at level of transverse suture and inside postpronotal lobes. Scutum was generally uniformly black, with at most small red–brown markings anterior to lateral postsutural vittae, inside postpronotal lobes and sometimes at the level of prescutellar setae, in *B. fuscitibia* (Figure 5), *B. latilineola* (Figure 9), *B. paraare-*

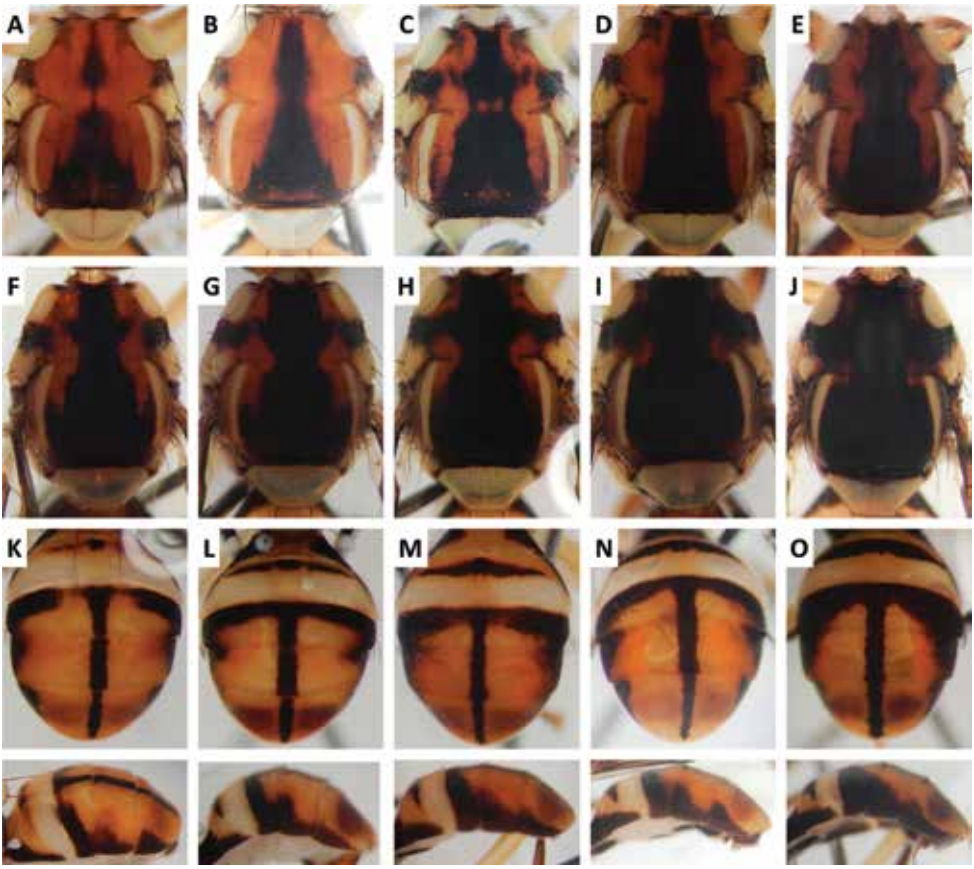

Figure 12. Variation in color pattern of scutum and abdomen in *Bactrocera propinqua* (Hardy and Adachi) (49 specimens examined and 24 sequenced). Voucher codes are: **A** ms4324 **B** ms4331 **C** ms4322 **D** ms3568 **E** ms3571 **F** ms3833 **G** ms3572 **H** ms3567 **I** ms2041 **J** ms1170 **K** ms4331 **L** ms3765 **M** ms3757 **N** ms3572 **O** ms3566.

cae (Figure 11), *B. thailandica* (Figure 13), and *B. usitata* (Figure 14H), and frequently with more extensive red–brown markings along transverse suture in *B. kanchanaburi* (Figure 6) and *B. melastomatos* (Figure 9 F–J). The shape and width of lateral postsutural vittae was relatively constant for all species except *B. thailandica* (Figure 13).

Abdomen color for almost all species and variants followed the basic "T-shaped" pattern typical of the *B. dorsalis* complex, i.e. a black band across the base of tergum III, a narrow to broad medial longitudinal black band covering the entire length of terga III to V, and narrow to broadly expanded lateral black markings on terga III to V. Medial band was broad and lateral markings generally broad along margins of tergum III and narrower on terga IV and V in *B. bhutaniae* (Figure 2) and *B. propinqua* (Figure 12), or the markings on terga III and IV expanded and pointed at apex in *B. bivittata* (Figure 3). Medial band was broad and extended to the base of tergum II and lateral markings broad on terga III, IV, and base of tergum V in *B. latilineola* (Figure 9 C–E). Medial band was

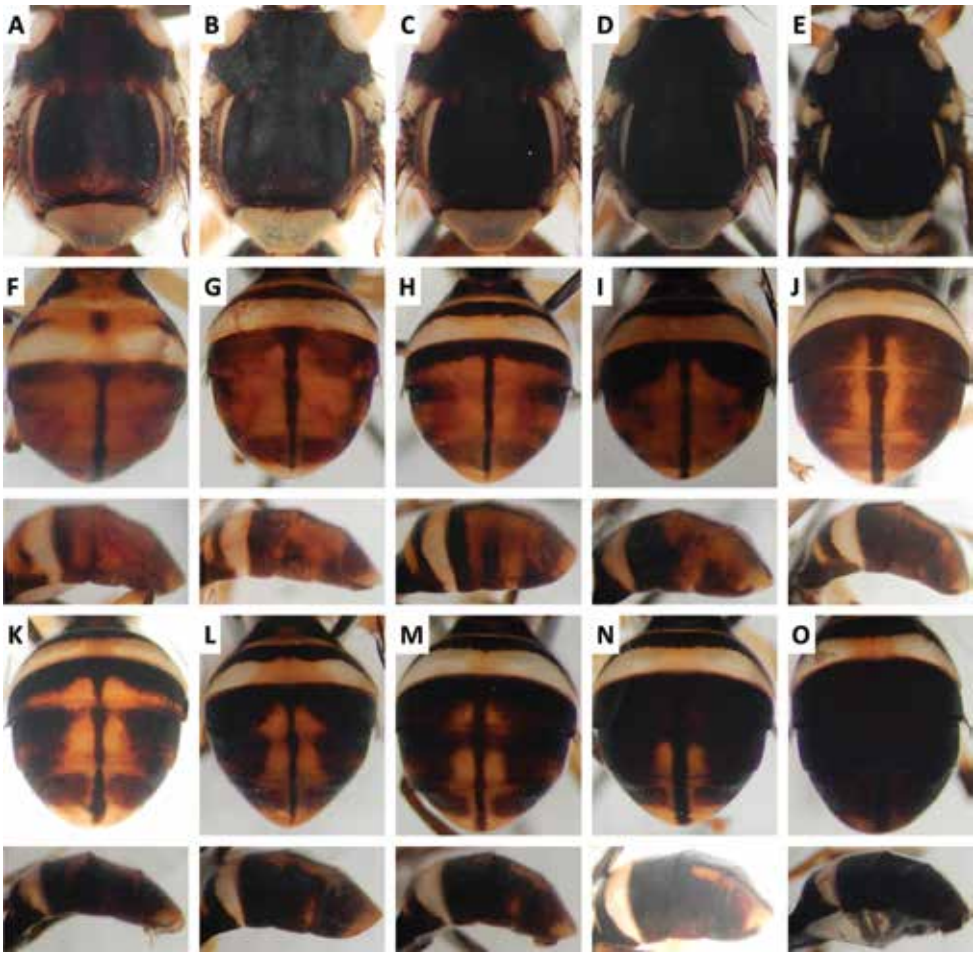

Figure 13. Variation in color pattern of scutum in *Bactrocera thailandica* Drew and Romig (712 specimens and 56 sequenced). Voucher codes are: **A** ms3587 **B** ms3588 **C** ms3586 **D** ms3525 **E** ms1952 **F** ms3576 **G** ms3586 **H** ms3736 **I** ms3585 **J** ms3539 **K** ms3581 **L** ms3538 **M** ms3695 **N** ms3582 **O** ms1949.

narrow (broad in *B. usitata*) and lateral markings usually broad along terga III–IV and basal half of tergum V in *B. cacuminata* (Figure 4), *B. kanchanaburi* (Figure 6), and *B. usitata* (Figure 14 G). Medial band was broad and lateral markings moderately to very broad on tergum III and IV, and shining spots on tergum V usually black (fuscous to dark fuscous in most other species) and continuous with lateral black markings in *B. fuscitibia* (Figure 5). Medial band was narrow (broad in *B. paraarecae*) and lateral markings moderately to very broad but diffuse, rather than well defined (as in previous species), in *B. kohkongiae* (Figure 8), *B. melastomatos* (Figure 9 K–O), *B. osbeckiae* (Figure 10), and *B. paraarecae* (Figure 11). In *B. thailandica*, medial band was narrow and the extent of lateral markings varied considerably, from very limited to almost entirely covering the terga except traces of red–brown on tergum V, on either side of medial band (Figure 13).

Figure 14. Scutum and abdomen of: **A** *Bactrocera dongnaiae* Drew and Romig (ms1158; 7 specimens examined and 3 sequenced) **B** *B. laithieuiae* Drew and Romig (ms3762; 1 specimen examined and sequenced) **C** *B. lombokensis* Drew and Hancock (ms1548; 1 specimen examined and sequenced) **D** *B. quasiinfulata* Drew and Romig (ms3455; 4 specimens examined and sequenced) **E** *B. raiensis* Drew and Hancock (ms1331; 2 specimens examined and 1 sequenced) **F** *B. rubigina* (Wang and Zhao) (ms3543; 259 specimens examined and 27 sequenced) **G** *B. usitata* Drew and Hancock (ms2039; 27 specimens examined and 6 sequenced).

Discussion

The concatenated tree demonstrates that the OFF complex is a highly polyphyletic assemblage of unrelated species. Consistent with other published studies, the methyl eugenol responsive *B. dorsalis*, *B. carambolae*, *B. occipitalis*, *B. cacuminata*, and *B. raiensis*, form a well-defined monophyletic unit (Krosch et al. 2012, San Jose et al. 2013, Boykin et al. 2013, Virgilio et al. 2015). Because the phylogeny is based on a relatively limited (24 %) proportion of all the species included in the OFF complex, adding more species and using multiple genes may reveal scattered clusters of related species, but the proportion of unrelated clades including OFF complex species is likely to remain high.

The widespread conformity of unrelated species to the *dorsalis*-like appearance is unclear. Color patterns in Dacine fruit flies are assumed to mimic wasps (White 2000), though few actual wasp mimic examples exist in Dacine fruit flies, and the OFF complex appearance is not a particularly convincing wasp imitation when compared to other groups of mimics (sesiid moths, syrphid flies, etc.). Whether the similarity represents convergent evolution or a retained ancestral state requires further investigation.

Except for a handful of well-studied species (e.g. *B. dorsalis*, *B. carambolae*, *B. cacuminata*), the definitions and concepts for the majority of the OFF complex species were based on morphology (mainly color patterns), lure response, and generally limited host fruit records. Only now are we starting to better characterize these species with molecular tools. Most of the non-economic species described by Drew and Hancock (1994) included in our study appear to be valid, confirmed by molecular data and comparison of morphological intraspecific variation with large series of specimens in QDAF (L.L., unpublished observations).

Attraction of *B. osbeckiae* to cue-lure is a new lure record. Morphological variation in our cue-lure trapped specimens (Figure 10) closely matched that observed in the QDAF and Bishop Museum (Honolulu, Hawaii, USA) series, which consist of host-reared specimens without male lure records.

Four species, consistent in appearance with the definition of the OFF complex, could not clearly be identified and are referred to here as numbered species. Species 54 (from Chiang Mai, Thailand) and 55 (Luang Nam Tha, Laos and Jinghong, China) look very similar (Figure 15A–F), yet are genetically distinct (8.85% *COI* pair-wise difference). They both key to *B. irvingiae* in Drew and Hancock (1994), but neither can be confidently matched to that species, even after comparison with series of pinned specimens of *B. irvingiae* and other OFF complex species in the QDAF collection. Also, *B. irvingiae* was collected further south in Thailand (Khao Yai) than the samples we have. Until fresh host-reared specimens of *B. irvingiae* can be obtained from the type locality and sequenced, we will defer from describing new species that may in the future turn out to be synonyms. Species 59 (Luang Nam Tha, Laos) and 60 (Jinghong, China) (Figure 15G–L) could not be definitely determined to species using available resources (Drew and Hancock 1994, Drew and Romig 2013), and did not match any of the OFF complex species examined in QDAF. They are likely new species, but not

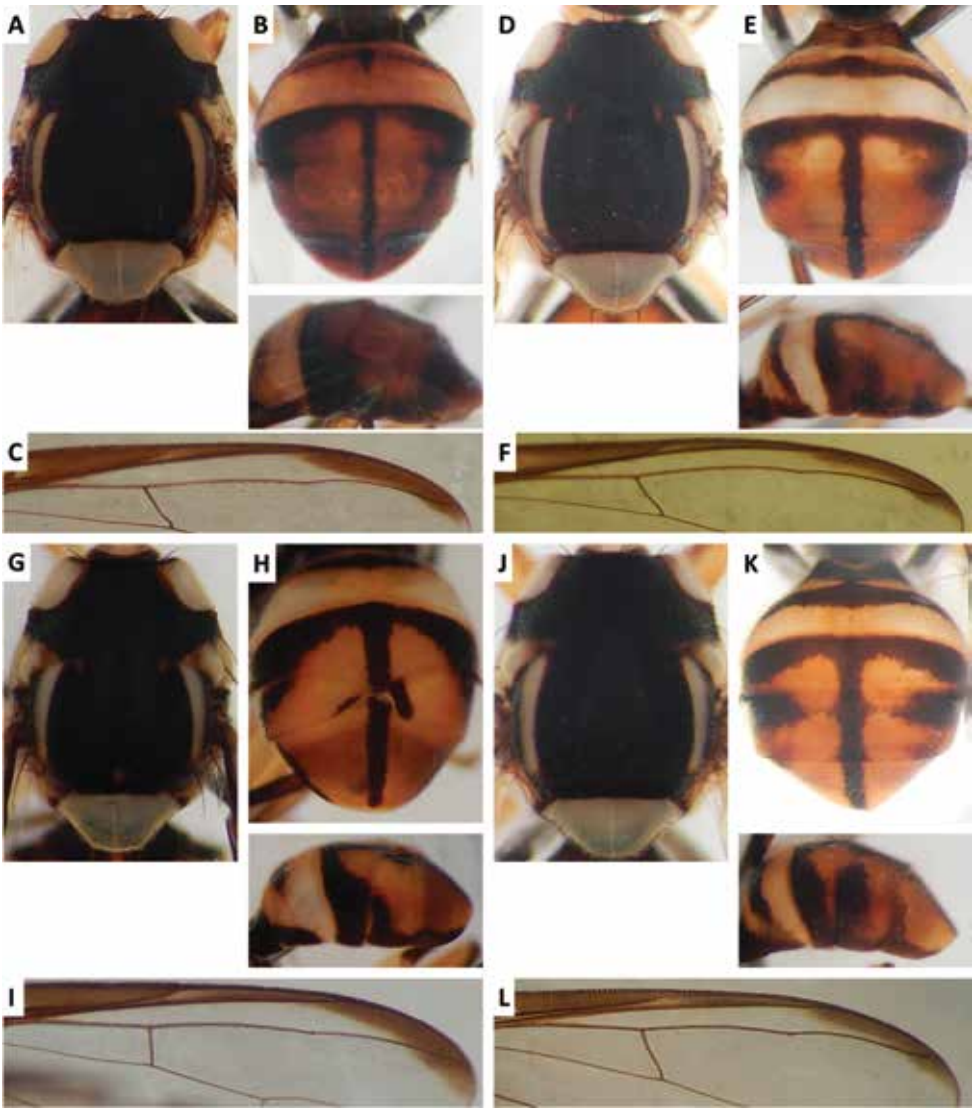

Figure 15. Scutum, abdomen and wing costal region of: **A–C** *Bactrocera* species 54 (ms1798 (wing, scutum), ms3777 (abdomen); 7 specimens examined and sequenced) **D–F** *B.* species 55 (ms3575; 7 specimens examined and sequenced) **G–I** *B.* species 59 (ms1164; 1 specimen examined and sequenced) **J–L** *B.* species 60 (ms3730; 3 specimens examined and sequenced).

described here, due to the lack of distinctive characters and the very small number of specimens available (1 of species 59 and 3 of species 60). With additional survey work and genetic sequencing, a number of additional cryptic species likely will appear.

Bactrocera dorsalis, *B. invadens* and *B. papayae* were recently declared conspecific, and are genetically indistinguishable (Schutze et al. 2014a), despite what some consider

diagnosable differences (Drew and Romig 2013). We have found a similar genetically indistinguishable situation for *B. osbeckiae*, *B. melastomatos* and *B. rubigina* (Wang and Zhao) in our phylogeny, despite them being very distinct from each other in color pattern (Figures 9F–O, 10, 14F). This suggests that these species, if distinct, may or may not differ at gene loci other than those sequenced in our study. *Bactrocera osbeckiae* and *B. rubigina* are sympatric in Thailand and Southern China (Leblanc, unpublished) and differ in color patterns and wing costal band expansion (Drew and Romig 2013), while *B. melastomatos* is confined to Peninsular Malaysia, Borneo, Java and Sumatra. *Bactrocera osbeckiae* and *B. melastomatos* are biologically close and peculiar, both breeding on flowers rather than fruits of Melastomataceae (Drew and Hancock 1994, Allwood et al. 1999), while the host range of *B. rubigina* is not well documented. Similarly, sympatric *B. tryoni* and *B. neohumeralis* in Australia are currently genetically inseparable, yet are likely valid biological species, isolated by time of mating (Clarke et al. 2011).

The high degree of intraspecific variation in color pattern severely limits the reliability of dichotomous and interactive keys. The range of variation differs considerably among species, with extreme cases like the scutum of *B. dorsalis* (Leblanc et al. 2013), and the abdomen of *B. thailandica* (Figure 13). Also, variants of unrelated species, such as *B. bhutaniae* (Figure 2) and *B. bivittata* (Figure 3), can overlap and make them hard to distinguish. The full extent of observed variation is easier to demonstrate in plates rather than words when describing a species. We suggest that descriptions of new species in the future should be accompanied by extensive plates showing variation, included in publications or posted as supplementary online material.

Conclusion

The OFF complex was defined by Hardy (1969) and the definition refined by Drew and Hancock (1994). The species and specimens examined in this study fit their definition in all respects, except for scutum color, said to be mostly black (Drew and Hancock 1994) or black (Drew and Romig 2013). Several species included in the complex consistently have extensive pale markings on the scutum (e.g. *B. arecae* (Hardy and Adachi), *B. bivittata*, *B. cacuminata*, *B. osbeckiae*). *Bactrocera dorsalis* has a broad range of variation, from entirely black to extensively or almost entirely pale (Schutze et al. 2014b, Leblanc et al. 2013), a form that was described as the now-synonymized, *B. invadens* (Drew et al. 2005), which was not included in the OFF complex by Drew and Romig (2013). It is likely that at least some of the 21 other species complexes (Drew 1989, Drew and Romig 2013) are also polyphyletic and their morphological diagnostic characters not robust. Nonetheless, the *B. dorsalis* complex is likely to remain entrenched for some time in future literature, as an informal group referred to as a "collective group" in the International Code of Zoological Nomenclature (http://iczn.org/iczn/index.jsp). Caution must be exercised in literature to not refer to the group as a biological or evolutionary unit.

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Supplementary material 1

Figure S1

Authors: Luc Leblanc, Michael San Jose, Norman Barr, Daniel Rubinoff

Data type: TIF File Format

Explanation note:Maximum likelihood tree, based the *CO*I gene dataset. Support values above branches are Maximum Likelihood Bootstrap values / Bayesian Posterior Probabilities. Scale bar indicates the number of substitutions per site. Species in the Oriental fruit fly complex are outlined in red.

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Supplementary material 2

Figure S2

Authors: Luc Leblanc, Michael San Jose, Norman Barr, Daniel Rubinoff

Data type: TIF File Format

Explanation note: Maximum likelihood tree, based the *EF-1a* gene dataset. Support values above branches are Maximum Likelihood Bootstrap values / Bayesian Posterior Probabilities. Scale bar indicates the number of substitutions per site. Species in the Oriental fruit fly complex are outlined in red.

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Supplementary material 3

Figure S3

Authors: Luc Leblanc, Michael San Jose, Norman Barr, Daniel Rubinoff

Data type: TIF File Format

Explanation note: Maximum likelihood tree, based the *period* gene dataset. Support values above branches are Maximum Likelihood Bootstrap values / Bayesian Posterior Probabilities. Scale bar indicates the number of substitutions per site. Species in the Oriental fruit fly complex are outlined in red.

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RESEARCH ARTICLE

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Effects of laboratory colonization on *Bactrocera dorsalis* (Diptera, Tephritidae) mating behaviour: 'what a difference a year makes'

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Abstract

Laboratory-reared insects are widely known to have significantly reduced genetic diversity in comparison to wild populations; however, subtle behavioural changes between laboratory-adapted and wild or 'wildish' (i.e., within one or very few generations of field collected material) populations are less well understood. Quantifying alterations in behaviour, particularly sexual, in laboratory-adapted insects is important for mass-reared insects for use in pest management strategies, especially those that have a sterile insect technique component. We report subtle changes in sexual behaviour between 'wildish' *Bactrocera dorsalis* flies (F1 and F2) from central and southern Thailand and the same colonies 12 months later when at six generations from wild. Mating compatibility tests were undertaken under standardised semi-natural conditions, with number of homo/heterotypic couples and mating location in field cages analysed via compatibility indices. Central and southern populations of *B. dorsalis* displayed positive assortative mating in the 2010 trials but mated randomly in the 2011 trials. 'Wildish' southern Thailand males mated significantly earlier than central Thailand males in 2010; this difference was considerably reduced in 2011, yet homotypic couples from southern Thailand still formed significantly earlier than all other couple combinations. There was no significant difference in couple location in 2010; however, couple location significantly differed among pair types in 2011 with those involving southern Thailand females occurring significantly more

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often on the tree relative to those with central Thailand females. Relative participation also changed with time, with more southern Thailand females forming couples relative to central Thailand females in 2010; this difference was considerably decreased by 2011. These results reveal how subtle changes in sexual behaviour, as driven by laboratory rearing conditions, may significantly influence mating behaviour between laboratory-adapted and recently colonised tephritid fruit flies over a relatively short period of time.

Keywords
Oriental fruit fly, sexual compatibility, isolation indices, laboratory adaptation

Introduction

While now debated as to whether it is a driver of speciation, or a secondary effect flowing from population divergence (de Queiroz 1998), the ability to mate and produce viable offspring (sensu the Biological Species Concept (Mayr 1957)) remains, to many biologists, the central test of whether two populations belong to the same or different species (The Marie Curie Speciation Network 2012). Experiments evaluating mating compatibility come with caveats, especially as bringing two populations together under artificial circumstances may influence one or more critical steps of the mate recognition process. Only when random mating between known conspecifics is demonstrated in choice mating tests, and incompatibility with a second putative species, then strong inference as to species limits can be drawn (Walter 2003).

For tephritid fruit flies, cross-species mating in small cages results in forced matings that produce viable hybrids (Cruickshank et al. 2001), and so are inappropriate for use in delimiting species boundaries. In contrast, mate choice experiments in large, walk-in field cages containing a host plant (i.e., semi-natural conditions) have proven useful tools in discriminating among closely related sibling species (Petit-Marty et al. 2004, Vera et al. 2006, Cáceres et al. 2009, Schutze et al. 2013, Bo et al. 2014). As reviewed by Juárez et al. (2015), protocols for such trials are now well established and widely applied. Where it is logistically feasible to bring populations together, such mating tests are a recommended component of integrative taxonomic studies (sensu Schlick-Steiner et al. 2010) on frugivorous tephritids (Clarke and Schutze 2014).

International protocols for tephritid mating trials were initially designed to test competiveness and compatibility among flies mass-reared for the sterile insect technique (SIT) and their wild counter-parts, or to compare the competitiveness and compatibility of populations from different mass-rearing facilities in different geographical areas (FAO/IAEA/USDA 2003). As such, they were factory quality assurance tests that were developed with a need for clearly defined, easily repeatable, and statistically comparable data sets. The key parameters now commonly used for tephritid mating trials (the Index of Sexual Incompatibility (ISI) and the Male and Female Relative Performance Indices (MRPI and FRPI, respectively)) meet these needs, but when used alone may hide potentially critical biological information. Most importantly, these indices report who eventually mated with whom, but not why, or more tellingly, why they did not. While the collection and reporting of secondary behavioural data (e.g., mating time

and location in the field cage) in tephritid mating studies is recommended in the international protocol, its importance appears often downplayed in the literature when compared to the reporting of the main mating indices.

In this paper, we report on two crossing experiments using the same populations of *Bactrocera dorsalis* (Hendel), conducted under identical experimental conditions exactly 12 months apart. The first cross used nearly wild flies (F1-F2 generation), while the second cross used flies from the same colony when six generations in culture (F6). Mating compatibility between the two populations was assessed between the trials, and this example was used to discuss: i) the importance of collecting secondary behavioural data in mating trials; ii) the importance of understanding subtle differences in courtship behaviour which may occur between wild populations of the same species; and iii) the implications of using 'wildish' versus laboratory-adapted populations for integrative taxonomic research.

Methods

Source material

We evaluated mating behaviour of *B. dorsalis* from central and southern Thailand. All flies were sourced directly from the wild via host-rearing and sent to the FAO-IAEA Insect Pest Control Laboratory (IPCL), Seibersdorf Austria, in March 2010. Collection locations were not privately owned and no endangered or protected species were involved in the study. No specific permits were required for the described field studies or for the import of live material into the IPCL. The central Thailand population was reared from *Mangifera indica* L. (Anacardiaceae) in Saraburi and sent as a batch of approximately 500 pupae; the southern population was reared from *Carica papaya* L. (Caricaceae) in Nakhon Si Thammarat and sent as a batch of approximately 200 pupae.

Flies were morphologically examined for external and internal genitalic characters to confirm their identity in accordance with taxonomic descriptions (Drew and Hancock 1994). Professor R.A.I. Drew confirmed the identity of the cultures as *B. dorsalis* based on pinned material, diagnostic micrographs, and genitalia measurements. Further, material from these colonies were used in subsequent integrative taxonomic studies examining molecular and morphological characters that further confirmed both colonies as *B. dorsalis* (Krosch et al. 2013, Boykin et al. 2014). Representative voucher samples were preserved as dried (pinned) and wet (> 95% alcohol) material at the IPCL and Queensland University of Technology, Brisbane, Australia.

General rearing protocol

Adult flies were provided a standard diet of enzymatic yeast hydrolysate and sugar (1:3) together with water *ad libitum*. Sexually mature flies were exposed to egg-cups dosed

with commercial guava juice (Rubricon, Rubricon Products, Middlesex, U.K.) as an oviposition stimulant. Eggs were incubated overnight (25 ± 2 ºC, 65% R.H.) on moist filter paper placed on wet sponges in Petri dishes and then transferred to carrot diet (Tanaka et al. 1970) for larval development (27-28 °C, 55% R.H.). Pupae were collected into and sifted from moistened teak sawdust, and transferred to either experimental (20 cm diameter × 27 cm height) or colony cages (50 cm × 50 cm × 50 cm).

Mating compatibility tests

The first mating compatibility tests were conducted in June and July of 2010 when Saraburi and Nakhon Si Thammarat colonies were at the F1/F2 laboratory colony generation. Eight replicates were completed, consisting of five using F1 generation flies and three using F2 generation flies. The second series of mating compatibility tests were undertaken one-year later in July 2011 when both cultures had reached F6 (eight replicates completed). Experimental protocols were identical for 2010 and 2011 trials, as outlined below.

Flies were sexed within four days of emergence; this is well before male and female sexual maturation which occurs 15-20 days post emergence based on personal observation (MKS; data not shown) and previous studies (McInnis et al. 1999, Wee and Tan 2000). Flies were maintained under low-stress conditions of 100-200 flies per cylindrical cage (20 cm diameter × 27 cm height). General procedures followed the FAO/IAEA/USDA (2003) Manual for Product and Quality Control. A small dot of coloured water-based paint was applied to the dorsal surface of each fly's thorax using a soft paint-brush (colours were randomized among tests) for identification of each population. Painting was done at least 48 hours prior to each field cage test to allow paint to dry and flies to become habituated to its presence. Mating tests were undertaken using flies between 20-30 days of age to ensure sexual maturity had been reached by the majority of individuals (> 90% of wild *B. dorsalis* reach maturity by 24 days (Wong et al. 1989)).

Field cage tests were conducted inside a glasshouse exposed to natural light and maintained at ~25 ºC and ~50% R.H. Replicates were undertaken inside one of four partitioned flight cages (2.0 m × 1.6 m × 1.9 m) within the glasshouse, with each cage containing a single, non-fruiting potted *Citrus sinensis* Osbeck (Rutaceae) tree of 2 m in height with a canopy of ~ 1.1 m in diameter.

Flies were released into the experimental field cage at a 1:2 male:female ratio. As this study was focussed on mating compatibility and not strictly competition, this ratio of males to females (as opposed to 1:1) was used to ameliorate the effect of potentially early-mating males from monopolising all females from one population and thus inflating isolation indices, as per Schutze et al. (2013). By providing twice as many females as males, potentially later starting males still have access to females from both populations. As *B. dorsalis* mates at dusk (Arakaki et al. 1984), 20 males of each of the two populations were released into a field cage one to two hours prior to sunset for each

replicate; 40 females of each of the same two populations were released 30 min later for a total of 40 males and 80 females per replicate. Experimental observations began immediately after females were released. Once couples formed, they were gently coaxed into sequentially numbered plastic vials (3.7 cm diameter × 4.0 cm). The following data were recorded for each pair: male origin; female origin; time of mating; and position (cage or tree). Periodic measurements of temperature (°C) and relative humidity in the cage were also made. Experiments concluded when flies became inactive, which occurred after sunset when light intensity dropped below 10 lux.

Data analysis

Relative percentages of each of the four possible couples (i.e., Saraburi ♂ × Saraburi ♀ [SS], Saraburi ♂ × Nakhon Si Thammarat ♀ [SN], Nakhon Si Thammarat ♂ × Saraburi ♀ [NS], and Nakhon Si Thammarat ♂ × Nakhon Si Thammarat ♀ [NN]) were calculated for each replicate. Proportion data were arcsine transformed prior to subsequent analysis; one-way ANOVA (with Tukey *post hoc* test where appropriate) was conducted to assess for significant differences among mating combinations within each year; paired t-tests were conducted to assess for significant differences in relative proportions of respective couple combinations across years.

Compatibility was determined using the Index of Sexual Isolation in conjunction with the Male Relative Performance Index and the Female Relative Performance Index (Cayol et al. 1999). Values of ISI may range from +1 (complete positive assortative mating, i.e., males and females only mating with their respective population) to 0 (complete random mating) to -1 (complete negative assortative mating; i.e., all males of one population mating with all females of the opposite population and *vice versa*). Values of MRPI range from +1 (only males of one population mated; i.e., the first listed in the test) to 0 (males of both populations participated equally in mating) to -1 (only males of the reciprocal population mated; i.e., the second listed in the test). The FRPI is the equivalent of the MRPI but as applied to females.

Ninety-five percent confidence intervals of the used indices (ISI, MRPI, and FRPI) for each of 2010 (F1/F2 flies) and 2011 (F6 flies) were calculated to determine deviations from random mating (ISI = 0) or equal participation by the respective sexes (MRPI & FRPI = 0). Confidence intervals that included zero represent cases of random mating and equal participation between the populations. Heterogeneity chi-square analyses across replicates for each treatment were undertaken to determine if data could be combined prior to further analysis. Following heterogeneity tests, chi-squared tests of independence were applied to determine if males mated predominantly with females of one population over the other.

The mean time to begin mating (mating latency) was estimated by calculating how many minutes had elapsed between the time each couple initiated mating and the time of the first observed mating couple (= time zero) within each particular cage replicate (as per Schutze et al. 2013). Statistical analyses were conducted for each of the tests,

with latency data for each of the four possible mating combinations combined prior to one-way ANOVA (with Tukey *post hoc* test where appropriate) to determine significant differences ($\alpha = 0.05$) in latency among mating combinations. We conducted a one-way ANOVA (with Tukey *post hoc* test where appropriate) on arcsine transformed percentage data of couples collected on the tree for each trial to determine if there was a significant difference in couple location (cage or tree).

All values reported represent mean ± 1 s.e. unless otherwise stated.

Results

Eight replicates of mating compatibility tests were completed for each of the 2010 and 2011 trials; 84.7 ± 2.1% and 78.1 ± 4.3% flies mated in 2010 and 2011, respectively.

Total numbers and mean percentages of each of the four possible mating-pair combinations (i.e., SS, SN, NS, and NN) varied considerably between years (Figure 1A). There was a highly significant difference among the percentages of mating combinations in 2010 ($F_{(3,28)} = 56.59$, $p < 0.0001$), with significantly more NN couples (43.99 ± 1.70%) forming relative to all other couples, together with significantly more SN heterotypic couples forming relative to the reciprocal NS. There was a significant difference among the percentages of mating combinations in 2011 ($F_{(3,28)} = 4.44$, $p < 0.05$), with significantly more NN couples (32.82 ± 2.62%) forming relative to SS and NS couples. While in 2010 there was an increased difference in the numbers of couples other than NN (i.e., SS, SN, and NS), there was no such difference among these couple combinations in 2011, with heterotypic couples (SN and NS) equally represented as SS homotypic couples; indeed, numbers of SN couples was not statistically different to NN couples. Across years, there was a significant change in relative proportion of NN and NS couples from 2010 to 2011 ($t = 3.539$, df = 14, $p < 0.01$; $t = -3.410$, df = 14, $p < 0.01$, respectively); however, there was no significant change in the relative proportion of SN and SS couples across years ($t = 0.326$, df = 14, $p = 0.749$; $t = -1.250$, df = 14, $p = 0.232$, respectively).

Analysis of latency (time to mate since first couple formed) revealed further differences between populations of *B. dorsalis* from Nakhon Si Thammarat and Saraburi (Figure 1B). There was a significant difference in latency between couples involving males from Saraburi and males from Nakhon Si Thammarat in 2010 ($F_{(3,266)} = 61.18$, $p < 0.0001$). Couples with males from Saraburi mated significantly later (SS = 74.34 ± 3.93 min and SN = 65.99 ± 3.46 min) than those from Nakhon Si Thammarat (NS = 35.03 ± 3.32 min and NN = 29.94 ± 1.73 min). While this trend continued in 2011 with significant differences in latency among couple combinations ($F_{(3,246)} = 5.562$, $p < 0.05$), the difference was nevertheless reduced with males from Saraburi mating sooner after sunset compared with those involved in the F1/F2-generation 2010 trials (2011 latency for SS = 50.35 ± 2.96 min; SN = 48.10 ± 4.17 min) while latency of couples involving males from Nakhon Si Thammarat either increased slightly or remained approximately the same (NS = 43.96 ± 3.65 min; NN = 33.95 ± 2.74 min). Overall,

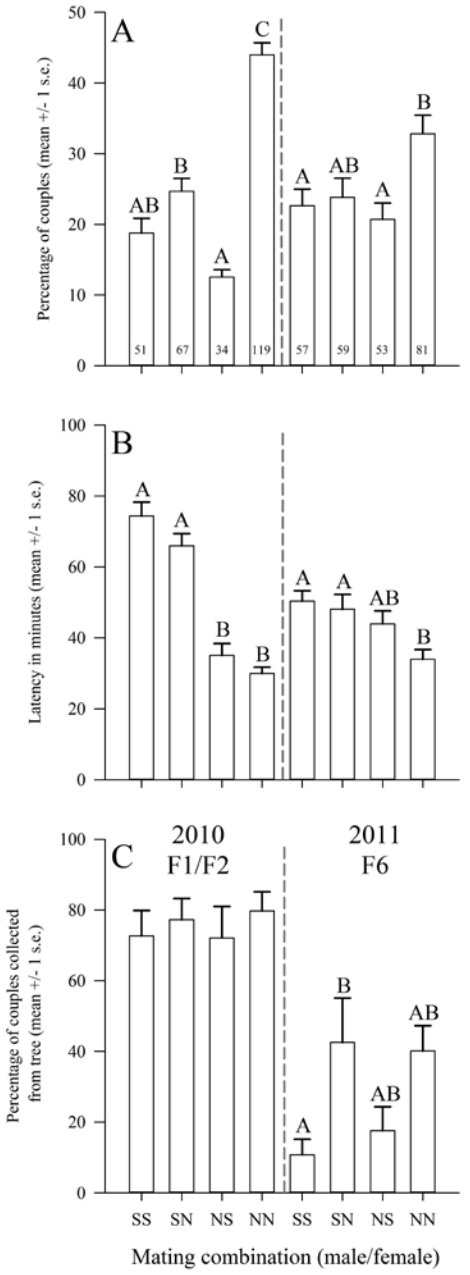

Figure 1. Behavioural parameters of *Bactrocera dorsalis* flies from Saraburi (S) and Nakhon Si Thammarat (N) (Thailand) during mating compatibility trials in 2010 and 2011. **A** Relative percentages and total numbers of each possible couple formed. Numbers in bars are total numbers of each couple formed summed across replicates **B** Mating latency as average time since first couple observed for couples formed **C** Average percentage of respective couples collected from the tree for each of the six mating compatibility comparisons. For all graphs, columns surmounted by the same letter within a year are not significantly different at $\alpha = 0.05$.

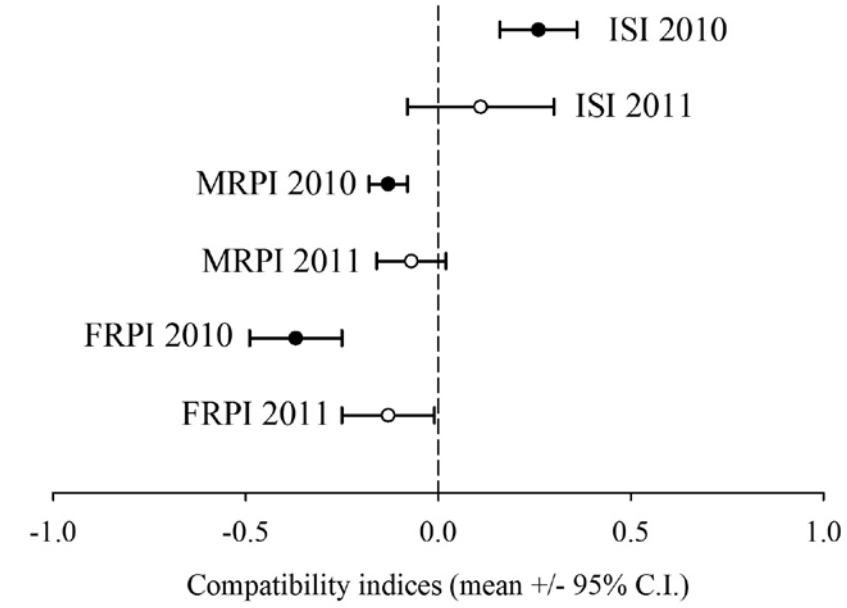

Figure 2. Index of Sexual Isolation (ISI) and relative performance indices for males (MRPI) and females (FRPI) with associated 95% confidence intervals calculated for 2010 and 2011 mating compatibility comparisons between *Bactrocera dorsalis* from Saraburi and Nakhon Si Thammarat, Thailand. Dotted line (0.00) represents random mating (ISI) or equal participation by the sexes (MRPI and FRPI).

there were no significant differences in latency among SS, SN, or NS couples in 2011; however, NN couples still mated significantly sooner than SS and SN (i.e., those involving Saraburi males) as for the 2010 trial.

There were no significant differences among mating combinations with respect to position on the tree or cage wall for the 2010 trial ($F_{(3,28)} = 0.134$, $p = 0.939$); however, there was a significant difference among couples in the 2011 trial ($F_{(3,28)} = 3.902$, $p < 0.05$) (Figure 1C). In 2011, SS couples mated significantly more on the cage wall (10.76 ± 4.37% on the tree) compared to SN couples of which 42.52 ± 12.58% of couples mated on the tree. While statistically non-significant, other combinations displayed similar trends, with only 17.59 ± 6.75% of NS couples mating on the tree in contrast to 40.11 ± 7.16% of NN couples on the tree. Taken together, and calculated based on summed replicates, 2011 couples that involved females from Nakhon Si Thammarat mated on trees 37% of the time relative to 15% of couples involving females from Saraburi.

As chi-squared tests of independence were homogeneous across replicates for both years (2010 $\chi^2 = 3.49$, df = 7, $p = 0.836$; 2011 $\chi^2 = 11.18$, df = 7, $p = 0.131$), data were summed prior to analysis of mating indices ISI, MRPI and FRPI. While there was a significant bias towards assortative mating in 2010 ($\chi^2 = 13.64$, df = 1, $p < 0.0001$; ISI = 0.26 ± 0.19 [95% C.I.]), this effect was largely lost by the time F6 flies were crossed in 2011, despite the consistent and significant increase in number of NN couples ($\chi^2 = 2.32$, df = 1, $p = 0.128$; ISI = 0.11 ± 0.10 [95% C.I.]) (Figure 2).

The FRPI significantly deviated from random (FRPI = -0.37 ± 0.12 [95% C.I.] in 2010, reinforcing that considerably more Nakhon Si Thammarat females mated (n = 186 summed across reps for SN and NN) relative to those from Saraburi (n = 85 summed across reps for SS and NS). While this trend continued in 2011, there was a considerably reduced difference in female participation (n = 140 *versus* n = 110, resp.) as reflected in the FRPI measure approaching zero (FRPI = -0.13 ± 0.12 [95% C.I.]). While less dramatic, there were also significantly more males from Nakhon Si Thammarat mating (n = 153 summed across reps for NS and NN) relative to those from Saraburi (n = 118 summed across reps for SS and SN) in 2010 with a mean MRPI (± 95% C.I.) of -0.13 ± 0.05 (Figure 2); yet in 2011 this difference in relative male participation was, as for females, considerably reduced with a total of 134 Nakhon Si Thammarat males mating (NS and NN summed across reps) compared to 116 from Saraburi (SS and SN summed across replicates).

Discussion

Changes in mating behaviour over a year

Our results show that F1/F2 (= 'wildish') *B. dorsalis* from Saraburi and Nakhon Si Thammarat demonstrated significant positive assortative mating: i.e., like was more likely to mate with like than expected by chance. This assortative mating was lost by the 6th generation, when random mating occurred between the two populations. The change from positive assortative to random mating was most likely due to two factors: latency and relative participation of the sexes. In 'wildish' populations Nakhon Si Thammarat males mated sooner than Saraburi males (i.e., their mating latency time was shorter) and Nakhon Si Thammarat females mated more than Saraburi females. The combination of the two attributes led to more Nakhon Si Thammarat × Nakhon Si Thammarat matings. By the 6th generation, the temporal difference in male latency was lost, as was the increased 'precociousness' of the Nakhon Si Thammarat females, leading to random mating between the populations.

Differences in latency in male mating behaviour may be the results of local environmental conditions from where respective populations of *B. dorsalis* originated. Time of sunset, for example, may be a potential causal factor, considering Nakhon Si Thammarat is located approximately 600 km south of Bangkok and time of sunset (and time of mating) would correspondingly vary. However, despite their geographic distance, time of sunset differs little between these locations across the year: the sun sets approximately 10-12 minutes later in Nakhon Si Thammarat relative to Bangkok in January, yet in July it sets approximately 6 minutes earlier (based on 2014 sunset data; www.sunrise-and-sunset.com). Nevertheless, this slight difference may be sufficient to influence mating latency in early-generation laboratory colony flies. Complexity in circadian rhythm patterns and differences in mating latency between wild and mass-reared colonies have been investigated in other tephritid species, such as the melon fly *Bactrocera cucurbitae* (Coquillett)

(Matsuyama and Kuba, 2009); exemplifying how subtle, but significant, differences in the onset of mating behaviour can be readily manipulated by changes in daily light patterns (Miyatake et al. 2002). We cannot rule out other factors besides time of sunset, however, and further work into the driving mechanisms of *B. dorsalis* mating latency are warranted.

What influenced variation in female mating in our trials remains open to speculation. Drivers of sexual propensity are varied, and may include both intrinsic and extrinsic (e.g., temperature, food, density, and sex ratios) factors (Spiess, 1970). Differences in mating propensity may also be affected by body size, as demonstrated in other tephritids such as *C. capitata*, for which mating frequency was dependant on the relative sizes of males and females (Churchill-Stanland et al. 1986). We did not, however, record additional attributes (e.g., size) of flies used in these trials; therefore, we are unable to account for the contribution of any of these factors towards differences in mating propensity of either sex.

'Wildish' or laboratory adapted flies

These results pose a conundrum for mating trials. It is generally considered that the use of 'wildish' populations (i.e., within one or very few generations of field collected material) is more desirable than using flies that already have been cultured for a long time because laboratory selection may alter key behavioural and physiological traits (Miyatake 1998, 2011, Meats et al. 2004, Gilchrist et al. 2012). However, in our case the use of 'wildish' populations led to a result which, when taken alone, was contrary to additional evidence that *B. dorsalis* constitutes a single biological species within Thailand (Schutze et al. 2012, Krosch et al. 2013, Aketarawong et al. 2014, Boykin et al. 2014). That is, we observed significant assortative mating behaviour driven by behavioural differences which may support a two-species hypothesis; yet, all other forms of data (e.g., molecular, morphological, cytogenetic, and chemoecological) strongly infer conspecificity across Thailand. Nevertheless, subtle differences in mating latency between populations of other *Bactrocera* species are known, even when there is no suggestion of sibling species. For example, differences in mating latency between populations of *B. cucurbitae* have been recorded (Miyatake 1998, 2011), but there is no evidence for cryptic taxa within that species (Hu et al. 2008, Virgilio et al. 2010).

Based on this experiment alone we are not in a position to make strong statements about using 'wildish' versus older cultures for mating tests in *B. dorsalis*, but we do highlight that even within a single biological species, local adaptation and drift may lead to subtle but potentially important differences in some aspects of the mating system, as documented in other organisms (Schmitt and Gamble 1990, Berlocher and Feder 2002, Nosil et al. 2005, Cocroft et al. 2010). Perhaps a compromise to ameliorate potential short term behavioural 'hangovers' from wild populations is to rear populations for a pre-determined number of generations to standardise colonies in the laboratory, so that any differences found are more likely to reflect underlying genetic causes and not short-term environmental influences. That said, long-term rearing does

lead to significant laboratory adaptation, and as demonstrated for some species this bottleneck can occur within the first few generations (Frankham and Loebel 1992, Hoffmann et al. 2001, Gilchrist et al. 2012). The significant reduction in the number of matings occurring on foliage versus cage walls from the F1/F2 flies to the F6 flies is perhaps behavioural evidence of laboratory adaptation, and for within *B. dorsalis* we suggest conducting compatibility studies on colonies greater than two generations from the wild, but not greater than six.

Mating indices as a factory QA measure versus a research tool

As a factory quality assurance measure, mating indices serve a valuable function by allowing repeatable and quantifiable measures of the quality of factory flies, thereby forming an effective tool allowing SIT action programme managers to determine if sterile males are fit for purpose to compete with wild males and are compatible with wild females. But there is no doubt that these indices, by focusing exclusively on the 'end product' of copulation, may lead some researchers to potentially under appreciate biologically important steps in the courtship process. Where mating is 100% random, or 100% positive-assortative, these prior steps may be less critical for interpreting meaning from the trials. But where the results fall in between these extremes, as has been found in several *Bactrocera* and *Anastrepha* studies (Schutze et al. 2013, Vera et al. 2006), the absence of more detailed courtship or mating data makes interpretation difficult.

Conclusion

The use of very recently established colony material is widely considered ideal for determining mating compatibility among strains, populations, or putative species. Our results clearly demonstrate that subtle behavioural characteristics may 'carry-over' from the wild and may result in inflated measures of incompatibility that are soon lost following colony establishment. For future tephritid research where mating is used to help delimit cryptic species, we therefore encourage the use of detailed courtship behaviour in field cage mating studies that is quantified by isolation and additional indices that dissect specific behavioural attributes among populations or putative species.

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Analyses of volatiles produced by the African fruit fly species complex (Diptera, Tephritidae)

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Abstract

Ceratitis fasciventris, *Ceratitis anonae* and *Ceratitis rosa* are polyphagous agricultural pests originating from the African continent. The taxonomy of this group (the so-called *Ceratitis* FAR complex) is unclear. To clarify the taxonomic relationships, male and female-produced volatiles presumably involved in pre-mating communication were studied using comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC-TOFMS) followed by multivariate analysis, and gas chromatography combined with electroantennographic detection (GC-EAD). GC×GC-TOFMS analyses revealed sex specific differences in produced volatiles. Male volatiles are complex mixtures that differ both qualitatively and quantitatively but share some common compounds. GC-EAD analyses of male volatiles revealed that the antennal sensitivities of females significantly differ in the studied species. No female volatiles elicited antennal responses in males. The results show clear species-specific differences in volatile production and provide complementary information for the distinct delimitation of the putative species by chemotaxonomic markers.

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Keywords
Ceratitis FAR complex, chemotaxonomy, male and female-borne volatiles, GC×GC-TOFMS, GC-EAD

Introduction

The fruit fly family Tephritidae (Diptera) consists of four major genera, *Ceratitis*, *Bactrocera, Anastrepha* and *Rhagoletis*, which are considered important insect pests worldwide (Aluja and Norrbom 2001). The genus *Ceratitis* is the most studied, with *Ceratitis capitata (*Wiedemann) as species most frequently monitored and used as the model pest organism due to its global distribution. The Afro-tropical group of fruit flies *Ceratitis fasciventris (*Bezzi), *C. anonae* Graham and *C. rosa* Karsch (the so-called FAR complex) is widespread in a number of African countries: *C. fasciventris* and *C. anonae* occur sympatrically in both East and West Africa while *C. rosa* is more restricted to southern and eastern Africa where its distribution partially overlaps with that of *C. fasciventris* but not with *C. anonae* (Barr and McPheron 2006, Copeland et al. 2006). *Ceratitis rosa* is now feared to be a global threat due to its tolerance to lower temperatures (Duyck and Quilici 2002). It may expand not only within Africa, but also across Europe, Asia, Australia and the North and South American continents (De Meyer et al. 2008).

Females of the FAR complex species cause extensive damage on commercially produced fruits from 24 plant families (De Meyer et al. 2002, Copeland et al. 2006), by puncturing the fruits during oviposition and by the feeding larvae inside the fruit that generally result in premature fruit abortion. Reproduction behavior in genus *Ceratitis* is initiated by males that aggregate in leks on vegetation to lure females by releasing long-range pheromones. Females attracted by male pheromone visit leks and choose males to mate based on complex visual, acoustical and chemical stimuli (Aluja and Norrbom, 2001). Chemical communication, that involves both long-range pheromones and close-range cuticular hydrocarbons, is integral part of the fruit fly courtship. Nevertheless, there are no records available on the composition of the long-range volatiles released by the FAR complex species.

Despite its economic importance, the taxonomy of this group is not clear and taxonomical classification is not easy (De Meyer and Freidberg 2006). It is particularly important to resolve invasive agricultural pest species, because inadequate morphological/molecular characterisation of the species might have serious economic consequences, resulting in inept ecological models and/or pest control strategies (Virgilio et al. 2013, Vaníčková et al. 2014).

The need to develop a precise pest-detection technique, diagnostic tools and management strategies for these pest species initiated large scale morphological and genetic studies, the investigation of their evolutionary relationships as well as the characterisation of the variation of cuticular hydrocarbon profiles within and between the species (De Meyer 2001, De Meyer and Freidberg 2006, Virgilio et al. 2012, Delatte et al. 2013, Virgilio et al. 2013, Vaníčková et al. 2014a,b, Vaníčková et al. 2015). The species of the FAR complex can only be identified based on specific small differences between

the morphological characters of adult male leg patterns (larvae, pupae and females, are even more difficult to distinguish) (De Meyer and Freidberg 2006). While it is possible to identify the females of *C. anonae*, the females of *C. rosa* and *C. fasciventris* are almost indistinguishable from each other and show only very subtle differences in their scutellar colour patterns. The absence of clear diagnostic morphological features to identify individual species emphasises the need for unambiguous identification applying molecular and/or chemical tools (Baliraine et al. 2004, Vaníčková 2012, Virgilio et al. 2013, Vaníčková et al. 2014a,b). Molecular approaches for species recognition were developed in the past (Virgilio et al. 2008, Barr and Wiegmann 2009, Delatte et al. 2013). Recently, Virgilio and co-workers have provided clear data on the specification of five different morphotypes using a comparison of allelic variations at 16 microsatellite loci (Virgilio et al. 2013). Nevertheless, the use of microsatellite loci for cryptic species identification is rather laborious and expensive. Recent studies on the cuticular hydrocarbon profiles extracted from the body surface of males and females of *C. fasciventris*, *C. anonae*, *C. rosa* and *C. capitata* have supported the existence of more than three genotypes in the FAR complex (Vaníčková 2012, Vaníčková et al. 2014a,b, Vaníčková et al. 2015). The authors pinpointed several chemotaxonomic markers whose presence/absence can be used for the identification of the putative species from the FAR complex.

To understand the detailed taxonomical relationships within the FAR complex and to support the evidence on cryptic speciation presented in the aforementioned studies, we aimed to analyse the chemical composition of the volatiles emitted by males and females. The communication signals are highly species-specific and are extremely important in the reproduction isolation of different species. Therefore we assume that the specific volatiles, examined in the present study, together with cuticular hydrocarbons could serve as an effective diagnostic tool.

Methods

Insects

The laboratory-reared populations of *Ceratitis fasciventris*, *C. anonae* and *C. rosa* R2 type were obtained from the International Centre of Insect Physiology and Ecology (ICIPE, Nairobi, Kenya). The pupae were kept under identical laboratory conditions at the Institute of Organic Chemistry and Biochemistry (IOCB, Prague, Czech Republic). Adult flies were fed on an artificial diet consisting of sugarcane:yeast (3:1) and mineral water and were kept at a relative humidity of 60%, at 25 °C, and a 12L:12D photoperiod.

The collection of volatiles

Male and female-borne volatiles of all three species were trapped by the standard dynamic headspace procedures. A group of five virgin 20-day-old male and/or female flies

of each species was placed into round-bottom flasks (250 mL) adapted for volatile collection (Verkon, Praha, Czech Republic). Air was sucked by a pump (Pocket Pump 210 Series, SKC Inc., PA, USA) at 100 mL min^{-1} from a flask through a glass pipette-shaped filter with a sieve located at its thinner end. The filter was filled with a layer of silanised cotton (Applied Science Laboratories, Inc. Bedford, Massachusetts, USA), followed by a SuperQ® (copolymer of ethylvinylbenzene and divinylbenzene, Alltech ARS Inc., Gainesville, Florida, USA) adsorbent layer (*m* = 30 mg), and finished with another layer of glass wool and Teflon ring. The adsorbed volatiles were subsequently rinsed from the filter by 500 µL of freshly distilled HPLC quality *n*-hexane (Lachner, Neratovice, Czech Republic). The extracts were stored in the freezer until chemical analyses.

GC×GC-TOFMS analysis

The male and female-borne headspace volatiles were identified using a LECO Pegasus 4D instrument (LECO Corp., St. Joseph, Michigan, USA). The first dimension column was a weak-polar DB-5 (J & W Scientific, Folsom, California; 30 m × 250 µm i.d. × 0.25 µm film), and the second dimension column was polar BPX-50 (SGE, Austin, Texas; 2 m × 100 µm i.d. × 0.1 µm film). 1 µL of the sample was injected in splitless mode into a constant flow of helium (1 mL min^{-1}), which was used as carrier gas. Injector temperature was 220 °C; temperature in the first dimension was held for 2 min at 40 °C followed by an increase of 5 °C min^{-1} to the target temperature of 270 °C which was held for 10 min. In the second dimension the temperature program was 10 °C higher. The modulation period was 4 s, the hold pulse time was 0.6 s and the cold pulse time between the stages was set to 1.4 s. The modulation temperature offset relative to the GC oven temperature was 30 °C. The temperature of the transfer line connecting the secondary column to the TOFMS detector source was operated at 280 °C. The source temperature was 250 °C with a filament bias voltage of –70 V. The data acquisition rate was 100 scans s^{-1}, with a mass range of 29–400 amu and a detector voltage of 1 650 V. The first to be analysed under the given conditions was a mixture of *n*-alkanes C_8-C_{22} (1×10^{-3} µg μL^{-1}, Sigma-Aldrich), followed by the pheromone samples. LECO ChromaTOF™ is equipped with a retention index (*RI*) calculation function. The identification of analytes was based on a comparison of their mass spectra fragmentation patterns obtained by electron impact ionisation, two-dimensional retention times and retention indices with the standards available and/or previously published data. Not all the authentic standards were available though. In such cases, the identifications were carried out using the reference spectra in the NIST library, the Wiley/NBS Registry of Mass Spectral Data and published *RIs* as well as available literature (Jang et al. 1989, Light et al. 1999, Adams 2007, Vaníčková 2012, Vaníčková et al. 2012, Faťarová 2013). With regards to quantification, raw-area percentages obtained by GC were used to report the relative ratios of active compounds in the pheromone blend.

Chemicals

The following synthetic standards were purchased from Sigma-Aldrich and tested in the concentration 5×10^{-3} µg µL^{-1}: methyl (*E*)-hex-3-enoate, methyl (*E*)-hex-2-enoate, 6-methylhept-5-en-2-one, ethyl hexanoate, ethyl (*E*)-hex-3-enoate, methyl (*E*)-oct-2-enoate, geranyl acetone, (*E,E*)-α-farnesene, methyl (2*E*,6*E*)-farnesoate, linalool, (*E*)-non-2-enal, (*Z*)-non-3-enol, and (*Z*)-non-2-enol. (*Z*)-Non-3-enal and (*Z*)-non-2-enal were prepared in our laboratory from the corresponding alcohols (Hazzard 1973). The purchased and synthesised chemicals were of analytical grade purity.

GC-EAD analyses

1–3 µL of headspace pheromone samples were injected splitless into a HP 5890 A chromatograph (Hewlet Packard, Palo Alto, CA, USA) with a Rxi-5Sil MS column (Restek, Bellefonte, PA; 30 m × 0.25 µm i.d. × 0.25 µm film). The end of the GC column was split into two arms by a Graphpack 3D/2 four-arm splitter (Gertsel Inc., Baltimore, MD, USA), directing the eluate to two detectors working simultaneously – a flame ionisation detector (FID) and an antenna (EAD). Volatile compounds were separated in a continuous helium stream (1 mL min^{-1}). The parameters of the GC oven were similar to the temperature program applied at GC×GC-TOFMS and were as follows: the injector temperature was set to 200 °C and the FID filament temperature was 260 °C. The GC column was operated at a temperature program starting at 40 °C for 2 min, followed by a 10 °C min^{-1} increase until the temperature reached 270 °C, which was held for 10 min. In order to correlate GC×GC-TOFMS and GC-FID/EAD data, RI_{EAD} were calculated using a standard mixture of *n*-alkanes C_8 - C_{22} (1×10^{-3}µg µL^{-1}) injected and analysed under the same conditions as the pheromone samples in both GC×GC-TOFMS and GC-FID/EAD systems.

The fruit fly antennal detector (EAD) was prepared by cutting off the head of a narcotised fly (virgin, 20 days old) and fixing it between two Ag/AgCl glass microelectrodes containing Ringer's solution. The reference electrode was inserted into the head capsule and the recording one was positioned to make a contact with the sensory epithelium on the last antennomere surface. The antennal preparation was then placed in a continual air stream (1 L min^{-1}) blowing from a glass tube (8 mm in diameter), in which the split GC eluate was directed. The electrical signal generated by the antennal preparation was led to a high impedance pre-amplifier (10^{14} Ω; 10x amplification, SYNTECH Equipment and Research, Kirchzarten, Germany) and fed to a PC. The data were evaluated using Syntech GC-EAD software, where signals from FID and EAD were displayed and analyzed simultaneously. Not all FID peaks elicited EAD responses. When some FID peaks were associated with EAD activity in at least 3 independent GC-EAD experiments, the compound was classified as biologically active.

To determine the antennal specificities of the three species studied, subsequent GC-EAD analyses were performed on *C. fasciventris*, *C. anonae* and *C. rosa* with equal doses of synthetic standards (10 ng). From these experiments, FID/EAD ratios were calculated from FID and EAD peak areas and compared for different compounds and species.

Statistics

The data obtained from the chemical analysis ($N = 7$) of male emanations for each species were statistically evaluated. For the statistical analyses, the peak areas of the 22 common compounds identified in the volatile mixtures released by 20 day-old males of all three studied species of the FAR complex were used. For further analysis, only the 12 antennally active compounds were used. The differences in the chemical composition of the samples from all of the three species were analysed by principal component analysis (PCA). Prior to the PCA analysis, the peak areas were subjected to logarithmic transformation, scaling was focused on inter-species correlation, each species score was divided by its standard deviation and the data were centered by species. In the PCA analyses, samples with similar chemical profiles cluster together and segregate from those that are different. PCA was employed for unimodal data while correspondence analyses (CA) were used for linear data. The multivariate data analysis software CANOCO 4.5 (Biometris, Plant Research International, Wageningen UR, The Netherlands) was used for both the PCA and CA.

Results

Chemical analyses of headspace extracts of the FAR complex

Fruit fly male-borne volatiles of *Ceratitis fasciventris*, *C. anonae*, *C. rosa* R2 type were highly complex, qualitatively and quantitatively diverse mixtures (Figure 1). GC×GC-TOFMS analysis identified 35 compounds in *C. fasciventris*, 18 compounds in *C. anonae* and 26 compounds in *C. rosa* R2 type (Suppl. materials 1–3: Table1–3). The mean of the total chromatographic peak areas of the identified male volatiles of *C. fasciventris, C. anonae* and *C. rosa* R2 type reached 2.27×10^8, 6.35×10^7, and 4.06×10^7, respectively. The volatile blends contained diverse chemical structures, including alcohols, aldehydes, terpenes, and esters. Out of all identified male-specific pheromone compounds, 11 were found in all studied species in varying abundance and 22 compounds were common for at least 2 species. In *C. fasciventris* the major component (≥ 8%) was ethyl (*E*)-3-hexenoate (71.5 %) together with methyl (*E*)-hex-3-enoate (13.6 %) and minor components (1-8%) were methyl (2*E*,6*E*)-farnesoate (4.9 %), nonan-2-ol (1.6 %), γ-valerolactone (1.1 %), and ethyl (*E*)-hex-2-enoate (1 %) (Suppl. material 1: Table 1). In *C. anonae*, the major compounds were (*E*,*E*)-α-farnesene (67 %) and methyl (*E*)-hex-2-enoate (14.5 %). The minor components identified comprised

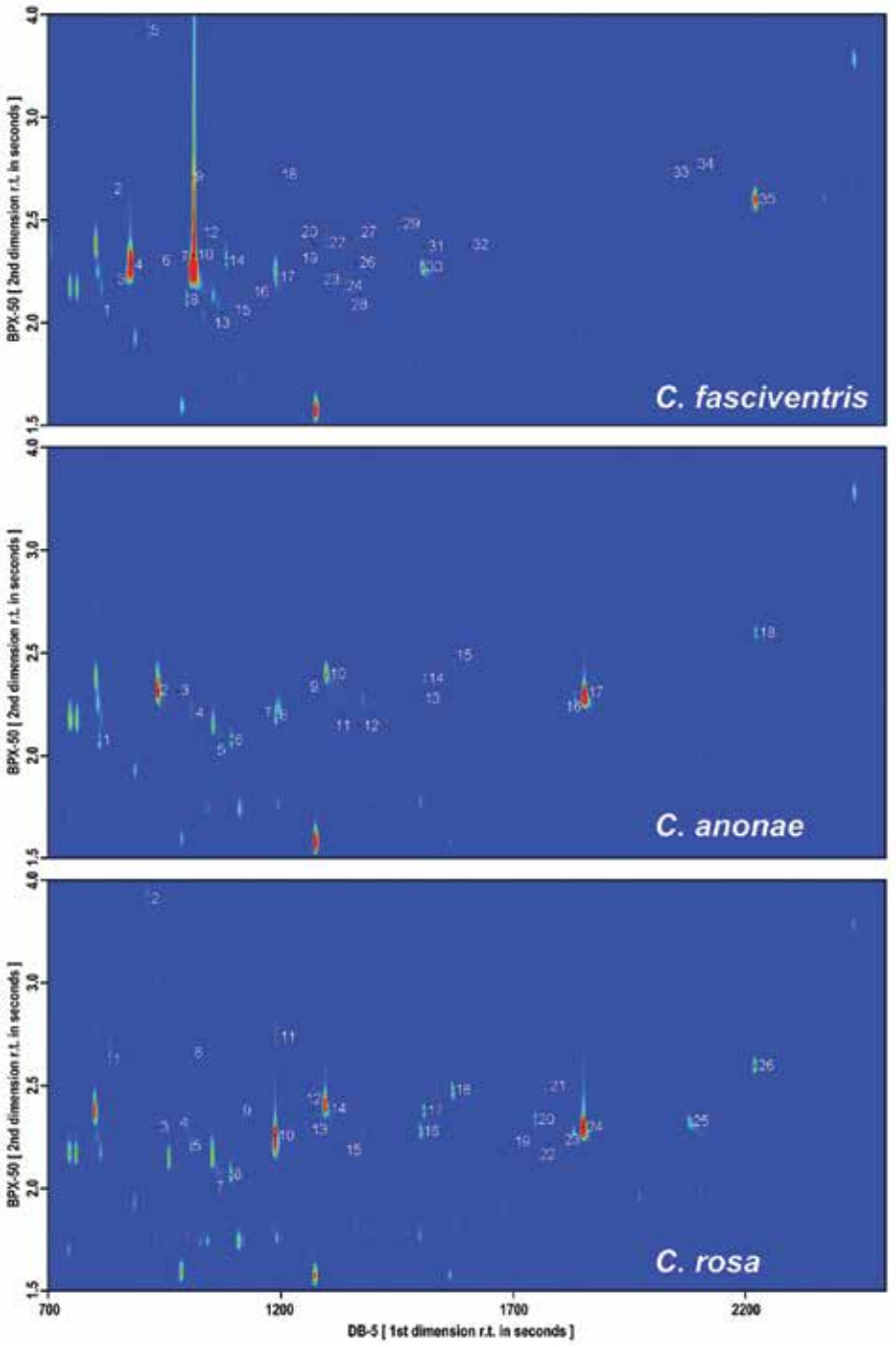

Figure 1. GC×GC-TOFMS chromatograms (TIC mode) of the male ($N = 5$) volatiles of *C. fasciventris*, *C. anonae* and *C. rosa*. Each spot represents one compound; the identified compounds are numbered in each chromatogram, with the numbering corresponding to the respective Table 1 of compounds. The intensity of each spot is colour-coded (blue - 0, red - maximum).

(*E*)-non-2-enal (6.9 %), methyl (2*E*,6*E*)-farnesoate (1.9 %), linalool (1.7 %), (*E*)-β-ocimene (1.6%), heptan-2-ol (1.4 %), (*Z*,*E*)-α-farnesene (1.1 %), and octen-3-ol acetate (1 %) (Suppl. material 2: Table 2). In *C. rosa*, the major components were linalool (47.8 %) and (*E*)-non-2-enal (15.9 %). Its minor compounds were e.g. (*E*,*E*)-α-farnesene (6.4 %), (*E*)-β-ocimene (5.2 %) and 6-methylhept-5-en-2-one (5 %) (Suppl. material 3: Table 3). The remaining compounds identified in the male emanation of the three species studied were present in trace amounts (Suppl. materials 1-3: Table 1–3). Qualitative analysis proved that male pheromone components were not present in female volatile emanations. No female specific volatiles were detected by GC×GC-TOFMS or by GC-EAD qualitative analysis.

Ceratitis fasciventris was found to have the highest number of species-specific compounds that were not found in other two species (Table 1, Suppl. material 1: Table 1). Pheromone specificity in this fruit fly was based on the presence of 17 compounds, e.g. saturated and unsaturated esters of isomers of hexenoic acid, specifically methyl (*E*)-hex-3-enoate (*RI* = 932), ethyl hexanoate (*RI* = 997), ethyl (*E*)-hex-3-enoate (*RI* = 1003), ethyl (*E*)-hex-2-enoate (*RI* = 1045), and methyl (*Z*)-oct-3-enoate (*RI* = 1131). These esters were absent in *C. rosa* and *C. anonae* male pheromone emanations (Table 1, Suppl. materials 2 and 3: Table 2, 3). The only ester of hexenoic acid shared by all three species studied was methyl (*E*)-hex-2-enoate (*RI* = 968). Furthermore, *C. fasciventris* did not emit isomers of α or β-farnesenes when compared with the other two species (Suppl. material 1: Table 1). Interestingly, *C. anonae* had no specific compounds. *C. rosa* had six species-specific compounds: (*E*)-oct-2-enal (*RI* = 1062), (*Z*)-non-3-enol (*RI* = 1158), β-elemene (*RI* = 1406), β-caryophyllene (*RI* = 1442), geranyl acetone (*RI* = 1456), and (*Z*)-β-farnesene (*RI* = 1458).

Electrophysiological analyses

The biological activity of the male volatile components present in the headspace samples was examined using female and male antennae and the GC-EAD technique. The antennal depolarisation was triggered by 12 compounds in total (Table 1, Figure 2). Out of these, five components, methyl (*E*)-hex-2-enoate (RI_{EAD} = 966), 6-methylhept-5-en-2-one (RI_{EAD} = 989), linalool (RI_{EAD} = 1104), (*E*)-non-2-enal (RI_{EAD} = 1163) and methyl (2*E*,6*E*)-farnesoate (RI_{EAD} = 1799), elicited biological activity in all of the species studied (Table 1, Figure 2). Five EAD-active compounds were specific for *C. fasciventris*, methyl (*E*)-hex-3-enoate (RI_{EAD} = 937), ethyl hexanoate (RI_{EAD} = 999), ethyl (*E*)-hex-3-enoate (RI_{EAD} = 1006), ethyl (*E*)-hex-2-enoate (RI_{EAD} = 1045) and methyl (*Z*)-oct-3-enoate (RI_{EAD} = 1131), whereas *C. rosa* had one EAD-specific compound – geranyl acetone (RI_{EAD} = 1459). (*E*,*E*)-α-Farnesene (RI_{EAD} = 1507) was EAD-active compounds shared by *C. anonae* and *C. rosa*.

There was no correlation between the amplitude of the EAD response and the relative abundance of the volatiles identified from the headspace male pheromone analysis. Among the three FAR complex species, the ranking of the relative EAD responses was

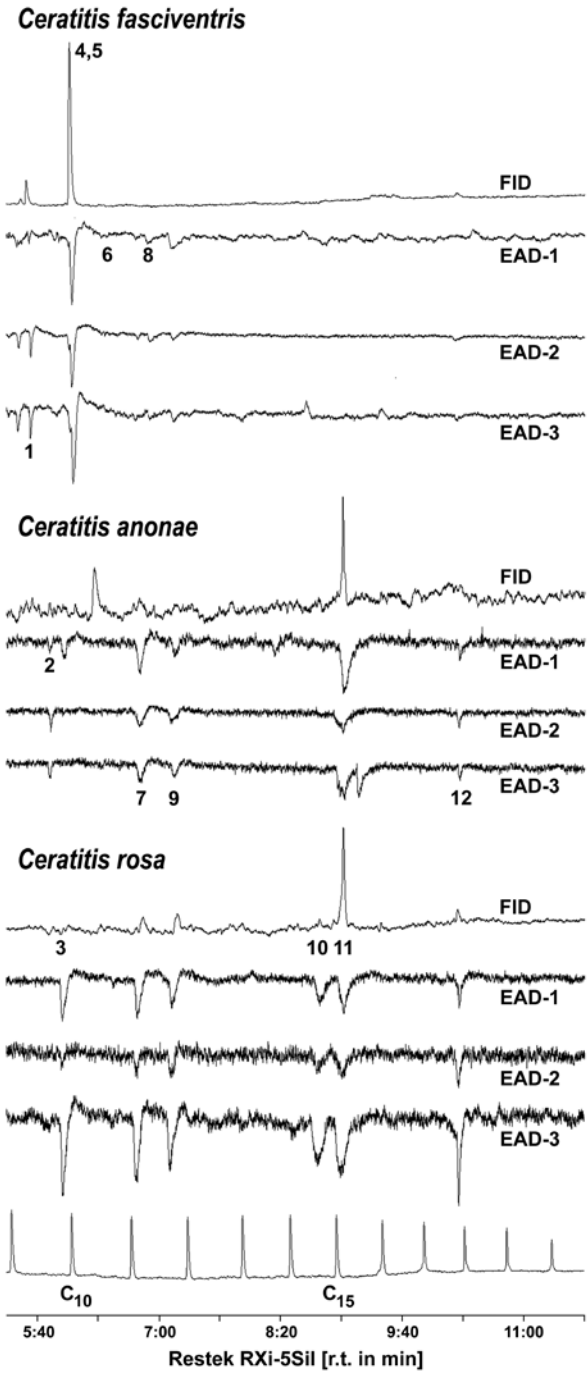

Figure 2. GC-FID/EAD analyses of the *Ceratitis fasciventris*, *C. anonae*, and *C. rosa* male-borne volatiles using a conspecific female antenna as an EAD detector. The numbers indicate EAD-active compounds and correspond to Table 1. The symbols EAD-1-3 denote the three independent repetitions of the GC-EAD analyses.

Table 1. Male-borne volatiles and their relative percentage (Area±SD) of antennaly active compounds found in the emanations of *Ceratitis fasciventris*, *C. anonae* and *C. rosa* (100% is represented by the total area of all antennaly active compounds in each respective species).

No	Compound	*RI*	RI_{EAD}	*C. fasciventris*	*C. anonae*	*C. rosa*
1	Methyl (*E*)-hex-3-enoate	932	937	14.54 ± 2.17	-	-
2	Methyl (*E*)-hex-2-enoate	968	966	0.17 ± 0.02	14.08 ± 3.99	0.36 ± 0.45
3	6-Methylhept-5-en-2-one	988	989	0.03 ± 0.01	0.07 ± 0.08	6.59 ± 4.77
4	Ethyl hexanoate	997	999	0.93 ± 0.06	-	-
5	Ethyl (*E*)-hex-3-enoate	1003	1006	76.48 ± 4.38	-	-
6	Ethyl (*E*)-hex-2-enoate	1045	1045	1.12 ± 0.28	-	-
7	Linalool	1104	1104	0.55 ± 0.04	1.98 ± 0.57	62.44 ± 12.41
8	Methyl (*Z*)-oct-3-enoate	1131	1131	0.10 ± 0.02	-	-
9	(*E*)-Non-2-enal	1167	1163	0.83 ± 0.39	6.81 ± 1.56	20.82 ± 18.70
10	Geranyl acetone	1456	1459	-	-	0.62 ± 0.01
11	(*E,E*)-α-Farnesene	1507	1507	-	74.81 ± 20.93	8.31 ± 0.86
12	Methyl (2*E*,6*E*)-farnesoate	1798	1799	5.26 ± 0.91	2.25 ± 0.82	0.86 ± 0.26

RI retention index identified by GC×GC-TOFMS; RI_{EAD} retention index of antennaly active compounds identified using GC-FID/EAD.

specific for the respective species (Figure 3). The effectiveness of individual compounds is expressed as an area ratio of the respective FID and EAD responses obtained from GC-EAD analyses. The higher the FID/EAD ratio, the higher the antennal sensitivity to the respective synthetic compound ($N = 3$). In *C. fasciventris*, females respond with the highest sensitivity to ethyl (*E*)-hex-3-enoate, the most abundant compound in the *C. fasciventris* male pheromone. Methyl (*E*)-hex-3-enoate is the second most abundant compound eliciting the second highest antennal response in this species, followed by ethyl hexanoate, linalool, and (*E*)-non-2-enal. Methyl (2*E*,6*E*)-farnesoate was the least effective compounds among all the synthetic standards tested in *C. fasciventris*. Among antennaly active components of *C. anonae* pheromone, (*E,E*)-α-farnesene is the most abundant, but it does not yield prominent response in *C. anonae* males. Instead, the *C. anonae* responded almost equally to ethyl-(*E*)-hex-2-enoate and ethyl-(*E*)-hex-3-enoate, followed by linalool, (*E*)-non-2-enal, methyl (*E*)-hex-2-enoate, and methyl (*E*)-hex-3-enoate. The less efficient compound along with (*E,E*)-α-farnesene is methyl (2*E*,6*E*)-farnesoate. The antennae of *C. rosa* females revealed prominent response to (*E*)-non-2-enal. Other compounds like ethyl (*E*)-hex-2-enoate, ethyl hexanoate, methyl (*E*)-hex-2-enoate, methyl (*E*)-hex-3-enoate, linalool, methyl (*Z*)-oct-3-enoate, geranyl acetone, 6-methylhept-5-en-2-one, (*E,E*)-α-farnesene, and methyl (2*E*,6*E*)-farnesoate were less active.

The female antennae of the three species have distinct specificities related to a conspecific pheromone (Figure 3). Nevertheless, they can also perceive pheromone components of the other two species. Both male and female antennae respond to the male-borne volatiles (data not presented).

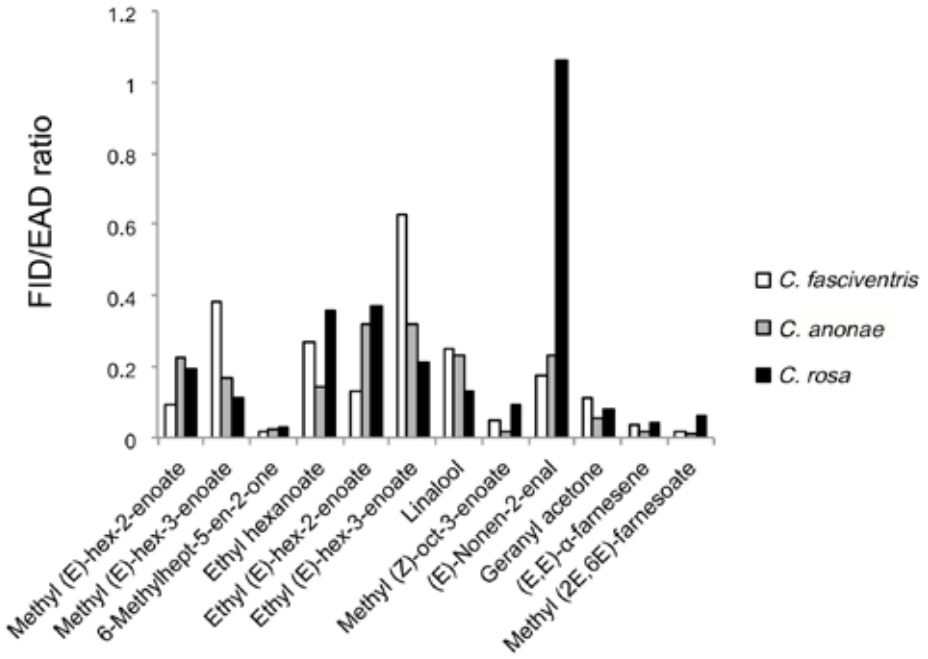

Figure 3. A comparison of female antennal responses of *Ceratitis fasciventris*, *C. anonae*, and *C. rosa* to standard solutions. The FID/EAD on the y-axis represents the ratio between an electroantennographic response and a conventional detector. The higher the number, the higher the response ($N = 3$).

PCA and CA analyses

The results of the principal component analyses are depicted in Figure 4A. The PCA shows a clear separation of the three species, indicating that the composition of the male pheromones is specific in each of the species. The two principal components (PC1 and PC2) together accounted for 97% of the total variability. The 22 common pheromone compounds are presented by the numbers in italics, which stand for the particular retention indices (Suppl. materials 1-3). The compounds specific for *C. fasciventris* male emanations were 2,5-dimethylpyrazine (*RI* = 914), γ-valerolactone (*RI* = 956), ethyl (*E*)-hex-3-enoate (*RI* = 1106), 2,3,5-trimethylpyrazine (*RI* = 1108) and (Z)-β-ocimene (*RI* = 1040). The *C. anonae* pheromone was characterised by octanal (*RI* = 1006), (E)-non-2-enal (*RI* = 1167), oct-3-enyl acetate (*RI* = 1292), methyl geranate (*RI* = 1329), (*Z,E*)-α-farnesene (*RI* = 1491), and (*E,E*)-α-farnesene (*RI* = 1507). 6-Methylhept-5-en-2-one (*RI* = 988), (*E*)-β-ocimene (*RI* = 1051), linalool (*RI* = 1104) and (*Z*)-non-2-enal (*RI* = 1151) were the compounds specific for the *C. rosa* male pheromone.

The multivariate correspondence analyses (CA) of the 12 EAD-active compounds identified by GC×GC-MS and GC-FID/EAD are depicted in Figure 4B. The results reveal species-specific EAD compounds identified by their retention indices. The FAR complex species formed three segregated groups. The CA analyses have revealed exclusive

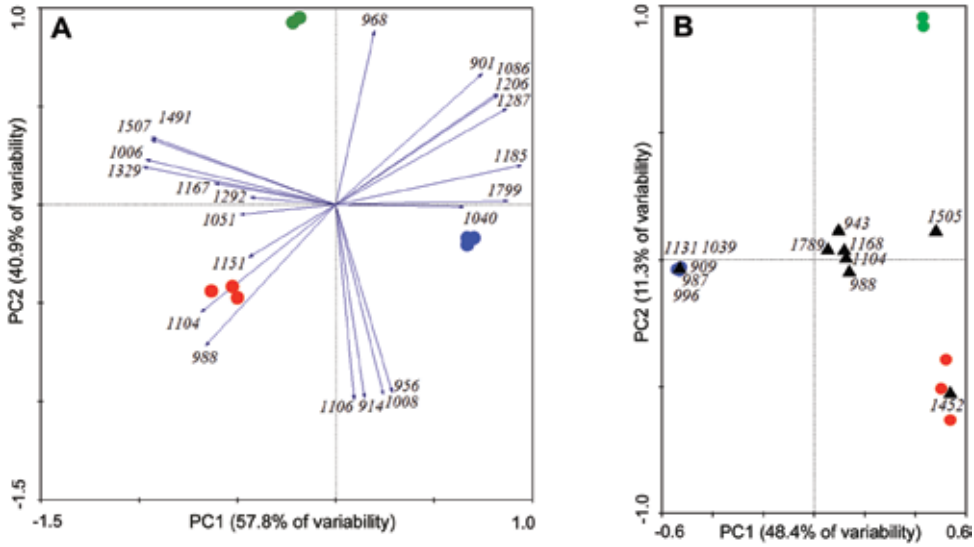

Figure 4. The results of statistical analyses of the male-borne volatiles produced by *Ceratitis fasciventris* (blue), *C. anonae* (green) and *C. rosa* (red). (**A**) Multivariate principal component analysis (PCA) of the 22 common compounds identified in the pheromone of the males of the FAR complex. (**B**) Multivariate correspondence analysis (CA) of the 12 antennal active compounds. The three species are clearly segregated. Each symbol on the plot represents one sample. The numbers in italics denote the retention indices (*RI*) of the species-specific compounds. For the structural identification of the compounds see the Suppl. materials 1–3: Tables 1–3.

compounds for particular species. Methyl (*E*)-hex-3-enoate (RI_{EAD} = 932), ethyl hexanoate (RI_{EAD} = 996), ethyl (*E*)-hex-2-enoate (RI_{EAD} = 1045) and methyl (*Z*)-oct-3-enoate (RI_{EAD} = 1131) are characteristic for the aeration extract from *C. fasciventris*. The *C. anonae* emanation also has typical compounds: methyl (*E*)-hex-2-enoate (RI_{EAD} = 968), (*E*)-non-2-enal (RI_{EAD} = 1163), (*E*,*E*)-α-farnesene (RI_{EAD} = 1507), and methyl (2*E*,6*E*)-farnesoate (RI_{EAD} = 1799). *C. rosa* is defined by geranyl acetone (RI_{EAD} = 1459) and 6-methylhept-5-en-2-one (RI_{EAD} = 988). Terpene linalool (RI_{EAD} = 1104) is shared by *C. anonae* and *C. rosa.*

Discussion

The present study provides the first identification and biological evaluation of volatiles produced by the *Ceratitis* FAR complex species. Our data show that pheromones in the study species are produced exclusively by males and are, like in other fruit fly species (Aluja and Norrbom 2001), highly complex species-specific mixtures characterized by specific qualitative and quantitative profiles of diverse chemical structures, including alcohols, aldehydes, terpenes, and esters.

The techniques applied in the present study (headspace collection of insect volatiles, GC×GC-TOFMS, GC-EAD) allowed for detail analyses and identification of

the specific volatiles produced by the FAR complex species. The headspace technique using the glass filter with the adsorbent is suitable when the extract from the same aeration/sample needs to be analysed by different approaches, e.g. GC-MS, GC×GC-TOFMS, GC-EAD, and behavioral assays (Vaníčková et al. 2012, Břízová et al. 2013, Gonçalves et al. 2013, Milet-Pinheiro et al. 2015). On the other hand the SPME fiber could be also use for the qualitative analyses of the fruit fly emanation (Alfaro et al. 2011, Cruz-López et al. in press). Nevertheless due to impossible storage of the SPME fiber for further laboratory bioassays, the dynamic headspace technique was preferred.

Our GC×GC-TOFMS analysis of the *Ceratitis* FAR complex pheromones resulted in the identification of 35 compounds produced by *C. fasciventris* males, 18 compounds released by *C. anonae* males and 26 volatiles emitted by *C. rosa* R2 type males. The composition of the male sex pheromones in the three species partially overlaps (11 compounds were shared among all three species, but were present in species-specific quantities). In addition to common compounds, the three respective species released also species-specific compounds. *Ceratitis fasciventris* had the highest number of specific compounds. The pheromone specificity in this fruit fly is based on the presence of saturated and unsaturated methylated and ethylated esters of hexenoic acid, specifically methyl (*E*)-hex-3-enoate, ethyl hexanoate, ethyl (*E*)-3-hexenoate and ethyl (*E*)-2-hexenoate. These esters are absent from *C. rosa* and *C. anonae* male pheromone emanations. The only ester of hexenoic acid shared by all three studied species is methyl (*E*)-hex-2-enoate. Furthermore, the males of *C. fasciventris* do not emit isomers of α or β-farnesene. *Ceratitis anonae* has no specific compound. The male pheromone of *C. rosa* has 6 species-specific compounds: (*E*)-oct-2-enal, (*Z*)-non-3-enol, β-elemene, β-caryophyllene, geranyl acetone and (*Z*)-β-farnesene.

Among all identified compounds in the *Ceratitis* FAR complex species, only a relatively small set of 12 compounds elicited antennal responses suggesting their prominent roles in pheromone communication. Biological activity was elicited by four compounds found in emanations of all three species studied, namely methyl (*E*)-hex-3-enoate, 6-methylhept-5-en-2-one, linalool, (*E*)-non-2-enal and methyl (2*E*,6*E*)-farnesoate. These shared compounds are present in the respective species in quite different amounts. In addition to the shared antennally active compounds, (*E,E*)-α-farnesene triggered antennal responses in the pheromones of *C. anonae* and *C. rosa* and geranyl acetone was an active component of the *C. rosa* pheromone.

GC-EAD data have shown that the females of the three investigated species of the *Ceratitis* FAR complex perceive the components of conspecific pheromones, but are also able to perceive the pheromone components of the other two species. The antennal responses of individual species differ significantly and are species-specific. Both males and females can perceive the male pheromone.

Many of the *Ceratitis* FAR complex male-borne volatiles identified here [e.g. 2,5-dimethylpyrazine, 6-methyhept-5-en-2-one, ethyl (*E*)-oct-3-enoate, (*Z*)-β-ocimene, (*E*)-β-ocimene, linalool, geranyl acetone, and α-farnesenes] have been previously reported as a part of male emanations of other fruit fly pheromones (Jang et al. 1989, Flath et al. 1993, Light et al. 1999, Cossé et al. 1995, Alfaro et al. 2011,

Vaníčková 2012, Vaníčková et al. 2012). Nevertheless, some of the components, e.g. methyl (2*E*,6*E*)-farnesoate and (*E*)-non-2-enal, are reported here for the first time as constituents of male emanations in the genera *Ceratitis.* Among antennaly active compounds, saturated and unsaturated ethylated and methylated esters of hexenoic acid represent volatile components of many kinds of fruits and food. Some of them were reported as constituents of the pheromones of the Mediterranean fruit fly, *C. capitata* (Jang et al. 1989, Light et al. 1999, Vaníčková 2012, Vaníčková et al. 2012), *Anastrepha ludens* and *A. obliqua* (Robacker 1988, López-Guillén et al. 2011). An ubiquitous plant compound, 6-methyhept-5-en-2-one was also determined as an antennally active component of the male sex pheromone in *C. capitata* (Vaníčková 2012, Vaníčková et al. 2012). A widely distributed plant volatile, (*E*,*E*)-α-farnesene, is an antennally and behaviourally active pheromone component in four tephritid species *C. capitata* (Jang et al. 1989, Heath et al. 1991, Vaníčková 2012, Vaníčková et al. 2012), *A. ludens*, *A. suspensa* (Rocca et al. 1992, Lu and Teal 2001) and *A. fraterculus* (Lima-Mendonça et al. 2014, Milet-Pinheiro et al. 2015). Isoprenoid geranyl acetone, aliphatic volatile (*E*)-non-2-enal and methyl (2*E*,6*E*)-farnesoate have not yet been reported in any fruit fly male sex pheromone. Geranyl acetone and (*E*)-non-2-enal are widely distributed plant volatiles that might be sequestrated from the plants to become part of the pheromone. What is very interesting is the presence of methyl (2*E*,6*E*)-farnesoate, which is a Crustacean reproductive hormone that is structurally similar to the insect juvenile hormone. In Crustacea, methyl (2*E*,6*E*)-farnesoate is responsible for enhancing reproductive maturation, maintaining juvenile morphology, and influencing male sex determination (Olmstead and LeBlanc 2007, Nagaraju and Borst 2008). In insects, methyl (2*E*,6*E*)-farnesoate is an immediate precursor of the insect juvenile hormone III (Teal et al. 2014) with the same function as in Crustacea. As a semiochemical, methyl (2*E*,6*E*)-farnesoate was reported only once in the pentatomid bug pheromone (Millar et al. 2002).

Conclusion

Our data show that the male-borne volatile profiles of the studied species of *C. fasciventris*, *C. anonae* and *C. rosa* differ both qualitatively and quantitatively. Also antennal responses to volatile compounds are species-specific in the three species studied. Therefore, the pheromone composition as well as electroantennography may be used as specific tools for the FAR complex species delimitation. Our findings are in agreement with recent studies on the cuticular hydrocarbon profiles of *C. fasciventris*, *C. anonae*, *C. rosa* R1 and R2 type and *C. capitata*, which shows that the cuticular fingerprints are species and sex-specific (Vaníčková 2012, Vaníčková et al. 2014a,b, Vaníčková et al. 2015). Like the composition of cuticular hydrocarbons, also pheromone composition and antennal specificity suggest that the *Ceratitis* FAR complex species are taxonomically well-defined entities.

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Supplementary material 1

Table 1

Authors: Radka Břízová, Lucie Vaníčková, Mária Faťarová, Sunday Ekesi, Michal Hoskovec, Blanka Kalinová

Data type: Adobe PDF file

Explanation note: Compounds, their relative percentage (Area±SD), and chemical characteristics identified by GC×GC-TOFMS and GC-FID/EAD in the headspace extracts of the calling males of *Ceratitis fasciventris*.

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Supplementary material 2

Table 2

Authors: Radka Břízová, Lucie Vaníčková, Mária Faťarová, Sunday Ekesi, Michal Hoskovec, Blanka Kalinová

Data type: Adobe PDF file

Explanation note: Compounds, their relative percentage (Area±SD), and chemical characteristics identified by GC×GC-TOFMS and GC-FID/EAD in the headspace extracts of the calling males of *Ceratitis anonae*.

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Supplementary material 3

Table 3

Authors: Radka Břízová, Lucie Vaníčková, Mária Faťarová, Sunday Ekesi, Michal Hoskovec, Blanka Kalinová

Data type: Adobe PDF file

Explanation note: Compounds, their relative percentage (Area±SD), and chemical characteristics identified by GC×GC-TOFMS and GC-FID/EAD in the headspace extracts of the calling males of *Ceratitis rosa*.

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An integrative approach to unravel the *Ceratitis* FAR (Diptera, Tephritidae) cryptic species complex: a review

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Abstract

This paper reviews all information gathered from different disciplines and studies to resolve the species status within the *Ceratitis* FAR (*C. fasciventris*, *C. anonae*, *C. rosa*) complex, a group of polyphagous fruit fly pest species (Diptera, Tephritidae) from Africa. It includes information on larval and adult morphology, wing morphometrics, cuticular hydrocarbons, pheromones, microsatellites, developmental physiology and geographic distribution. The general consensus is that the FAR complex comprises *C. anonae*, two species within *C. rosa* (so-called R1 and R2) and two putativte species under *C. fasciventris*. The information regarding the latter is, however, too limited to draw final conclusions on specific status. Evidence for this recognition is discussed with reference to publications providing further details.

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Keywords
Taxonomy, *Ceratitis rosa*, *Ceratitis fasciventris*, *Ceratitis anonae*, Africa, fruit fly

Introduction

Historical background and systematic position

Ceratitis MacLeay is an Afrotropical genus of tephritid fruit flies comprising close to 100 species found in Sub-Saharan Africa and the islands of the Western Indian Ocean. Phylogenetically it belongs to the subtribe Ceratitidina within the tribe Dacini. The latter tribe includes all main pest genera occurring naturally in Africa, i.e. *Bactrocera* Macquart, *Capparimyia* Bezzi, *Ceratitis*, *Dacus* Fabricius, *Neoceratitis* Hendel and *Trirhithrum* Bezzi. However, the monophyly of the genus is not supported and some species appear phylogenetically more closely related to *Trirhithrum*, while other *Ceratitis* species probably do not belong to the genus (Barr and McPheron 2006, Virgilio et al. 2015). Contrary to other indigenous African fruit fly pest groups that have a particular host niche width attacking particular plant families, species of the genus *Ceratitis* demonstrate a very variable host specificity. Some species are largely monophagous, while others form monophyletic stenophagous clusters specialized on particular host genera. Yet, others are polyphagous species attacking a wide variety of unrelated plants (De Meyer et al. 2002, Erbout et al. 2011). For example, the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), is the most extreme case of polyphagy, with 304 different host plants belonging to 154 genera in 57 families considered to be suitable host plants (Liquido et al. 2014).

The *Ceratitis* FAR complex is a group of polyphagous species comprising three morphologically similar species: *C. fasciventris* (Bezzi), *C. anonae* Graham and *C. rosa* Karsch (Figure 1). Although the FAR complex is recognized as a group of closely related species, their monophyly is not always supported in phylogenetic studies. Barr and Wiegmann (2009) presented a molecular analysis for a number of *Ceratitis* (*Pterandrus*) species including one specimen for each *Ceratitis* FAR complex species. They state that CAD1 and ND6 markers supported the monophyly of the complex, while *tango* and *period* markers placed the three species in different clusters within the so-called 'Pterandrus A' group. A majority rule Bayesian tree for the combined molecular data supported the monophyly of the complex. Yet, a cladistics analysis based on adult morphological characters could not recognize them as a monophyletic cluster within the subgenus *Pterandrus* (De Meyer 2005).

Current taxonomic status of recognized species and sexual dimorphism

The Natal fruit fly, *C. rosa*, was originally described from 'Delagoabai' (nowadays Baia de Maputo, Mozambique and probably referring to a location near Maputo) by Karsch in 1887, based on a single male specimen (Karsch 1887). Bezzi (1920) described *fas-*

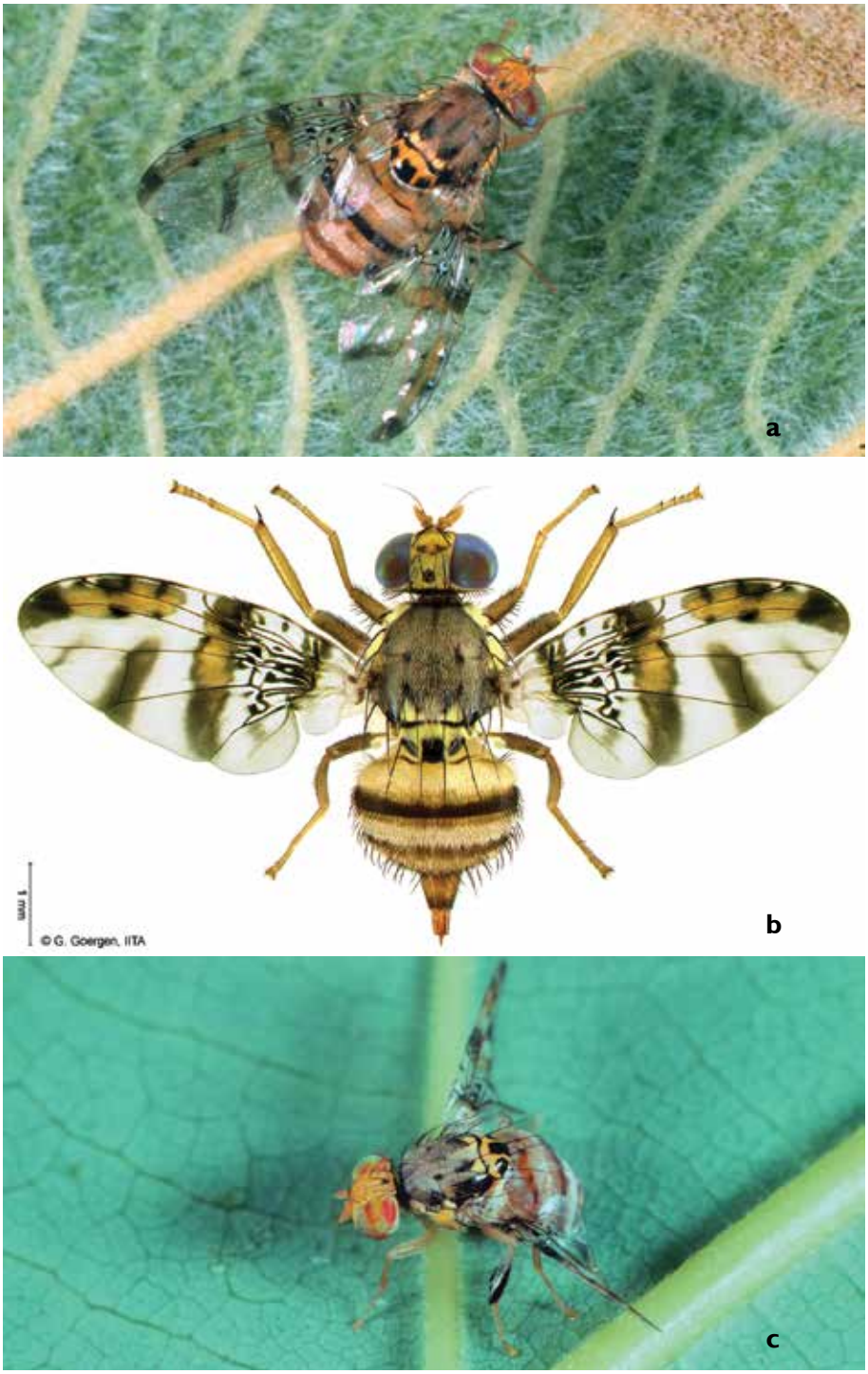

Figure 1. Habitus image of **a** *Ceratitis fasciventris* **b** *C. anonae* **c** *C. rosa* (**a**, **c** copyright R.S. Copeland, **b** copyright G. Goergen).

Figure 2. Mid leg of male *Ceratitis anonae*, anterior view of femur and tibia (copyright I.M. White, NHM).

civentris as a variety of *Pterandrus rosa*, based upon material from Uganda (unknown locality). De Meyer (2001) considered it to be a separate entity with specific status. *Ceratitis anonae* was described by Graham from material reared from an *Annona* fruit in Ashanti, Ghana (Graham 1908).

All three species show remarkable sexual dimorphism, with the males having leg ornamentation that is absent in females. *Ceratitis anonae* males have the mid leg (Figure 2) with a row of long dark, flattened setae (so-called 'feathering') ventrally along the entire length of the mid femur. The mid tibia is broadened with feathering dorsally along distal 0.9 and ventrally along the distal 0.8. The mid leg is largely brownish to brownish black in colour. In *C. rosa* males (Figure 3), the ventral feathering on mid femur is absent (at most there are a few thin and dispersed setulae ventrally). The mid tibiae is moderately broadened, anteriorly black with a conspicuous silvery reflection seen when kept under a certain angle and black feathering dorsally along distal 0.75 and ventrally along distal 0.66–0.75. Otherwise, the leg is yellow. *Ceratitis fasciventris* males have the mid leg shaped similarly to *C. rosa*, except that the mid tibia is not distinctly broadened and the black feathering is restricted to the distal 0.5 at most (Figure 4). The leg is coloured uniformly yellow, except in some specimens where the anterior part is partially brownish in the distal 0.3 (De Meyer 2001). Preliminary courtship behavior studies by Quilici et al. (2002) give some indication that, for at least in *C. rosa*, the mid legs are used in precopulatory behavior.

Figure 3. Mid leg of male *Ceratitis rosa*, anterior view (copyright I.M. White, NHM).

Figure 4. Mid leg of male *Ceratitis fasciventris*, anterior view (copyright I.M. White, NHM).

Unlike the males, females, are morphologically almost indistinguishable. *Ceratitis anonae* females differ from the other two taxa in the pilosity of the anepisternum and fore femur. In *C. anonae* the anepisternum has some few dark setulae medioventrally, and the fore femur has dispersed short dark setulae between the ventral setae and posterior row of setae. In *C. rosa* and *C. fasciventris*, the anepisternal pilosity is completely pale and the fore femur usually only has pale setulae present between the ventral setae and posterior row of setae (De Meyer and Freidberg 2006). Females of *C. rosa* and *C. fasciventris* females cannot be reliably differentiated on morphological characters.

Distribution patterns throughout Africa

The occurrence of the three species throughout Africa shows different distribution patterns with only a partial overlap between some of the species. Although some species do occur sympatrically or parapatrically, nowhere do all three species co-occur. *Ceratitis rosa* is found throughout South (from western Cape onwards) to East Africa with the northernmost records from the Central Highlands in Kenya (Figure 5). The latter occurrence seems to be a recent expansion (Copeland and Wharton 2006) with its previous extension reaching till the Kenyan coast only. It has been introduced to the Indian Ocean Islands of La Réunion and Mauritius in the 20th Century (White et al. 2000). The single record of *C. rosa* (one male) from Cameroon in West Africa is probably due to mislabeling, while the presence of the species in Ivory Coast (N'dépo et al. 2010, 2013) is an erroneous record. No reliable records from western Africa have been found. *Ceratitis fasciventris* has a wider distribution and is found throughout western Africa, with isolated records from Central Africa, and extensive distribution along the Albertine and Gregory Rifts in eastern Africa, as far north as Ethiopia (Figure 5). *Ceratitis anonae* has a predominantly equatorial belt distribution, being widespread through West (from Senegal onwards) and Central Africa (Figure 6). Its easternmost distribution seems to be confined to the western side of the Gregory Rift in Kenya (Copeland et al. 2006) (records further to the east need confirmation).

Sympatric and allopatric occurrence is found between *C. anonae* and *C. fasciventris* in western Africa (Vayssières et al. 2004), and western Kenya (Copeland and Wharton 2006), while *C. rosa* and *C. fasciventris* occur together in parts of Kenya and Tanzania (Figure 5) (Copeland et al. 2006, Mwatawala et al. 2006). *Ceratitis anonae* and *C. rosa* were not found together.

Indications of inconsistencies

Despite the morphological differences in adult males and the partially disjunct distribution, there are indications that cryptic speciation occurs within the three currently recognized morphospecies.

Figure 5. Geographical distribution of *Ceratitis rosa* (blue triangles) and *C. fasciventris* (yellow circles).

Figure 6. Geographical distribution of *Ceratitis anonae.*

When comparing sequences of mitochondrial and nuclear markers, Virgilio et al. (2008) could not recover the three morphospecies as monophyletic groups, although different markers recovered well-supported clades within the representatives of *C. fasciventris* from West and East Africa. So, although the molecular data did not contradict or support the morphological separation, it did suggest that *C. fasciventris* is itself a complex of cryptic species. This study, as well as Douglas and Haymer (2001) and Barr et al. (2006) indicated moreover the existence of other separate clusters within the complex (although not always with high support).

Finally, correlative ecological niche modeling showed that *C. rosa* prefers climatic conditions with lower temperatures when compared to *C. capitata* (De Meyer et al. 2008). This was corroborated by findings on the island of La Réunion where *C. rosa* occupies a colder and more humid climate niche than *C. capitata* (Duyck et al. 2006). However, these results were contradicted by Grout and Stoltz (2007) who studied the developmental thresholds for a number of *Ceratitis* species, based on South African populations and concluded that the situation in South Africa is different from that in La Réunion. This led to the suggestion that *C. rosa* could comprise entities with different biological requirements.

Objective

The inclusion of the *Ceratitis* FAR complex in the Coordinated Research Project (CRP) on cryptic species, therefore, was to establish whether the three morphological entities actually represent three distinct species, or if the relationship can be resolved in a different manner. The authors opted for an integrative approach, using different methodologies, including population genetics, larval morphology, wing morphometrics, cuticular hydrocarbons, developmental physiology and pre- and postzygotic mating compatibility. The results of these different approaches are presented in this volume or have been published elsewhere (Virgilio et al. 2013, Vaníčková et al. 2014). We herewith summarize the main results obtained by each of these approaches and provide a synthesis on the current knowledge and status of the different entities within the *Ceratitis* FAR complex.

Results

Microsatellite analysis

Using microsatellites, Baliraine et al. (2004) revealed significant differentiation with respect to genotypic frequencies between *C. rosa* specimens from the African mainland and the Indian Ocean islands, and between *C. fasciventris* from Kenya and from Uganda. Delatte et al. (2013) isolated and characterized 16 microsatellite markers us-

ing representatives of the three species from 11 localities in Africa. Subsequently, Virgilio et al. (2013) used these microsatellites to survey the allelic variation in 27 African populations of the three morphospecies. Their main finding was that the complex comprises five genotypic clusters, based upon the individual Bayesian assignments of STRUCTURE (Pritchard et al. 2000): a single *C. anonae* genotypic cluster, two clusters for *C. fasciventris* (allo- and parapatric) and two clusters, of *C. rosa* (allo- and sympatric) (Figure 7). The sympatric *C. rosa* clusters were observed in two South African sampling sites (from a sample coverage including *C. rosa* from Malawi, Mozambique, Kenya, la Réunion and Tanzania). The two *C. fasciventris* genotypic clusters roughly corresponded to a western African and an eastern African group, except for a single population from Tanzania that was more genetically similar to the West African samples. The two phylogenetic *C. fasciventris* clades (Virgilio et al. 2008) corresponded to the two microsatellite genotypic clusters. Unfortunately, voucher material of Baliraine et al. (2004) could not be traced (Malacrida, personal communication) so that their microsatellite data could neither be fitted into the patterns observed in Virgilio et al. (2013) nor used for morphological analysis. The results of the microsatellite analysis were used to corroborate the morphospecies hypotheses and to generate new hypotheses which were then further tested through an integrative taxonomical approach.

Adult morphology

The 399 male specimens of the 27 African populations used for the microsatellite analysis were, in light of the above findings, re-examined. Preliminary observations indicated a difference for *C. fasciventris* in the coloration of the male mid tibia with specimens from western Africa demonstrating darker coloration (De Meyer 2001) (henceforth referred to as "F1"; Figure 8a), whereas specimens from eastern populations have completely yellow mid tibia ("F2"; Figure 8b).

Re-examination of the *C. rosa* specimens revealed slight morphological differences with one type bearing a broader and more stout mid tibia with black coloration reaching the lateral margins of the tibia throughout ("R1"; Figure 8c), while the other morphotype bearing a more slender tibia, gradually tapering towards the base, and with the black coloration not reaching the lateral margins throughout the full length ("R2"; Figure 8d).

All males were scored blindly (i.e. without reference to the genotypic cluster they belong to) for these morphological characters. Morphological assignment for R2 and F2 was 100% in alignment with the genotypic clustering while R1 and F1 had, respectively, 3.4% and 10% uncertain assignment (i.e. could not be assigned unambiguously to any of the genotypic clusters) because of doubtful interpretation of the morphological character. None of the specimens examined scored contradictorily to the genotypic cluster. It was concluded that the morphological differences observed in the male mid leg shape and coloration are a reliable character state when combined with genotyping to differentiate the different populations of *C. rosa* and *C. fasciventris*.

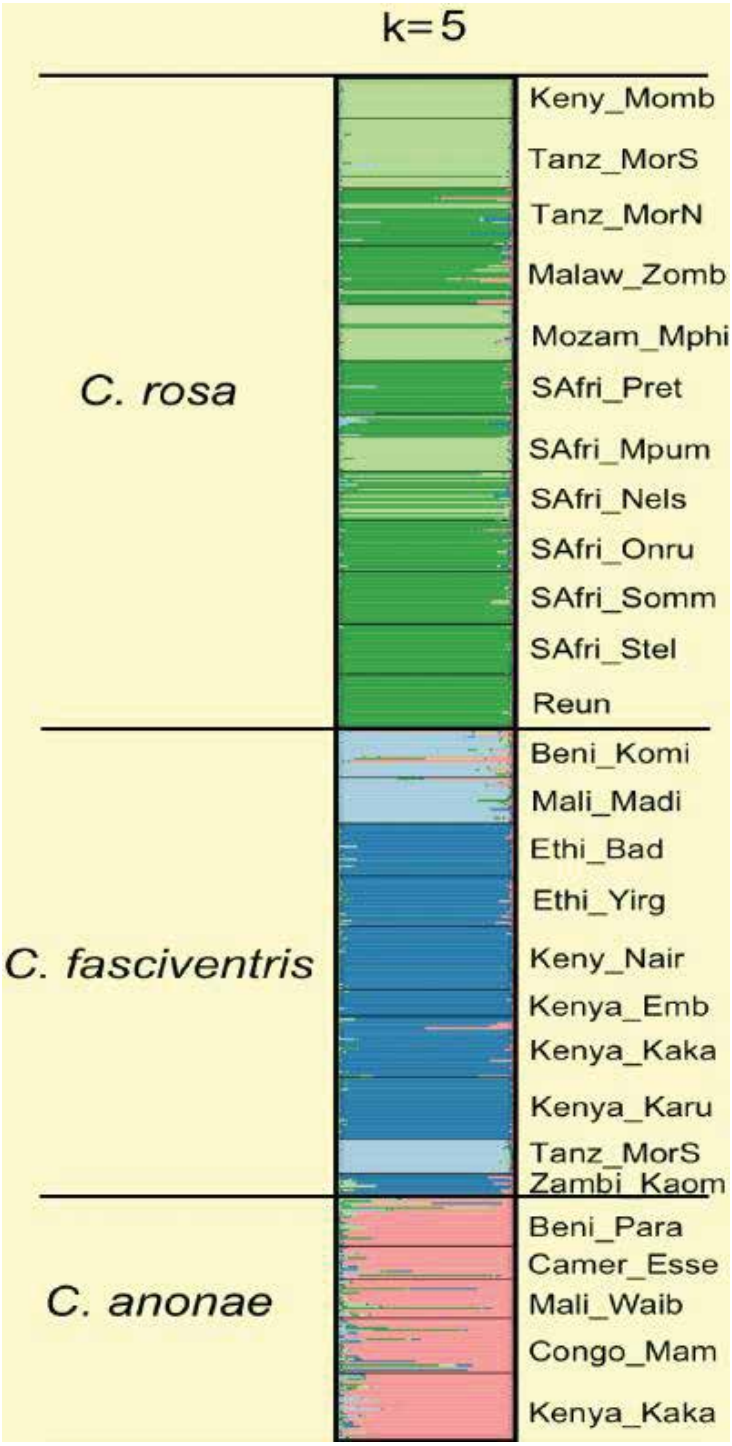

Figure 7. Structure of admixture proportions of 621 specimens of the FAR complex, assigned to five genotypic clusters (from Virgilio et al. 2013).

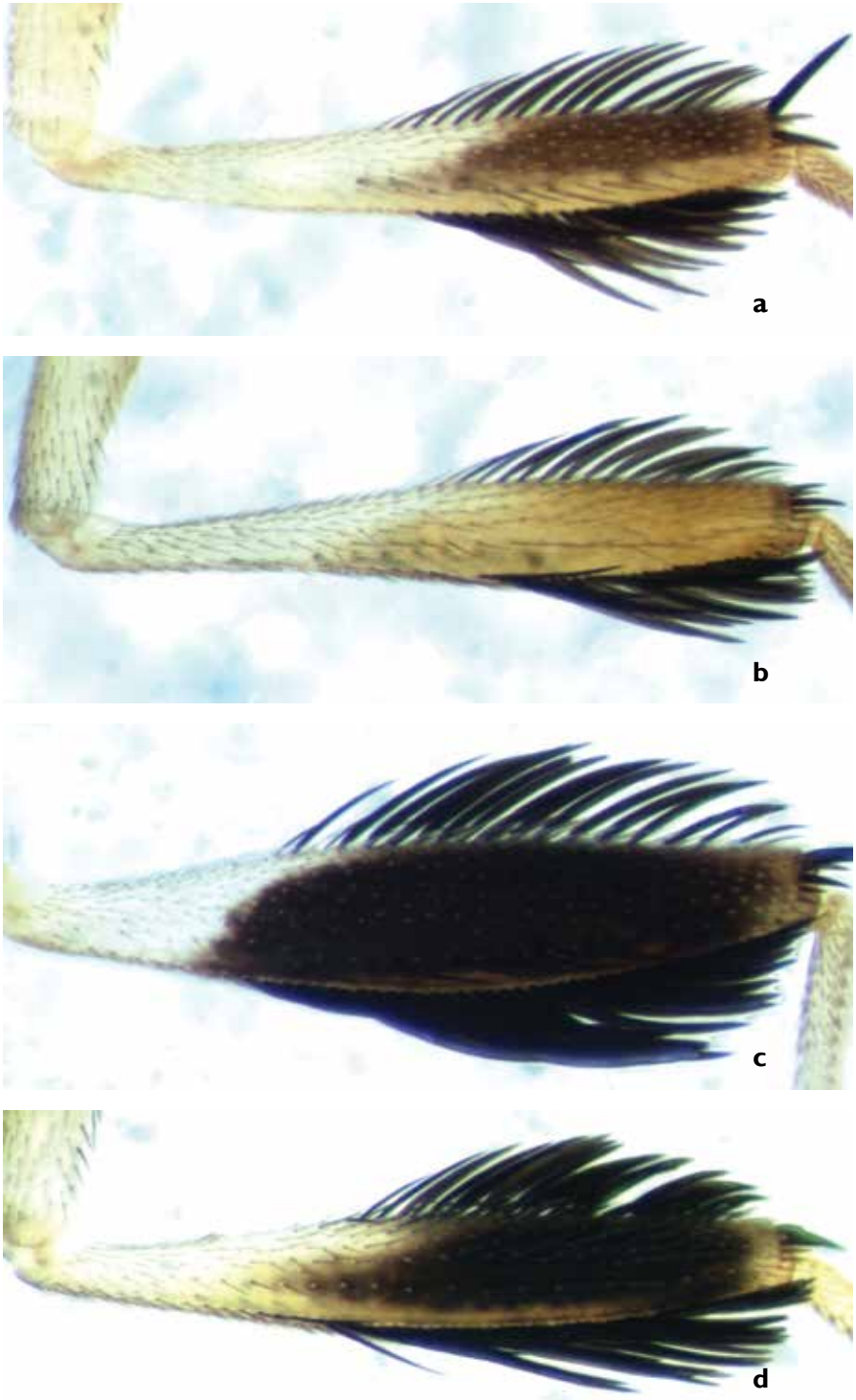

Figure 8. Anterior view of male mid tibia in *Ceratitis fasciventris* (**a** F1, **b** F2) and *C. rosa* (**c** R1, **d** R2).

Wing morphometrics

Since no morphological characters were found to reliably differentiate females neither of the different morphospecies nor of the five genotypic clusters recognized by the microsatellite analysis, a morphometric study was conducted on the wings of the same specimens used for the microsatellite (males and females) and the adult morphology studies (males only). Two techniques were used by Van Cann et al. (2015): landmarking based on intersections between wing veins and cross-veins, and measurement of wing band areas. In total 227 specimens, previously morphologically identified and genotyped at 16 microsatellite loci, were used. Seventeen wing landmarks and six wing band areas were used for morphometric analyses, mainly from unambiguous points such as intersections between veins and cross-veins, or between the former and bands. Significant differences were found both among the three morphospecies as well as among the five genotypic clusters when Permutational Multivariate Analysis of Variance (PERMANOVA) was conducted. Unconstrained and constrained ordinations could not resolve these groups but posterior group membership probabilities (PGMPs) of the Discriminant Analysis of Principal Components (DAPC) showed that wing landmarks (and, to a less extent, wing band areas) could be used to consistently assign a relevant proportion of specimens to morphospecies (range 96.8–98.2%) and genotypic cluster (range 87.5–96.4%). This indicates that wing morphometrics does reflect to a certain extent the clustering and division in different genotypic clusters.

Larval morphology

Recognition of larval stages of fruit fly pests is a necessity given the fact that most quarantine interceptions are larvae inside imported fruits during regulatory checkpoints. However, identification of fruit fly larvae at species level is notoriously difficult because of the limited morphological characters and the high intraspecific variability in character states. The study by Steck and Ekesi (2015) on representatives of the *Ceratitis* FAR complex and comparison with *C. capitata*, has confirmed the difficulties in this matter. Among the different populations studied of *C. fasciventris* (1), *C. anonae* (1) and *C. rosa* (5, including the two genotypic clusters from different geographical regions, i.e. Kenya and South Africa), only larvae of *C. fasciventris* could be unambiguously distinguished from the other species of the FAR complex, based on differences in quantitative measures of numerous larval morphological characters (at least as observed in the single population studied). Although there was also variation observed in diagnostic morphological characters among the larvae of the different *C. rosa* populations studied, the two genotypic clusters (R1 and R2) could not be differentiated consistently. Moreover, characters once considered reliable to separate the larvae of *C. capitata* from *C. rosa* (Carroll, 1998), or even to separate the genus *Ceratitis* from the genus *Bactrocera* (White and Elson-Harris, 1992) have shown to be variable among the different species and populations studied here and not diagnostic for a particular taxon.

Cuticular hydrocarbons

Cuticular hydrocarbons (CHCs) comprise a majority of the components of the cuticular waxes in many insects and may include *n*-alkanes, *n*-alkenes, terminally monomethylalkanes, dimethylalkanes among others (Blomquist and Jackson 1979). The long-chain CHCs play central roles in waterproofing of the insect cuticle and function extensively in chemical communication as sex pheromones, and species and sex recognition cues among others (Blomquist and Bagnères 2010). As a result of their species-specificity, CHCs are widely used for identification of sibling or cryptic species (Kather and Martin 2012). Recent studies on CHC profiles of drosophilid (Jennings et al. 2014) and tephritid flies (Vaníčková 2012, Vaníčková et al. 2015) evaluated the use of CHCs in delineating groups within supposedly cryptic taxa. An initial analysis included one population each of the three morphospecies within the FAR complex and *C. capitata* as a comparative taxon (Vaníčková 2012, Vaníčková et al. 2014). Male and female specimens were obtained from the colonies kept at the International Centre for Insect Physiology and Ecology, ICIPE (for FAR complex) and the FAO/International Atomic Energy Agency Agriculture and Biotechnology Laboratories in Seibersdorf (for *C. capitata*) and were genotyped, following Delatte et al. (2013) and Virgilio et al. (2013). The *C. rosa* population represented the R2 genotypic cluster, while the *C. fasciventris* population represented the F2 genotypic cluster. Using two-dimensional gas chromatography with mass spectrometric detection, differences in the CHC profiles between all three species (but also between sexes) were observed with twelve compounds indicated as possible chemotaxonomical markers to differentiate the three types (Figure 9). After recognition of the two *C. rosa* genotypic clusters (cf above), the analysis was repeated for one population each of the R1 and R2 type obtained from ICIPE colonies (Vaníčková et al. 2015b). This resulted in four potential markers that allow differentiation between the R1 and R2 genotypic clusters. CHCs as such provide indication of qualitative differentiation in their profiles to allow distinction between at least four of the five genotypes (the F1 genotype could not be included because of lack of an established colony that could provide material for analysis). These findings should preferably be further explored by using populations from other regions for the same genotypes as there is evidence that also elements such as diet can have an impact on CHC intraspecific variability.

Pheromones

Pheromones in investigated FAR complex species are produced exclusively by males and are, similarly as in other fruit fly species, highly complex species-specific mixtures characterized by specific qualitative and quantitative profiles of diverse chemical structures, including alcohols, aldehydes, terpenes, and esters. GCxGC-TOFMS analysis of male *Ceratitis* FAR complex pheromones observed in single population samples for each species, resulted in the identification of 35 compounds produced by *C. fasciventris* F2 type, 18 compounds released by *C. anonae* and 26 volatiles emitted by *C. rosa* R2

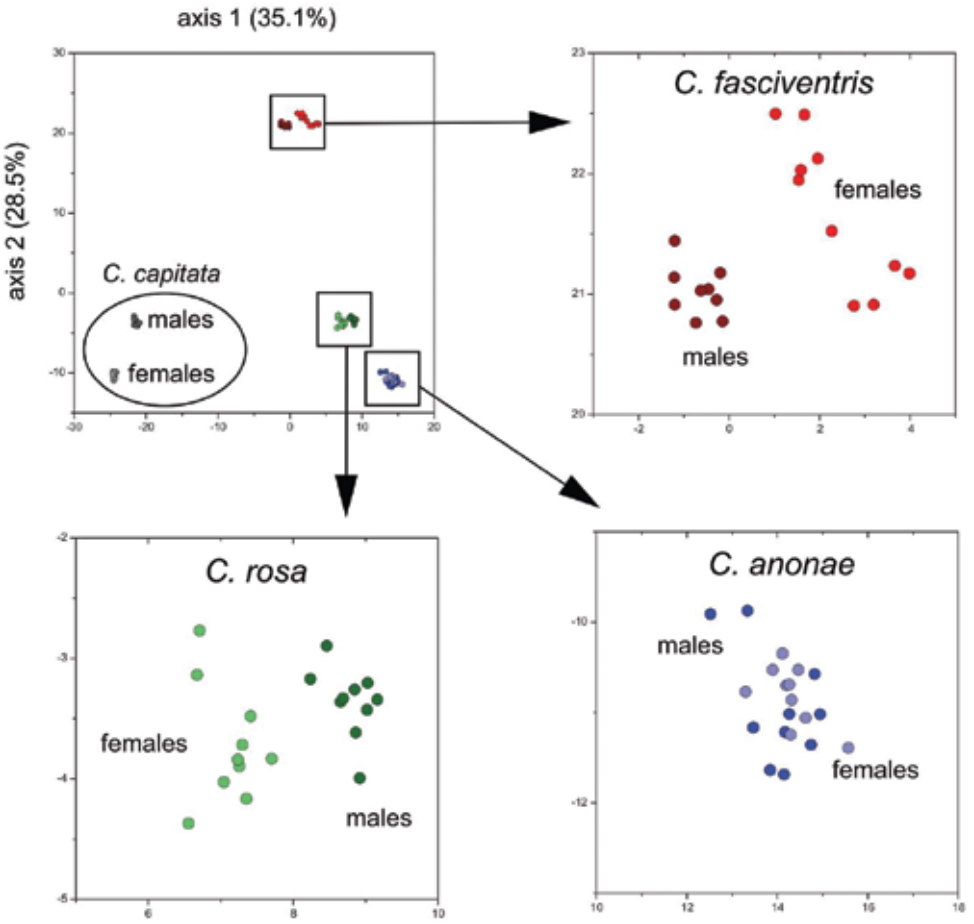

Figure 9. Principal components analysis (PCA) of Euclidean distances between males and females *Ceratitis capitata*, *C. fasciventris*, *C. anonae* and *C. rosa* (as calculated from peak areas of 59 cuticular hydrocarbons (CHCs)).

type. The composition of male pheromones partially overlapped, but contained also species-specific chemical compounds. Only 12 compounds elicited antennal responses suggesting a prominent role in pheromone communication. Four of the compounds were found in emanations of all three studied species (Břizová et al. 2015). However these shared compounds are present in the respective species in different concentrations. Electrophysiological data showed that females of the *Ceratitis* FAR complex species perceive components of conspecific pheromones specifically, e.g. they are specifically tuned to conspecific pheromones, but antennae respond also to pheromone components of other species. Both males and females can perceive male pheromones. However to decide whether there are sex-specific differences, further electrophysiological experiments are needed.

Many volatiles identified in *Ceratitis* FAR male emanation have been previously identified in other tephritid pheromones (Cruz-López et al. 2006, Heath et al. 1991, Lu and Teal 2001, Milet-Pinheiro et al. 2014, Robacker 1988, Rocca et al. 1993, Vaníčková 2012, Vaníčková et al. 2012). Others, specifically isoprenoid geranyl acetone, aliphatic volatile (*E*)-non-2-enal, and methyl (2*E*,6*E*)-farnesoate, have not been reported before. Interestingly, the latter is a crustacean reproductive hormone, structurally similar to insect juvenile hormone, which is responsible for enhancing reproductive maturation, maintaining juvenile morphology, and influencing male sex determination (Olmstead and LeBlanc 2007, Nagaraju and Borst 2008). In insects, methyl (2*E*,6*E*)-farnesoate represents the immediate precursor of insect juvenile hormone III (Teal et al. 2014). As a semiochemical, methyl (2*E*,6*E*)-farnesoate was reported in pentatomid bug pheromones (Millar et al. 2002).

The pheromone composition as well as electroantennography may be used for species identification. Similarly as for the composition of CHCs (Vaníčková et al. 2014), the pheromone composition and antennal specificity suggest that the three nominal species of the *Ceratitis* FAR complex correspond to taxonomically well-defined entities. It is recommended, however, that this work should be expanded by including pheromone studies of F1 and R1 and further sampling and analysis of different populations for all types, to determine inter-population variability.

Developmental physiology

Developmental physiology studies can assist in detecting differences between species with regard to biological requirements. Only the developmental physiology of the two *C. rosa* types was studied in detail (Tanga et al. 2015), because of the lack of established colonies for the other species and because it was the differences observed between developmental studies conducted in La Réunion (Duyck and Quilici 2002) and South Africa (Grout and Stolz 2007) that initiated the idea that *C. rosa* may consist of different biotypes with different climatic requirements. R1 and R2 populations were, therefore, studied simultaneously in Kenya and South Africa (Tanga et al. 2015). Depending on locality and temperature, marked differences were observed between R1 and R2 populations in the developmental duration of immature life stages. In both Kenyan and South African populations, R2 appears to be less adapted to hotter environments than R1. In Kenya, R2 appeared to be better adapted to colder environments, while in South Africa, both *C. rosa* types were able to tolerate lower temperatures. Despite discrepancies in temperature related developmental physiology of the two types in the two localities, results from Kenya and South Africa clearly demonstrate and support the existence of two genetically distinct populations of *C. rosa* that are divergent in their physiological response to temperature. Discrepancies between the localities with regards to observed and estimated developmental time parameters of the two *C. rosa* types can be due to geographic variation (populations from South Africa originated from temperate climates

and populations from Kenya originated from tropical climates). Other factors such as food quantity and quality, rearing conditions, acclimatization and generation age are also mentioned as factors influencing the developmental physiology.

Geographical distribution and altitudinal transect

The distribution of *C. fasciventris* and *C. rosa* was re-analyzed, taking into account the existence of two types for each of these species. As the known distribution was largely based upon museum specimens collected over a period of 130 years and the DNA retrieval from older specimens (i.e. >10yrs) is cumbersome and with low success rate, it was decided to re-assign specimens based only on morphological characters which excluded female specimens. In total, specimens from 218 localities were re-examined and assigned to one of the four types (F1, F2, R1, R2). The observed distributions are given in Figures 10–11. F1 is mainly represented in western Africa but extends its distribution throughout southern and eastern parts of the continent with records from Angola, Zambia, Malawi and Tanzania (Figure 10). F2 is confined to the Rift areas of Ethiopia, Kenya and eastern part of the Democratic Republic of Congo, with southern expansion into Katanga region of the Democratic Republic of Congo and Zambia. So far, no sympatric or parapatric occurrence of the two types has been observed. R1 and R2 on the other hand do not show a clear geographic isolation (Figure 11). Only in the Cape and central parts of South Africa is a single type (R2) present, as well as in the adventive populations on the Indian Ocean islands. In the northern part of South Africa and northwards there are records showing overlap of distributions.

A detailed study was conducted in the Uluguru Mountains near Morogoro (Tanzania) where both types are known to occur (Mwatawala et al. 2015). Along an altitudinal transect, ranging from 540 to 1650 masl the occurrence of both types was monitored using traps with EGO-lure. A gradual shift was observed with both types occurring at lower altitudes (with predominance of R1) while only R2 was observed at the highest elevations. Geurts et al. (2012) observed a temperature shift of 7-8°C along this transect. When looking at the developmental differences observed for, at least the Kenyan populations, temperature could well play a major role in the observed pattern, although it remains to be seen whether other aspects such as differential host range and availability also have an influence regarding the differences observed.

Pre- and postzygotic incompatibility

Although no detailed data are presented in this volume, preliminary data by S. Ekesi (pers. communication), indicate that in field cage studies there is a significant pre- and post-zygotic incompatibility both between *Ceratitis rosa* and *C. fasciventris* (F2), as well as between the two genotypic clusters of *C. rosa*: R1 and R2. None of the other genotypic clusters or morphospecies were included.

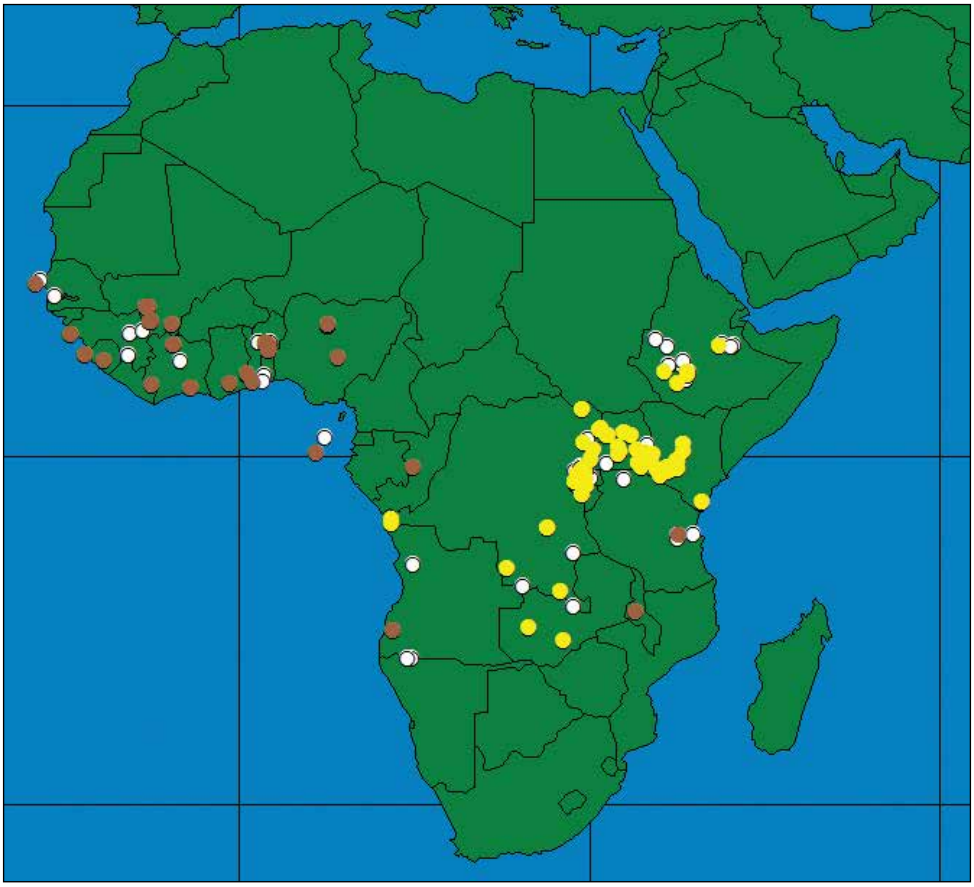

Figure 10. Geographical distribution of *Ceratitis fasciventris*, F1 (brown circles), F2 (yellow circles), unassigned (white circles).

Conclusion

The majority of the research approaches discussed here indicate that the *Ceratitis* FAR complex consists of at least the three recognized morphospecies, but possibly of five different species. All methodologies (except larval morphology) used confirm that *Ceratitis anonae*, *C. fasciventris* and *C. rosa* are well recognized groups. *Ceratitis anonae* is a uniform group showing no apparent morphological or genetic variability and has a well-defined distribution range. The two other entities consist each of two separate groups. For *C. rosa*, the two entities (called 'R1', 'lowland' or 'hot rosa' on one hand, and 'R2', 'highland' or 'cold rosa' on the other hand) can be distinguished morphologically in males, as well as by other means and demonstrate a different developmental physiology. They occur sympatrically in some regions, but also show a disjunct distribution that appears to be correlated with ambient temperature. It is concluded that both types should be considered as two different species. Taxonomically, the type

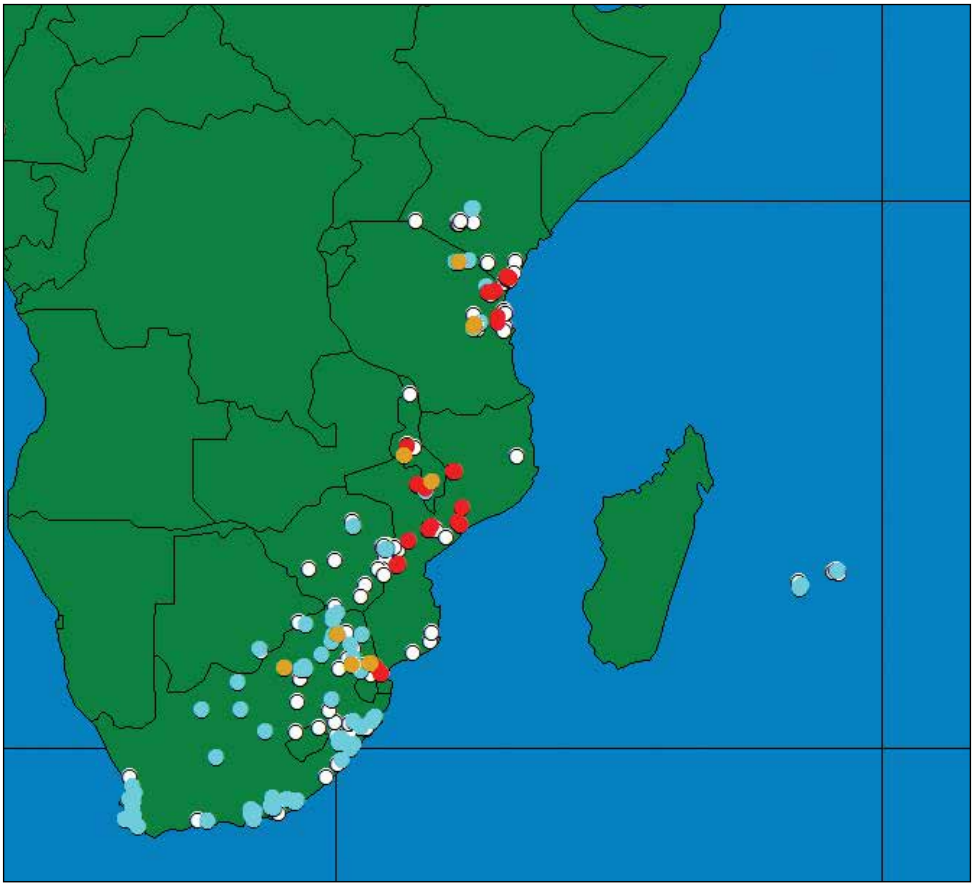

Figure 11. Geographical distribution of *Ceratitis rosa*, R1 (red circles), R2 (blue circles), sympatric occurrence (brown circles), un-assigned (white circles).

material of *C. rosa* belongs to the R1 type, which means that the R2 type should be considered as a new species, and a formal description will be published in the near future. Similarly, the review indicates that also *C. fasciventris* tends to be composed of two entities. Yet, because the data are currently insufficient to establish clearly whether they should also be considered two different species or not, we currently suggest to maintain the two types under one and the same species.

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Niche partitioning among two *Ceratitis rosa* morphotypes and other *Ceratitis* pest species (Diptera, Tephritidae) along an altitudinal transect in Central Tanzania

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Abstract

Two standard parapheromones, trimedlure (routinely used for monitoring *Ceratitis rosa* and *C. capitata*) and terpinyl acetate (routinely used for monitoring *C. cosyra*) were compared with enriched ginger root oil (EGO) lure for detecting and monitoring the presence and relative population abundance of these particular pest species. Standard yellow fruit fly traps were used for the comparison, which was conducted at 10 sites along an altitudinal transect ranging from 540 to 1650 masl on the Uluguru mountains, in Morogoro Region (Central Tanzania). A gradual change of relative occurrence of the two *C. rosa* morphotypes was clear from the EGO lure trapping. The morphotype R1 was predominant at lower altitudes while morphotype R2 was predominant at higher altitudes. Further experiments are needed to confirm the consistency of the observed pattern across regions, seasons and years as well as possible differences in the developmental physiology of both morphotypes. The mango fruit fly, *Ceratitis cosyra*, showed a distinct predominance at altitudes below 800 masl as shown in both the EGO lure and the terpinyl acetate trapping. The catches of all three target species were higher in traps with the EGO lure compared to the conventional lures trimedlure and terpinyl acetate. It is argued that for these species EGO lure can act as a suitable and more effective alternative for trimedlure and terpinyl acetate parapheromones. In addition, EGO lure has the added advantage that it combines the taxon spectrum for the two latter substances, thus requiring the use of only a single attractant.

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Keywords
EGO lure, terpinyl acetate, trimedlure, monitoring

Introduction

The Natal fruit fly, *Ceratitis rosa* Karsch, is an indigenous pest of significant importance to horticultural production in Africa. It is a member of the *Ceratitis* FAR complex, that is comprised of this and two other polyphagous, and morphologically similar species: *C. fasciventris* (Bezzi) and *C. anonae* Graham (Barr and McPheron 2006, Virgilio et al. 2013). The distribution of *C. rosa* in Africa ranges from South (from Western Cape in South Africa onwards) to eastern Africa, with the northernmost records from the Central Highlands in Kenya (De Meyer 2001). *Ceratitis rosa* can survive in a wide range of climates, but with less preference for drier areas (De Meyer et al. 2008, De Villiers et al. 2013). The pest can impact production of both tropical and temperate fruits because its population is relatively stable across altitudes (Geurts et al. 2012).

The climatic requirements and potential distribution of *C. rosa* have been subjects of controversy. This became more evident in studies that compare climatic niche of *C. rosa* and other *Ceratitis* species. De Meyer et al. (2008) reported that *C. rosa* and the Mediterranean fruit fly *C. capitata* (Wiedemann) appear to have broadly similar potential ranges in Africa and southern Europe, but the latter may be more tolerant to a wider range of climatic conditions. However, there have been contrasting reports about thermo-tolerance of *C. rosa*. A minimum thermal developmental threshold reported by Duyck and Quilici (2002) is substantially lower than what was reported by Grout and Stoltz (2007). The ensuing confusion is whether the species is more adapted to cooler or warmer climates. In another study, Nyamukondiwa et al. (2010) reported that *C. capitata* and *C. rosa* have similar levels of survival to acute high and low temperature exposures under common rearing conditions. However, the time to extinction is greater for *C. capitata* than for *C. rosa*, especially in habitats where temperatures frequently drop below 10°C.

The contrasting observations suggested the existence of two *C. rosa* biotypes with different climate requirements (Grout and Stoltz 2007). Recently, Virgilio et al. (2013) distinguished two *C. rosa* genotypes, designated as R1 and R2, that may occur in sympatry. The genotypes conform to two *C. rosa* morphotypes described by De Meyer et al. (2015). These new insights suggest revisions of current models of ecological niche requirements and invasion risk of *C. rosa* (Virgilio et al. 2013). Generally, R1 is abundant in the low land warm areas, while R2 is abundant at higher altitude cold areas. But the actual distribution of the two morphotypes is not well known and it is the focus of this study.

In studying the distribution of the two *C. rosa* morphotypes, it was desirable to understand niche partitioning between *C. rosa* and two other economically important *Ceratitis* species, *C. capitata* and marula fly *C. cosyra* (Walker). Male specimens of the three *Ceratitis* species are attracted to different lures. *Ceratitis capitata* and *C. rosa*

are attracted to trimedlure, while *C. cosyra* is attracted to terpinyl acetate (White and Elson-Harris 1994). Recently the Enriched Ginger Oil (EGO) Lure was found to be more effective than trimedlure for *C. rosa* (Mwatawala et al. 2012). The limited comparisons, which were done in low land warm areas, showed that *C. rosa, C. capitata* and *C. cosyra* can be attracted to EGO lure, making it a better, single substitute for multiple lures. However, the results contrast reports from Hawaii, where trimedlure was more attractive to *C. capitata* than EGO lure (Shelly and Pahio 2013), warranting further investigations. In this experiment we studied the ecological niche partitioning among three *Ceratitis* species across an altitudinal range while at the same time comparing effectiveness of three lures: EGO lure, trimedlure and terpinyl acetate.

Methods

Ten locations, spaced at similar altitudinal intervals along a transect extending from 550 to 1650 masl were selected in the Morogoro region, Tanzania, (Table 1a, b; see also Geurts et al. (2012) for altitudinal profile of the sampling area except for the lowest sampling point) and sampled for three times in June 2013 (1 Jun, 15 Jun, 29 Jun). The average difference in temperature between the highest and lowest sampling point was previously reported to range between 7–8 °C (June average temperatures 15–22.5 °C, see Geurts et al. 2012). Modified McPhail® traps (Scentry Co, Bilings, MT, USA) were hung on fruit trees, usually mango, except at the high-altitude sites where traps were hung either on peach, plum or apple. Traps were baited with one of three different parapheromones: terpinyl acetate (TA), trimedlure (TM) (both purchased from IPS, Elsmere Port, UK) and EGO lure (EGO) (purchased from Insect Science, Tzaneen, South Africa). In addition to the different lures, a killing agent DDVP (containing 20% W/W dichlorovos; purchased from IPS) was placed in each trap. Sticky glue "tangle foot" was applied on the branches on which traps were hung to prevent predatory ants from accessing insects caught in traps.

Three replicate traps for each lure were placed at each altitude (for a total of 90 traps). Traps were activated for a single week and fresh lures and killing agents were used at each sampling instance. To guarantee replicate interspersion, traps where randomly re-positioned on different tree branches before each sampling. Flies collected from each trap were placed in uniquely marked vials, and brought to the lab for identification, counting and preservation in 70% ethanol. Trapping followed guidelines given by the International Atomic Energy Agency and FAO (IAEA 2013). The identification of flies was done using keys and characters presented by White and Elson-Harris (1994). The two *C. rosa* morphotypes were sorted following characters given by De Meyer et al. (2015). Only males *C. rosa* R1 and R2 were sorted as there are no discriminating morphological characters known for females.

The R package GAD (Sandrini and Carmago 2012) was used for analysis of variance (ANOVA) of cumulative abundances of flies collected in each trap. ANOVAs allowed testing differences between (a) abundances of male *C. rosa, C. cosyra* or *C.*

Table 1a. Geographic position, altitudes of, and fruit trees present at trapping locations along the transect in Morogoro region, Tanzania.

S/N	Location	District, Division	Latitude	Longitude	Distance from preceding trapping location (kms)	Altitude (masl)
1	SUA	Morogoro, Municipality	S 06°50'00.0"	E 037°35'00.0"	-	550
2	Hobwe mlali	Mvomero, Mlali	S 06°59'09.5"	E 037°33'44.5"	34	654
3	Msikitini (PEHCOL)	Mvomero, Mlali	S 06°59'55.2"	E 037°34'18.0"	2.5	755
4	Kibundi	Mvomero, Mgeta	S 07° 00'21.8"	E 037°34'11.2"	2.4	843
5	Kidiwa	Mvomero, Mgeta	S 07°01'36.9"	E 037°34'34.8"	2.1	1034
6	Pinde	Mvomero, Mgeta	S 07°01'56.4"	E 037°34'45.1"	1.7	1094
7	Langali – Vosomoro	Mvomero, Mgeta	S 07°01'54.4"	E 037°34'10.8"	5.4	1170
8	Langali- Konrad	Mvomero, Mgeta	S 07°03'57.7"	E 037°34'57.3"	1	1268
9	Visada	Mvomero, Mgeta	S 07°04'03.8"	E 037°34'57.6"	0.5	1392
10	Nyandira	Mvomero, Mgeta	S 07°05'03.72"	E 037°34'46.1"	3.5	1650

Table 1b. Fruits trees recorded at lowest (SUA Horticulture Unit) and highest (Nyandira) trapping locations.

Location	Fruits grown
SUA Horticulture Unit	Mango, *Mangifera indica* L., tangerine* *Citrus reticulata* Blanco, sweet orange*, *Citrus sinensis* (L.) Osbeck., avocado*, *Persea americana* Miller., governors' plum, *Flacourtia indica* (Burman f.) Merr., guava*, *Psidium guajava* L., soursop*, *Annona muricata* L., cherimoya*, *Annona cherimola* Miller and loquat*, *Eriobotrya japonica* (Thunb.) Lindley,
Nyandira	Apple, *Malus* spp., peach, *Prunus persica* (L.) Batsch., coffee*, *Coffea canephora* Pierre ex A. Froehner, feijoa *Feijoa sellowiana* (O. Berg.), nectarines, *Prunus persica* (L.) Batsch, loquat*, cherimoya*, avocado* and guava*

*mature and ripe fruits recorded during the trapping period.

capitata (with lure as fixed and altitude as random orthogonal factors) and (b) abundances of the two *C. rosa* morphotypes (R1 and R2) (with type as fixed and altitude as random orthogonal factors). Before analyses, data were fourth root transformed and homogeneity of variances were verified through Cochran's C test (Mair and Eye 2014). Student-Neuman-Keuls (SNK) tests were used for *posteriori* comparisons of means (Hochberg 2014).

Results

A total of 836 male specimens of the three *Ceratitis* species were trapped along the transect (Table 2) (female specimens constituted less than 1% of all trappings and were not included in the analyses because of lack of diagnostic morphological features for the two *C. rosa* morphotypes). More specimens were caught in traps baited with EGO lure, than in traps baited with TA or TM (Figure 1). *Ceratitis cosyra* was the most abundant species constituting 61.6% of all trapped specimens, while *C. rosa* (33.3%) and *C. capitata* (5%) had lower abundances (Figure 2). A total of 279 *C. rosa* R1 and R2 were collected from EGO lure traps with R1 being more abundant (61.2% of. *C. rosa* morphotypes).

Ceratitis cosyra showed altitudinal differences in traps baited with different lures, with higher abundances at lower altitudes (550, 654, 755, 986 masl) in traps baited with EGO lure (Tables 3a and 3b).

C. rosa also showed significant differences between lures (EGO > TM) and altitudes (Tables 4a and 4b, Figure 3). The distribution of the *C. rosa* R1 and R2 types along the altitude is shown in Figure 4. Morphotype R1 is present throughout the altitudinal transect, with higher abundances at lower attitudes. Conversely, morphotype R2 was more abundant at higher altitudes, reaching a peak at the Langali – Konrad station (1268 m asl) while being absent at the lower station (SUA, 550 m asl). ANOVA (Table 5a, 5b) showed significantly higher abundances of morphotype R1 at 550 masl and of morphotype R2 at 1170, 1268, 1392 and 1644 masl.

The catches of *C. capitata*, were remarkably low with only 42 specimens trapped (Table 1, Figure 2).

Table 2. Number of specimens of the three species / morphotypes caught by the tree lures.

Species/ entity	Enriched ginger root oil (EGO)	Trimedlure (TM)	Terpinyl acetate (TA)	Total
C. rosa R1	165	6	0	171
C. rosa R2	95	13	0	108
C. capitata	30	12	0	42
C. cosyra	475	0	40	515
Total	765	31	40	836

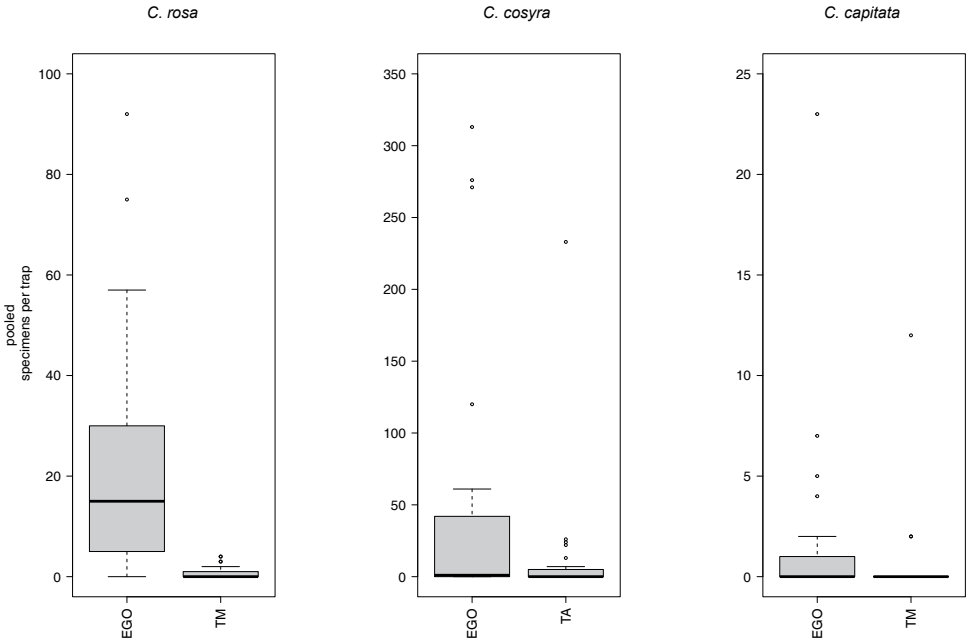

Figure 1. Catches of the three *Ceratitis* species by lures.

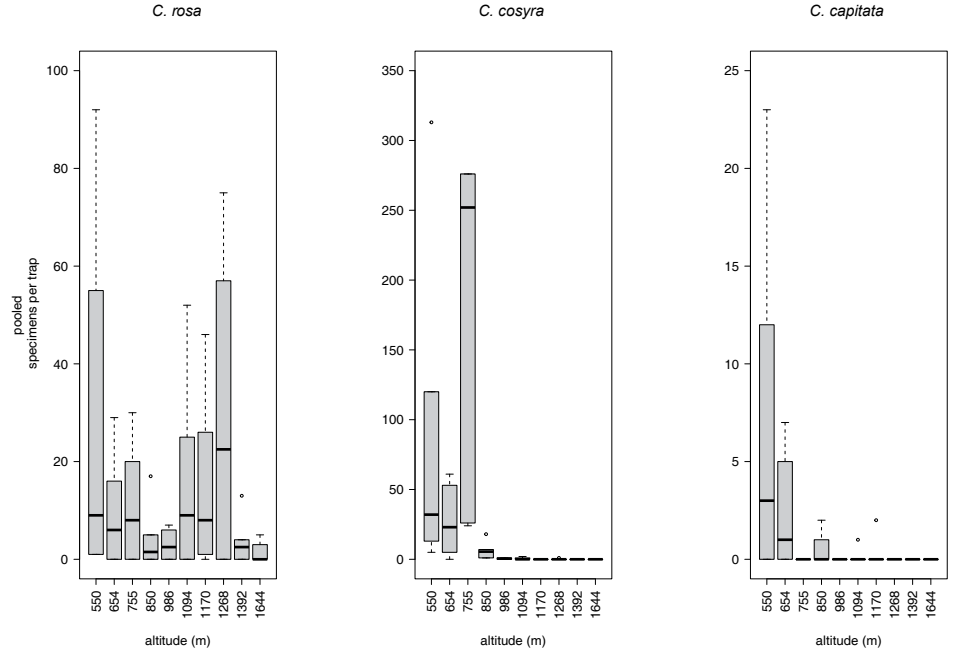

Figure 2. Catches of *Ceratitis* species along the transect.

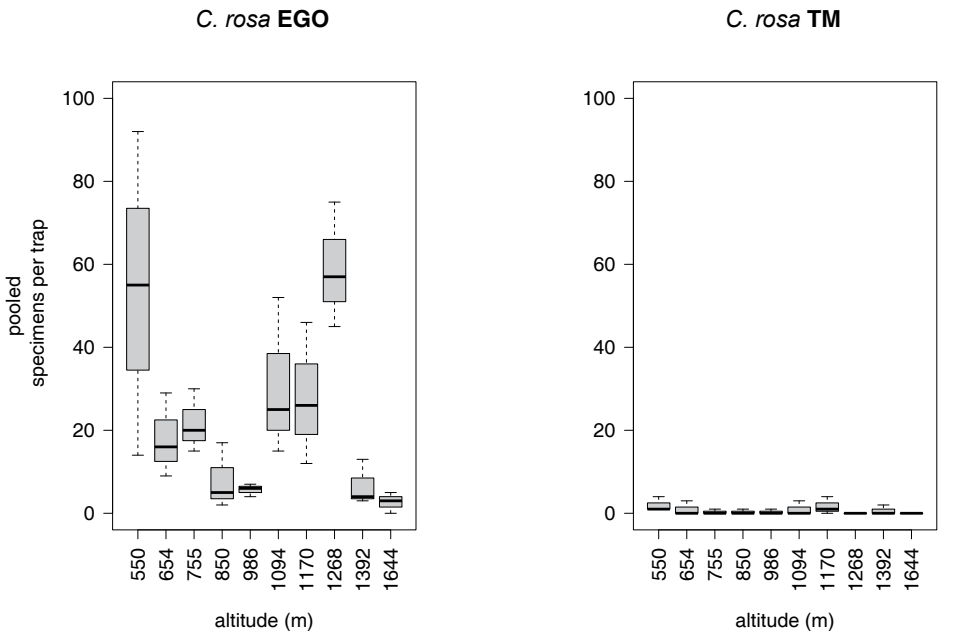

Figure 3. Catches of *C. rosa* along the transect (different lures).

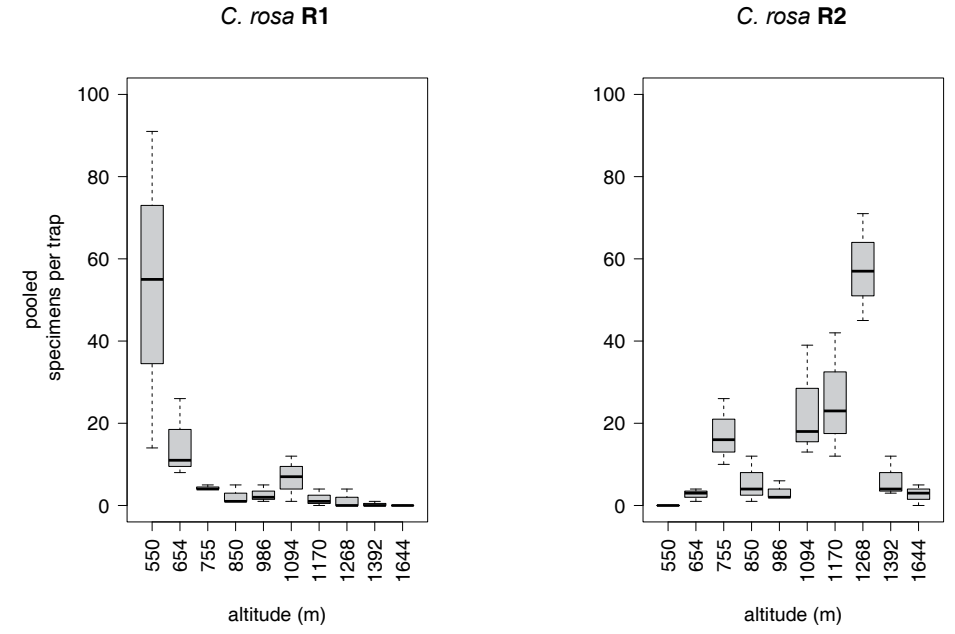

Figure 4. Catches of *C. rosa* morphotypes along the transect (EGO lure).

Table 3a. ANOVA verifying differences in abundances of *C. cosyra* trapped with different lures (EGO, TA) at 10 different altitudes.

	df	MS	F	P
Lure (L)	1	8.78	11.07	**
Altitude (A)	9	10.22	43.66	***
L x A	9	0.79	3.39	**
Residual	40	0.23		

d.f.: degrees of freedom; MS: mean squares; n.s.: not significant at p<0.05; ***: p<0.001, **: p<0.01; *: p<0.05. Data fourth root transformed. Homoscedasticity verified through Cochran's C test (C = 0.260, n.s.).

Table 3b. *Post hoc* SNK test for the interaction between lure and altitude on *C. cosyra* catches.

Altitude	Station	Lure
550	SUA	EGO > TA
654	Hobwe mlali	EGO > TA
755	Msikitini (PEHCOL)	EGO > TA
850	Kibundi	EGO = TA
986	Kidiwa	EGO > TA
1094	Pinde	EGO = TA
1170	Langali - Vosomoro	EGO = TA
1268	Langali - Konrad	EGO = TA
1392	Visada	EGO = TA
1644	Nyandira	EGO = TA

Table 4a. ANOVA verifying differences in abundances of *C. rosa* trapped with different lures (EGO, TM) at 10 different altitudes.

	df	MS	F	P
Lure (L)	1	35.63	88.86	***
Altitude (A)	9	0.88	3.57	**
L x A	9	0.40	1.62	ns
Residual	40	0.25		

d.f.: degrees of freedom; MS: mean squares; n.s.: not significant at p<0.05; ***: p<0.001, **: p<0.01; *: p<0.05. Data fourth root transformed. Homoscedasticity verified through Cochran's C test (C= 0.134, n.s.).

Table 4b. *Post hoc* SKN test on effects of lures and altitudes on abundance of *C. rosa*

Lure	EGO > TM
Altitude	550 = 654 = 755 = 850 = 986 = 1094 = 1170 = 1268 = 1392 = 1644

Discussion

Our results showed a gradual change in the relative abundance of the two *C. rosa* morphotypes, with R1 being predominant at lower altitudes and R2 being predominant at higher altitudes. Further experiments will have to show if these differences are

Table 5a. ANOVA verifying differences in abundances of the two *C. rosa* types (R1 hot and R2 cold) at 10 different altitudes.

	df	MS	F	P
C. rosa type (T)	1	2.54	0.97	ns
Altitude (A)	9	0.98	5.41	***
T x A	9	2.62	14.38	***
Residual	40	0.18		

d.f.: degrees of freedom; MS: mean squares; n.s.: not significant at p<0.05; ***: p<0.001, **: p<0.01; *: p<0.05. Data fourth root transformed. Homoscedasticity verified through Cochran's C test (C = 0.183, n.s.).

Table 5b. *Post hoc* SNK test for the interaction between *C. rosa* type and altitude

Altitude	Station	Morphotype
550	SUA	R1 > R2
654	Hobwe mlali	R1 = R2
755	Msikitini (PEHCOL)	R1 = R2
850	Kibundi	R1 = R2
986	Kidiwa	R1 = R2
1094	Pinde	R1 = R2
1170	Langali - Vosomoro	R1 < R2
1268	Langali - Konrad	R1 < R2
1392	Visada	R1 < R2
1644	Nyandira	R1 < R2

consistent across seasons and years and whether the different distributions are related to differences in temperature thresholds and developmental rates of the two morphotypes (Tanga et al. 2015). The results of this study may explain the differences observed between Grout and Stolz (2007) versus Duyck and Quilici (2002). The South African morphotype studied by Grout and Stoltz (2007) may well represent the morphotype R1 that is dominant in lower altitude areas. On the other hand, the population in Réunion could correspond to morphotype R2 predominant in the high altitude areas, as Virgilio et al. (2013) showed that the population studied from Réunion belonged exclusively to R2. In Mpumalanga and Kwa-Zulu Natal regions of South Africa, both types occur, but it is not clear what population was used by Grout and Stolz (2007) for their experiments. The climatic niche partitioning of these two morphotypes is not very clear as both morphotypes were present throughout the altitudinal transect, albeit at contrasting population levels, and it still remains to be explored what biotic and/or abiotic factors exactly determine their distribution. It can be further inferred that the impact of morphotype R2 might be more pronounced on temperate fruits like peach, avocado and apple, while morphotype R1 might have a more important impact on tropical and subtropical fruits. Of course, these hypotheses need further experimental validation including sampling at different fruit phenological states.

Captures of *C. cosyra*, and possibly of *C. capitata*, were higher in the lower altitude areas, where tropical fruits are grown, but low at high altitudes. The distributions of these two species in the field conform to the laboratory results by Duyck and Qulici (2002) and Grout and Stoltz (2007), in Réunion and South Africa respectively. According to Geurts et al. (2012) the presence of suitable hosts and the competition between fruit fly species seem decisive for diversity along the altitudinal transect, although climatic suitability cannot be neglected. The competitive ability of *Bactrocera dorsalis* (Hendel) affects the abundance of *Ceratitis* species. The presence of *B. dorsalis* has impacted the abundance of *Ceratitis* species, notably *C. cosyra*. Fruits infestation by *C. cosyra* seems to be negatively affected by *B. dorsalis* especially in hosts like mango (*Mangifera indica* L.). In Benin, Vayssières et al. (2005) reported a decrease in density of *C. cosyra* as the density of *B. dorsalis* increases. The evidence of competitive displacement of *C. cosyra* by *B. dorsalis* was provided by Ekesi et al. (2009) with *B. dorsalis* having stronger competitive traits than *C. cosyra* (Salum et al. 2013). The latter is now mostly confined to hosts of the family Annonaceae in this study area (Geurts et al. 2012). On the contrary, the abundance and infestation of *C. rosa* do not seem to be significantly affected by the abundance of *B. dorsalis* (Geurts et al. 2012). *Bactrocera dorsalis* is not yet established in high altitude areas (Geurts et al. 2013), where R2 is dominant. However, the competition between morphotype R1 and *B. dorsalis* can be expected. So far, data collected from the same region do not suggest the displacement of *C. rosa* by *B. dorsalis*.

The population of *C. capitata* recorded in this study was very low. This species is more restricted in this study area to hosts like *Fortunella margarita* (Thunb.) Swingle (Mwatawala et al. 2009), and *Capsicum* spp. (Mziray et al. 2010). There are no data on distribution and abundance of *C. capitata* prior to the introduction of *B. dorsalis* in the study region, hence competitive displacement cannot be ascertained. The distribution of *Ceratitis* species along the altitude has an implication of management programs. As *C. rosa* of morphotype R2 is the predominant pest species at higher altitude areas, any fruit flies management program in this particular region should target morphotype R2.

Of the three male lures tested, EGO lure attracted more flies than TM with regard to *C. rosa* and *C. capitata* (and higher catches than TA with regard to *C. cosyra*). In a previous study, the catches of *C. rosa* and *C. capitata* by EGO lure were equal or superior to TM (Mwatawala et al. 2013). The present study showed that EGO lure is a significantly stronger attractant for the males of *C. rosa*, *C. capitata* and *C. cosyra*.

The findings of this study support the results of Cunningham (1989) who reported that alpha-copaene is 2–5 times more attractive for male Mediterranean fruit flies than TM. This is in contrast to Shelly and Pahio (2002) and Shelly (2013) who observed higher catches of *C. capitata* in traps baited with TM than EGO lure, especially as time progressed. They went on to suggest that neither capilure (not a subject of the current study) nor EGO lure can be an adequate substitute for TM. According to Shelly (2013) the discrepancy in the results for *C. capitata* between Hawaii and Africa could reflect differences in the composition of the (ginger) oils used in the two regions. The presence and concentration of sesquiterpenes other than α-copaene may affect Mediterranean fruit fly response to natural oils. Also variation in the chemical composition

of ginger root oils from different suppliers could generate different results in trapping studies (Shelly 2013) and should be studied.

Despite the observed discrepancies, EGO lure has an added advantage of attracting a wider spectrum of pest fruit flies, which allows deployment of a single lure trap rather than two different ones. TM is an effective lure for surveying and monitoring activities for male Mediterranean fruit flies (Grout et al. 2011) and members of the *Ceratitis* FAR complex (Virgilio et al. 2008) including *C. rosa*. *Ceratitis cosyra* males are not attracted to TM but to TA (White and Elson-Harris 1994). This study showed that *C. cosyra* responds more to EGO lure than TA. It is concluded that EGO lure should be considered as a suitable alternative for TM in detection, monitoring and control programs for African fruit flies of the genus *Ceratitis*. The major drawback at the present moment is, however, the cost of EGO lure which is currently about tenfold of that for either TM or TA, when purchased from commercial suppliers. As such, the purchase of EGO lure by poor farmers is currently a financial restraint if no additional financial aid is provided.

Further studies are currently being carried on across diverse ecologies in Africa (Manrakhan pers. comm.) in order to verify the current observations, before EGO lure can be generally regarded as a better substitute for other attractants. Such studies should include a wide range of attractants for *Ceratitis* species. Probably, EGO lure from different sources should also be tested within the same framework. More advanced studies like capture-mark-release studies (see also Manrakhan et al. 2014) can be conducted to test the sensitivity of these *Ceratitis* species to EGO lure. This information is necessary to verity the effectiveness of EGO lure as part of management program for *Ceratitis* pest species.

Conclusion

This study has presented the distribution of two *C. rosa* morphotypes across an altitudinal transect. Morphotype R1 is more dominant in lower altitude, warmer areas while morphoptype R2 is prevalent in high altitude, cooler areas. However, both morphotypes occur throughout the transect. EGO lure attracted all the three *Ceratitis* species, including the two *C. rosa* morphotypes, more effectively than TA and TM. It is suggested that the use of EGO lure as a single attractant for the combined capture of these important *Ceratitis* species should be further explored.

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Description of third instar larvae of *Ceratitis fasciventris*, *C. anonae*, *C. rosa* (FAR complex) and *C. capitata* (Diptera, Tephritidae)

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Abstract

Third instar larvae of members of the *Ceratitis* FAR complex, including *Ceratitis fasciventris* (Bezzi), *Ceratitis anonae* Graham, and *Ceratitis rosa* Karsch are described and compared with those of *Ceratitis capitata* (Wiedemann). Diagnostic characters, such as presence vs. absence of a secondary tooth on the mandibles, previously used to separate *C. capitata* from *C. rosa*, are shown to vary in each species. Significant variation in diagnostic morphological characters among populations of *C. rosa* from east and south Africa is documented; however, the differences are not simply congruent with the R1 and R2 designations based on other studies. Quantitative measures of numerous morphological characters are consistently smaller in the larvae of *C. fasciventris* and distinguish them from other species of the FAR complex. Larvae of *C. capitata* can be distinguished from those of the FAR complex by characters such as absence of accessory plates of the oral ridges, the shape of the anterior spiracle, and the pattern of dorsal spinules. Previous studies indicated that absence of accessory lobes separate the genus *Ceratitis* from *Bactrocera*, but this is shown to be incorrect, as accessory lobes are in fact present in several species of *Ceratitis*.

Keywords

Mediterranean fruit fly, immature stages, taxonomy, identification

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Introduction

Members of the *Ceratitis* FAR complex, including *Ceratitis fasciventris* (Bezzi), *Ceratitis anonae* Graham, and *Ceratitis rosa* Karsch, are serious agricultural pests in large parts of Africa. Understanding of the species taxonomy is important to determining host plant relationships, pest management practices, knowledge of geographical distribution, and quarantine and related plant protection issues. In recent years, the taxonomy of species of the *Ceratitis* FAR complex has been clarified by careful study of adult morphology, and on-going morphological, genetic, and physiological studies suggest that additional, previously unrecognized species may be present (De Meyer 2001, De Meyer and Freidberg 2006, Virgilio et al. 2008, Virgilio et al. 2013). The present morphological study of the immature stages is intended to augment and possibly corroborate the differences seen among adult and various other biological parameters. In particular, this study was originally prompted by a problem in identifying larvae that had been intercepted alive infesting fresh peppers (*Capsicum chinense* Jacq. 'Habanero') that had been shipped from the Netherlands and intercepted in Miami, Florida in August, 2004. It wasn't clear whether they were *C. rosa* or *Ceratitis capitata* (Wiedemann) because of poorly documented overlapping morphological variation in these two species that confounded the identification process. Years later, in a telling of this dilemma during general discussion at the first meeting of the International Atomic Energy Agency's Coordinated Research Project on fruit fly cryptic species complexes in Vienna in 2010, the senior author learned that there existed research colonies of various *Ceratitis* species at ICIPE from which immature stages could be obtained, and a collaboration to do a morphological study was developed. It was quickly discovered that larvae from the Kenyan colony of *C. rosa* differed in a significant feature from those of a South African colony as described by Carroll (1998). This prompted a broader study of immature stages of all three species of the FAR complex and their various populations as described here.

Materials and methods

One goal of this paper is to describe morphological variation among geographic populations of *C. rosa*. To that end and for ease of comparison, Carroll's (1998) well-written description of mature 3rd instar larvae is repeated verbatim below (in italics) and any differences observed in the populations in the present study are given in parentheses in bold, normal font. Descriptions of other instars, pupae and eggs of most fruit fly species, including important pests, are generally unavailable for comparison. Full data is given in corresponding tables. Some features detailed by Carroll (1998) were not examined in this study and those portions of her description are not repeated. These include most of the sensillae (on the stomal organ, maxillary palp, cephalic segment, Keilin's organ, and other body parts), and vestigial spiracular openings. Most of these sensillae are only seen with a scanning electron microscope (SEM), they are often difficult to find, and may be variably expressed. They are generally highly conserved among

cyclorrhaphan Diptera and have not been found useful in alpha taxonomy. Carroll did not provide measurement data on the cephalopharygneal skeleton, but these data are provided here because they do show differences among species, and they reliably separate the instars. The descriptions and measurements follow the terminology of Teskey (1981), Steck and Malavasi (1988), Steck and Wharton (1988), and Carroll (1998). New figures are provided for most of the same features illustrated in Carroll (1998).

Corresponding descriptions were made of larvae of other species of the FAR complex, namely *C. anonae* (minimally described by Silvestri (1914) and included in the key of White and Elson-Harris (1992)) and *C. fasciventris* (not described previously). A further goal is to provide means of identifying larvae of these species morphologically, if possible. Additionally, equivalent observations were made of *C. capitata* larvae, as this important pest is likely to be encountered in the same host plants and geographic regions as members of the FAR complex.

The samples in this study include

C. rosa R1 ("hot"): KENYA, from a colony at the International Centre of Insect Physiology and Ecology (ICIPE) 20 May 2013, originating in coastal region: Mwanjamba, 04°18'21"S; 39°29'88"E, 106 m above sea level; host plant: guava; 5th generation. SOUTH AFRICA: from a colony at Citrus Research International (CRI), originating from Nelspruit, 25°27'08.19"S; 30°58'11.27"E, 11 Nov 2013, host: loquat, *Eriobotrya japonica* (Thunb.) Lindley, collector J-H. Daneel; 9th generation.

C. rosa R2 ("cold"): KENYA: from a colony at ICIPE 3 April 2001 (originating in highlands, unknown number of generations in laboratory), and December 2010, originating in highlands: Kithoka, 00°05'59"N; 037°40'40"E and 1425 m above sea level; host plant: mango; 6th generation; SOUTH AFRICA: from a colony at CRI, originating from Pretoria, 25°45'13.7"S; 28°13'45"E, 25 Feb 2014, host: jambos, *Syzygium jambos* (L.) Alston, collector J-H. Daneel; 6th generation; and from a colony at Stellenbosch University, originating from Stellenbosch, 33°56'10.99"S; 18°51'56.186"E, April 2013, host: kei apples, *Aberia caffra* Hook f. & Harv., collector Pia Addison.

C. anonae: KENYA: from a colony at ICIPE, 4 Feb 2011, originating in Kakamega forest, Western Kenya, 0°16'0"N; 34°52'60"E and altitude of 1603 m above sea level; host plant: *Antiaris toxicara* (Pers.) Lesh (Moraceae); 101st generation.

C. fasciventris: KENYA: from a colony at ICIPE, 7 Feb 2011, originating in Ruiru, Central Kenya, 1°8'31.9"S; 36°57'23.5"E and altitude of 1612 m above sea level; host plant: *Coffea arabica* L. (Rubiaceae); 92nd generation.

C. capitata: KENYA: from colonies at ICIPE, 3 April 2001 (source material unknown) and 4 Feb 2011, originating in Ruiru, Central Kenya, 1°8'31.9"S; 36°57'23.5"E and altitude of 1612 m above sea level; host plant: *Coffea arabica* L. (Rubiaceae); 231st generation; GUATEMALA: from a colony (probably USDA-APHIS), July 1987; USA, Hawaii: from a colony, Steiner laboratory, 1957; USA, Florida: Mi-

ami via Netherlands, 2004 ex habanero peppers (*Capsicum chinense* Jacq. 'Habanero'), Division of Plant Industry accession # E2004-6626; and various samples of dead, cold-treated larvae intercepted in clementines (*Citrus reticulata*) from Spain.

Voucher specimens of all African FAR colonies were verified morphologically based on adult males (De Meyer et al. 2015) and the Kenyan colonies were also genotyped for both sexes (De Meyer, personal communication 31 March 2015).

Larvae were killed in hot water and preserved in 70% ethanol or isopropanol. Specimens intended for SEM examination were sonicated for 30 seconds, then dehydrated in an ethanol series, followed by ethyl acetate, then air-dried, mounted on stubs, sputter-coated with gold-palladium, and examined in a JEOL JSM-5510LV SEM at FDACS/DPI, Gainesville, FL. Measurements derived from stub-mounted specimens were made from SEM photographs and calibrated using the embedded scale bar.

Specimens intended for examination under dissecting and compound optical microscopes were macerated overnight in 10% NaOH at room temperature. Once cleared, they were temporarily slide mounted in glycerin and positioned to allow measurements as needed. Most measurements were made manually using an eyepiece reticle calibrated for conversion to mm. Some measurements were made using a Zeiss AxioCam ICc 5 digital camera and ZEN 2 software (Blue edition, 2011). The optical microscopes used were a Zeiss Discovery V8 dissecting microscope, Nikon Labophot compound microscope, and Olympus BX51 compound microscope. Some imagery was obtained using a Leica Z16 APO lens, JVC KY-F75U digital camera, and Synchroscopy Auto-Montage v. 5.01.0005 software. Finally, specimens were mounted in Euparol or Canada balsam as permanent vouchers deposited at the Florida State Collection of Arthropods (FSCA) in Gainesville.

Sample sizes from which data were derived are provided with each species description. Not all character states could be observed nor was it possible to make all measurements on each specimen, as some were prepared for SEM examination and others were slide-mounted with varying success.

The length and width data presented for spiracles are based on cleared, slide-mounted specimens only. The range of measurement made on the same structures as seen under the SEM often do not overlap. We consider the measurements made on slide-mounted specimens to be more accurate, as it is much easier using this preparation to determine whether or not the structure is lying flat when measured.

The data from these descriptions will be incorporated into an interactive system based on that of Carroll et al. (2004) to improve the accuracy of fruit fly larval identifications.

Descriptions

Ceratitis rosa (partial text of Carroll (1998) in italics; new text and data based on observations of all additional populations in this study combined are shown in parentheses and bold, standard font)

Diagnosis of third instar.

Medium-sized muscidiform larvae with **(mandibular tooth ventrally grooved), (usually)** *with minute subapical mandibular tooth: usually with 9-11 (rarely 8 or 12) oral ridges; accessory plates* **(present or)** *absent; leaf-like secondary stomal lobes present, sclerotized stomal guards absent; dorsal spinules present on segments T1-A1* **(T1-T2 only; not T3, A1)***; anterior spiracles usually with 9-10 (rarely 7- 8 or 11-12)* **(usually 10-13, rarely 8 or 15)** *tubules in a single straight* **(to slightly curved or sinuous)** row; base of anterior spiracle cylindrical, **(ca.)** *half as wide as apical width; posterior spiracles with rimae 2.75-3.8 times as wide as long; spiracular processes mostly unbranched* **(to mostly branched, bases narrow to wide)***; caudal ridge present; anal lobes entire* **(or grooved, posterior lobe often larger than anterior lobe)**.

Description of third instar.

Length 7.7–9.6 mm **(newly molted 3rd instars estimated at ca. 3.5–4.0 mm)***; creamy-white, subcylindrical, tapering gradually to cephalic segment.*

Head **(Figure 1c–g)***: with cephalic lobes moderately developed, in lateral view more rounded and protuberant than in C. capitata* **(true for some *C. capitata* samples, but in others the difference is very subtle at best; observed differences may be due, at least in part, to the method used to kill and preserve larvae)***; antenna 2-segmented, both segments with sclerotized walls, the distal segment apically thin-walled and conical; maxillary palp with ... sensilla ... visible by SEM as 3 papilla sensilla and 2 knob sensilla, the remainder as pits; dorsolateral group of sensilla with 2 papilla sensilla and a pit sensillum, adjacent to but distinct from palp; stomal organ* **(Figure 1c–g)** *with primary lobe small, bearing 3-4 unbranched peg sensilla, ...; 6 secondary lobes present: a broad, flat subtending lobe and a lobe medial to it; usually 2 additional lobes immediately surrounding the primary lobe anteriorly, and usually 2 lobes anteromedial to these, all with edges entire; none of these secondary lobes is strikingly similar to oral ridges; sclerotized stomal guards absent; labium short, triangular, with narrow lateral lobes; .... Oral ridges* **(Figure 2c–h)** *usually 9-11 (but two had 8 and three had 12 ridges on one side only)* **(same range)***; well developed with margins scalloped to 1/4–1/5 of their depth (visible by SEM), located on a semicircular region laterad of mandible; accessory plates (supernumerary ridges) and other reticulation absent* **(accessory plates present or absent, variably developed in different populations) (Figure 2c–h)**.

Cephalopharyngeal skeleton **(CPS) (Figure 3c–g)** *well developed; mandible black to dark brown, apical tooth pointed, with a small subapical tooth (visible in slide-mounted material)* **(present or absent, mandible ventral surface smooth and concave between tip and subapical tooth (Figure 4c–d)**; *hypopharyngeal sclerite black in anterior half, bridge and posterior processes brown; tentoropharyngeal sclerite with dorsal and ventral cornua broadly joined, with strongly pigmented anterior and* **(unpigmented)** *posterior margins, becoming less pigmented dorsally and ventrally; dorsal cornu split posteriorly; ventral cornu with a slight hump midway along dorsal margin, pigmented along dorsal margin and ventrally to slightly more than half its length, with an incomplete window; parastomal sclerite long, stout, brown, slightly hooked* **(straight)** *apically; other sclerites as follows: dental sclerite dark brown, narrow in profile, free from and distinctly posterior to base of*

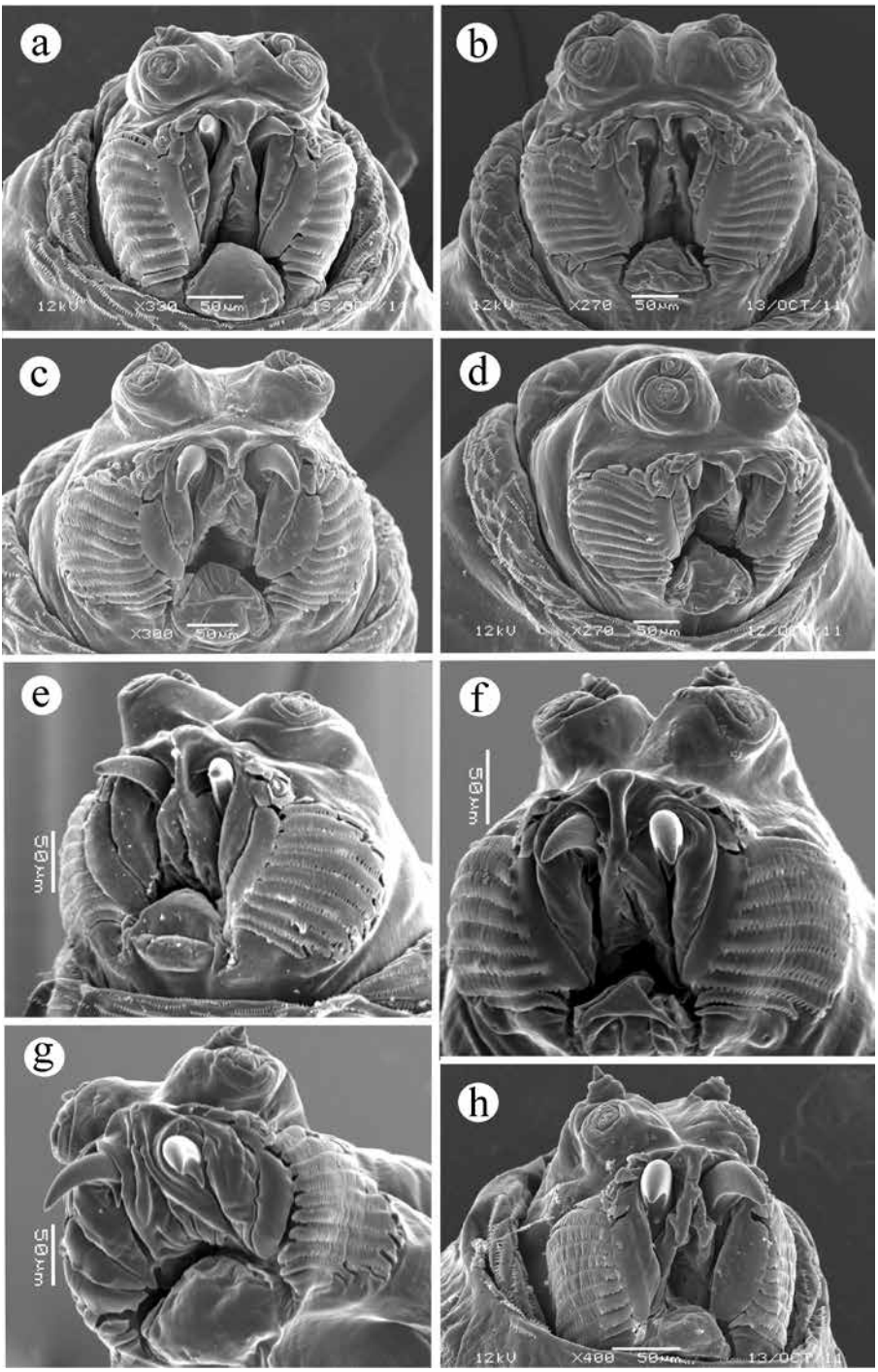

Figure 1. Head, ventral view. **a** *Ceratitis fasciventris* **b** *Ceratitis anonae* **c** *Ceratitis rosa* R1, Kenya **d** *Ceratitis rosa* R2, Kenya **e** *Ceratitis rosa* R1, S. Africa, Nelspruit **f** *Ceratitis rosa* R2, S. Africa, Pretoria **g** *Ceratitis rosa* R2, S. Africa, Stellenbosch **h** *Ceratitis capitata*, Hawaii.

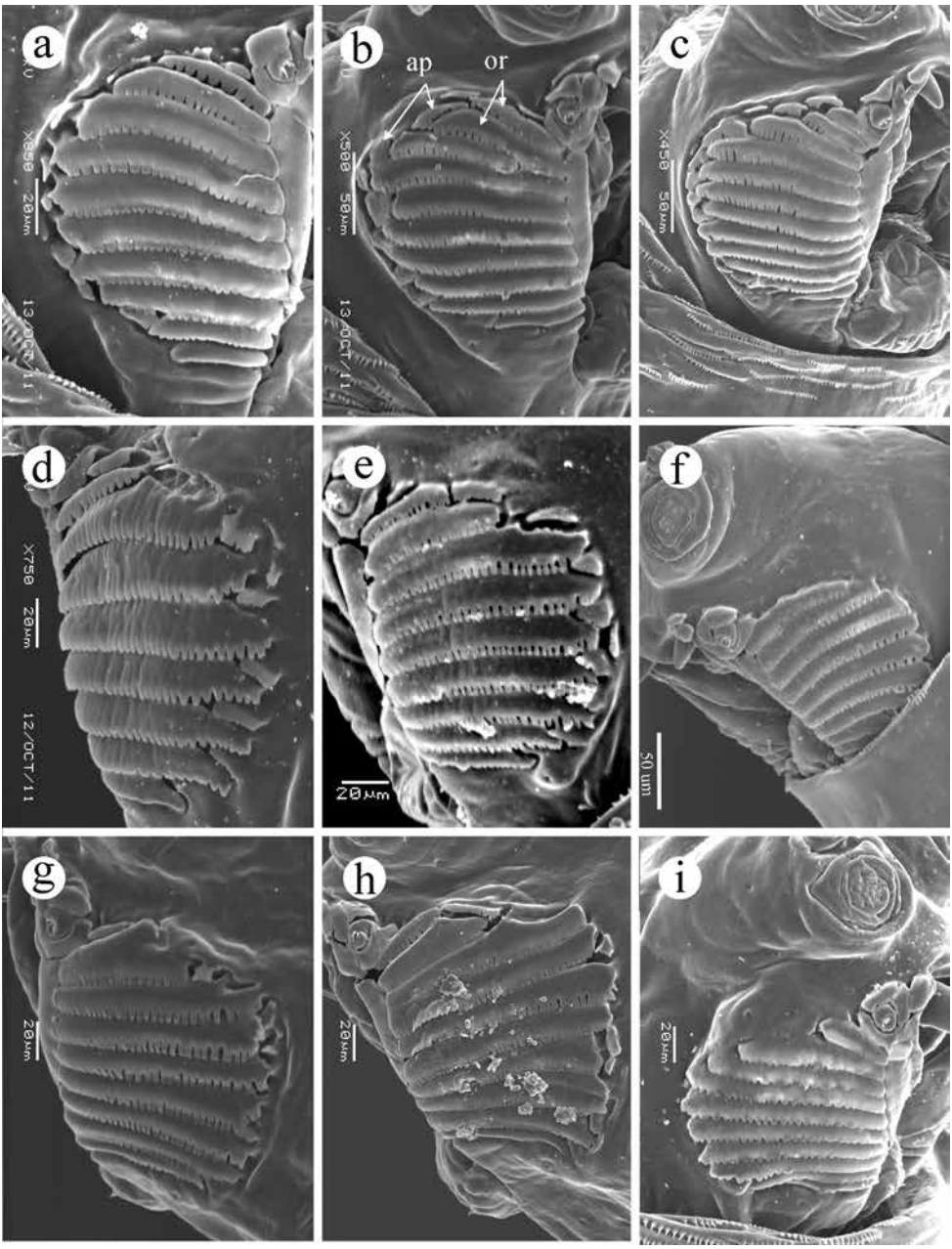

Figure 2. Oral ridges, third instar. **a** *Ceratitis fasciventris* **b** *Ceratitis anonae* **c** *Ceratitis rosa* R1, Kenya **d** *Ceratitis rosa* R2, Kenya **e** *Ceratitis rosa* R1, S. Africa, Nelspruit **f** *Ceratitis rosa* R2, S. Africa, Pretoria **g** *Ceratitis rosa* R2, S. Africa, Stellenbosch **h** *Ceratitis rosa* R2, S. Africa, Stellenbosch **i** *Ceratitis capitata*, Guatemala. Abbreviations: **ap** accessory plate, **or** oral ridge.

mandible; labial sclerites dark brown, slightly shorter than length of hypopharyngeal sclerite bridge, broadly connected to one another to form a pale W-shaped or quadrate sclerite; epipharyngeal sclerite small, faintly pigmented, amorphous **(not observed)***; anterior sclerite ... present in mature larvae; pharyngeal filter present, with 7* **(not counted)** *lamellate ridges extending the length of the pharynx.* **CPS length 0.99–1.34 mm, mandible tip to notch 0.55–0.75 mm, dorsal cornu length 0.35–0.50, ventral cornu length 0.67–0.95 mm; mandible length a 0.21–0.26 mm, mandible length b 0.22–0.27 mm, mandible length c 0.14–0.19 mm, mandible height 0.15–0.18 mm; hypopharyngeal sclerite length 0.16–0.22 mm, dorsal arch height 0.21–0.33 mm.**

Anterior spiracle **(Figure 5c–g)** *pale golden brown, projecting, usually with 8-10* **(10-13)** *tubules (but one had 7 and one had 11 on one side only; one had 11 on both sides)* **(rarely 8 or 15)** *closely spaced in a single straight* **(to curved or sinuous)** *row; distal width 0.145—0.166 mm (n = 4)* **(0.16–0.24 mm, n = 18)***, base cylindrical, about half as wide as distal width; tubules about as long as wide, rounded apically, each with a slitlike opening; felt chamber as in Figure 10; unpigmented ecdysial scar posterior to tubules. Segments T1-T3 and usually A1* **(T1-T2, but not T3 and A1)** **(Figure 6c–g)** *with broken rows of weak, conical spinules on dorsal anterior margin, with 3-5, 3-5, 1-4* **(0)***, and 1-2* **(0)** *rows of spinules, respectively, at dorsal midline; on Tl and T2 the spinulose area encircles the body* **(T1 only)***, while on T3* **(T2)** *the ventral spinulose area is separated from that of the dorsum; dorsal spinules absent on A2-A8* **(T3-A8)***; ventral spinulose areas on Tl with 10-12 rows, T2 and T3 with 3-7 rows each, and Al with 4-7 rows of posteriorly directed spinules; ventrally, segments A2 - A7 with 9-11 rows and A8 with 6-9 rows of spinules that are alternately arranged in groups of anteriorly and posteriorly directed rows, typically arranged as follows: 1-3 rows of small (approximately 0.005 mm long), anteriorly directed spinules that appear to overlap segmental lines and actually pertain to the preceding segment; 2-4 rows of small, posteriorly directed spinules; 1-2 rows of anteriorly directed spinules, and 2- 5 rows of posteriorly directed spinules, some of which may reach 0.014 mm in length.*

Caudal segment **(Figure 7c–g)** *with a caudal ridge on the intermediate region, without a dark transverse line in the medial region; 10 pairs of sensilla present as follows: dorsal area with Dl and D2 on separate papillae very close to one another; lateral area with I3 and L on separate papillae; intermediate area with intermediate tubercle well developed, bearing the following sensilla: I1a and I1b (on the same papilla or very close together, near medial end of caudal ridge), and I2 (below caudal ridge); ventral area with 3 V sensilla (one as a papilla sensillum and two as pit sensilla). Posterior spiracle* **(Figure 8c–g)** *above midline, with 3 slit-like openings, dorsal and central slit subparallel, ventral slit more medial and at an angle to the other two; rimae about 2.75-3.8 times longer than wide (0.065-0.082* **(0.06–0.09)** *mm long; 0.021–0.025 mm wide), separated from midline by approximately 2-3 times the length of the rima; spiracular processes well-developed, about half as long as rimae, mostly unbranched* **(to mostly branched)***, some with 1-2 branches; numbers of trunks and tips as follows; I (dorsal) (8-9, 10-12)* **(7-24, 11-38)***, II (3, 5-6)* **(3-7, 5-15)***, III (4-7, 7-11)* **(3-13, 5-21)***, IV (ventral) (9-10, 11-14)* **(4-17, 8-25)***. Anal lobes* **(Figure 9c–g)** *well-developed, pro-*

truding, entire (rarely grooved) **(usually grooved, posterior lobe often larger than anterior lobe)**, *surrounded by 2-4 broken rows of spinules.*

Specimens examined.—SOUTH AFRICA: ex culture, Brian Barnes (n = 40, USNM, TAMU) [Specimens described by Carroll (1998) came from a laboratory colony that originated with material from Stellenbosch, Western Cape, ex guava, ca. 1972-1973; Brian Barnes, personal communication, 28 Oct 2011. They were not re-examined in this study]. **R1 (hot type): KENYA n = 12 SEM + 5 slide, SOUTH AFRICA Nelspruit n = 5 SEM + 5 slide. R2 (cold type): KENYA n = 15 SEM + 5 slide, SOUTH AFRICA Pretoria n = 5 SEM + 5 slide, SOUTH AFRICA Stellenbosch n = 4 SEM + 5 slide.**

Variation among populations. There is considerable variation among populations in various quantitative characters, and some ranges do not overlap. See accompanying tables of comparative data for various measures and counts taken on the oral ridges, accessory plates, stomal organ, and anterior spiracles (Table 1), cephalopharyngeal skeleton (Table 2; mandible abbreviated as MH), posterior spiracles and anal lobes (Table 3), and mandible secondary tooth (Table 4).

Some notable differences among populations include:

Dorsal spinules–Carroll (1998) described a Stellenbosch-derived colony as having dorsal spinules present on segments T1-T3 and usually A1. In all populations observed in this study, however, including a newly derived colony from Stellenbosch, larvae have dorsal spinules present on T1 and T2 only, but none on T3, A1 or beyond.

Oral ridge accessory plates–Carroll (1998) described a Stellenbosch-derived colony as lacking accessory plates. However, among populations observed here, larvae of R1-Kenya, R2-Kenya, and R1-Nelspruit have accessory plates present, numerous and well-developed to the point of having serrate edges. Alternatively, larvae of R2-Pretoria have accessory plates lacking or minimally present as a few thin ridges or nubs, without serrate edges, and on R2-Stellenbosch they range from a single well-developed serrate accessory plate plus a few additional nubs, to numerous nubs, to completely lacking.

Anal lobes–Carroll (1998) described a Stellenbosch-derived colony as having anal lobes entire (rarely grooved). In all populations observed in this study, however, including a newly derived colony from Stellenbosch, larvae have grooved anal lobes.

Quantitative measures that do not (or minimally) overlap among samples include the trachea diameter at base of anterior spiracle (larger in Kenyan populations than South African populations regardless of hot or cold type); length from tip of mandible to notch in CPS, length from tip of mandible to tip of ventral prominence, and height of dorsal arch (larger in R1- and R2-Kenya + R2-Stellenbosch vs. smaller in R1-Nelspruit and R2-Pretoria). Various other individual pairings of samples do not overlap for some measures and counts especially those associated with the posterior spiracles (See Table 3). These differences in quantitative characters may merely reflect relatively small sample sizes or the result of artificial selection in laboratory colonies.

Table 1. Oral ridges and anterior spiracle quantitative data (length in mm).

Species	no. Oral Ridges	no. Accessory Plates	Stomal Organ: no. petals	Anterior spiracle: # tubules	Anterior spiracle: apical width	Anterior spiracle: tracheal width
rosa R1 Kenya	8–12	7-11, well-developed, numerous serrate	2-3 subtending + 2-3 medial	10–12	0.17–0.21	0.10–0.12
rosa R2 Kenya	8–11	4-7, well-developed, some serrate	3-4 subtending + 2-3 medial	10–11	0.18–0.22	0.10–0.12
rosa R1 S.A. Nelspruit	9–12	7-8, well-developed, some serrate	2-4 subtending + 2-3 medial	8–15	0.16–0.21	0.08–0.10
rosa R2 S.A. Pretoria	8–10(+)	0-3 nubs or short linear	4 subtending + 2 medial	11–13	0.19–0.21	0.08–0.10
rosa R2 S.A. Stellenbosch	8–10	0-1 serrate + 4-7 nubs	3-4 subtending + 2-3 medial	12–13	0.19–0.24	0.09–0.11
anonae	10–11	8-12, well-developed, some serrate	4 subtending + 2 medial	10–13	0.19–0.24	0.10–0.12
fasciventris	10	3-8, small or nubs	3-4 subtending + 1-2 medial	9–12	0.14–0.17	0.09–0.12
capitata	8–12	absent	1-4 subtending + 1-2 medial	9–12	0.16–0.19	0.06–0.12

Table 2. Cephalopharyngeal skeleton quantitative data (length in mm).

Species	CPS total length	MH tip to notch	dorsal cornu length	ventral cornu length	MH secondary tooth	length (a) MH tip to posterior prominence	length (b) MH tip to dorsal prominence	length (c) MH tip to ventral prominence	height MH dorsal prominence to ventral prominence	Hypopharyngeal sclerite length	dorsal arch
rosa R1 Kenya	1.16–1.23	0.61–0.65	0.44–0.45	0.77–0.87	present	0.24–0.25	0.25–0.25	0.16–0.17	0.15–0.16	0.16–0.19	0.30–0.33
rosa R2 Kenya	1.15–1.27	0.60–0.75	0.35–0.49	0.72–0.81	present/ absent	0.21–0.25	0.22–0.26	0.16–0.16	0.16–0.18	0.16–0.17	0.29–0.33
rosa R1 Nelspruit	0.99–1.26	0.55–0.60	0.35–0.44	0.67–0.88	present	0.21–0.25	0.23–0.25	0.14–0.16	0.15–0.16	0.16–0.20	0.21–0.28
rosa R2 Pretoria	1.16–1.34	0.59–0.60	0.40–0.47	0.75–0.95	present	0.22–0.25	0.24–0.27	0.14–0.16	0.15–0.18	0.18–0.22	0.25–0.26
rosa R2 Stellenb.	1.14–1.20	0.60–0.64	0.44–0.50	0.73–0.78	present	0.24–0.26	0.23–0.26	0.16–0.19	0.15–0.17	0.16–0.18	0.30–0.31
anonae	1.16–1.23	0.59–0.63	0.43–0.44	0.75–0.80	present	0.20–0.25	0.25–0.26	0.16–0.16	0.16–0.17	0.16–0.18	0.29–0.31
fasciventris	0.89–1.14	0.52–0.60	0.37–0.43	0.52–0.72	present	0.21–0.23	0.21–0.25	0.15–0.16	0.14–0.16	0.12–0.17	0.25–0.30
capitata	1.06–1.11	0.53–0.57	0.27–0.43	0.69–0.73	present/ absent	0.21–0.23	0.20–0.22	0.14–0.15	0.14–0.16	0.16–0.17	0.25–0.29

Table 3. Posterior spiracle quantitative measures and anal lobes (length in mm).

Species	Post spiracle slit length	Post spiracle slit width	Post spiracle tracheal width	SP-I no. trunks / no. tips	SP-I ratio tips/ trunks	SP-I basal width	SP-I ratio width/ slit length
rosa R1 Kenya	0.08–0.09	0.02– 0.03	0.16–0.21	10-18 / 14-29	1.0–2.0	0.02–0.04	0.25–0.44
rosa R2 Kenya	0.07–0.09	0.02– 0.03	0.17–0.19	7-11 / 11-20	1.2–2.3	0.01–0.02	0.15–0.24
rosa R1 S.A. Nelspruit	0.06–0.08	0.02– 0.03	0.14–0.17	13-14 / 13-24	1.0–1.7	0.02–0.04	0.27–0.52
rosa R2 S.A. Pretoria	0.07–0.07	0.02– 0.03	0.12–0.16	8-14 / 13-20	1.2–1.8	0.01–0.02	0.28–0.30
rosa R2 S.A. Stellenb.	0.08–0.09	0.02– 0.03	0.15–0.17	12-24 / 24-38	1.6–2.1	0.04–0.05	0.34–0.63
anonae	0.08–0.09	0.02– 0.03	0.18–0.21	9-12 / 13-21	1.3–1.9	0.02– 0.04	0.22–0.32
fasciventris	0.07–0.07	0.02– 0.03	0.15–0.18	2-9 / 3-14	1.5–2.2	0.01–0.02	0.07–0.28
capitata	0.07–0.08	0.02– 0.03	0.16–0.21	6-15 / 10-17	1.1–1.7	0.01–0.02	0.17–0.33
	SP-II no. trunks / no. tips	**SP-III no. trunks / no. tips**	**SP-IV no. trunks / no. tips**	**SP-IV ratio tips/ trunks**	**SP-IV basal width**	**SP-IV ratio width/ slit length**	**Anal lobe shape**
rosa R1 Kenya	4-7 / 7-12	6-13 / 7-20	6-14 / 11-23	1.3–2.1	0.02–0.04	0.18–0.41	grooved
rosa R2 Kenya	3-5 / 5-8	3-6 / 5-9	4-8 / 8-16	1.2–3.2	0.01–0.02	0.11–0.20	grooved
rosa R1 S.A. Nelspruit	4-6 / 7-12	6-8 / 9-15	10-15 / 10-24	1.0–1.9	0.02–0.03	0.19–0.49	grooved
rosa R2 S.A. Pretoria	3-6 / 5-8	3- 9 / 6-11	8-12 / 8-12	1.2–1.7	0.01–0.03	0.13–0.38	grooved
rosa R2 S.A. Stellenb.	4-7 / 9-15	6-12 / 10-21	10-17 / 18-25	1.5–2.1	0.03–0.04	0.24–0.42	grooved
anonae	3-6 / 6-9	4-8 / 5-11	6-11 / 11-16	1.2–2.2	0.01–0.03	0.15–0.27	grooved
fasciventris	2-4 / 4-7	2-6 / 4-9	4-8 / 6-12	1.5–2.0	0.01–0.01	0.07–0.19	grooved
capitata	3-4 / 4-5	2-6 / 4-11	2-10 / 3-14	1.2–1.5	0.01–0.01	0.17–0.17	entire to grooved

Table 4. Development of mandible secondary tooth.

	***rosa* R2 Kenya**	***rosa* R1 Kenya**	***rosa* R1 Nelspruit**	***rosa* R2 Pretoria**	***rosa* R2 Stellenbosch**	***capitata* Kenya**	***capitata* Hawaii**	***capitata* Guatemala**	***capitata* E2004-6626**	***anonae***	***fasciventris***
Well developed	6	6	2	2	8	4	2	0	4	7	8
Poorly developed	2	0	0	0	0	1	5	6	0	0	0
Present/Absent	2	0	0	0	0	0	0	0	0	1	0
Absent	1	0	0	0	0	8	6	3	0	0	0
N =	11	6	2	2	8	13	13	9	4	8	8

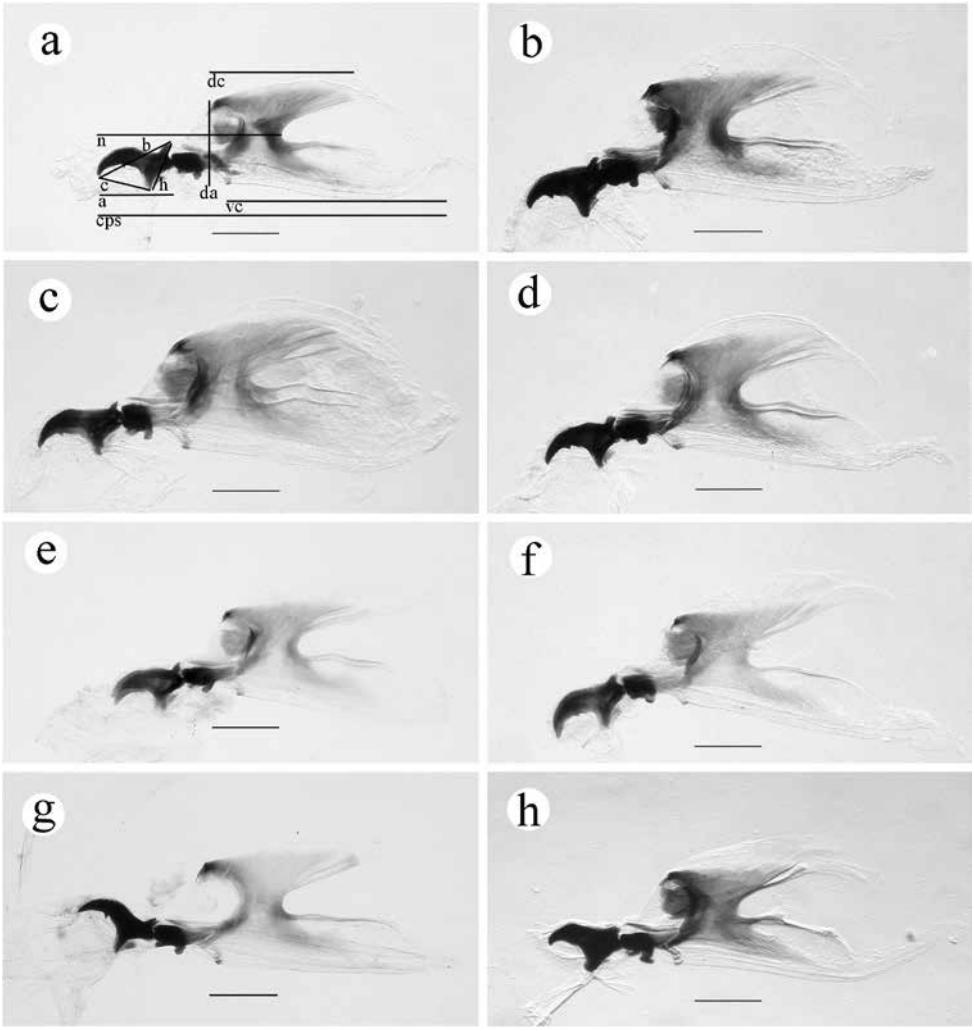

Figure 3. Cephalopharyngeal skeleton. **a** *Ceratitis fasciventris* **b** *Ceratitis anonae* **c** *Ceratitis rosa* R1, Kenya **d** *Ceratitis rosa* R2, Kenya **e** *Ceratitis rosa* R1, S. Africa, Nelspruit **f** *Ceratitis rosa* R2, S. Africa, Pretoria **g** *Ceratitis rosa* R2, S. Africa, Stellenbosch **h** *Ceratitis capitata*, Hawaii. Scale bars 0.20 mm (**a–h**). Abbreviations: **a** mandible tip to posterior prominence, **b** mandible tip to dorsal prominence, **c** mandible tip to ventral prominence, **cps** total length, **da** dorsal arch, **dc** dorsal cornu, **h** mandible height, **n** mandible tip to notch, **vc** ventral cornu.

Ceratitis anonae

Figures 1b, 2b, 3b, 4b, 5b, 6b, 7b, 8b, 9b

Diagnosis of third instar. Medium-sized muscidiform larvae with mandibular tooth ventrally grooved, with minute subapical mandibular tooth; with 10-11 oral ridges; accessory plates present; petal-like secondary stomal lobes present, sclerotized stomal

guards absent; dorsal spinules present on segments T1-T2; anterior spiracles with 10-13 tubules in a single straight to slightly curved or sinuous row; base of anterior spiracle cylindrical, ca. half as wide as apical width; posterior spiracles with rimae ca. 3 times longer than wide; spiracular processes mostly unbranched to mostly branched with narrow bases; caudal ridge present; anal lobes grooved, posterior portion often larger than anterior portion.

Description of third instar (differences from *C. rosa* description above are noted in bold font). Similar to *C. rosa*, except length **3.8–8.9 mm**; oral ridges **10–11**; accessory plates **present, well-developed**; parastomal sclerite **straight to slightly curved**, **not hooked apically**; **CPS length 1.16–1.23 mm, mandible secondary tooth present, mandible tip to notch 0.59–0.63 mm, dorsal cornu length 0.43–0.44, ventral cornu length 0.75–0.80 mm; mandible length a 0.20–0.25 mm, mandible length b 0.25–0.26 mm, mandible length c ca. 0.16 mm, mandible height 0.16–0.17 mm; hypopharyngeal sclerite length 0.16–0.18 mm, dorsal arch height 0.29–0.31 mm.** Anterior spiracle with **10–13** tubules, closely spaced in a single **slightly sinuous** row, apical width **0.19–0.24 mm (n = 7)**; segments **T1-T2** with broken rows of weak, conical spinules on dorsal anterior margin; on **T1** the spinulose area encircles the body, while on **T2** the ventral spinulose area is separated from that of the dorsum; dorsal spinules **absent on T3-A8;** posterior spiracle rimae **0.08–0.09** mm long, ca. 0.025 mm wide; spiracular processes **mostly unbranched to mostly branched, base of SP-I and SP-IV narrow**, numbers of trunks and tips as follows; I (dorsal) (**9-12, 13-21**), II (**3-6, 6-9**), III (**4-8, 5-11**), IV (ventral) (**6-11, 11-16**); anal lobes **grooved, posterior portion often larger than anterior portion**.

Specimens examined. n = 4 (SEM) + 5 (slide).

Ceratitis fasciventris

Figures 1a, 2a, 3a, 4a, 5a, 6a, 7a, 8a, 9a

Diagnosis of third instar. Medium-sized muscidiform larvae with mandibular tooth ventrally grooved, with minute subapical mandibular tooth; usually with 10 oral ridges; accessory plates weakly developed; petal-like secondary stomal lobes present; sclerotized stomal guards absent; dorsal spinules present on segments T1-T2; anterior spiracles with 9-12 tubules in a single sinuous row; base of anterior spiracle cylindrical, ca. half as wide as apical width; posterior spiracles with rimae ca. 3 times longer than wide; spiracular processes mostly unbranched to mostly branched, with narrow bases; caudal ridge present; anal lobes grooved, lobes subequal or posterior lobe larger.

Description of third instar (differences from *C. rosa* description above are noted in bold font). Similar to *C. rosa*, except length **3.2–6.8 mm**; oral ridges usually **10**; accessory plates **weakly developed**; parastomal sclerite straight to curved, not hooked apically; anterior sclerite **present or absent**; **CPS length 0.89–1.14 mm, mandible secondary tooth present, mandible tip to notch 0.52–0.60 mm, dorsal cornu**

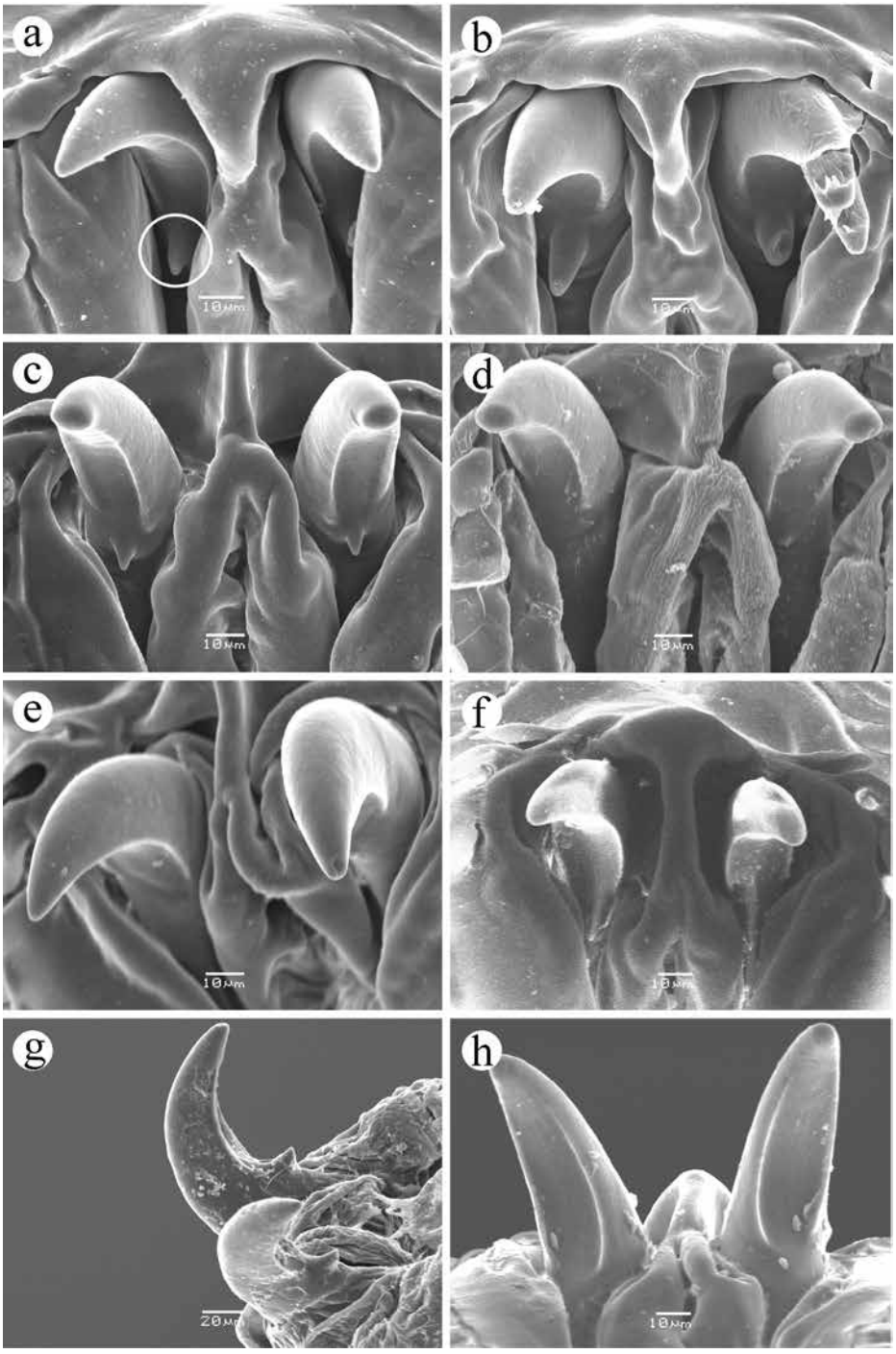

Figure 4. Mouthhooks. **a** *Ceratitis fasciventris* (secondary tooth circled) **b** *Ceratitis anonae* **c** *Ceratitis rosa* R2, Kenya **d** *Ceratitis rosa* R2, Kenya **e** *Ceratitis capitata*, Hawaii **f** *Ceratitis capitata*, Hawaii **g** *Ceratitis capitata*, ex habaneros peppers via Netherlands **h** *Ceratitis capitata*, Guatemala.

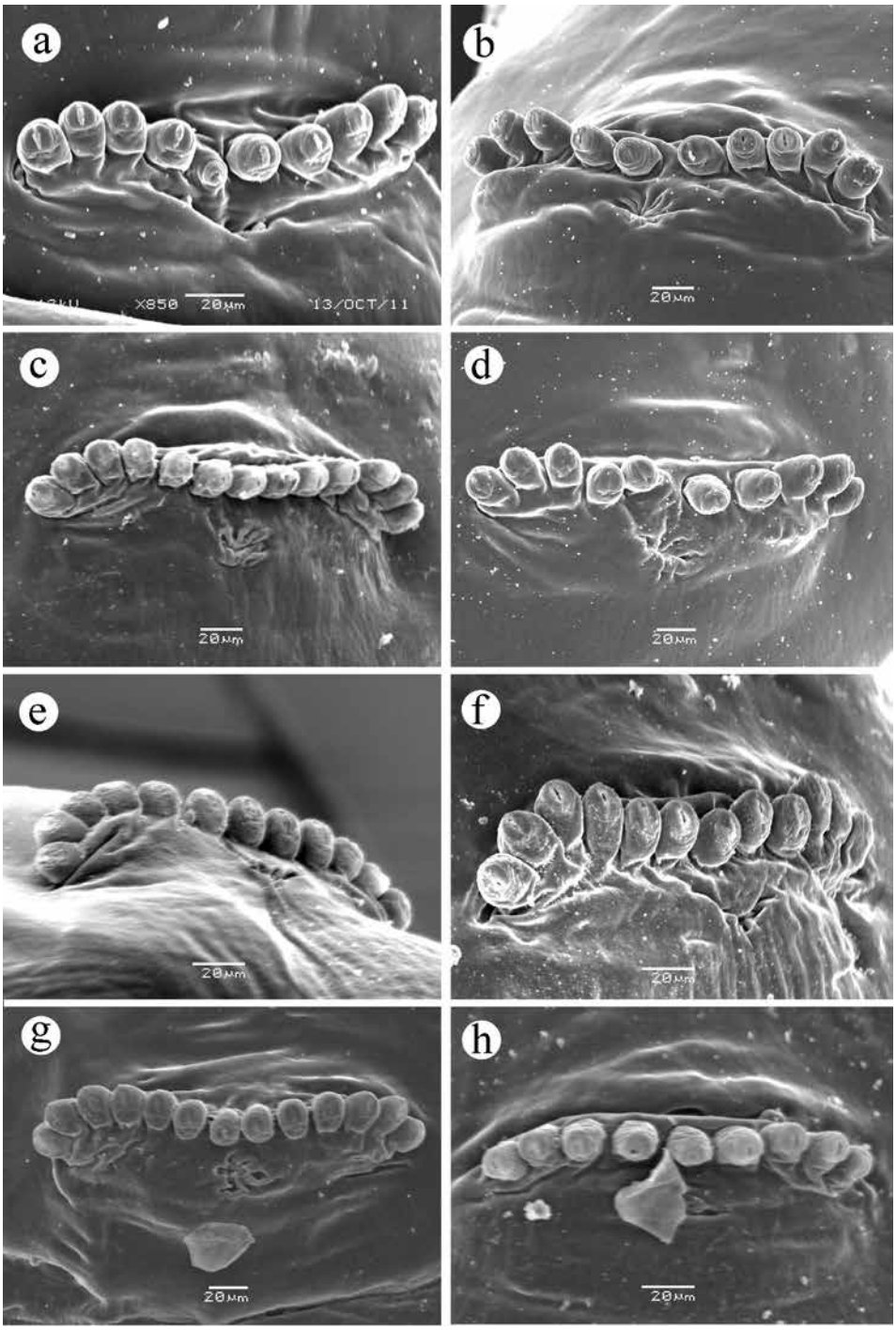

Figure 5. Anterior spiracles. **a** *Ceratitis fasciventris* **b** *Ceratitis anonae* **c** *Ceratitis rosa* R1, Kenya **d** *Ceratitis rosa* R2, Kenya **e** *Ceratitis rosa* R1, S. Africa, Nelspruit **f** *Ceratitis rosa* R2, S. Africa, Pretoria **g** *Ceratitis rosa* R2, S. Africa, Stellenbosch **h** *Ceratitis capitata*, Guatemala.

length 0.37–0.43 mm, ventral cornu length 0.52–0.72 mm; mandible length a 0.21–0.23 mm, mandible length b 0.21–0.25 mm, mandible length c 0.15–0.16 mm, mandible height 0.14–0.16 mm; hypopharyngeal sclerite length 0.12–0.17 mm, dorsal arch height 0.25–0.30 mm; anterior spiracle with **9–12** tubules, tubules closely spaced in a single **slightly sinuous** row; distal width **0.14–0.17 mm (n = 5)**; segments **T1-T2** with broken rows of weak, conical spinules on dorsal anterior margin; on **T1** the spinulose area encircles the body, while on **T2** the ventral spinulose area is separated from that of the dorsum; dorsal spinules **absent on T3-A8**; posterior spiracle rimae **ca. 0.07** mm long; spiracular processes mostly unbranched to mostly branched, base of SP-I and SP-IV narrow, numbers of trunks and tips as follows; I (dorsal) (**2-9, 3-14**), II (**2-4, 4-7**), III (**2-6, 4-9**), IV (ventral) **(4-8, 6-12**). Anal lobes **grooved**, lobes subequal or posterior lobe larger.

Specimens examined. n = 5 (SEM) + 10 (slide).

Ceratitis capitata

Figures 1h, 2h, 3h, 4e-h, 5h, 6h, 7h, 8h, 9h

Diagnosis of third instar. Medium-sized muscidiform larvae with mandibular tooth ventrally grooved, minute subapical mandibular tooth present or absent; with 8-12 oral ridges; accessory plates absent; petal-like secondary stomal lobes present; sclerotized stomal guards absent; dorsal spinules present on segments T1-T3; anterior spiracles with 9-12 tubules in a single sinuous row; base of anterior spiracle cylindrical, ca. half as wide as apical width; posterior spiracles with rimae ca. 3 times longer than wide; spiracular processes mostly unbranched, with narrow bases; caudal ridge present; anal lobes entire or grooved, lobes subequal.

Description of third instar (differences from *C. rosa* description above are noted in bold font). Similar to *C. rosa*, except length **3.9–8.7 mm**; oral ridges **9–11 (rarely 8 or 12)**; accessory plates **absent**; parastomal sclerite straight to curved; **CPS length 1.06–1.11 mm, mandible secondary tooth present or absent, mandible tip to notch 0.53–0.57 mm, dorsal cornu length 0.27–0.43 mm, ventral cornu length 0.69–0.73 mm; mandible length a 0.21–0.23 mm, mandible length b 0.20–0.22 mm, mandible length c 0.14–0.15 mm, mandible height 0.14–0.16 mm; hypopharyngeal sclerite length 0.16–0.17 mm, dorsal arch height 0.25–0.29 mm**; anterior spiracle with **9–12** tubules, tubules closely spaced in a single **slightly sinuous** row; distal width **0.16–0.19 mm (n = 8)**; segments **T1-T3 (rarely A1)** with broken rows of weak, conical spinules on dorsal anterior margin; on **T1** the spinulose area encircles the body, while on **T2-T3** the ventral spinulose area is separated from that of the dorsum; dorsal spinules **absent on A1-A8**; posterior spiracle rimae **0.07–0.08** mm long; spiracular processes mostly unbranched, numbers of trunks and tips as follows: I (dorsal) (**6-15, 10-17**), II (**3-4, 4-5**), III (**2-6, 4-11**), IV (ventral) **(2-10, 3-14**). Anal lobes **entire or grooved**, lobes subequal.

Specimens examined. n = 41 (SEM) + 9 (slide).

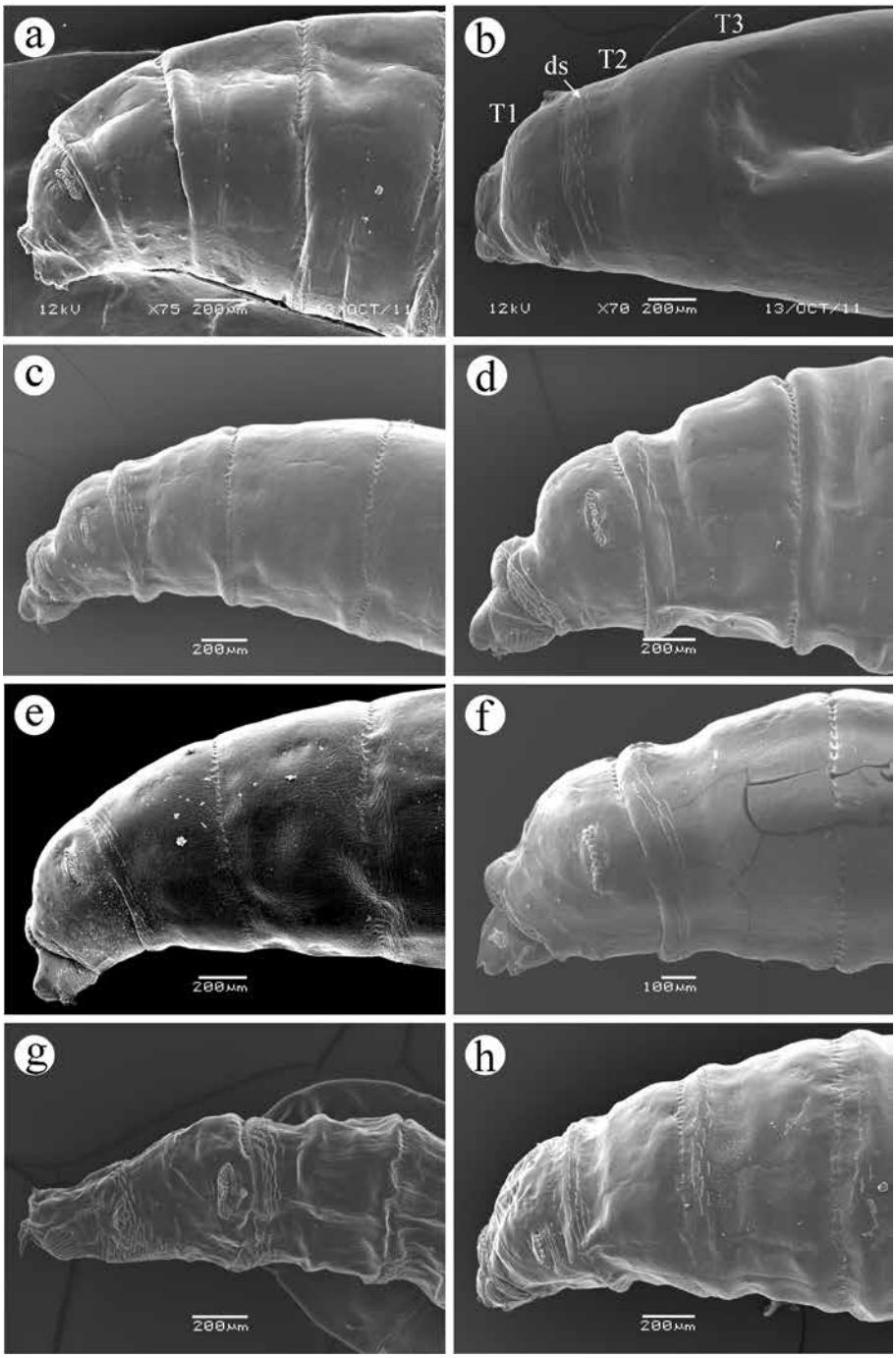

Figure 6. Segments T1-T3. **a** *Ceratitis fasciventris* **b** *Ceratitis anonae* **c** *Ceratitis rosa* R1, Kenya **d** *Ceratitis rosa* R2, Kenya **e** *Ceratitis rosa* R1, S. Africa, Nelspruit **f** *Ceratitis rosa* R2, S. Africa, Pretoria **g** *Ceratitis rosa* R2, S. Africa, Stellenbosch **h** *Ceratitis capitata*, Guatemala. Abbreviations: **T1**, **T2**, **T3** thoracic segments 1 to 3, **ds** dorsal spinules.

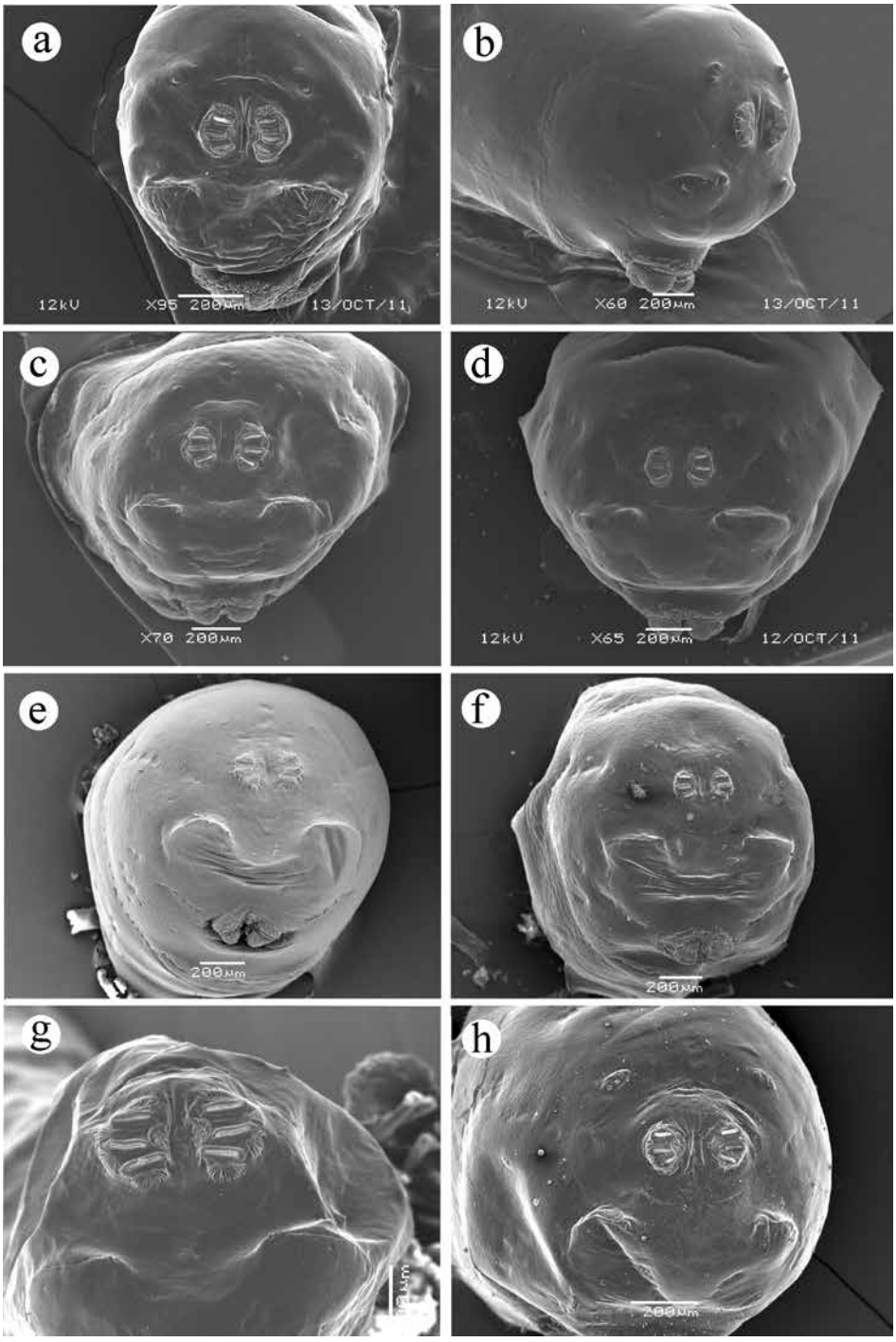

Figure 7. Caudal segment. **a** *Ceratitis fasciventris* **b** *Ceratitis anonae* **c** *Ceratitis rosa* R1, Kenya **d** *Ceratitis rosa* R2, Kenya **e** *Ceratitis rosa* R1, S. Africa, Nelspruit **f** *Ceratitis rosa* R2, S. Africa, Pretoria **g** *Ceratitis rosa* R2, S. Africa, Stellenbosch **h** *Ceratitis capitata*, Kenya.

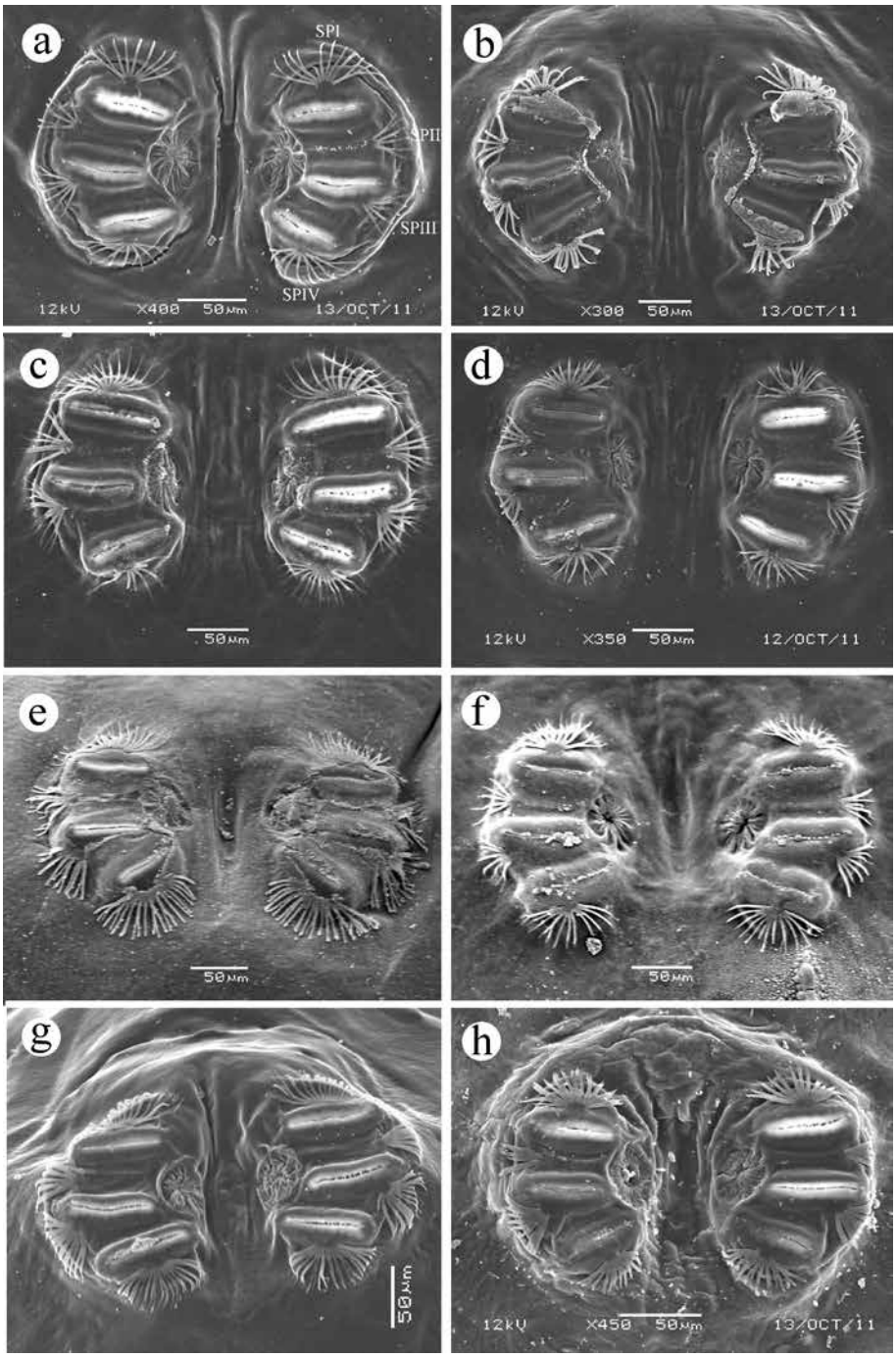

Figure 8. Posterior spiracles. **a** *Ceratitis fasciventris* **b** *Ceratitis anonae* **c** *Ceratitis rosa* R1, Kenya **d** *Ceratitis rosa* R2, Kenya **e** *Ceratitis rosa* R1, S. Africa, Nelspruit **f** *Ceratitis rosa* R2, S. Africa, Pretoria **g** *Ceratitis rosa* R2, S. Africa, Stellenbosch **h** *Ceratitis capitata*, Kenya. Abbreviations: **SPI**, **SPII**, **SPIII**, **SPIV** spiracular processes 1 to 4.

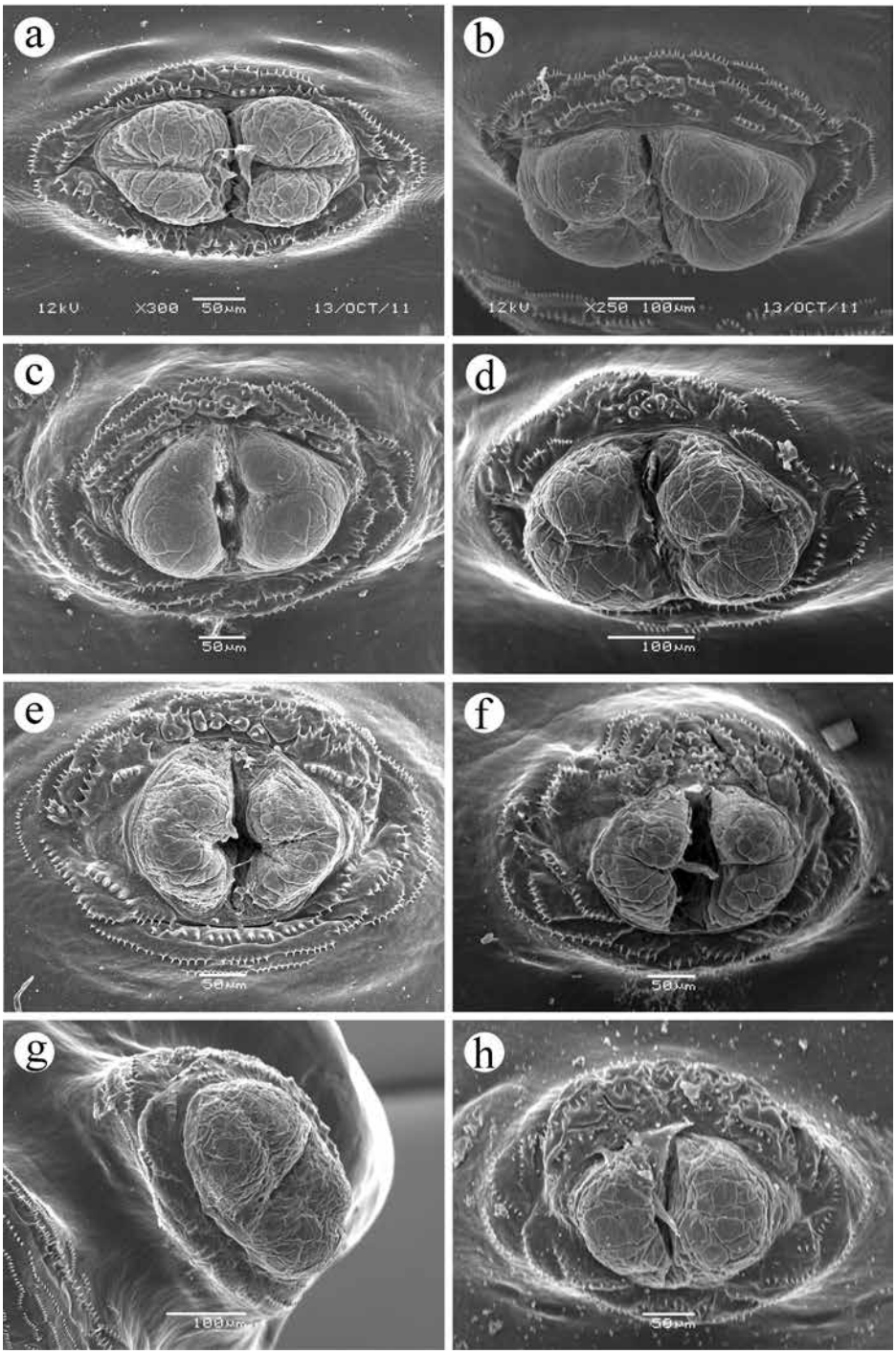

Figure 9. Anal lobes. **a** *Ceratitis fasciventris* **b** *Ceratitis anonae* **c** *Ceratitis rosa* R1, Kenya **d** *Ceratitis rosa* R2, Kenya **e** *Ceratitis rosa* R1, S. Africa, Nelspruit **f** *Ceratitis rosa* R2, S. Africa, Pretoria **g** *Ceratitis rosa* R2, S. Africa, Stellenbosch **h** *Ceratitis capitata*, Kenya.

Discussion

Although larval stages of numerous fruit fly species have been described, very few are based on wide geographic sampling, and often they are based on colony material. The extent to which these descriptions reflect actual variation in nature is generally unknown. Specimens from laboratory colonies are probably more homogenous than those collected directly from the wild. Sample sizes used in this study are small, so we have to expect that the range of measurements presented here is less than that in nature.

There are consistent morphological differences in larval character states among some of the *C. rosa* populations studied here. Some of these would be considered key diagnostic characters to recognize different species in other genera, e.g. *Anastrepha* (Steck et al. 1990). Whether they represent intra-specific variation or diagnostic differences among biologically distinct taxa of *Ceratitis* cannot be answered using these data alone. The larval morphological differences observed among populations examined here are not congruent in any simple way with the R1 and R2 designations. The diagnosis and description of *C. rosa s.l.* given here incorporates data from all of the populations observed. If some of these eventually are determined to represent different taxa, then the diagnosis and description of *C. rosa s.s.* may require alteration, as some character states do not overlap among populations. Additional data of other types (e.g., genetic, behavioral, etc.) are required to determine the taxonomic interpretation of larval morphology data.

Even beyond the question of possible cryptic species among *C. rosa s.l.*, it is maddeningly difficult to find reliable diagnostic differences in larval morphology among species of the FAR complex. Many of the quantitative larval characters seem to be little constrained and their ranges vary wildly. However, *Ceratitis fasciventris* can generally be distinguished from *C. rosa s.l.* and *C. anonae* by its smaller dimensions of the CPS and anterior spiracle apical width, and lower counts of spiracular processes and narrowness of their bases.

C. capitata larvae can be separated from most individuals of the FAR complex by the absence of oral ridge accessory plates and the presence of dorsal spinules on T3. Also the shape of the anterior spiracle seems consistently different (smoothly expanded from the base to the tubules), as described and illustrated by Carroll (1998). Presence vs. absence of a small secondary tooth on the mandible is not a reliable character state to separate them. While the secondary tooth is typically present and easy to see on FAR larvae, it may be absent or poorly developed as seen in the *C. rosa* R2 colony at ICIPE. Conversely, the secondary tooth is often present on larvae of *C. capitata*, although it frequently is poorly developed and not likely to be noticed except by use of SEM.

It should be noted that presence vs. absence of oral ridge accessory plates has been used as a key character to separate larvae of the genus *Ceratitis* from those of *Bactrocera* (White and Elson-Harris 1992). It is now clear that this distinction was falsely based on too limited taxon sampling within *Ceratitis*. Further studies are needed to determine reliable characters to separate these genera.

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Comparative analysis of development and survival of two Natal fruit fly *Ceratitis rosa* Karsch (Diptera, Tephritidae) populations from Kenya and South Africa

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Abstract

Comparative analysis of development and survivorship of two geographically divergent populations of the Natal fruit fly *Ceratitis rosa* Karsch designated as *C. rosa* R1 and *C. rosa* R2 from Kenya and South Africa were studied at seven constant temperatures (10, 15, 20, 25, 30, 33, 35°C). Temperature range for development and survival of both populations was 15–35°C. The developmental duration was found to significantly decrease with increasing temperature for *C. rosa* R1 and *C. rosa* R2 from both countries. Survivorship of all the immature stages of *C. rosa* R1 and *C. rosa* R2 from Kenya was highest over the range of 20–30°C (87–95%) and lowest at 15 and 35°C (61–76%). Survivorship of larvae of *C. rosa* R1 and *C. rosa* R2 from South Africa was lowest at 35°C (22%) and 33°C (0.33%), respectively. Results from temperature summation models showed that *C. rosa* R2 (egg, larva and pupa) from both countries were better adapted to low temperatures than R1, based on lower developmental threshold. Minimum larval temperature threshold for Kenyan populations were 11.27°C and 6.34°C (R1 and R2, respectively) compared to 8.99°C and 7.74°C (R1 and R2, respectively) for the South African populations. Total degree-day (DD) accumulation for the Kenyan populations were estimated at 302.75 (*C. rosa* R1) and 413.53 (*C. rosa* R2) compared to 287.35 (*C. rosa* R1) and 344.3 (*C. rosa* R2) for the South African populations. These results demonstrate that *C. rosa* R1 and *C. rosa* R2 from both countries were physiologically distinct in their re-

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sponse to different temperature regimes and support the existence of two genetically distinct populations of *C. rosa*. It also suggests the need for taxonomic revision of *C. rosa*, however, additional information on morphological characterization of *C. rosa* R1 and *C. rosa* R2 is needed.

Keywords
Ceratitis rosa, comparative demography, developmental thresholds, survivorship

Introduction

Amongst the Afro-tropical group of tephritid fruit flies (Diptera: Tephritidae), *Ceratitis rosa* Karsch is considered a serious pest of cultivated fruit (White and Elson-Harris 1992, De Meyer 2001a, Copeland et al. 2006). *Ceratitis rosa* is highly polyphagous being recorded on over 90 species of wild and cultivated crops (De Meyer et al. 2002). In mainland Africa, *C. rosa* is known only from southern and eastern Africa, being absent from the western and central parts of the continent (De Meyer 2001a). Outside this native range, *C. rosa* has also been reported from the Indian Ocean islands of Mauritius and La Réunion where it is regarded as a major pest of quarantine importance (Orian and Moutia 1960, De Meyer 2001b, White et al. 2001, Duyck and Quilici 2002). The phytophagous nature of *C. rosa* and its ability to expand its distribution beyond its native range raises major concern for the horticulture industry in Africa and beyond (De Meyer et al. 2008, Li et al. 2009, Mwatawala et al. 2009, de Villiers et al. 2012).

In Kenya, *C. rosa* was originally thought to be restricted to the coastal region (De Meyer 2001a). However, on 7th December 2001, Copeland and Wharton (2006) reported the occurrence of *C. rosa* from fruit of five indigenous and exotic plants in the central highlands of Kenya at an altitude of 1,533-1,771 m above sea level. Prior to this, there were no records of *C. rosa* in Central Kenya following several wild fruit collections in that area by Copeland and Wharton (2006), which led the authors to conclude that *C. rosa* was a recent colonizer of the central highlands of Kenya. Subsequent surveys in the area have led to continuous recovery of the pest from mango (S. Ekesi et al. unpublished data). In South Africa, *C. rosa* is widely distributed across the country but is either scarce or absent in the drier inland regions (De Meyer 2001a, De Villiers et al. 2013). In a review on the fruit fly fauna of South Africa, Bezzi (1924) reported on the collection of two *C. rosa* groups: (1) "*C. rosa*" from the northern parts of South Africa and "*C. fasciventris*" (formerly *C. rosa*) from the southern and eastern parts of the country. Reports of *C. rosa* in different parts of the Western Cape was also claimed by some researchers (Hepburn and Bishop 1954).

Ceratitis rosa is morphologically very similar to two other species within the same subgenus *Pterandrus*: *C. fasciventris* (Bezzi) and *C. anonae* Graham (De Meyer and Freidberg 2006). The 3 species form a complex known as the FAR complex (Virgilio et al. 2013) and are sexually dimorphic (De Meyer and Freidberg 2006). The males within the FAR complex can be readily separated based on differences on their leg and anepisternal pilosity patterns (De Meyer and Freidberg 2006). For example, morphological comparisons of the two *C. rosa* clusters: *C. rosa* R1 and *C. rosa* R2 showed

differences in the shape and ornamentation of the mid-tibia of the males (Virgilio et al. 2013). The males of the two *C. rosa* groups can be distinguished from each other as follows: The black area of the mid tibia of *C. rosa* R1 reaches the lateral margins while the black area of the mid tibia of *C. rosa* R2 does not reach the lateral margins (De Meyer et al. 2015).

Recent genetic analysis has shown that the FAR complex is probably five entities, rather than the three taxonomic species (Virgilio et al. 2013). A neighbor Joining tree from these studies showed that morphospecies of *C. rosa* and *C. fasciventris* was represented by two well-supported clusters of populations depicted as R1 and R1 (for *C. rosa*), and F1 and F2 (for *C. fasciventris*). The authors recommended a thorough assessment of the different ecological requirements (e.g. host preference, thermal tolerance etc) of the two populations of *C. rosa* given their huge economic significance. The possibility of two forms of *C. rosa* was earlier suggested in molecular studies by Barr et al. (2006) who associated the forms with geographical distribution of the pest (South Africa and La Reunion form versus Kenyan form). Moreover, differences in thermal developmental rates between *C. rosa* from La Reunion and South Africa were found in studies conducted separately in the respective countries (Duyck and Quilici 2002, Grout and Stoltz 2007) leading Grout and Stoltz (2007) to suggest the possibility of existence of two biotypes of *C. rosa*, one being more cold tolerant than the other.

Temperature is the single most important environmental factor determining development and survival of tephritid fruit flies (Fletcher 1989). Temperature effects on development and stage-specific survival have been shown to influence both the quantity and quality of tephritid fruit flies produced (Vargas et al. 1996, Vargas et al. 1997, Brévault and Quilici 2000, Vargas et al. 2000, Duyck and Quilici 2002, Trudgill et al. 2005, Grout and Stoltz 2007, Rwomushana et al. 2008, Vayssières et al. 2008, Liu and Ye 2009, Salum et al. 2013). Various tephritid species have specific optimal temperature range for development limited by lower and upper thresholds (base temperature and upper limit). Below and above these temperature limits, development does not occur and this can vary both with developmental stage and geographical origin (Honék and Kocourek 1990). Information on the thermal requirements of insect groups forms an important basis in understanding and predicting the geographical distribution of the different insect groups.

Given the recent evidence of existence of the two groups of *C. rosa*, studies were undertaken separately in Kenya and South Africa, spanning across the geographical distribution of the pest in mainland Africa, to determine the thermal developmental rates and thresholds of the two *C. r*osa types.

Materials and methods

Fruit fly cultures

The colonies of the two *C. rosa* groups (*C. rosa* R1 and *C. rosa* R2, "hereafter referred to as R1 and R2") from Kenya were established at the Animal Rearing and Containment

Unit (ARCU) of the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya. The *C. rosa* R1 colony was started with 93 flies (47 males and 46 females) reared from infested fallen guava fruits collected from a farm in Kibarani, Msambweni district, Kenya (S 04°19.628'; E 039°32.411'; 34 m a.s.l). The *C. rosa* R2 colony was initially started with 29 individuals (14 males and 15 females) recovered from infested mango fruits collected at a smallholder farm in Kithoka, Imenti North district, Kenya (N 00°05'58.9"; E 037°40'39.5"; 1,425 m a.s.l).

Stock cultures of the South African *C. rosa* groups came from infested jambos, *Syzygium jambos* L. (Alston), and loquat, *Eriobotrya japonica* (Thunb.) Lindl. Collected, respectively, from the following locations: Nelspruit: S 25° 27' 08.19" E30° 58' 11.27", approx 612 m) and Pretoria: S25° 45' 13.7" E28° 13' 45", approx 1,368 m a.s.l). The flies originating from Nelspruit were designated as *C. rosa* R1 by M. De Meyer (Royal Museum for Central Africa) and those from Pretoria were assigned as *C. rosa* R2

Procedures for obtaining the wild fruit fly populations from infested fruits in both countries were carried out according to the methodology described by Rwomushana et al. (2008). The larvae of the two *C. rosa* populations reared from the host fruits in each countries were subsequently transferred to carrot-based artificial diet after two generations on fruits (Ekesi et al. 2007).

On the artificial diet, the two *C. rosa* populations from each country were reared for 5-8 generations before the start of the experiments. In both countries R1 colony was kept at 28 ± 1°C, 50 ± 8% RH and photoperiod of L12: D12, while the R2 colony was kept at 23 ± 1°C, 60 ± 10% RH and photoperiod of L12: D12).

Egg collection

Newly emerged adults were held in well ventilated Perspex cages (30 cm length x 30 cm width x 30 cm height). The eggs of each *C. rosa* population were collected by offering ripe fruit domes (fruit skin that has the seed and pulp scooped out) to mature adult flies. Numerous small holes were made on the fruit domes made using pins (0.8 mm diameter) to facilitate oviposition by the adult flies. The eggs were collected within a uniform time interval of 1 h after oviposition using a moistened fine camel's hair brush

Effect of temperature on development and survival of eggs, larvae and pupae of *C. rosa*

Egg: Using a fine brush, one hundred (100) eggs were randomly selected, counted and carefully lined on moistened sterilized black cloth, which were thereafter placed on top of ≈ 60 g of diet inside a Petri dish. The Petri dishes were immediately transferred to thermostatically controlled environmental chambers (MIR-554-PE, Sanyo/Panasonic cooled incubators, Japan and modified Conviron CMP3023 incubators, Manitoba, Canada were used in Kenya and South Africa, respectively) set at seven constant tem-

peratures of 10, 15, 20, 25, 30, 33 and 35°C (± 0.03°C) and 50 ± 8% RH, 12:12 L:D photoperiod. Duration of egg stage was observed at 6-hourly intervals under a binocular microscope to determine the time and percentage hatch. The start time was taken as the time when the eggs were collected from the mango dome or apple and developmental time and survival for each replicate were estimated. The experiments were replicated 5 to 6 times. The required temperatures inside the incubators were regularly monitored using standard thermo-hygrometers and experiments in which temperatures fluctuated more than ±0.03°C were discarded and not included in the analysis.

Larva: One hundred neonate larvae of ~1 h old were randomly obtained from the fruit fly cultures and carefully transferred to squares (either 1cm^2 or 2 cm^2) of filter paper. The square filter paper containing neonate larvae were placed on top of a 150 g carrot-based larval diet in either a Petri dish or a plastic container. The Petri dish or plastic container was then placed in a rectangular plastic rearing container carrying a thin layer (~ 0.5 cm) of sterilized sand at the bottom for pupation and then transferred to the thermostatically controlled environmental chamber. The top of the plastic container was screened with light cloth netting material for ventilation. Larvae fed *ad libitum*, and mature larvae were allowed to freely leave the Petri dish into rectangular plastic containers for pupation. The sand was observed daily for newly formed pupae and puparia were separated from the pupation medium by gentle sifting. Records of larval durations were kept for each *C. rosa* group at each temperature regime. The experiments were replicated 5 times.

Pupa: One hundred newly formed pupae (~ 1 h old) were randomly obtained from the fruit fly cultures kept at the rearing conditions described previously. The pupae used were from larvae kept at the same temperature being studied. Pupae used were placed in Petri dishes (8.6 cm diameter) and transferred into aerated Perspex cages (30 cm x 30 cm x 30 cm) to allow for adult emergence. The cages were monitored on a daily basis for adult emergence and pupal developmental time and survival were recorded. The experiments were replicated 5 times.

Data analyses

The developmental time and percentage survival of each immature life stage of the two *C. rosa* groups in each country were compared using a two-way analysis of variance (ANOVA). Prior to analysis, the developmental time data and percentages of survivorship were subjected to [log (x + 1)] and arcsine-square-root transformation [Arcsin square root (x+1)], respectively, to meet the assumption of homogeneity (Sokal and Rohlf 1981). Means were further compared where appropriate, by the Student-Newman-Keuls (SNK) (Steel and Torrie 1980) multiple range tests at $\alpha = 0.05$.

Linear model: The linear model expressed as $r(T) = a + bT$ was used to estimate the relationship between relevant temperatures and developmental rate of *C. rosa*. In this model, r is the rate of development [=1/Development time (D) in days], T is ambient temperature (°C); intercept (a) and slope (b) are the model parameters. Thermal con-

stant, K (=1/b) is the number of degree-days (DDs) or heat units above the threshold needed for completion of a developmental stage. Lower temperature threshold (T_{min}) was determined using the inverse slope of the fitted linear regression line as the x-intercept (= - a/b), and is the estimated lower temperature at which the rate of development is either zero or no measurable development occurs (Campbell et al. 1974). Campbell et al. (1974), provide statistics for the standard error (SE) of the lower developmental threshold (T_{min}) and this was used as follows:

$$s.e.(t_{\min}) = \frac{\bar{y}}{b}\sqrt{\frac{s^2}{N(\bar{y})^2} + \left[\frac{s.e.(b)}{b}\right]^2}$$

where s^2 is the residual mean square of y, $\bar{y}$ is the sample mean, and N is the sample. Additionally, the size of the SE_K for the thermal constant K for the linear model having slope b is expressed as:

$$s.e.(k) = s.e.(b) / b^2$$

Nonlinear model: Several empirical nonlinear models were fitted to the instar specific developmental rate data to estimate the optimum temperature threshold (T_{opt}) and upper temperature threshold (T_{max}). T_{opt} is the threshold temperature at which developmental rate is maximal, while T_{max} is the lethal threshold at which development ceases. Among the various non-linear models applied to assess the nonlinear relationship, Brière 1 model provided an excellent description of the temperature-dependent development of lowland and highland populations of *C. rosa* across all temperatures tested for all developmental stages, permitting the estimation of the upper and lower developmental thresholds (Brière et al. 1999). The Brière -1 model is given by the expression:

$$r(T) = aT\,(T - T_{min}) \times (T_{max} - T)^{1/2}$$

where, r is the developmental rate as a function of temperature (T), and 'a' is an empirical constant. The following equation from Brière et al. (1999) was used to calculate the optimum temperature:

$$T_{opt} = [4T_{max} + 3T_{min} + (16T_{max}^2 + 9T_{min}^2 - 16T_{min}T_{max})^{1/2}]/10$$

The mean values for T_{min}, T_{opt}, and T_{max} were determined for each life stage for each group of *C. rosa* using the results generated by the developmental rate models.

For both the linear and non-linear models, the following statistical items were used to assess the goodness-of-fit: the coefficient of determination (for linear model; R^2) or the coefficient of nonlinear regression and residual sum of squares (RSS) (for nonlinear models; R^2). Higher values of R^2 and lower values for RSS reveal a better fit. For the linear regression, data which deviated from the straight line through the other points were rejected for correct calculation of regression (Campbell et al. 1974).

Results

Kenya - Effect of temperature on development of immature life stages

Egg: For R1, egg development was longest at 15°C and shortest at 35°C (F = 108.2; df = 5, 50; P=0.0001) (Table 1). For R2, the time required for eggs to hatch decreased from 7.10 ± 0.77 d at 15°C to 1.83 ± 0.34d at 33°C (F = 43.25; d.f. = 4, 51; P = 0.0001). There were significant differences in egg developmental duration at 15°C (F = 9.803; d.f. = 1, 29; *P*=0.0040), 20°C (F = 13.84; d.f. = 1, 21; P = 0.0013) and 30°C (F = 4.859; d.f. = 1, 17; P = 0.0416) between the two *C. rosa* groups. However, no significant differences in egg developmental duration were observed between the two *C. rosa* groups at 25°C (F = 2.075; d.f. = 1, 15; P = 0.1700) and 30°C (F = 0.946; d.f. = 1, 12; P = 0.3500). The eggs of both *C. rosa* groups did not develop at 10°C. At 35°C, eggs of R2 also failed to hatch.

Larva: At larval stage, the trend was similar to egg with developmental duration decreasing from 28.71 ± 0.65 d at 15°C to 6.77 ± 0.52 d at 35°C (F = 705.6; d.f. = 5, 72; P = 0.0001) for R1 and from 23.93 ± 0.64 d at 15°C to 9.36 ± 0.30 d at 33°C (F = 422.5; d.f. = 4, 60; P = 0.0001) for R2 (Table 1). There were significant differences in the duration of larval development at all tested temperatures between the two *C. rosa* groups at 15-35°C (F = 7.2 – 84.1; d.f. = 1, 25; P = 0.0135 – < 0.0001) (Table 1). At 15 and 20°C, the larval developmental duration of R1 was significantly longer than that of R2. In contrast, the larval developmental duration of R1 was significantly shorter at 25, 30, 33 and 35 °C compared to R2. The larvae of both *C. rosa* groups failed to develop at 10°C. Also at 35°C no development occurred for the R2.

Pupa: At 10, 33 and 35°C no eclosion was observed for both *C. rosa* groups (Table 1). Pupal developmental duration of R1 and R2 varied significantly between the other temperatures (F = 455.9; d.f. = 3, 47; P < 0.0001 and F = 945.5; d.f. = 3, 48; P = 0.0001, respectively). Moreover, the pupal developmental duration varied significantly between the two *C. rosa* groups at 15°C (F = 54.7; d.f. = 1, 25; P < 0.0001), 20°C (F = 28.66; d.f. = 1, 24; P < 0.0001), 25°C (F =69.64; d.f. = 1, 22; P< 0.0001) and 30°C (F = 41.09; d.f. = 1, 24; *P* < 0.0001). The longest pupal developmental duration occurred at 15°C for both R1 and R2 . It took 8.31 ± 0.4 days for R1 and 10.85 ± 0.48 days for R2 to reach eclosion at 30°C.

Egg-adult: Total developmental duration from egg to adult for R1 and R2 was longest at 15°C and shortest at 30°C. Significant differences were found between the two *C. rosa* groups when egg to adult developmental durations were compared across all the temperatures (Table 1) (F = 57.6 – 143.6; d.f. = 1, 25; P = 0.0037 – < 0.0001).

Kenya - Temperature-dependent developmental rates

Estimated parameter values of the linear and nonlinear models are presented in Table 2. For each *C. rosa* group, a strong and positive linear relationship was observed between temperature and development rates for egg, larval and pupal stages (Table 2).

Table 1. Mean ± SE developmental time (days) of immature stages of *C. rosa* R1 and *C. rosa* R2 from Kenya at different constant temperatures.

Temperature (°C)	Egg		Larva		Pupa		Total (days)	
	***C. rosa* R1**	***C. rosa* R2**	***C. rosa* R1**	***C. rosa* R2**	***C. rosa R1***	***C. rosa* R2**	***C. rosa* R1**	***C. rosa* R2**
10	-	-	-	-	-	-	-	-
15	$8.91 \pm 0.51^{a}B$	$7.10 \pm 0.77^{a}A$	$28.71 \pm 0.65^{a}B$	$23.93 \pm 0.64^{a}A$	$32.54 \pm 0.85^{a}B$	$27.79 \pm 0.64^{a}A$	$68.64 \pm 1.79^{a}B$	$58.85 \pm 1.39^{a}A$
20	$5.50 \pm 0.59^{b}B$	$3.82 \pm 0.34^{b}A$	$14.92 \pm 0.56^{b}B$	$12.36 \pm 0.51^{b}A$	$19.31 \pm 0.53^{b}B$	$16.77 \pm 0.55^{b}A$	$39.0 \pm 0.85^{b}B$	$32.67 \pm 0.59^{b}A$
25	$2.43 \pm 0.24^{c}A$	$2.90 \pm 0.33^{bc}A$	$8.92 \pm 0.52^{c}B$	$10.17 \pm 0.50^{c}A$	$9.92 \pm 0.43^{c}B$	$13.82 \pm 0.59^{c}A$	$19.60 \pm 1.33^{c}B$	$27.20 \pm 0.81^{c}A$
30	$1.90 \pm 0.33^{c}B$	$2.56 \pm 0.24^{bc}A$	$7.75 \pm 0.58^{d}B$	$9.71 \pm 0.41^{c}A$	$8.31 \pm 0.42^{d}B$	$10.85 \pm 0.48^{d}A$	$17.30 \pm 1.33^{c}B$	$23.0 \pm 0.81^{d}A$
33	$1.50 \pm 0.24^{c}A$	$1.83 \pm 0.34^{c}A$	$7.36 \pm 0.50^{d}B$	$9.36 \pm 0.30^{c}A$	No emergence	No emergence	-	-
35	$1.38 \pm 0.23^{c}B$	$0.00 \pm 0.00A$	$6.77 \pm 0.52^{d}B$	$0.00 \pm 0.00A$	No emergence	No emergence	-	-

Means in the same column followed by the lower case and in the same row followed by the same upper case letter are not significantly different by Student – Newman – Keul's (SNK) test, $P < 0.05$.

Table 2. Parameter estimates and their approximate standard errors for linear and Brière-1 nonlinear models describing the relationship between temperature and development rate (1/D) of *C. rosa* R1 and *C. rosa* R2 from Kenya

Model	Parameters	***C. rosa* R1**			***C. rosa* R2**		
		Egg	**Larva**	**Pupa**	**Egg**	**Larva**	**Pupa**
Linear	*a*	-0.412	-0.077	-0. 080	-0.270	-0.041	-0. 043
	b	0.035	0.008	0.007	0.0263	0.006	0.005
	K	28.57 ± 2.68	133.33 ± 7.24	140.85 ± 33.13	37.04 ± 1.96	172.41 ± 37.75	204.08 ± 30.28
	T_{min}	11.77 ± 1.50	10.27 ± 2.54	11.31 ± 2.26	10.0 ± 0.83	7.07 ± 3.99	8.73 ± 2.15
	RSS	2.6×10^{-5}	4.2×10^{-5}	1.4×10^{-4}	2.0×10^{-3}	8.1×10^{-5}	1.3×10^{-4}
	R^2	0.999	0.991	0.936	0.899	0.908	0.895
Brière-1	T_{min}	14.23 ± 1.08	11.27 ± 0.71	11.66 ± 0.47	9.66 ± 1.45	6.34 ± 0.84	8.09 ± 0.69
	T_{max}	37.0 ± 0.22	37.0 ± 8.71	33.0 ± 1.28	35.0 ± 2.64	35.0 ± 1.19	33.0 ± 1.26
	T_{opt}	31.44	30.98	27.87	29.16	28.70	27.35
	R^2	0.945	0.896	0.835	0.992	0.898	0.905

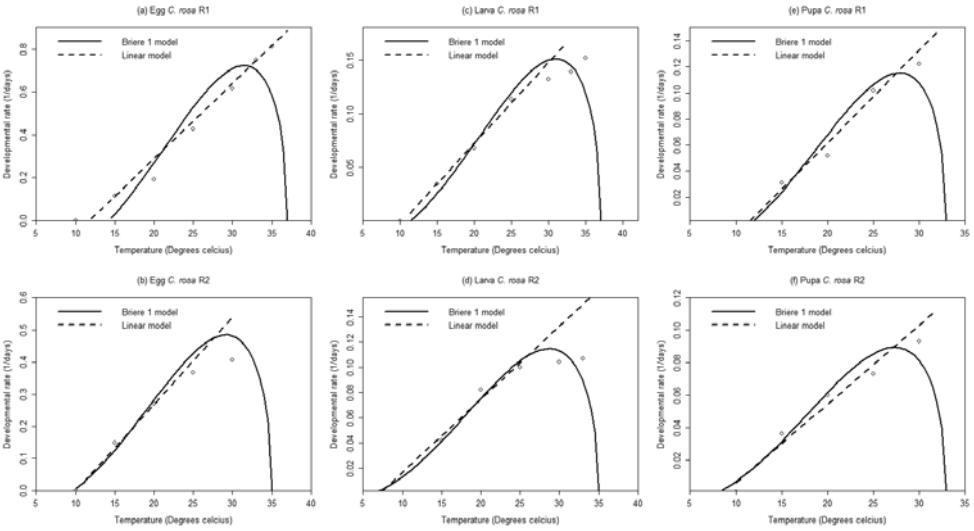

Figure 1. Linear and non-linear regressions of temperature related developmental rates of immature stages of two groups of *C. rosa* from Kenya

Using the linear model, the lowest developmental threshold for eggs was estimated at 11.8°C for R1 and 10.0°C for R2. The egg stage required 28.57 degree-days (DD) to complete development in the R1 and 37.04 DD in the R2. *Ceratitis rosa* R1 required 133.33 DD above the development threshold of 10.27°C to complete development from larval stage to the pupal stage while R2 took 172.41 DD to develop above a threshold of 7.07°C (Table 2). The lower developmental thresholds for pupae of R1 and R2 were calculated as 11.3 and 8.7°C, respectively. The corresponding thermal constants of the pupal stage were 140.85 DD for R1 and 204.08 DD for R2.

For R1, the low developmental thresholds generated by the Brière-1 model were found to be slightly higher for egg, larva and pupa compared to those estimated by the linear regression model while for R2 the lower developmental thresholds estimated were slightly lower for egg, larva and pupa (Figure 1 and Table 2). The model estimated optimum temperature range of 27.9–31.4°C for R1 and 27.4-29.2°C for R2 (Table 2). The lethal temperatures for R1 and R2 were estimated to range from 33.0–35.0°C and 33.0–37.0°C, respectively, for the various developmental stages (Table 2).

Kenya - Survival of immature stages

At the egg stage, percentage survival ranged from 76.8 ± 4.3% at 35°C to 93.8 ± 2.0% at 25°C in R1 ($F = 4.75$; d.f. = 5, 24; $P = 0.0037$) and 80.4 ± 3.2% at 33°C to 91.8 ± 1.8% at 20°C ($F = 5.17$; d.f. = 4, 20; $P = 0.0050$) in R2 (Table 3).

For R1, survival rate was lowest at 35°C and highest at 25°C ($F = 13.22$; d.f. = 5, 24; $P < 0.0001$) while for R2, survivorship at larval stage ranged between 67.6 ± 1.6%

Table 3. Mean ± SE survivorship (%) of immature stages of *C. rosa* R1 and *C. rosa* R2 from Kenya at different constant temperatures

Temperature(°C)	Egg		Larva		Pupa	
	***C. rosa* R1**	***C. rosa* R2**	***C. rosa* R1**	***C. rosa* R2**	***C. rosa* R1**	***C. rosa* R2**
10	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
15	81.6 ± 2.94^{ab}A	87.4 ± 1.50^{a}A	75.8 ± 2.22^{b}A	84.6 ± 4.66^{a}A	90.6 ± 2.09^{a}A	92.4 ± 2.20^{a}A
20	85.8 ± 2.46^{ab}A	91.8 ± 1.80^{a}A	80.8 ± 3.88^{ab}A	86.8 ± 3.09^{a}A	91.6 ± 1.44^{a}A	95.2 ± 1.16^{a}A
25	93.8 ± 2.01^{a}A	91.6 ± 1.03^{a}A	87.6 ± 1.44^{a}A	83.6 ± 2.29^{a}A	94.2 ± 1.07^{a}A	91.4 ± 1.78^{a}A
30	92.2 ± 1.98^{a}A	89.4 ± 1.99^{a}A	85.4 ± 2.25^{ab}A	81.8 ± 2.27^{a}A	81.2 ± 2.89^{b}A	78.2 ± 3.56^{b}A
33	88.2 ± 3.57^{ab}A	80.4 ± 3.23^{b}A	78.4 ± 1.29^{ab}B	67.6 ± 1.63^{b}A	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
35	76.8 ± 4.26^{b}B	0.00 ± 0.00A	60.6 ± 2.96^{c}B	0.00 ± 0.00A	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}

Means in the same column followed by the same lower case and in the same row followed by the same upper case letter are not significantly different by Student – Newman – Keul's (SNK) test, $P < 0.05$.

Table 4. Mean ± SE developmental time (days) of immature stages of *C. rosa* R1 and *C. rosa* R2 from South Africa at different constant temperatures.

Temperature (°C)	Egg		Larva		Pupa		Total days	
	***C. rosa* R1**	***C. rosa* R2**	***C. rosa* R1**	***C. rosa* R2**	***C. rosa* R1**	***C. rosa* R2**	***C. rosa* R1**	***C. rosa* R2**
10	-	-	-	-	-	-	-	-
15	7.53 ± 0.10^{a}A	7.40 ± 0.06^{a}A	17.73 ± 0.19^{a}B	20.74 ± 0.40^{a}A	36.56 ± 0.28^{a}A	36.18 ± 0.29^{a}A	61.80 ± 0.24^{a}A	64.72 ± 0.45^{a}B
20	3.22 ± 0.00^{b}A	3.06 ± 0.00^{b}A	11.38 ± 0.12^{b}B	13.63 ± 0.36^{b}A	17.14 ± 0.36^{b}A	19.02 ± 0.87^{b}A	31.75 ± 0.43^{b}A	35.71 ± 1.06^{b}B
25	2.14 ± 0.01^{c}A	2.11 ± 0.03^{d}A	7.59 ± 0.11^{d}B	8.44 ± 0.12^{d}A	11.81 ± 0.22^{c}A	12.09 ± 0.24^{c}A	21.54 ± 0.20^{c}A	22.67 ± 0.23^{c}B
30	1.61 ± 0.00^{e}A	1.60 ± 0.02^{e}A	7.90 ± 0.03^{c}B	9.92 ± 0.61^{c}A	7.53 ± 0.02^{d}	-	17.04 ± 0.05^{d}	-
33	1.69 ± 0.01^{e}B	1.99 ± 0.08^{d}A	5.74 ± 0.06^{e}	6.73*	-	-	-	-
35	1.88 ± 0.03^{d}A	2.36 ± 0.03^{c}B	6.32 ± 0.05^{f}	7.85 ± 0.00**^{d}	-	-	-	-

Means in the same column followed by the same lower case and in the same row followed by the same upper case letter are not significantly different by Student – Newman – Keul's (SNK) test, $P < 0.05$.

at 33°C to 86.8 ± 3.1% at 25°C (F = 5.19; d.f. = 4, 20; P = 0.0049) (Table 3). No significant differences between the two *C. rosa* groups were observed when larval survival was compared over a range of 15 - 30°C except at 33 and 35°C.

No pupae survived at 10, 33 and 35°C for both *C. rosa* groups (Table 3). For R1, survival ranged from 81.2 ± 2.9% at 30°C to 94.2 ± 1.1% at 25°C (F = 8.097; d.f. = 3, 16; P = 0.0017). Survival ranged from 78.2 ± 3.6% at 30°C to 95.2 ± 1.2% at 20°C in R2 (F = 10.43; d.f. = 3, 16; P = 0.0005). Survival did not differ significantly between the two *C. rosa* groups at all temperatures.

South Africa - Effect of temperature on development of immature life stage

Egg: The time required for eggs to hatch ranged from 7.53 ± 0.10 d at 15°C to 1.69 ± 0.01d at 33°C (F = 1701.32; d.f. = 5, 44; P < 0.0001) for R1. On the other hand, the egg developmental time of R2 was longest at 15°C and shortest at 30°C (F = 742.34; d.f. = 5, 46; P < 0.0001). However, no significant differences in egg developmental duration were observed between the two groups of *C. rosa* at 15, 20, 25, and 30°C. The eggs of both *C. rosa* groups failed to develop at 10°C.

Larva: At larval stage, developmental duration was generally shorter for R1 compared to R2 at temperatures ranging from 15°C to 35°C (Table 1). The developmental duration of R1 decreased from 17.73 ± 0.19 d at 15°C to 5.74 ± 0.06 d at 33°C (F = 1765.82; d.f. = 5, 17; P < 0.0001) while that of R2 decreased from 20.74 ± 0.40 d at 15°C to 6.73 d at 33°C (F = 133.58; d.f. = 4, 13; P < 0.0001). The larvae of both *C. rosa* groups did not develop at 10°C (Table 4).

Pupa: For R1 no eclosion was observed at 10, 33 and 35°C while for R2, no eclosion was recorded at 10, 30, 33 and 35°C. Pupal developmental duration of both R1 (F = 2578.64; d.f. = 3, 12; P < 0.0001) and R2 (F = 495.54; d.f. = 2, 9; P < 0.0001) varied significantly when compared across the tested temperatures. Between the two *C. rosa* groups, again no significant differences in pupal development were observed at 15, 20 and 25°C (Table 4). The longest pupal developmental duration for R1 was 36.56 ± 0.28 d at 15°C and that of R2 was 36.18 ± 0.29 d at the same temperature.

Egg-adult: Total developmental duration from egg to adult for R1 was longest at 15°C and shortest at 30°C. For R2, in contrast, there was no complete development of the immature life stages at 30°C. Total developmental duration from egg to adult for R2 was longest at 15°C and shortest at 25°C. Significant differences were found between the two *C. rosa* groups when egg to adult developmental durations were compared across all the temperatures (Table 4) (R1: F =179.48, d.f. = 1, 11, P = < 0.0001; R2: F =669.34, d.f. = 1, 8, P < 0.0001). The egg-adult development of R1 was significantly faster than that of R2 at temperatures ranging from 15°C to 25°C (Table 4).

Table 5. Parameter estimates and their approximate standard errors for linear and Brière-1 nonlinear models describing the relationship between temperature and development rate (1/D) of *C. rosa* R1 and *C. rosa* R2 from South Africa.

Model	Parameters	*C. rosa* R1			*C. rosa* R2		
		Egg	**Larva**	**Pupa**	**Egg**	**Larva**	**Pupa**
Linear	a	-0.469	-0.080	-0.078	-0.323	-0.073	-0.056
	b	0.041	0.009	0.007	0.032	0.008	0.006
	K	24.29 ± 3.29	117.12 ± 9.04	145.94 ± 14.0	31.47 ± 0.89	131.34 ± 10.6	181.49 ± 9.68
	T_{min}	11.39 ± 1.51	9.42 ± 0.76	11.44 ± 1.19	10.18 ± 0.34	9.61 ± 0.78	10.15 ± 0.57
	RSS	7.8×10^{-3}	5.4×10^{-5}	1.2×10^{-4}	2.0×10^{-4}	9.4×10^{-5}	4.3×10^{-6}
	R^2	0.931	0.982	0.973	0.997	0.981	0.994
Brière-1	T_{min}	12.47 ± 3.11	8.99 ± 2.44	10.97 ± 4.50	9.60 ± 1.65	7.74 ± 4.01	10.47 ± 1.92
	T_{max}	36.53 ± 1.05	31.86 ± 6.28	33.0 ± 0.00	36.5 ± 2.64	32.57 ± 1.37	30.0 ±0.00
	T_{opt}	30.79	26.57	27.67	30.36	26.96	25.32
	R^2	0.976	0.993	0.952	0.997	0.997	0.990

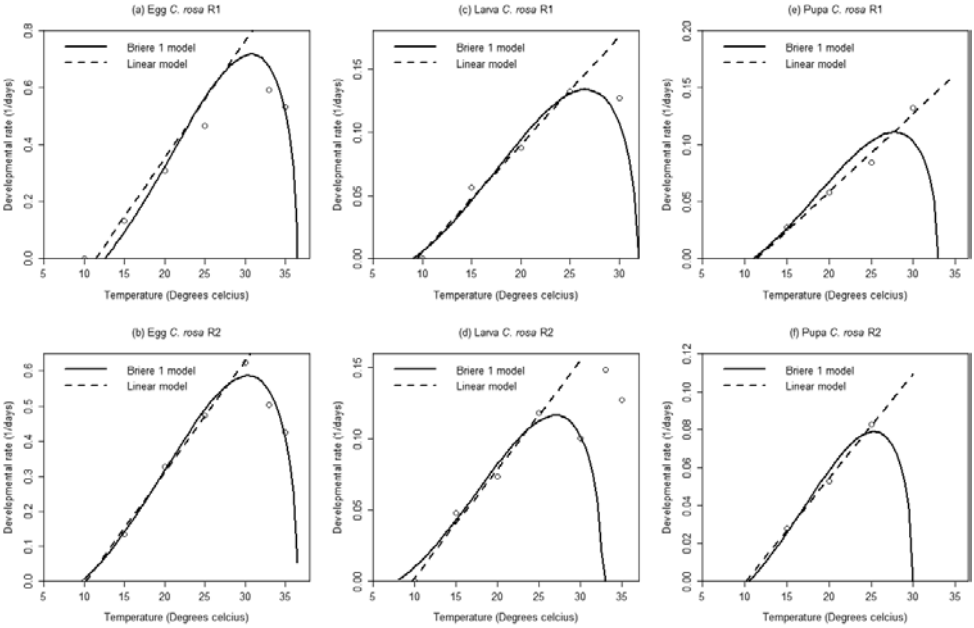

Figure 2. Linear and non-linear regressions of temperature related developmental rates of immature stages of two groups of *C. rosa* from South Africa

South Africa - Temperature-dependent developmental rates

Estimated parameter values of the linear and nonlinear models are presented in Table 5. A positive linear relationship was observed between temperature and development rates for egg, larval and pupal stages for both *C. rosa* groups.

For the egg, the lowest developmental threshold was estimated to be 11.39°C for R1 and 10.18°C for R2. The egg stage required 24.29 DD to complete development in R1 and 31.47 DD in R2. The R1 group required 117.12 DD to develop above a threshold of 9.42°C from larval stage to the pupal stage while R2 required 131.34 DD to develop above a threshold of 9.61°C (Table 5). The lower developmental thresholds for the pupal stages of R1 and R2 were estimated at 11.44 and 10.15°C, while the corresponding thermal constants were 145.94 DD and 181.49 DD, respectively.

The low developmental threshold values generated by the Brière-1 model for larva and pupa stages for both *C. rosa* groups were found to be lower compared to values estimated by the linear regression model (Table 5). For R1 the lower developmental thresholds of egg, larva and pupa were slightly different compared to that of R2 (Figure 2 and Table 5). An optimum temperature range of 26.57 – 30.79°C was estimated for R1 and 26.96 – 30.36°C for R2 for the various developmental stages. The lethal temperatures for R1 and R2 were estimated to range from 31.86 – 36.53°C and 30.0 – 36.5, for the various developmental stages (Table 5).

Table 6. Mean ± SE survivorship (%) of immature stages of *C. rosa* R1 and *C. rosa* R2 from South Africa at different constant temperatures.

Temperature (°C)	Egg		Larva		Pupa	
	C. rosa R1	***C. rosa* R2**	***C. rosa* R1**	**C. rosa R2**	**C. rosa R1**	**C. rosa R2**
10	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	-	-
15	$77.00 \pm 4.99^{b}A$	$54.67 \pm 8.74^{b}B$	$61.33 \pm 4.41^{b}A$	$73.67 \pm 2.91^{a}A$	$84.10 \pm 3.91^{a}A$	$90.06 \pm 1.94^{a}A$
20	$96.00 \pm 2.08^{a}A$	$58.00 \pm 2.31^{ab}B$	$61.67 \pm 4.06^{b}A$	$41.67 \pm 1.86^{b}B$	$62.71 \pm 5.15^{b}A$	$69.37 \pm 6.47^{b}A$
25	$90.67 \pm 0.99^{a}A$	$61.33 \pm 1.20^{ab}B$	$72.67 \pm 1.45^{b}A$	$23.33 \pm 2.85^{c}B$	$68.18 \pm 8.67^{b}A$	$52.38 \pm 9.52^{c}A$
30	$93.22 \pm 0.89^{a}A$	$75.11 \pm 2.23^{a}B$	$87.00 \pm 2.65^{a}A$	$39.33 \pm 8.76^{b}B$	68.33 ± 2.73^{b}	0.00 ± 0.00^{d}
33	$79.50 \pm 3.48^{b}A$	$66.80 \pm 3.12^{ab}B$	$36.33 \pm 4.10^{c}A$	$0.33 \pm 0.33^{d}B$	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}
35	$74.00 \pm 1.00^{b}A$	$57.33 \pm 2.92^{b}B$	$22.25 \pm 3.22^{d}A$	$0.80 \pm 0.58^{d}B$	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}

Means in the same column followed by the same lower case and in the same row followed by the same upper case letter are not significantly different by Student – Newman – Keul's (SNK) test, $P < 0.05$.

Survival of immature life stages

At the egg stage, the percentage survival of R1 ($F = 92.63$; d.f. = 6, 47; $P < 0.0050$) was significantly higher compared to that R2 ($F = 22.94$; d.f. = 6, 49; $P = 0.0070$) across the temperature range of 15°C to 35°C (Table 6). The highest survival rate of R1 was recorded at 20°C, while that of R2 was recorded at 30°C.

For R1, percentage survival of the larval stage ranged between 22.25 ± 3.22% at 35°C to 87.0 ± 2.65% at 30°C ($F = 77.55$; d.f. = 6, 25; $P < 0.0001$), while that for R2 ranged between 0.33 ± 0.33% at 33°C to 73.67 ± 2.91% at 15°C ($F = 86.56$; d.f. = 6, 22; $P < 0.0001$). For both *C. rosa* groups, no significant difference in larval survival was observed at 15°C. However at temperatures ranging from 20°C to 35°C, percentage larval survival of R1 was significantly higher compared to R2 (Table 6).

For R1, no eclosion was recorded at 10, 33, 35°C while for R2, no pupa survived at 10, 30, 33 and 35°C. The highest pupal survival rate for both R1 and R2 was recorded at 15°C. For R1, the lowest survival rate was recorded at 20°C, while that of R2 was recorded at 25°C. Percentage pupal survival of R1 ($F = 94.25$; d.f. = 5, 18; $P < 0.0001$) and R2 ($F = 145.06$; d.f. = 5, 18; $P < 0.0001$) were significantly different when compared across the test temperatures.

Discussion

The study on the developmental rates of the two parapatric *C. rosa* groups across the geographical range of the species in question - *Ceratitis rosa* showed different trends according to the area of occurrence. In the north eastern limit of *C. rosa*, R1 was more heat tolerant and less cold tolerant than R2. In the southern limit of the pest, R1 was more heat tolerant compared to R2 but not necessary less cold tolerant than R2.

Duyck and Quilici (2002) published the first report on development of *C. rosa* at different constant temperatures. The authors reported that immature stages of *C. rosa* were unable to complete development at 35°C and survivorship was also extremely low at 30°C for all the immature life stages. Our results showed that egg and larval stages of Kenyan population of R1 was able to successfully complete development at 30, 33 and 35°C, although no emergence was observed from puparia at 33 and 35°C. The performance of the immature stages of the Kenyan population of R2 however mirrored the results obtained by Duyck and Quilici (2002). In South Africa, egg and larva of both *C. rosa* groups were able to complete development at the upper temperatures of 30, 33 and 35°C and deviates from results obtained for the Kenya population of R2.

The reasons for these differences are unclear but nutritional elements of the diet, the biological traits of the two populations and adaptations resulting from the fact that both populations of *C. rosa* from South Africa were reared for 5-8 generations at similar experimental conditions (25 ±1°C, 60 ± 10% RH and photoperiod of L12: D12) before the start of the experiment may have contributed to the observed variations. Indeed, populations of tephritids from different geographical regions may differ

in various reproductive and life history traits (Vargas and Carey 1989, Dimantidis et al. 2011). The observed differences may contribute to the invasion potential of the different *C. rosa* populations, since population growth rates influences basic population processes that operate during invasion events (Liebhold and Tobin 2008). The results obtained also support the genetic differentiation of geographically isolated *C. rosa* populations as has been demonstrated in previous studies (Virgilio et al. 2013).

The developmental duration of the immature life stages of the two *C. rosa* groups decreased as temperature increased. This observation is consistent with earlier studies with other tephritid species (Carey et al. 1985, Fletcher 1987, Vargas et al. 1996, Vargas et al. 1997, Brèvault and Quilici 2000, Duyck and Quilici 2002, Grout and Stoltz 2007, Rwomushana et al. 2008, Salum et al. 2013, Liu and Ye 2009, Vayssières et al. 2008, Vargas et al. 2000). However, in the South African *C. rosa* populations (R1 and R2), developmental duration of the egg and larva were found to decrease with increasing temperature up to 30°C, followed by a slight increase in developmental duration beyond 30°C. At the larval stage, both groups of the South African *C. rosa* developed faster than the Kenyan populations. Also, the R2 from the Kenyan highland had a faster development of immature life stages at lower temperatures (15 and 20°C) and the situation was reversed at the upper temperatures (25, 30, 33°C) with R1 from the lowland emerging sooner than R2 from the highland. However, in the South African populations, immature larval stages of R1 tended to develop faster across all temperatures than the highland population. The larval survivorship of R1 on the artificial diet at all temperatures was significantly higher than that of R2 and could have led to higher metabolic heat within the diet and therefore faster development. To date, little is known of the selective pressures shaping the life history of immature tephritids in geographically isolated locations (Diamantidis et al. 2011a, b) and additional research focusing on the selective pressure that shape the life history of immature life stages of the different *C. rosa* populations is warranted.

For both the Kenyan and South Africa *C. rosa* populations, the values for the temperature threshold and thermal constant were not always consistent with previous studies. In La Réunion, Duyck and Quilici (2002) reported lower developmental thresholds for egg, larval and pupal stages as 9.8, 3.1, and 11.0°C, respectively. Our lower temperature thresholds for egg and pupa are within the range reported by the previous authors. However, the estimated lower development thresholds from the linear models for larva in the Kenyan (7.1–10.3) and South African (7.8-8.6) populations are well above estimated values from La Réunion. Overall, R2 population from the highlands of Kenya tended to tolerate lower temperatures than the lowland R1 but the reverse was the case in South Africa. Duyck and Quilici (2002) reported total value of 405 DD for *C. rosa* in La Réunion. Thermal constant values for total development of the immature life stages of the Kenyan R1 and R2 were 302.75 DD and 413.53 DD, respectively. Our highland value is in agreement with data from La Réunion but differ sharply with that of the lowland population (Duyck and Quilici 2002). According to Virgilio et al. (2013) the Réunion population of *C. rosa* is referred to as R2. Therefore, the fact that our results of R2 are more in accordance with that reported by Duyck and

Quilici (2002) is thus not surprising. This confirms that the highland population of R2 in Kenya might likely be of the same genotype as the Réunion population. The total thermal constant values for the South Africa population were low for the two *C. rosa* populations (R1: 342 DD, and R2: 380 DD). Fletcher (1989) noted that large differences in thermal requirements among various species of tephritids can be attributed to difference in experimental methodologies and geographic variation of populations. Besides geographic origin, factors such as food quantity and quality and larval density in the rearing chambers have been reported to influence the thermal requirements of larval stages of tephritids (Vargas et al. 1996, Duyck and Quilici 2002).

No previous studies are available in literature with regard to upper developmental threshold for *C. rosa*. However, Brière-1 nonlinear model used in this study predicted that immature stages of R1 were more tolerant to heat than R2 and this irrespective of the area of origin. Observed values clearly showed higher survivorship and faster development for R1 compared to R2 for the South African populations. In non linear models differences seemed were very small. *Ceratitis rosa* R2 did not complete development at 30 degrees. Lethal temperature values generated here may be relevant for future development regarding post harvest dis-infestation treatments for the two populations of *C. rosa*.

Both populations of *C. rosa* from Kenya and South Africa survived at temperatures of 15, 20, 25, 30 and 33°C but no adult emerged from puparia at 10, 33 and 35°C. In the Kenyan populations, survival of all developmental stages at temperatures other than 10 and 35°C was > 50% which is consistent with previous studies assessing the effect of constant temperatures on development and survival of tephritids (Vargas et al. 1996). In contrast, survivorship of the South African populations was < 40% at the larval stage at the upper temperatures of 30 and 33°C and is in agreement with earlier studies (Duyck and Quilici 2002). Overall, high survival of both populations from Kenya across a wide range of temperatures suggest that *C. rosa* from this part of the world could potentially have higher invasive powers than the South African populations and warrant careful attention in terms of monitoring and surveillance to minimize its advertent translocation and potential establishment.

Conclusion

In conclusion, our results clearly demonstrates and support the existence of two genetically distinct populations of *C. rosa* that are divergent in their physiological response to temperature with potential consequent implications in the invasion dynamics of the pest. Difference in parameters measured between the Kenyan and South African populations may reflect certain attributes such as the diet used in the experiments, rearing procedures and adaptation processes of the insects. The findings suggest the need for taxonomic revision of *C. rosa* but additional information from integrative morphological, molecular, cytogenetic, behavioural and chemoecological data may be needed to accomplish this task.

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Wing morphometrics as a possible tool for the diagnosis of the *Ceratitis fasciventris*, *C. anonae*, *C. rosa* complex (Diptera, Tephritidae)

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Abstract

Previous attempts to resolve the *Ceratitis* FAR complex (*C. fasciventris*, *C. anonae*, *C. rosa*, Diptera, Tephritidae) showed contrasting results and revealed the occurrence of five microsatellite genotypic clusters (A, F1, F2, R1, R2). In this paper we explore the potential of wing morphometrics for the diagnosis of FAR morphospecies and genotypic clusters. We considered a set of 227 specimens previously morphologically identified and genotyped at 16 microsatellite loci. Seventeen wing landmarks and 6 wing band areas were used for morphometric analyses. Permutational multivariate analysis of variance detected significant differences both across morphospecies and genotypic clusters (for both males and females). Unconstrained and constrained ordinations did not properly resolve groups corresponding to morphospecies or genotypic clusters. However, posterior group membership probabilities (PGMPs) of the Discriminant Analysis of Principal Components (DAPC) allowed the consistent identification of a relevant proportion of specimens (but with performances differing across morphospecies and genotypic clusters). This study suggests that wing morphometrics and PGMPs might represent a possible tool for the diagnosis of species within the FAR complex. Here, we propose a tentative diagnostic method and provide a first reference library of morphometric measures that might be used for the identification of additional and unidentified FAR specimens.

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Keywords
Ceratitis anonae, *Ceratitis fasciventris*, *Ceratitis rosa*, fruit flies, cryptic species, integrative taxonomy, wing morphometrics, Posterior Group Membership Probability

Introduction

"True" fruit flies (Diptera, Tephritidae) are represented by more than 4,000 phytophagous species of which 25–30% feed on fruits. Many of them include major agricultural pests affecting crop production (Aluja and Norrbom 1999). Fruit fly pests also include a number of economically important species complexes that cannot be adequately resolved by morphological or molecular characters (Schutze et al. 2012, Schutze et al. 2015a, Selivon et al. 2005, Virgilio et al. 2008, Virgilio et al. 2013) and for which pest management proves to be problematic (e.g. see Schutze et al. 2015a). These include the so-called *Ceratitis* FAR complex, a small group of morphologically similar species including *C. fasciventris* (Bezzi, 1920), *C. anonae* Graham, 1908 and the main agricultural pest *C. rosa* Karsch, 1887 (the Natal fruit fly). Males of these species can be morphologically separated using leg ornamentation patterns (De Meyer and Freidberg 2006) while females of *C. rosa* and *C. fasciventris* cannot be morphologically resolved (De Meyer 2001b). The diagnosis of the three species is further complicated by their partially overlapping geographic distributions (Copeland et al. 2006, De Meyer 2001a) and by the fact that *C. fasciventris* and *C. rosa* might not even comply to the biological species concept as they produce fertile hybrids under laboratory conditions (Erbout et al. 2008).

Previous attempts to separate the three morphospecies using alternative diagnostic methods showed contrasting results. Both PCR - RFLP (Barr et al. 2006) and sequencing of mitochondrial and nuclear DNA markers (Virgilio et al. 2008) could not resolve the three morphospecies as separate entities. Analyses of cuticular hydrocarbon profiles (Vaníčková et al. 2014), however, showed differences between colony samples of *C. fasciventris, C. anonae* and *C. rosa* (a single population for each species) and suggested that this might also reflect differences between morphospecies. Microsatellites (Virgilio et al. 2013) failed to separate the three species as individual entities but showed the existence of five genotypic clusters largely corresponding to the morphospecies *C. anonae* (cluster A), *C. fasciventris* (clusters F1 and F2) and *C. rosa* (clusters R1 and R2). Interestingly, *a posteriori* morphological analyses of *C. rosa* types R1 and R2 (Virgilio et al. 2013) revealed differences in male secondary sexual characters (shape of, and extent of coloration on, midtibia). Additionally, the two *C. rosa* types showed significantly different distributions along an altitudinal transect in Tanzania (Mwatawala et al. 2015) thus suggesting possible ecological differentiation between genotypic clusters.

This paper aims at exploring morphometric differentiation between FAR morphospecies and genotypic clusters in an integrative taxonomic framework (see Schutze et al. 2015b and references therein). In particular, we used wing morphometrics to verify the diagnosis of a group of *Ceratitis* FAR specimens that were characterised previously by morphology and microsatellite genotyping.

Methods

Study material

We considered a set of 227 specimens genotyped at 16 polymorphic microsatellites (Virgilio et al. 2013). The set included 80 *C. anonae*, 97 *C. fasciventris* and 50 *C. rosa* individuals of both sexes assigned to five genotypic clusters (see Suppl. material 1 and 2 for specimen details). Only specimens with at least one intact wing were used. Wings were mounted on a glass slide (dorsal side facing up) and a 2mm ladder was used for scaling. Digital images were taken using a Micropublisher 5.0 RTV camera (QImaging, Canada) mounted on a Leica MZ12.5 microscope. Two pictures (10x magnification) were taken with the first picture focusing on the wing and the second on the scale placed over the glass slide. The two pictures were then merged using the program Auto-Montage (Syncroscopy, Cambridge, UK). Seventeen homologous type I landmarks (Bookstein 1997) consisting of points at which a wing vein meets the edge of the wing, or wing vein and cross-vein intersections were selected. Thirteen of the landmarks were homologous to those of Schutze et al. (2011), and an additional four were chosen to try to obtain a higher resolution of species and of genotypic clusters. No landmarks were chosen in the anterior and posterior regions of the wing because these areas are most easily damaged. Six partial wing band areas were also selected and digitalised (Suppl. material 3, 4). All wing band areas were entirely within a single wing cell or were separated by wing veins and cross-veins. Some wing band areas did not border a vein (e.g. anterior part of area 3), and thus should be considered as semi-landmarks according to Gunz et al. (2005). ImageJ (Abramoff et al. 2004) was used to score wing landmark coordinates and measure wing band areas. Wing landmark coordinates were scaled, translated, and rotated against the consensus configuration in MorphoJ (Klingenberg 2011) according to the generalized least squares Procrustes superimposition method (Rohlf 1999).

Statistical analyses

A preliminary methodological experiment aimed at quantifying morphometric differences between sexes (see Gidaszewski et al. 2009), left and right wings as well as of possible biases related to measurement errors (Arnqvist and Martensson 1998). For this purpose, a subset of 7 male and 7 female *C. rosa* specimens were randomly selected and morphometric measures (both of wing landmarks and wing band areas) were replicated by taking two digital images of each left and right wing and by scoring each digital image twice. Permutational Multivariate Analysis of Variance (PERMANOVA, Anderson 2001, 2005) was used to test the effects of (a) sex (as fixed factor), (b) wing (fixed orthogonal factor), (c) digital image (random factor nested in wing) and (d) measurement (random factor nested in the interaction of wing x image). As this test confirmed the occurrence of morphometric differences between sexes (see Results), we decided to

separately consider males (163 specimens) and females (64 specimens see discussion). Whenever possible, right wings were used for digital imaging (this regardless of the lack of correlation between wing side and morphometrics, see results) however, right wings were damaged in 19.3% of specimens and in those cases left wings were used. Principal Component Analysis (PCA), as implemented by the R package *adegenet* 1.4-2 (Jombart 2008), was used to visualise differences in male and female wing landmarks and wing band areas across morphospecies (*C. fasciventris*, *C. anonae* and *C. rosa*) and genotypic clusters (F1, F2, R1, R2 A). Prior to PCA, data were centred by the *scaleGen* function of *adegenet*. Morphospecies and genotypic clusters were then considered as prior groups and specimens ordinated by maximising between-group variances through Discriminant Analysis of Principal Components (DAPC). The number of Principal Components (PCs) retained in DAPCs was optimised through the *xvalDapc* function of *adegenet* (Jombart et al. 2010). *XvalDapc* was then used to calculate average individual posterior group membership probabilities (PGMPs) to morphospecies and genotypic clusters (for both wing landmarks and wing band areas). *XvalDapc* performs replicated DAPCs on training sets including 90% of specimens (selected through stratified random sampling) and calculates PGMPs of the remaining 10%. For these analyses, 1000 replicates were carried out at each level of PC retention. The proportion of consistently assigned specimens, i.e. of specimens for which the highest PGMP corresponded to the prior specimen grouping (with respect to morphospecies and genotypic clusters) were quantified by considering (a) the highest PGMP of each specimen (no probability threshold) and (b) two arbitrary thresholds corresponding to PGMP = 0.95 and PGMP = 0.99. In this latter case, only specimens with PGMPs higher than the thresholds were assigned, the other ones were discarded. PERMANOVAs (Anderson 2001) were performed on male and female specimens (for wing landmarks and wing band areas) and independently tested differences across (a) morphospecies (fixed factor) and (b) genotypic cluster (fixed factor). In order to avoid biases associated to the unbalanced experimental design (see Anderson and Walsh 2013), PERMANOVAs were performed on random subsets of data including balanced numbers of replicates (ranging from 3 to 31 replicate specimens). Tests were based on 10^4 unrestricted permutations of Euclidean distances between untransformed Procrustes coordinates. *A posteriori* pair-wise comparisons on significant terms were also implemented using the PERMANOVA t-statistic (Anderson 2005). Probability values of repeated pair-wise tests were corrected for Type I errors using the False Discovery Rate (FDR) procedure (Benjamini and Hochberg 1995).

Results

Preliminary methodological control

PERMANOVAs revealed differences in multivariate patterns of 7 male and 7 female *C. rosa* specimens for both wing landmarks and wing band areas (Tab. 1) while it neither detected

significant effects related to wing (left or right) nor to measurement error (both for repeated images of the same wing or for repeated scoring of the same image). Similarly, PCAs of wing landmarks of the *C. rosa* specimens used in the methodological test (Suppl. material 5) showed separation between males and females, while measures of (a) left and right wings, (b) repeated images and (c) repeated scoring were largely overlapping (see 95% confidence ellipses). PCAs of wing band areas (Suppl. material 6) did not allow separating male from female control specimens. A PCA of wing landmarks of all 227 specimens also suggested sex related differences across morphospecies (see further and Suppl. material 7).

Morphometrics of morphospecies and genotypic clusters

The first two PC axes of PCAs including all specimens accounted for 57.9% and 47.6% of variation in males and females, respectively. PCAs of both wing landmarks (Suppl. material 8) and wing band areas (Suppl. material 9) did not resolve either morphospecies or genotypic clusters. DAPC analyses were based on 10 and 20 PCs (for males and females, respectively) when considering wing landmarks and morphospecies as a prior group, on 10 (for males) and 5 (for females) PCs when considering wing landmarks and genotypic cluster and prior group and on 6 PCs (for both males and females) when using wing band areas and morphospecies or genotypic cluster as prior variables. Stressing the ordination of points by using prior groups did not provide a better resolution of both morphospecies and genotypic clusters and DAPC 95% confidence ellipses were largely overlapping (Suppl. material 10, Suppl. material 11).

The average individual PGMP within morphospecies (Figure 1, Suppl. material 12) ranged from 0.85 (SD = 0.33, females *C. rosa*) to 0.95 (SD = 0.13, males *C. anonae*) for wing landmarks and from 0.68 (SD = 0.35, males *C. rosa*) to 0.89 (SD = 0.24, males *C. anonae*) for wing band areas. Average PGMP of genotypic groups (Suppl. material 12, Suppl. material 13) was more variable and ranged from 0.27 (SD = 0.2, females R1) to 0.94 (SD = 0.19, males A) for wing landmarks and from 0.11 (SD = 0.04, females R1) to 0.86 (SD = 0.26, males A). When using wing landmarks, the proportion of specimens consistently assigned to morphospecies (Figure 2) ranged from 0.45 (*C. rosa* males, probability threshold = 0.99) to 0.98 (*C. anonae* males, no threshold), when considering wing band areas from 0.10 (*C. rosa* males, threshold = 0.99) to 0.93 (*C. anonae* males, no threshold). The proportion specimens consistently assigned to genotypic cluster (Figure 2) was highly variable and ranged from 0.0 (females A, threshold 0.95, females F1 and R1, threshold 0.99) to 1.0 (females F2, no threshold) when considering wing landmarks and from 0.0 (females R1, no threshold, females F1, threshold 0.95, males F1, F2, R1, females A, F2, R2, threshold 0.99) to 94.9 (males F2, no threshold) when considering wing band areas.

PERMANOVAs evidenced significant interspecific differences in both wing landmarks and wing band areas (for both males and females, Table 2, Suppl. material 14). *A posteriori* comparisons showed significant pairwise differences across all species in male and female wing band areas and in female wing landmarks. Conversely, male

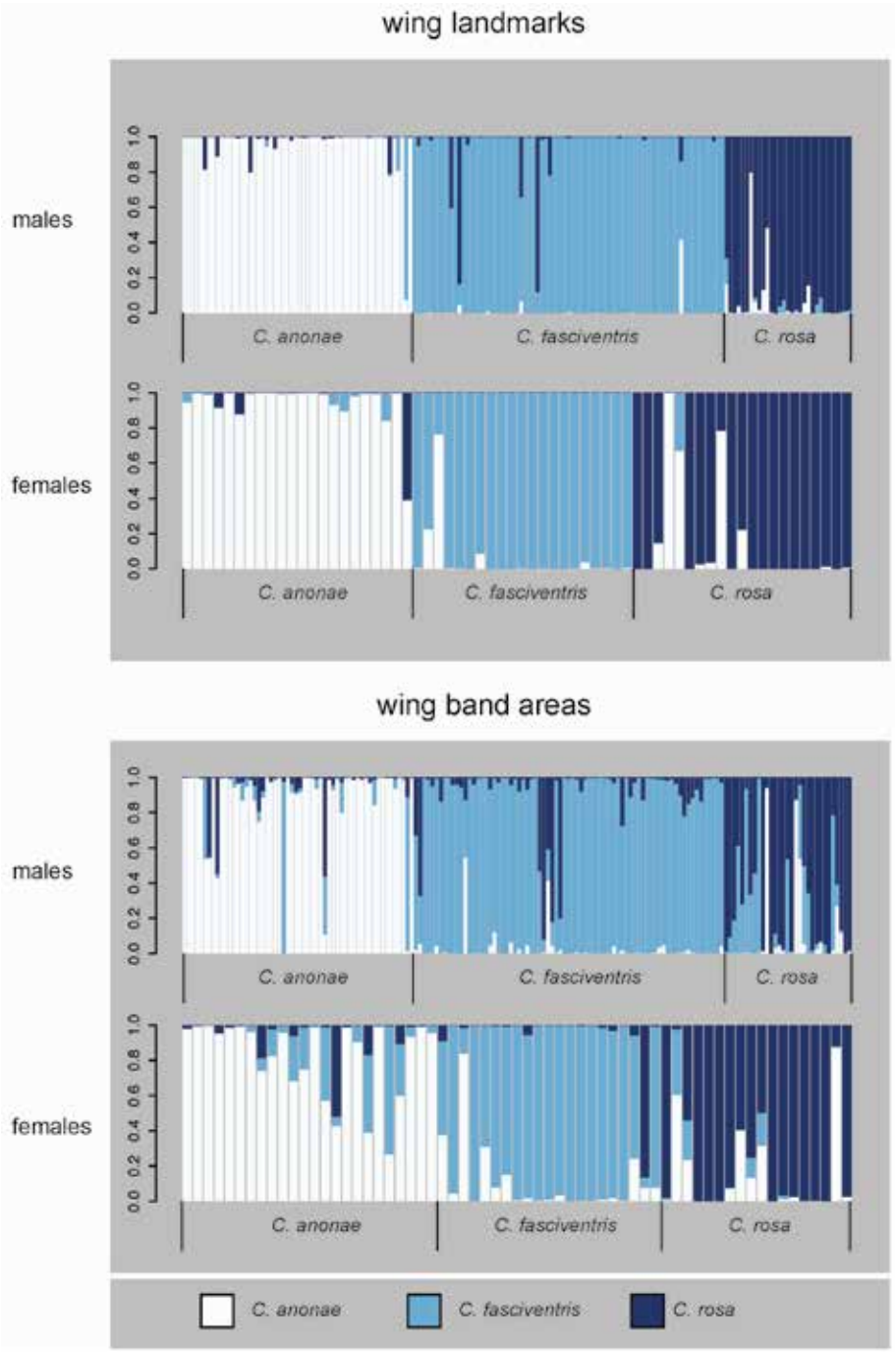

Figure 1. Individual assignments to morphospecies. Posterior group membership probabilities (PGMPs) of male and female specimens as resulting from Discriminant Analysis of Principal Coordinates of wing landmarks (upper) or wing band areas (lower). Prior groups: *C. anonae* (white), *C. fasciventris* (light blue), *C. rosa* (dark blue).

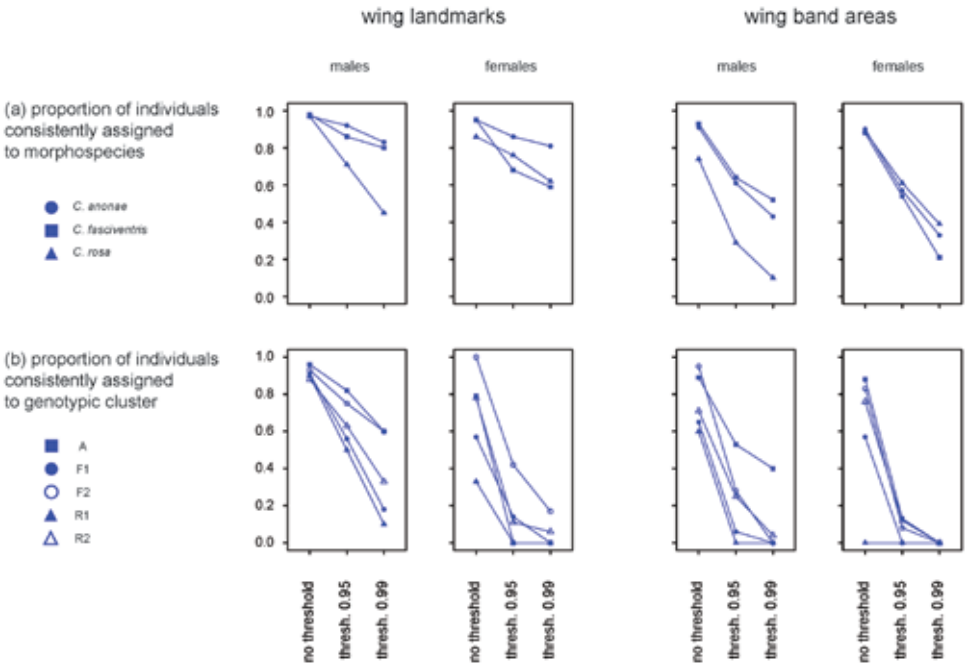

Figure 2. Diagnostic performance at different identification thresholds. Proportions of male and female specimens consistently assigned (**a**) to morphospecies (i.e. of specimens for which the highest posterior group membership probabilities (PGMP) corresponds to the prior morphospecies grouping) and (**b**) to genotypic cluster (i.e. of specimens for which the highest PGMP corresponds to the prior A, F1, F2, R1, R2 genotypic grouping) when considering wing landmarks (left) or wing band areas (right) and by using different assignment thresholds (no threshold, PGMP = 0.95, PGMP = 0.99).

wing landmarks differed only between *C. anonae* and *C. rosa*. PERMANOVA also showed differences across genotypic clusters (but with different patterns in *a posteriori* pairwise comparisons within males and females and between wing landmarks and wing band areas (Table 3, Suppl. material 15).

Discussion

The preliminary methodological experiment showed significant differences between male and female *C. rosa* wing morphometrics and suggests that these differences are consistent across species. Wing size and shape is supposed to have a main role in visual and vibrational courtship displays of tephritid fruit flies and sexual dimorphism in wing morphometrics has been already shown in tephritids (e.g. Marsteller et al. 2009). Once established the occurrence of relevant sexual variation (in at least one of the target species), we separately considered males and females. This approach allowed (a)

Table 1. Preliminary methodological control. PERMANOVA testing differences in multivariate patterns of wing landmarks and wing band areas of 14 *C. rosa* specimens in response to: sex (male / female), wing (right / left), image (1, 2) and measure (A, B). d.f.: degrees of freedom; MS: mean square estimates; F: pseudo-F. Probability of Monte Carlo simulations: n.s.: not significant a P<0.05; ***: P<0.001.

		wing landmarks			wing band areas		
	d.f.	**MS**	**F**		**MS**	**F**	
Sex = S	1	0.0220	68.64	***	0.1390	66.23	***
Wing = W	1	0.0001	0.30	n.s.	0.0006	0.28	n.s.
Image = I(W)	2	0.0000	0.10	n.s.	0.0011	0.52	n.s.
Measure = M(IxW)	4	0.0000	0.03	n.s.	0.0001	0.05	n.s.
S x W	1	0.0001	0.45	n.s.	0.0006	0.29	n.s.
S x I(W)	2	0.0000	0.06	n.s.	0.0001	0.03	n.s.
S x M(WxI)	4	0.0000	0.04	n.s.	0.0001	0.04	n.s.
Residual	96	0.0003			0.0021		

Table 2. Morphometric differences across morphospecies (wing landmarks).

PERMANOVA and *a posteriori* comparisons (t-statistic) testing differences in multivariate patterns of wing landmarks among morphospecies (*C. anonae*, *C. fasciventris*, *C. rosa*). d.f.: degrees of freedom; MS: mean square estimates; F: pseudo-F. Probability of Monte Carlo simulations: n.s.: not significant a P<0.05; ***: P<0.001, **: P<0.01; *: P<0.05 (after False Discovery Rate Correction for repeated *a posteriori* comparisons).

	males				**females**			
	d.f.	**MS**	**F**		**d.f.**	**MS**	**F**	
Morphospecies	2	0.0021	2.34	*	2	0.0039	6.63	***
Residual	90	0.0009			54	0.0006		

Pair-wise *a posteriori* comparisons (males and females: lower and upper diagonal matrix, respectively).

	C. anonae	***C. fasciventris***	***C. rosa***
C. anonae	-	***	*
C. fasciventris	n.s.	-	***
C. rosa	*	n.s.	-

disentangling sexual differences in wing shape from morphospecies / genotypic related variation and (b) maximising the number of balanced male and female replicates used in PERMANOVA.

PERMANOVAs showed that *C. fasciventris, C. anonae, C. rosa* as well as their five genotypic clusters (Virgilio et al. 2013), differ in their wing shape and banding patterns. These differences did not seem particularly pronounced, as (un)constrained ordinations neither resolved morphospecies nor genotypic clusters. However, PGMPs of wing landmarks (and particularly of male wing landmarks) proved to be effective in consistently assigning a large proportion of individuals to morphospecies. Interestingly, compared to microsatellite genotyping (Virgilio et al. 2013), wing landmarks seem more efficient in resolving the three FAR morphospecies as separate entities. Wing

Table 3. Morphometric differences across genotypic clusters (wing landmarks).

PERMANOVA and *a posteriori* comparisons (t-statistic) testing differences in multivariate patterns of wing landmarks among genotypic clusters (A, F1, F2, R1, R2). d.f.: degrees of freedom; MS: mean square estimates; F: pseudo-F. Probability of Monte Carlo simulations: n.s.: not significant a P<0.05; ***: P<0.001, **: P<0.01; *: P<0.05 (after False Discovery Rate Correction for repeated *a posteriori* comparisons).

	males				**females**			
	d.f.	MS	F		d.f.	MS	F	
Genotypic clusters	4	0.0037	6.10	***	4	0.0013	2.30	**
Residual	45	0.0006			10	0.0006		

Pair-wise *a posteriori* comparisons (males and females: lower and upper diagonal matrix, respectively)

	A	**F1**	**F2**	**R1**	**R2**
A	-	n.s.	n.s.	n.s.	n.s.
F1	***	-	*	n.s.	n.s.
F2	***	n.s.	-	*	n.s.
R1	n.s.	**	**	-	n.s.
R2	**	***	**	**	-

landmarks allowed a consistent species identification of a relevant proportion of males (97–98%, depending on morphospecies) even if the percentage of consistently identified females was lower (86–95%). The performance of wing band areas for species identification is poorer, particularly for male *C. rosa* (where only 74% of specimens could be consistently identified). The disagreement between morphological, morphometric and genotypic data was further evidenced by the tentative diagnosis of the genotypic clusters which showed large variations in the proportion of consistently assigned specimens and much poorer identification performances.

Distance thresholds are currently implemented in DNA barcoding identification of species (Ratnasingham and Hebert 2013, Sonet et al. 2013) where they can heavily affect identification performances (Virgilio et al. 2012). Similarly to what previously proposed for DNA barcoding, implementing PGMP thresholds, might help reducing the proportion of false positives (i.e. of individuals erroneously assigned to a morphospecies / genotypic cluster) as it would only allow the morphometric identification of specimens with reasonably high membership probabilities (e.g. > 0.95 or > 0.99), while for all other ones, alternative / complementary identification methods might be considered. Considering PGMP thresholds in the morphometric identification of specimens also caused a marked reduction of the proportion of consistently identified specimens (due to individuals that were discarded from identification as not reaching the threshold). It would now be interesting to verify if relationships between PGMP thresholds and identification success are similar to what has already been observed between distance thresholds and DNA barcoding identification performance, where more restrictive identification thresholds could increase identification precision but negatively affect accuracy (Virgilio et al. 2012).

Conclusion

This study suggests that wing morphometrics might represent a possible tool for the diagnosis of species within the *Ceratitis* FAR complex. In this respect, PGMP of individuals might be calculated and used to quantify the proximity of individuals to each morphospecies (see Jombart et al. 2010). The morphometric measures collected for the 227 FAR specimens considered here (and provided as supplementary material) might be used as a reference library to calculate group memberships of additional and unidentified FAR specimens. A larger reference dataset, including more representatives of each morphospecies and genotypic cluster, is now required to properly quantify relationships between PGMP thresholds and identification accuracy and precision.

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Supplementary material 1

Morphometric data

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: morphometric data

Explanation note: Specimen list and details and raw morphometric data (for both wing landmarks and wing band areas).

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Supplementary material 2

Map of sampling locations

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: occurence

Explanation note: Number of sampled specimens for each morphospecies are indicated in parentheses.

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Supplementary material 3

Wing landmarks and wing band areas

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: species data

Explanation note: List of wing landmarks and wing band areas considered in this study.

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Supplementary material 4

Wing landmarks and wing band areas

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: species data

Explanation note: Position of wing landmarks and wing band areas (numbers according to Suppl. material 3).

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Supplementary material 5

Preliminary methodological experiment: unconstrained ordination of wing landmarks

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: species data

Explanation note: Principal component analysis (PCA) showing morphometric differences in wing landmarks of 14 C. rosa specimens across sexes (M, F), wings (LW: left wing, RW: right wing), repeated images of the same wing (1, 2), repeated measures of the same image (A, B).

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Supplementary material 6

Preliminary methodological experiment: unconstrained ordination of wing band areas

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: species data

Explanation note: Principal component analysis (PCA) showing morphometric differences in wing band areas of 14 C. rosa specimens across sexes, wings (LW: left wing, RW: right wing), repeated images of the same wing (1, 2), repeated measures of the same image (A, B).

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Supplementary material 7

Unconstrained ordination of wing landmarks across sexes of each morphospecies

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: species data

Explanation note: Principal component analysis (PCA) showing morphometric differences in wing landmarks between sexes of each morphospecies (*C. anonae*, *C. fasciventris*, *C. rosa*). (all 227 specimens included).

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Supplementary material 8

Unconstrained ordination of wing landmarks

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: species data

Explanation note: Principal component analysis (PCA) showing morphometric differences in wing landmarks between males and females (a) *C. anonae*, *C. fasciventris* and *C. rosa* and (b) genotypic clusters A, F1, F2, R1, R2.

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Supplementary material 9

Unconstrained ordination of wing band areas

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: species data

Explanation note: Principal component analysis (PCA) showing morphometric differences in wing band areas between males and females (a) *C. anonae*, *C. fasciventris* and *C. rosa* and (b) genotypic clusters A, F1, F2, R1, R2.

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Supplementary material 10

Constrained ordination of wing landmarks

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: species data

Explanation note: Discriminant analysis of principal coordinates (DAPC) maximising morphometric differences in wing landmarks between males and females (a) *C. anonae*, *C. fasciventris* and *C. rosa* and (b) genotypic clusters A, F1, F2, R1, R2.

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Supplementary material 11

Constrained ordination of wing band areas

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: species data

Explanation note: Discriminant analysis of principal coordinates (DAPC) maximising morphometric differences in wing band areas between males and females (a) *C. anonae*, *C. fasciventris* and *C. rosa* and (b) genotypic clusters A, F1, F2, R1, R2.

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Supplementary material 12

Average individual assignments

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: species data

Explanation note: Average individual posterior group membership probabilities (PGMPs) of male and female specimens of three morphospecies (*C. anonae, C. fasciventris* and *C. rosa*) and five genotypic clusters (A, F1, F2, R1, R2) as resulting from the analysis of wing landmarks (light grey) and wing band areas (dark grey).

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Supplementary material 13

Individual assignments to genotypic clusters A, F1, F2, R1, R2

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: molecular data

Explanation note: Posterior group membership probabilities (PGMPs) of male and female specimens as resulting from Discriminant Analysis of Principal Coordinates of wing landmarks (upper) or wing band areas (lower). Prior groups: A, F1, F2, R1, R2 (from white to dark blue).

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Supplementary material 14

Morphometric differences across morphospecies (wing band areas)

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: morphometric data

Explanation note: PERMANOVA and a posteriori comparisons (t-statistic) testing differences in multivariate patterns of wing band areas among morphospecies (*C. anonae, C. fasciventris, C. rosa*). d.f.: degrees of freedom; MS: mean square estimates; F: pseudo-F. Probability of Monte Carlo simulations: n.s.: not significant a P<0.05; ***: P<0.001, **: P<0.01; *: P<0.05 (after False Discovery Rate Correction for repeated a posteriori comparisons).

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Supplementary material 15

Morphometric differences across genotypic clusters (wing band areas)

Authors: Joannes Van Cann, Massimiliano Virgilio, Kurt Jordaens, Marc De Meyer

Data type: morphometric data

Explanation note: PERMANOVA and a posteriori comparisons (t-statistic) testing differences in multivariate patterns of wing band areas among morphospecies (*C. anonae, C. fasciventris, C. rosa*). d.f.: degrees of freedom; MS: mean square estimates; F: pseudo-F. Probability of Monte Carlo simulations: n.s.: not significant a P<0.05; ***: P<0.001, **: P<0.01; *: P<0.05 (after False Discovery Rate Correction for repeated a posteriori comparisons).

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RESEARCH ARTICLE

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Cuticular hydrocarbons corroborate the distinction between lowland and highland Natal fruit fly (Tephritidae, *Ceratitis rosa*) populations

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Abstract

The cuticular hydrocarbons (CHs) and morphology of two *Ceratitis rosa* Karsch (Diptera: Tephritidae) populations, putatively belonging to two cryptic taxa, were analysed. The chemical profiles were characterised by two-dimensional gas chromatography with mass spectrometric detection. CHs of *C. rosa* that originated from the lowlands and highlands of Kenya comprised of *n*-alkanes, monomethylalkanes, dimethylalkanes and unsaturated hydrocarbons in the range of the carbon backbone from C_{14} to C_{37}. Hydrocarbons containing C_{29}, C_{31}, C_{33} and C_{35} carbon atoms predominated in these two populations. 2-Methyltriacontane was the predominant compound in both populations. Quantitative differences in the distribution of hydrocarbons of different chain lengths, mainly the C_{22}, C_{32}, C_{33} and C_{34} compounds of these two populations, were observed despite indistinct qualitative differences in these hydrocarbons. Morphological analyses of male legs confirmed that the flies belong to different morphotypes of *C. rosa* previously labelled as R1 and R2 for lowland and highland populations, respectively. A statistical analysis of the CH compositions of the putative R1 and R2 species showed distinct interspecific identities, with several CHs specific for each of the lowland and highland populations. This study supports a hypothesis that the taxon *C. rosa* consists of at least two biological species.

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Keywords
Ceratitis rosa, cryptic species, chemotaxonomy, GC×GC/MS, integrative taxonomy

Introduction

Sexual selection within populations can play an important role in speciation when divergence in mating signals and corresponding mate preference occur along different evolutionary trajectories in different populations (Jennings et al. 2014). In fruit flies (Diptera, Tephritidae), one potential target of sexual selection may be the blend of hydrophobic compounds on their cuticle, which often show intra- and interspecific variation, sexual dimorphism and may act as short-range pheromones (Carlson and Yocom 1986, Goh et al. 1993, Sutton and Carlson 1993, Vaníčková et al. 2012b, Vaníčková et al. 2014, Vaníčková et al. 2015). These compounds, cuticular hydrocarbons (CHs), play a major role in desiccation resistance, waterproofing, and/or mate choice, and may be under selection if particular components confer a mating advantage or increase the fitness of the resulting offspring (Howard and Blomquist 2005, Blomquist and Bagnères 2010, Gibbs 2011, Jennings et al. 2014). Characteristics of the CHs blend can vary with the diet, sex, age and geographic origin of a species and population (Blomquist and Bagnères 2010, Jennings et al. 2014).

In species of the fruit fly genus *Ceratitis*, courtship generally includes visual, auditory, tactile and olfactory cues (Shelly 2000, Aluja and Norrbom 2001, Yuval and Hendrichs 2001, Shelly et al. 2007). During courtship, male-borne volatiles are recognised in the initial phase and are detected by olfactory sensillae on the fly's antennae, while less volatile compounds, such as CHs, may be exchanged during later courtship stages, when the male touches the female with legs and proboscis (Aluja and Norrbom 2001). The courted female chooses whether or not to mate with the male based on the quality of the various signals that he emits. Signal-preference co-evolution may provide mechanisms for both mate recognition and sexual selection in the early stages of population divergence, which may eventually lead to speciation (Jennings et al. 2014).

The Natal fruit fly, *Ceratitis rosa* Karsch (Diptera, Tephritidae), is a polyphagous species attacking a wide range of fruits on the African mainland. It has invaded some Indian Ocean islands, where it displaced the similarly introduced *C. capitata* (De Meyer 2000, De Meyer 2001, Duyck et al. 2004). *Ceratitis rosa*, together with *C. fasciventris* and *C. anonae*, are a closely related group of morphologically similar taxa known by researchers as the FAR species complex. The FAR complex has recently been studied by chemical, molecular genetic and morphological approaches to allow for discrimination of the putative species of this cryptic species complex (De Meyer 2001, De Meyer and Freidberg 2006, Virgilio et al. 2012, 2013, Vaníčková et al. 2014). Within the FAR complex, five genotypic groups have been identified and labeled as A (for *C. anonae*), F1 and F2 (for two *C. fasciventris* populations), and R1 and R2 (for two *C. rosa* populations) (Virgilio et al. 2013).

The chemical analyses of the cuticular hydrocarbon profiles of these putative species found significant differences between the A, F2 and R2 genotypes and characterised

chemotaxonomic markers to distinguish these groups (Vaníčková et al. 2014). More recently, research has focused on the two *C. rosa* types, largely because *C. rosa* is considered the most economically important species within the complex (De Meyer 2001, Quilici et al. 2002, Baliraine et al. 2004). Adult males of the two *C. rosa* types can be differentiated based on the characters of the male, but not female, mid tibia (De Meyer et al. 2015); while analysis of wing land-marks using geometric morphometrics gives only a partial separation of the five FAR complex genotypes (Van Cann et al. 2015). Additional markers for the R1 and R2 populations are therefore needed.

The literature provides conflicting information regarding developmental physiology and climatic niche for *C. rosa.* Some studies indicate that *C. rosa* might be more tolerant of colder and wetter conditions than *C. capitata* (Duyck et al. 2004), suggesting greater potential for establishment in temperate regions (De Meyer et al. 2008). However, Grout and Stoltz (2007) indicate that *C. rosa* prefers hot and wet conditions. A re-analysis of the distributional data and historical material in collections shows that this might be because of the failure to differentiate between the two types (R1 and R2) that were indicated by the microsatellite study (Virgilio et al. 2013). R2 appears to occur at lower latitudes on the African continent and at higher altitudes – hereafter referred to as 'highland' type. It might be more cold resistant than the R1 type, which is absent from the colder parts (lower latitudes, higher altitudes) within the geographic range of *C. rosa* – hereafter referred to as 'lowland' or 'coastal' type (Tanga et al. 2015). The cold resistance may be directly connected to the cuticle composition as previously reported for other Diptera e.g. *Drosophila* sp. (Gibbs et al. 1997, Rouault et al. 2001, Rouault et al. 2004), and *Anopheles* sp. (Wagoner et al. 2014). With respect to CHs amount/*n*-alkane length, it is assumed that a reduction in water loss is the outcome of lower surface-area-to-volume ratio and reduced cuticle permeability, respectively (Rouault et al. 2004, Blomquist and Bagnères 2010, Gibbs 2011). Combining this background knowledge, leads us to hypothesize that CHs are likely to vary between R1 and R2 populations.

The purpose of the present study was, therefore, to identify the chemical constituents of the CHs and to analyse their variation between two populations of *C. rosa* (one highland and one lowland - based on morphological differentiation) originating from Kenya. These two populations were chosen for this study because they had previously been shown to be sexually incompatible (Ekesi et al. unpublished data), as well as having distinct male-borne volatile profiles (Kalinová et al. unpublished data). Additional to inter-population differences, we also evaluated sexual dimorphism in CHs composition within each population.

Methods

Insects

Pupae of two laboratory populations of *C. rosa* were obtained from the International Centre of Insect Physiology and Ecology (ICIPE, Nairobi, Kenya). The source colonies

were established in 2012 and came from one lowland locality [Mwajamba, Msambweni, Coast Province, 04°18.21'S; 39°29.88'E, host fruit *Psidium guajava* (Myrtaceae), altitude 106 m, average temperature 28.1°C] and one highland locality [Kithoka, Meru, Central Province, 00°05.59'N; 37°40.40'E, host fruit *Mangifera indica* (Anacardiaceae), altitude 1425 m, average temperature 21.5°C] in Kenya (see Appendix). The pupae (F_2 generation) were kept under identical laboratory conditions at the Institute of Organic Chemistry and Biochemistry (IOCB, Prague, Czech Republic). Flies were separated by sex within 24 hours of eclosion, fed on an artificial diet consisting of cane sugar and enzymatic yeast hydrolysate (in the ratio 3:1) and mineral water and kept at a relative humidity of 60%, at 25 °C, and a 12L:12D photoperiod.

Chemical analyses

The extraction of the cuticular hydrocarbons of 20-day-old virgin males (N = 10) and females (N = 10) of the R1 and R2 morphotypes (resulting in N = 20 for R1 and N = 20 for R2) followed the methodology described in Vaníčková et al. (2012b) and Vaníčková et al. (2014). Flies were frozen at -18 °C and placed for 15 minutes into a desiccator to remove the surface moisture. In order to extract CHs from insect body surface individual fly was placed in small glass vials, which contained 0.5 mL of hexane (Fluka, Germany) and gently agitate for 5 minutes. 1-Bromdecane (Sigma-Aldrich, Czech Republic) was used as an internal standard for quantification (10 ng per 1 μL of the extract). Each extract was concentrated to approximately 100 μL by a constant flow of nitrogen and stored in a freezer (-5 °C) until analysis.

Two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC/MS) was used for the quantification and identification of CH profiles. The analyses were performed on a LECO Pegasus 4D instrument (LECO Corp., St. Joseph, MI, USA) equipped with a non-moving quad-jet cryomodulator. A DB-5 column (J&W Scientific, Folsom, CA, USA; 30 m × 250 μm i.d. × 0.25 μm film) was used for GC in the first dimension. The second-dimension analysis was performed on a polar BPX-50 column (SGE Inc., Austin, TX, USA; 2 m × 100 μm i.d. × 0.1 μm film). Helium was used as a carrier gas at a constant flow of 1 mL min^{-1}. The temperature program for the primary GC oven was as follows: 150 °C for 2 min, then 150–300 °C at 5 °C min^{-1}, and finally a 10 min hold at 320 °C. The program in the secondary oven was 10 °C higher than in the primary one and was operated in an iso-ramping mode. The modulation period, the hot-pulse duration and the cool time between the stages were set to 3.0, 0.4 and 1.1 sec, respectively. The transfer line to the TOFMS was operated at 260 °C. The source temperature was 250 °C with a filament bias voltage of –70 eV. The data-acquisition rate was 100 Hz (scans/sec) for the mass range of 29–400 amu. The detector voltage was 1750V. For each sample, 1μL was injected in splitless mode. The inlet temperature was 200 °C. The purge time was 60 sec at a flow of 60 mL min^{-1}. The data were processed and consecutively visualized on 2D and 3D chromatograms using LECO ChromaTOF™ software. The *n*-alkane standard (C_8–C_{38}; Sigma-Aldrich) was co-injected with authentic samples

to determine the retention indices (*RI*) of the analytes. The hydrocarbons were identified by a comparison of their mass spectra fragmentation patterns and *RI* (Van Den Dool and Kratz 1963, Carlson and Yocom 1986, Vaníčková 2012, Vaníčková et al. 2014).

Morphological identification

Male specimens were shipped to the Royal Museum for Central Africa (RMCA), Tervuren, Belgium, where identifications were confirmed by M. De M. based on the pilosity and coloration of mid tibia (Virgilio et al. 2013, De Meyer et al. 2015).

Statistics

The relative peak areas of 46 CH compounds (as identified by the GC×GC/MS in the deconvoluted total-ion chromatogram mode) were calculated in 10 replicate specimens for each sex of the two species ($N = 40$). Following Clarke (1993), we log-transformed the multivariate data in order to reduce the differences in scale between the variables while preserving information on the relative abundance of CHs across specimens.

A heat map was used to visualise the complex data sets organised as matrices. Heat maps make it possible to identify differences in the relative amounts of CHs between populations, with different compounds tending to form small clusters according to their quantities. To achieve this, the heat map performed two actions on a matrix of chromatographic peak areas. First, it reordered the rows and columns so that rows and columns with similar profiles were closer to one another, causing these profiles to be more visible to the eye. Second, each entry in the data matrix was displayed in a different colour, making it possible to view the patterns graphically. The dendrograms were created using correlation-based distances and the Ward method of agglomeration was applied in the present analysis (Key 2012).

To examine the differences between the two populations and sexes further, the percentage contribution of each compound to the average dissimilarity between the aforementioned factors was calculated with similarity percentage analysis (SIMPER) (Clarke 1993). All computations were performed with R 3.1.2 language and environment (R Core Team 2014) and the R packages *gplots* (Warnes et al. 2015) and *vegan* (Oksanen et al. 2015).

Results

CHs composition

The GC×GC/MS analyses identified 46 peaks. The chain-length of the carbon backbones ranged from C_{14} to C_{37}. The hydrocarbon profiles of the males and females

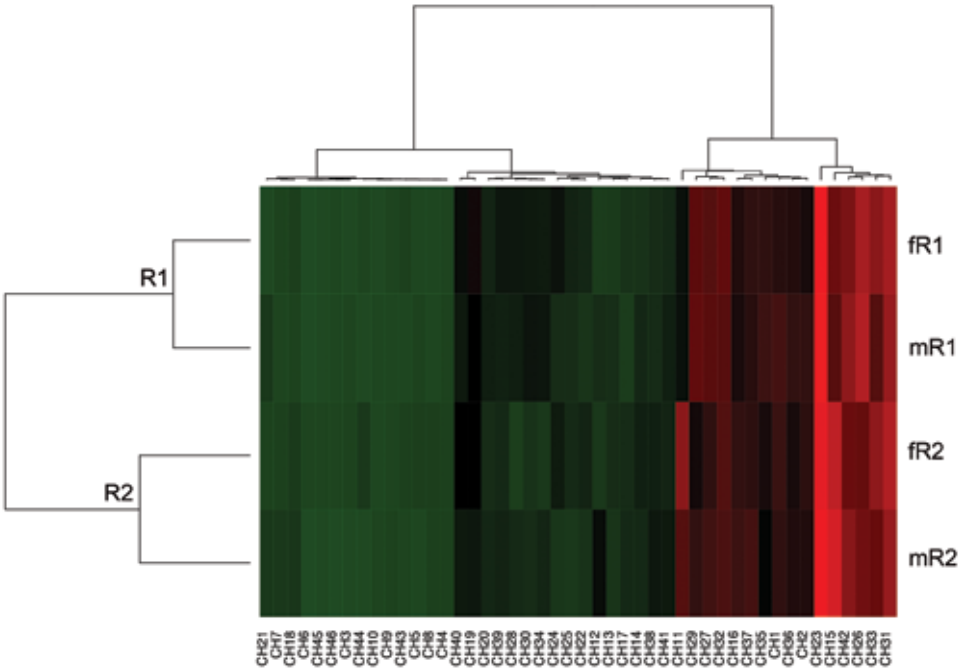

Figure 1. A heat map of the 46 cuticular hydrocarbons (columns, CH1-46) and the two *Ceratitis rosa* populations (rows, f-female, m-male) from the GC×GC/MS data set. The dendrograms are created using correlation-based distances and the Ward method of hierarchical clustering ($P < 0.05$). Putative morphotypes (R1 for the coastal population and R2 for the highland population) are depicted in the row dendrogram.

included 5 *n*-alkanes, 19 methylbranched alkanes, 19 unsaturated alkanes, squalene, 1 aldehyde and 1 unidentified compound. The heat map characterised differences in the relative amounts of CHs between the *C. rosa* flies originating from highland and coastal regions (Figure 1). Marked quantitative differences were observed in the peaks between the two populations and genders. The most prominent peaks in all of the chromatograms were 2-methyltriacontane (2-Me$C_{30,}$ *RI 3064*, CH23) and tritriacontene ($C_{33:1}$, *RI 3240*, CH31) (Figures 1–3).

Sexual dimorphism in CHs

The CH profiles of the virgin males and females differed qualitatively. SIMPER analyses, comparing conspecific males and females, revealed sex-specific compounds. In females the most abundant compounds were docosene ($C_{22:1}$, *RI 2182*, CH11), hentriacontene ($C_{31:1}$, *RI 3082*, CH24), 3-methyldotriacontane (3-MeC_{32}, *RI 3272*, CH32) and tritriacontene ($C_{33:1}$, *RI 3280,* CH33) (Table 1). In males, the compounds shared by coastal and highland flies were identified as tritriacontene ($C_{33:1}$, *RI 3292,*

Table 1. A comparison of the average abundance of important cuticular hydrocarbons between two morphotypes of *Creatitis rosa* [coastal R1, highland R2]. The compounds are listed in the order of their contribution (δ_i) to the average dissimilarity 5(δ_i) between the two groups, with a cut-off when the cumulative percent contribution ($\sum\delta_i$%) to δ_i reaches 70%. The numbering of the compounds corresponds to Figure 1.

No.	Compound	*RI*	Abundance		δ_i	δi /SD(δi)	% contr. diss.	$\sum\delta_i$%
			R1 male	R2 male				
15	2-MeC_{28}	2865	1.037	1.731	0.016	3.384	0.104	15
11	$C_{22:1}$	2182	0.425	0.995	0.015	1.592	0.096	11
35	diMeC_{31}	3297	0.883	0.447	0.010	2.884	0.065	35
26	diMeC_{28}	3105	1.575	1.147	0.010	4.198	0.064	26
29	3-MeC_{31}	3178	1.093	0.797	0.007	3.241	0.044	29
12	$C_{27:1}$	2622	0.198	0.419	0.006	1.531	0.039	12
16	diMeC_{26}	2902	0.613	0.869	0.006	2.443	0.039	16
30	diMeC_{29}	3205	0.395	0.196	0.005	2.623	0.030	30
37	MeC_{33}	3331	0.721	0.906	0.004	1.826	0.029	37
36	$C_{34:1}$	3308	0.815	0.650	0.004	1.389	0.028	36
38	$C_{34:1}$	3342	0.206	0.370	0.004	1.431	0.026	38
23	2-MeC_{30}	3064	2.045	1.882	0.004	1.633	0.026	23
2	unknown	1402	0.816	0.649	0.004	2.035	0.025	2
27	7-/9-MeC_{31}	3142	1.044	0.882	0.004	1.768	0.024	27
1	C_{14}	1400	0.934	0.771	0.004	1.826	0.024	1
34	$C_{33:1}$	3291	0.378	0.261	0.004	1.134	0.024	34
28	MeC_{31}	3152	0.317	0.227	0.004	1.695	0.023	28
No.	**Compound**	***RI***	**Abundance**		δ_i	**δi /SD(δi)**	**% contr.diss.**	$\sum\delta_i$%
			R1 female	**R2 female**				
11	$C_{22:1}$	2182	0.435	1.415	0.022	2.485	0.133	11
15	2-MeC_{28}	2865	1.186	1.768	0.013	2.817	0.079	15
29	3-MeC_{31}	3178	1.078	0.599	0.011	4.145	0.065	29
26	diMeC_{28}	3105	1.534	1.191	0.008	2.284	0.047	26
34	$C_{33:1}$	3291	0.352	0.112	0.007	1.789	0.044	34
28	MeC_{31}	3152	0.384	0.066	0.007	3.160	0.043	28
33	$C_{33:1}$	3280	1.353	1.444	0.005	1.337	0.029	33
30	diMeC_{29}	3205	0.362	0.148	0.005	2.727	0.029	30
27	7-/9-MeC_{31}	3142	1.043	0.835	0.005	1.875	0.029	27
36	$C_{34:1}$	3308	0.705	0.639	0.005	1.373	0.029	36
1	C_{14}	1400	0.732	0.925	0.005	1.791	0.028	1
42	$C_{35:2}$	3460	1.263	1.223	0.004	1.381	0.027	42
2	unknown	1402	0.623	0.811	0.004	1.831	0.027	2
35	diMeC_{31}	3297	0.803	0.629	0.004	1.384	0.026	35
16	diMeC_{26}	2902	0.689	0.858	0.004	1.512	0.026	16
38	$C_{34:1}$	3342	0.199	0.318	0.004	2.264	0.024	38
24	$C_{31:1}$	3082	0.414	0.354	0.004	1.426	0.024	24

No.	Compound	*RI*	Abundance		δ_i	δi /SD(δi)	% contr. diss.	$\sum\delta_i$%
			R1 male	R1 female				
33	$C_{33:1}$	3280	1.026	1.353	0.008	1.680	0.071	33
11	$C_{22:1}$	2182	0.425	0.435	0.005	1.118	0.049	11
24	$C_{31:1}$	3082	0.196	0.414	0.005	1.633	0.045	24
36	$C_{34:1}$	3308	0.815	0.705	0.005	1.387	0.043	36
1	C_{14}	1400	0.934	0.732	0.005	2.207	0.043	1
2	unknown	1402	0.816	0.623	0.004	2.217	0.041	2
15	2-MeC_{28}	2865	1.037	1.186	0.004	1.220	0.040	15
40	$C_{34:2}$	3371	0.311	0.242	0.004	1.376	0.034	40
42	$C_{35:2}$	3460	1.352	1.263	0.004	1.500	0.034	42
34	$C_{33:1}$	3291	0.378	0.352	0.003	1.160	0.031	34
35	diMeC_{31}	3297	0.883	0.803	0.003	1.759	0.031	35
28	MeC_{31}	3152	0.317	0.384	0.003	1.215	0.030	28
22	$C_{31:1}$	3047	0.155	0.258	0.003	1.958	0.027	22
32	3-MeC_{32}	3262	1.031	1.125	0.003	1.557	0.027	32
31	$C_{33:1}$	3240	1.406	1.516	0.003	1.614	0.027	31
19	MeC_{29}	2960	0.477	0.586	0.003	1.350	0.026	19
12	$C_{27:1}$	2622	0.198	0.101	0.003	0.915	0.026	12
13	MeC_{26}	2649	0.189	0.112	0.003	1.194	0.025	13
38	$C_{34:1}$	3342	0.206	0.199	0.003	1.058	0.024	38
26	diMeC_{28}	3105	1.575	1.534	0.003	1.439	0.024	26
16	diMeC_{26}	2902	0.613	0.689	0.002	1.200	0.023	16
No.	**Compound**	***RI***	**Abundance**		δ_i	**δi /SD(δi)**	**% contr. diss.**	$\sum\delta_i$%
			R2 male	**R2 female**				
11	$C_{22:1}$	2182	0.995	1.415	0.013	1.324	0.093	11
33	$C_{33:1}$	3280	1.095	1.444	0.008	1.904	0.058	33
31	$C_{33:1}$	3240	1.357	1.673	0.007	2.177	0.052	31
12	$C_{27:1}$	2622	0.419	0.115	0.007	1.604	0.050	12
34	$C_{33:1}$	3291	0.261	0.112	0.006	1.486	0.042	34
24	$C_{31:1}$	3082	0.141	0.354	0.005	1.491	0.038	24
35	diMeC_{31}	3297	0.447	0.629	0.005	1.451	0.033	35
29	3-MeC_{31}	3178	0.797	0.599	0.005	2.054	0.033	29
42	$C_{35:2}$	3460	1.275	1.223	0.004	1.211	0.030	42
2	unknown	1402	0.649	0.811	0.004	1.605	0.028	2
32	3-MeC_{32}	3262	0.942	1.070	0.004	1.422	0.028	32
1	C_{14}	1400	0.771	0.925	0.004	1.521	0.027	1
36	$C_{34:1}$	3308	0.650	0.639	0.004	1.574	0.027	36
23	2-MeC_{30}	3064	1.882	2.025	0.004	1.581	0.027	23
21	$C_{31:1}$	3029	0.227	0.066	0.004	2.473	0.027	21
37	MeC_{33}	3331	0.906	0.804	0.004	1.348	0.026	37
15	2-MeC_{28}	2865	1.731	1.768	0.003	1.085	0.025	15
41	$C_{34:2}$	3377	0.355	0.277	0.003	1.587	0.024	41
40	$C_{34:2}$	3371	0.348	0.482	0.003	1.816	0.023	40
19	MeC_{29}	2960	0.369	0.489	0.003	1.580	0.023	19

RI – retention index on the DB-5 column.

CH34), tetratriacontene ($C_{34:1}$, *RI 3308*, CH36) and pentatriacontadiene ($C_{35:2}$, *RI 2416*, CH42) (Table 1). Interestingly, the compounds *n*-tetradecane (C_{14}, *RI 1400*, CH1), unknown (*RI 1402*, CH2) and dimethylhentriacontane (diMeC_{31}, *RI 3297*, CH35) were found to be specific for both coastal males and highland females (Table 1, Figure 1).

Differences in the CH profiles between the highland and coastal *C. rosa*

Different patterns of CHs were detected between the two populations when constructing the heat map (Figure 1). The coastal (R1) population had higher amounts of dimethyloctacosane (diMeC_{28}, *RI 3105*, CH26), 7-/9-methylhentriacontane (7-/9-MeC_{31}, *RI 3142*, CH27), 3-methylhentriacontane (3-MeC_{31}, *RI 3178*, CH29), 3-methyldotriacontane (3-MeC_{32}, *RI 3272*, CH32) and pentatriacontadiene ($C_{35:2}$, *RI 2416*, CH42), whereas the highland (R2) flies had higher amounts of docosene ($C_{22:1}$, *RI 2182*, CH11), 2-methyloctacosane (2-$MeC_{28,}$ *RI 2865*, CH15) and dimethylhexacosane (diMeC26, *RI 2902*, CH16) on their cuticle. When the data were compared by SIMPER analyses, a pairwise comparison of the males or females between the two populations revealed the presence of two specific compounds that mostly contributed to the overall dissimilarity, suggesting these CHs to be potential chemotaxonomic markers. These compounds were identified as docosene ($C_{22:1}$, *RI 2182*, CH11) and 2-methyloctacosane (2-$MeC_{28,}$ *RI 2865*, CH15) (Table 1, Figures 1–3).

Discussion

Significant quantitative differences in the chemical CH profiles of the two populations of *C. rosa* have been demonstrated and complementary morphological analyses have confirmed that these two populations belong to two different morphotypes/genotypes, previously labelled by Virgilio et al. (2013) as R1 and R2.

The characteristic compounds of the lowland R1 type, diMeC_{28} and 3-$MeC_{31,}$ were present in higher relative amounts, whereas the highland R2 flies were characterised by high amounts of $C_{22:1}$ and 2-$MeC_{28.}$ The compounds found in the present study correspond to the estimated chain lengths of the CH clusters identified in our earlier work for *C. rosa*, *C. anonae*, *C. fasciventris* and *C. capitata*, where the *C. rosa* R2 type could be determined based on the presence of even methylbranched hydrocarbons and the absence of odd methylbranched CHs when compared with the other three *Ceratitis* species (Vaníčková et al. 2014).

The intraspecific variation in the CH profiles between the two types reported here might be a result of several different factors, such as the effects of temperature, the social context and diet (Ferveur 2005, Kather and Martin 2012, Bontonou and Wicker-Thomats 2014, Vaníčková et al. 2015). Considering that the R2 type of *C. rosa* appears to be more cold resistant than the R1 type (Tanga et al. 2015), we assume that temperature

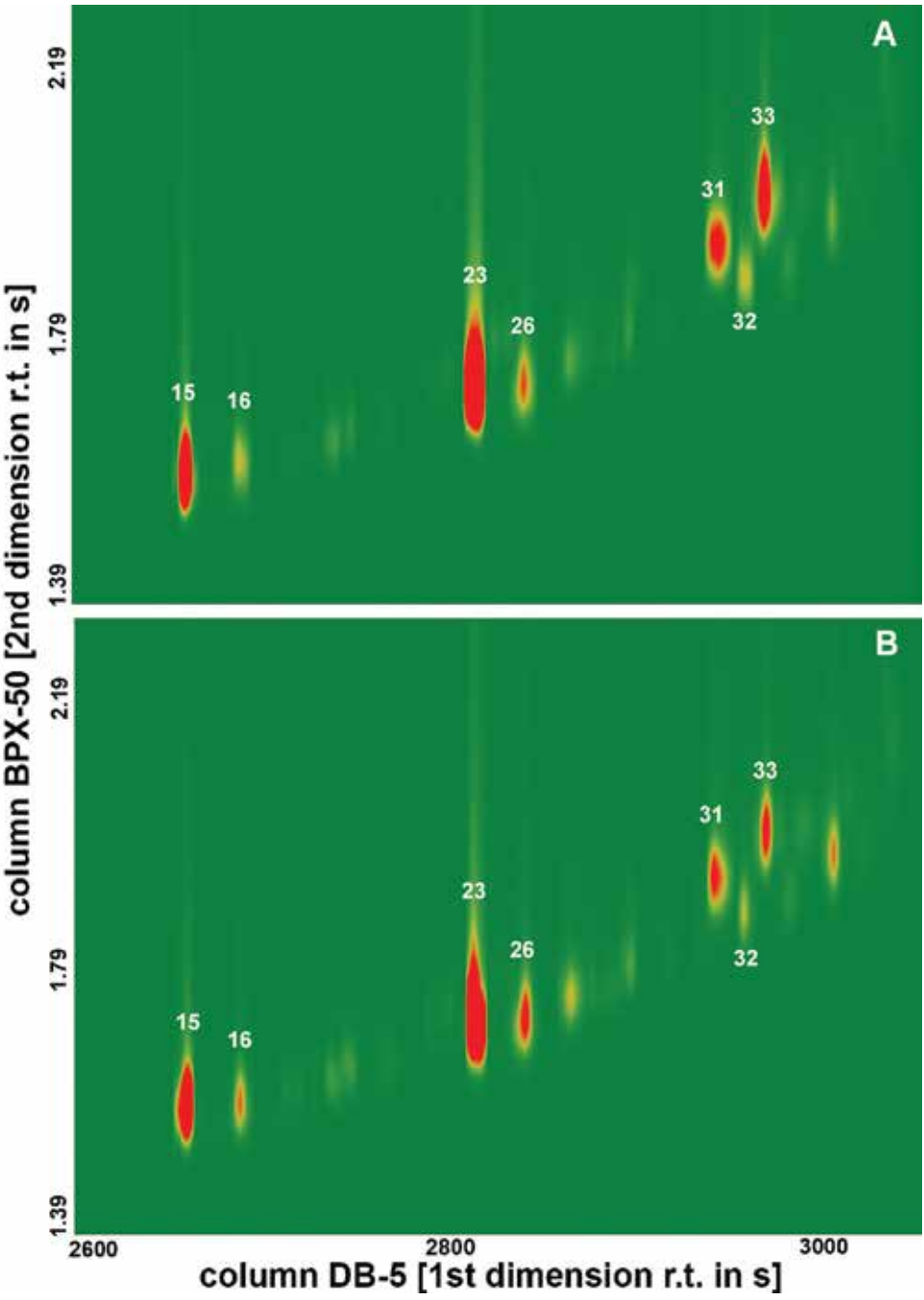

Figure 2. Section of the GC×GC/MS analysis of the female (**A**) and male (**B**) cuticular hydrocarbon profiles of the highland population (R2) of *Ceratitis rosa* from Kenya. The intensity of the signals is colour-coded from green (zero) to red (maximum). The compounds are assigned according to Table 1.

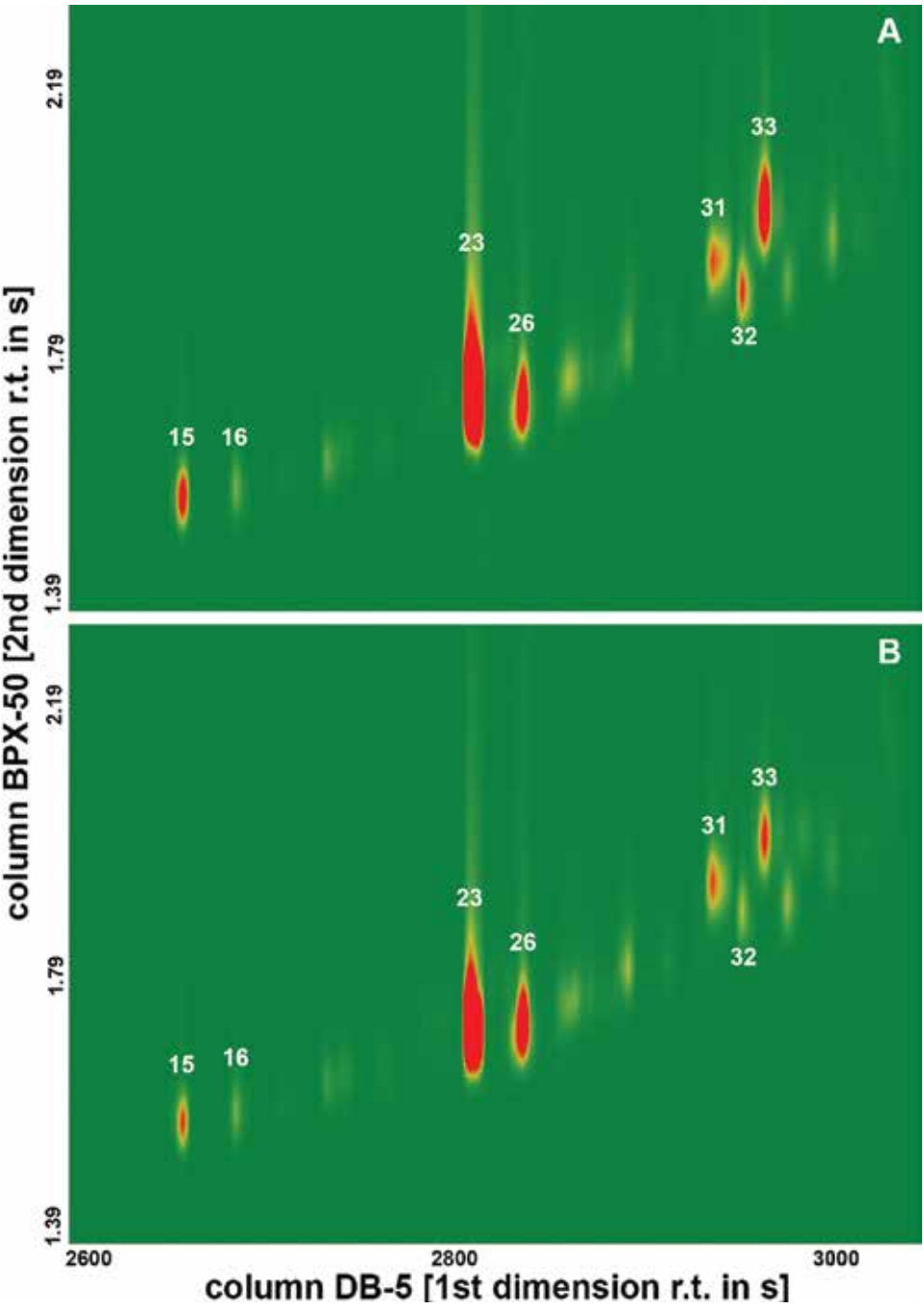

Figure 3. Section of the GC×GC/MS analysis of the female (**A**) and male (**B**) cuticular hydrocarbon profiles of coastal population (R1) of *Ceratitis rosa* from Kenya. The intensity of the signals is colour-coded from green (zero) to red (maximum). The compounds are assigned according to Table 1.

may be one of the main sources of variation in R1 and R2 CH profiles. The coastal population of *C. rosa*, living at an average temperature of ~28°C was characterised by greater amounts of long-chain CHs with carbon backbones from C_{30} to C_{35} when compared with the highland population living at an average temperature of ~21°C. Long-chain CHs have higher melting points, which give them a superior capacity to limit water loss as compared to short-chain CHs (Bontonou and Wicker-Thomas 2014) and insect species or populations living in warmer, drier environments loose water less rapidly and have longer-chain CHs than mesic ones (Ferveur 2005). A recent study of six South American fruit fly populations has shown that the CH profile varies significantly with relative temperature, relative humidity and altitude (Vaníčková et al. 2015).

In *C. rosa*, we found that the differences in cuticular hydrocarbon profiles between the two populations were greater than those between the sexes, although there was still a significant quantitative sexual dimorphism. Our findings are in agreement with studies conducted on *Drosophila* sp., where differences between *D. montana* populations were found to be considerably greater than those between the sexes (Veltsos et al. 2012, Jennings et al. 2014). Mating compatibility studies of the *C. rosa* flies from the same lowland and highland populations examined here have revealed a high degree of mating incompatibility between the two populations, where the index of sexual isolation (ISI) values ranged from 0.84 to 0.93, inferring reproductive isolation (Ekesi et al. unpublished data). The sex-specific differences in the quantitative composition of the *C. rosa* CH profiles identified in the present study indicate that these compounds might serve as short-range pheromones and thus could be directly involved in the mating compatibility/incompatibility within and between populations. Since the CHs involved in mating and courtship are not selectively neutral, reinforcing selection may cause closely related species to have distinct CH profiles (Blomquist and Bagnères 2010). A divergence in CH profiles between populations and sexes can lead to assortative mating and reproductive isolation, as shown in two populations of *D. mojavensis* (Stennett and Etges 1997, Etges 1998). Studies on *D. mojavensis* have demonstrated how even short-time isolation events can result in significant changes in CH composition (Stennett and Etges 1997, Etges 1998, Etges and Jackson 2001, Havens and Etges 2013).

It is important to note that the two populations of *C. rosa* studied here originate from different host plants, nevertheless they were reared during two generations on identical laboratory diet. The identified differences in the abundance of the CH between the populations and between the sexes may be, in addition to temperature and reproductive isolation factors, a result of the effects of host plants from which they originated (Stennett and Etges 1997, Vaníčková 2012, Vaníčková et al. 2012a). In *Drosophila* sp., the variation of CH profiles between closely related species of *D. mojavensis* on varied cactus plants or between populations of these species reflects the adaptation to different host plants (Etges and Jackson 2001). The ratio of the principal CHs changed rapidly with laboratory acclimation and influenced courtship mating in *D. mojavensis* (Stennett and Etges 1997). These CH changes depend on enzymes whose level could represent a metabolic adaptation to host-plant chemicals

(Higa and Fuyama 1993, Jones 2001, Houot et al 2010). In tephritids, it is not known how are the CHs modified by diet composition and/or laboratory acclimation and whether any observed changes may impact the attractiveness of CH profiles. Therefore, future work needs to be conducted in order to elucidate the complex mechanisms involved in these events.

Conclusion

Our data on cuticular hydrocarbon profiles, along with the previously published studies on morphology, genetics and sexual compatibility suggest that there exist two different entities, almost certainly unique biological species, within the taxa *C. rosa* from Kenya. In order to determine whether the different entities observed are consistent, the study needs to be extended to other populations of the two entities throughout their geographic and host ranges.

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Appendix

Map of the *Ceratitis rosa* collection area. Flies were picked up in Kenya, in Mwajamba, Msambweni (Coast Province) and in Kithoka, Meru (Central Province).

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Population structure and cryptic genetic variation in the mango fruit fly, *Ceratitis cosyra* (Diptera, Tephritidae)

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Abstract

The fruit fly *Ceratitis cosyra* is an important agricultural pest negatively affecting the mango crop production throughout Africa and also feeding on a variety of other wild and cultivated hosts. The occurrence of deeply divergent haplotypes, as well as extensive morphological variability, previously suggested possible cryptic speciation within *C. cosyra*. Here we provide the first large-scale characterisation of the population structure of *C. cosyra* with the main objective of verifying cryptic genetic variation. A total of 348 specimens from 13 populations were genotyped at 16 polymorphic microsatellite loci. Hardy-Weinberg equilibrium (HWE) deviations were observed in 40.4% of locus-population combinations and suggested the occurrence of genetic substructuring within populations. Discriminant Analysis of Principal Components (DAPC) showed genetic divergence between the vast majority of vouchers from Burundi and Tanzania (plus a few outliers from other African countries) and all other specimens sampled. Individual Bayesian assignments confirmed the existence of two main genotypic groups also occurring in sympatry. These data provided further support to the hypothesis that *C. cosyra* might include cryptic species. However, additional integrative taxonomy, possibly combining morphological, ecological and physiological approaches, is required to provide the necessary experimental support to this model.

Keywords

Ceratitis cosyra, fruit flies, cryptic species, microsatellites, morphology, sympatric speciation

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Introduction

The tephritid fly, *Ceratitis cosyra* (Walker, 1849), is possibly the most important indigenous pest of mango throughout sub-Saharan Africa. It is estimated that *C. cosyra* can reduce the mango crop yield between 20 and 30%, and the damage this pest causes affects the quality and market value of the fruit at both local and international markets (Lux et al. 2003). Although it has been partially displaced by the invasive *Bactrocera dorsalis* (Hendel, 1912) in recent years (Ekesi et al. 2009), it still has serious impact on the mango production, especially for early and mid-season cultivars in western Africa (Vayssières et al. 2009). The range of host records for *C. cosyra* is relatively large (but not as large as for other congeneric fruit flies such as *C. rosa* Karsch, 1887 or *C. capitata* (Wiedemann, 1824), see Copeland et al. 2006 for details) and includes guava as well as a variety of hosts from Anacardiaceae, Annonaceae, Apocynaceae and Rubiaceae among others (De Meyer 1998).

In Kenya, the mango fruit fly can be found in both lowlands and highlands at altitudes between 20 and 2100 m, while Geurts et al. (2012) observed predominance at lower altitudes in Tanzania. On the Kenyan coast, *C. cosyra* uses wild fruits, primarily the marula tree, *Sclerocarya birrea* (A. Rich.) Hochst., as an alternative host when mango is not available (Copeland et al. 2006). Similarly, in Tanzania *C. cosyra* shifts to soursop (*Annona muricata* Linnaeus) out of the mango season (Mwatawala et al. 2009). The distribution of *C. cosyra* in southern Africa is limited to the subtropical regions and its occurrence in this region coincides with the known distribution range of *S. birrea* (De Meyer 2001). *Ceratitis cosyra* is commonly intercepted in Europe (Li et al. 2009 and references therein) where the establishment of adventive populations raises serious concerns. Based on the observed altitudinal records, Copeland et al. (2006) suggested that *C. cosyra* may be pre-adapted to survive in the same subtropical and Mediterranean climatic areas as the cosmopolitan *C. capitata*, thus representing a potential risk of invasion and establishment of this pest to Europe and the US mainland (but see Grout and Stoltz 2007 for a less pessimistic view on its invasion potential). Similarly, Li et al. (2009) listed North, Central and South American and Middle Eastern, Asian and Australian countries as potential suitable areas for the establishment of adventive *C. cosyra* populations.

Barr et al. (2012) investigated the utility of DNA barcoding for molecular identification of several tephritid pests, including *C. cosyra*. They suggested that the mango fruit fly might include cryptic species. In fact, in their study, *C. cosyra* was represented by a larger haplotype group with vouchers from Mali (two sampling locations) and Kenya (two sampling locations) and by two *C. cosyra* outliers sampled at the coast of southern Kenya (Shimba Hills). Surprisingly, these two individuals, sharing the same COI haplotype, were separated from the main haplotype group by 52 mutational steps. Also on morphological grounds, *C. cosyra* has been the subject of confusion. Various taxa (now considered synonyms) have been described as separate species or varieties based on differences in cephalic and leg chaetotaxy and mesonotal patterns (see De Meyer 1998 for detailed discussion). The occurrence of cryptic species in *C. cosyra*

would pose relevant issues with respect to pest management, ecological modelling and estimation of its invasion potential. In this study, the population structure of *C. cosyra* was inferred across the species distributional range as a first step towards exploring its cryptic diversity.

Methods

A total of 348 specimens of *C. cosyra* from 13 populations ($13 < n < 32$) were collected in Africa from 2000 to 2012 (Table 1, see also supplementary file SF 1: Map of sampling locations). DNA was extracted from ethanol preserved adults by the DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer's instructions. Individual flies were genotyped at 16 polymorphic microsatellite loci developed by Delatte et al. (2014): Co1350, Co1444, Co2J, Co486, Co633, Co806, CoD4, CoES, CoKW, CoOI, CoP7, CoQT, CoRTA, CoWU, CoZ29, CoZW (see Delatte et al. (2014) for primer sequences and laboratory procedures). Electrophoretic analyses were conducted on an automated ABI Prism 3100 Genetic Analyzer (Applied Biosystem) with individuals declared non-amplifiable at a locus after two independent amplification failures. The genotypes of the 348 individual insects were analysed by the ADEGENET 1.4-2 package of the R statistical software (Jombart 2008) to ascertain the genetic variability and differentiation, among the *C. cosyra* populations, including number of alleles per locus (N_{all}), observed and expected heterozygosity (H_{obs}, H_{exp}) and deviations from the Hardy-Weinberg equilibrium (HWE). The function *genotype_curve* of the R package POPR (Kamvar et al. 2014) was used to calculate a genotype accumulation curve (this function randomly sample loci without replacement and count for the number of multilocus genotypes). Linkage disequilibrium was tested for each population across each pair of loci using the log likelihood ratio statistic implemented in GENEPOP 4.3 (Rousset 2008) and assessing significance through Markov-chain randomizations based on 1000 dememorizations, 100 batches, and 5000 iterations per batch. FreeNA 1.0 (Chapuis and Estoup 2007) was used to estimate null allele frequencies (per locus and population) according to Dempster et al. (1977). Probability values of repeated tests were corrected for Type I errors using the False Discovery Rate (FDR) procedure (Benjamini and Hochberg 1995). Isolation by distance (IBD) was verified in ADEGENET through Mantel test between Edwards' genetic distances and Euclidean geographic distances (1000 permutations). Principal Component Analysis (PCA) was used to ordinate specimens in multivariate space. Prior to PCA, the SCALEGEN function of ADEGENET was used to centre the data and replace missing genotypes with mean allele frequencies. Specimens from different populations were then ordinated by maximising between-group variances through Discriminant Analysis of Principal Components (DAPC). The number of Principal Components (PCs) retained in DAPC was optimised using XVALDAPC function of ADEGENET (Jombart et al. 2010).

STRUCTURE 2.3.4 (Pritchard et al. 2000) was used to calculate individual admixture coefficients (Q) across individuals and populations. STRUCTURE analyses

were based on the admixture model (individuals were allowed to have mixed ancestries from different clusters) with correlated allele frequencies (allele frequencies in different clusters were likely to be similar due to migration or shared ancestry) and the parameter of the Dirichlet distribution of allelic frequencies (λ) separately inferred for each population. We used STRUCTURE HARVESTER 0.6.94 (Earl and vonHoldt 2012) to infer the optimal number of clusters (K) using the Evanno et al. (2005) parameters. Since this method only detects the uppermost level of population structure when different hierarchical levels exist, we further investigated the genetic substructuring of our dataset by following the sequential clustering method described in Coulon et al. (2008). For this purpose, replicated STRUCTURE runs were performed by (a) dividing the main dataset in subsets of data including individuals assigned to the same cluster, (b) recalculating the optimal K value (Evanno et al. 2005) of each subset of data and (c) repeating the STRUCTURE analyses of each subset of data. We set Q = 0.7 as an arbitrary threshold for cluster assignment and individuals not reaching the threshold were discarded from further replicated runs. For each value of K, five iterations were run for 3 million generations (with 1.5 million generations as burn-in) and the posterior estimates of cluster memberships of the 3 runs with the highest estimated log probability of the data were summarized in CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) and visualized in DISTRUCT 1.1 (Rosenberg 2004).

Results

The amount of scored multilocus genotypes reached a plateau after 5-7 sampled loci, indicating that the genetic variability of *C. cosyra* was adequately sampled by the 16 microsatellites markers used (see supplementary file SF 2: Genotype accumulation curve). The total scored number of alleles (N_{all}) ranged from 67 (in the Burkina Faso population) to 124 (Mozambique), with an average proportion of missing data per population ranging from 2.2% (SE = 1.5%, Mali) to 30.9% (SE = 9.0%, Ivory Coast). H_{obs} ranged from 0.372 (Mali) to 0.504 (South Africa), while H_{exp} from 0.432 (Mali) to 0.637 (Tanzania) (Table 1). Pearson's Chi-squared test showed significant HWE deviations in 84 of 208 locus-population combinations, corresponding to 40.4% of observations (see supplementary files SF 3: Pearson's Chi-squared test for Hardy-Weinberg equilibrium and SF 4: Observed and expected heterozygosity). These HWE deviations are compatible with the occurrence of genetic substructuring within populations (Walhund effect) as described below. The average estimated proportion of null alleles was 8.1% (SE = 0.6%) (see supplementary file SF 5: Estimated null allele proportions) and linkage disequilibrium was observed in 16.7% of pairwise tests (see supplementary file SF 6: Linkage disequilibrium). Mantel test did not evidence significant correlation between individual geographic and genetic distances ($p > 0.05$, see supplementary file SF 7: Mantel test).

PCA was based on 28 PC axes that accounted for 70.6% of cumulative inertia. The first two PCs (Figure 1) represented a relatively low amount of variation (22.3%)

Table 1. Population locations and genetic variability. Sampling locations, geographic coordinates (decimal degrees) and summary of genetic variability in 13 populations of *C. cosyra* (see Figure 1) genotyped at 16 microsatellite loci. N: number of individuals per population, N_{all}: total number of alleles, H_{obs}: observed heterozygosity, H_{exp}: expected heterozygosity, null: mean null allele frequency based on Dempster et al. (1977). Standard deviations in parentheses.

	Locality		Latitude	Longitude	N	N_{all}	H_{obs}	H_{exp}	null
1	Burkina Faso	(interception)			29	67	0.412 (0.319)	0.484 (0.289)	0.060 (0.094)
2	Burundi	Isabu	-3.394	29.361	32	79	0.391 (0.312)	0.478 (0.304)	0.070 (0.090)
3	Ethiopia	Badano	9.317	41.217	13	74	0.430 (0.313)	0.475 (0.279)	0.065 (0.073)
4	Ivory Coast	Korhogo	9.450	-5.633	18	100	0.417 (0.327)	0.449 (0.366)	0.036 (0.059)
5	Kenya	Nairobi	-1.283	36.817	32	122	0.415 (0.276)	0.557 (0.266)	0.109 (0.062)
6	Malawi	Zomba	-15.383	35.333	29	118	0.481 (0.266)	0.601 (0.266)	0.082 (0.090)
7	Mali	(interception)			29	69	0.372 (0.308)	0.432 (0.331)	0.050 (0.073)
8	Mozambique	Cuamba	-14.816	36.535	32	124	0.478 (0.276)	0.581 (0.255)	0.076 (0.092)
9	Nigeria	Sokoto	13.051	5.231	26	93	0.421 (0.292)	0.482 (0.283)	0.059 (0.074)
10	South Africa	Constantia	-23.644	30.679	22	110	0.504 (0.284)	0.570 (0.278)	0.061 (0.075)
11	Senegal	Sané	12.750	-15.500	28	114	0.393 (0.262)	0.559 (0.259)	0.112 (0.112)
12	Sudan	Singa	13.150	33.850	32	113	0.376 (0.287)	0.532 (0.279)	0.123 (0.091)
13	Tanzania	Mzinga	-6.883	37.617	26	101	0.410 (0.255)	0.637 (0.186)	0.146 (0.113)

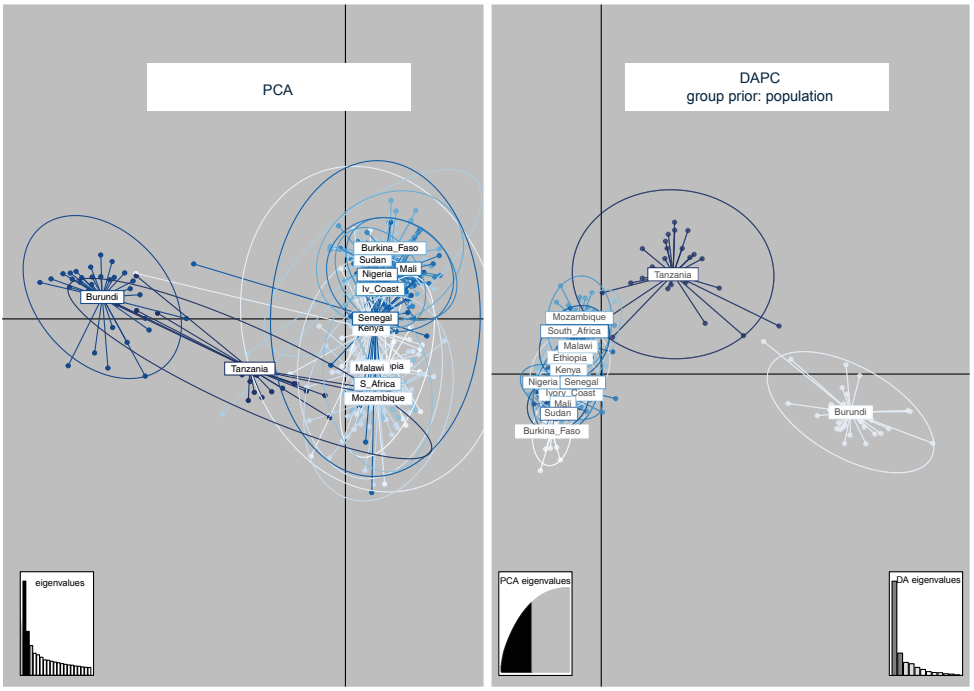

Figure 1. Unconstrained and constrained ordination. Principal Component Analysis (PCA) and Discriminant Analysis of Principal Components (DAPC) of 348 *C. cosyra* microsatellite genotypes. Specimen groups are labelled inside their 95% inertia ellipses and genotypes are connected to the corresponding group centroids.

and didn't allow proper resolution of populations (see 95% confidence ellipses). DAPC considered the populations as a priori defined groups and was based on 90 PCs. Stressing the ordination of points by using a prior group only allowed a better resolution of the population from Burundi and, possibly, of that from Tanzania. The latter was clearly separated from the other populations only when excluding 4 STRUCTURE outliers from DAPC (see below). Confidence ellipses of all other populations were largely overlapping (Figure 1).

The STRUCTURE analysis of the entire dataset (n=345, run 0, Figure 2) showed ΔK values (Evanno et al. 2005) peaking at K=2 indicating that the main hierarchical level of the population structure is based on 2 genotype groups. A first and smaller group (then separately analysed in run 1) included all specimens from Burundi (n=32) and 22 out of the 26 individuals from Tanzania. A second and larger group (subsequently analysed in run 2) included 98.6% of genotypes from all other populations combined (n=290), including the 4 outliers from Tanzania (see supplementary file SF 8: STRUCTURE sequential assignments). Run 1 (K=2) resolved all specimens from Burundi in one group (that also included one outlier from Kenya and one from Senegal), while specimens from Tanzania were partially assigned to the Burundi group (5 specimens) and, for a larger part, to a second group (17 specimens) together with

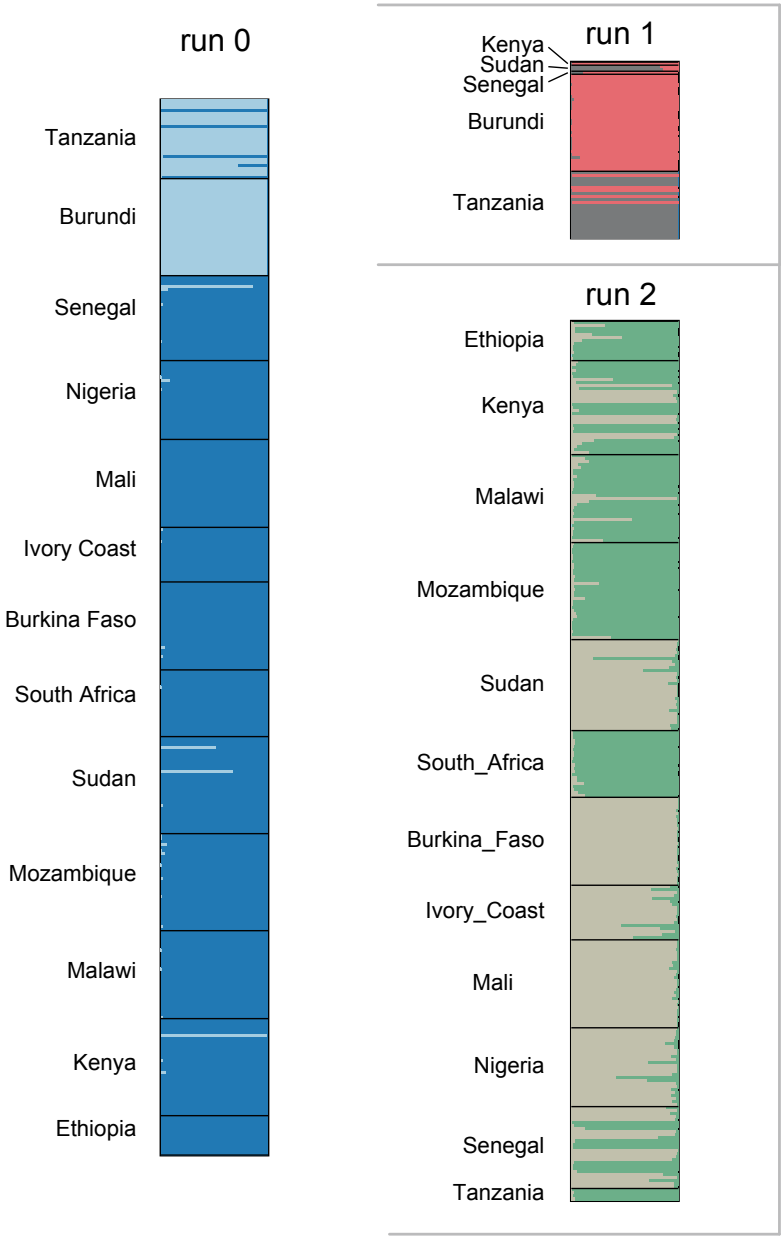

Figure 2. Individual Bayesian assignments. STRUCTURE sequential individual assignments of 348 specimens of *C. cosyra* from 13 African countries.

2 outliers from Sudan. Run 2 (K=2) resolved specimens from South Africa (100%), Mozambique (96.9%), Malawi (93.1) and Ethiopia (84.6%) in a first group and specimens from Burkina Faso (100%), Mali (100%), Nigeria (96.2%), Sudan (87.5%) and Ivory Coast (88.9%) in a second group. Populations from Senegal and Kenya included specimens that were assigned in part to the first group (35.7% and 62.5%, respectively) and in part to the second (60.7% and 31.3%).

Discussion

The morphospecies *C. cosyra* includes two groups of genetically well-differentiated individuals. The vast majority of vouchers from Burundi and Tanzania (plus a few outliers from other African countries) belong to the first of these two groups, all other specimens to the second. Specimens from the two groups were also found sympatrically in a number of populations from Kenya, Senegal, Sudan, and Tanzania. Interestingly, the two *C. cosyra* outliers described by Barr et al. (2012) were also sampled from the Kenyan coast. The fact that our Kenyan population included specimens from the two clusters suggests that Barr et al. might have genotyped representatives of both types. If this would be confirmed, the two genotypes resolved trough microsatellite markers might also show marked differentiation in the cytochrome oxidase I gene barcode region so that they might be more easily diagnosed through DNA barcoding rather than through microsatellite genotyping.

Further studies are needed to verify if specimens from the two genotypic clusters are also morphologically, ecologically and / or physiologically different and to which extent the two groups are connected by gene flow. A preliminary screening of thorax patterns of the samples used in this study (6 characters scored, data not shown) did not reveal straightforward morphological differences between groups. Wing morphometrics (Van Cann et al. 2015) might provide a more suitable tool to further investigate morphological cryptic variation in *C. cosyra*.

A wide variety of hosts have been described for *C. cosyra,* including Annonaceae (such as the introduced soursop) or Anacardiaceae (including the indigenous marula or the introduced mango). An intriguing hypothesis is that the two different *C. cosyra* types might also have different host preferences, similarly to what has been observed by McPheron et al. (1988) for *Rhagoletis pomonella* (Walsh, 1867). A separate host range characterization for the two types might provide useful information and help to understand if the observed genetic split has a recent evolutionary history (possibly related to the introduction of novel hosts in Africa) or a deeper evolutionary origin.

The sequential Bayesian assignment of genotypes also helped to disentangle the effects of cryptic speciation and of population structure within each of the genotypic groups. Specimens from Burundi and Tanzania are, to a less extent, genetically divergent, and among samples from the other African countries, two groups can be further resolved. In the latter case, specimens could be roughly subdivided between Western African samples (including Burkina Faso, Ivory Coast, Mali and Nigeria) and Eastern / Southern African samples (including Ethiopia, Tanzania, Malawi, Mozambique, South Africa) with

the notable exception of Sudan (which is genetically closer to the West African samples) and of Kenya and Senegal (that included a mix of individuals from both groups). Morphological differences were considered by De Meyer (1998) to represent a plausible (but incomplete) geographical split between western and eastern Africa, and this is also only partially corroborated by the genotypic clustering of this study. On the other hand the preliminary morphological screening of thoracic patterns does not provide any support for this division. Most importantly, it is not clear to what extent the mixed patterns of Kenya and Senegal and Tanzania can be related to historical evolutionary processes or to more recent events involving fruit trade and transport (Malacrida et al. 2007).

Conclusions

Marked and sympatric genetic splits are compatible with the occurrence of presumptive cryptic species, within *C. cosyra*. Additional integrative taxonomy, possibly combining morphological, ecological and physiological data (e.g., see Schutze et al. 2015) is now required to further support this model.

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Supplementary material 1

Map of sampling locations

Authors: Massimiliano Virgilio, Hélène Delatte, Yasinta Beda Nzogela, Christophe Simiand, Serge Quilici, Marc De Meyer, Maulid Mwatawala

Data type: species data

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Supplementary material 2

Genotype accumulation curve

Authors: Massimiliano Virgilio, Hélène Delatte, Yasinta Beda Nzogela, Christophe Simiand, Serge Quilici, Marc De Meyer, Maulid Mwatawala

Data type: data analysis

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Supplementary material 3

Pearson's Chi-squared test for Hardy-Weinberg equilibrium

Authors: Massimiliano Virgilio, Hélène Delatte, Yasinta Beda Nzogela, Christophe Simiand, Serge Quilici, Marc De Meyer, Maulid Mwatawala

Data type: data analysis

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Supplementary material 4

Observed and expected heterozygosity

Authors: Massimiliano Virgilio, Hélène Delatte, Yasinta Beda Nzogela, Christophe Simiand, Serge Quilici, Marc De Meyer, Maulid Mwatawala

Data type: species data

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Supplementary material 5

Estimated null allele proportions

Authors: Massimiliano Virgilio, Hélène Delatte, Yasinta Beda Nzogela, Christophe Simiand, Serge Quilici, Marc De Meyer, Maulid Mwatawala

Data type: data analysis

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Supplementary material 6

Linkage disequilibrium

Authors: Massimiliano Virgilio, Hélène Delatte, Yasinta Beda Nzogela, Christophe Simiand, Serge Quilici, Marc De Meyer, Maulid Mwatawala

Data type: data analysis

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Supplementary material 7

STRUCTURE sequential assignments

Authors: Massimiliano Virgilio, Hélène Delatte, Yasinta Beda Nzogela, Christophe Simiand, Serge Quilici, Marc De Meyer, Maulid Mwatawala

Data type: data analysis

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RESEARCH ARTICLE

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A review of the current knowledge on *Zeugodacus cucurbitae* (Coquillett) (Diptera, Tephritidae) in Africa, with a list of species included in *Zeugodacus*

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Abstract

This paper reviews all available information regarding the occurrence and biology of the melon fly, *Zeugodacus cucurbitae* (Coquillett), in the Afrotropical Region, including data on invasion history, distribution patterns, population genetics, host range, and interspecific competition. Although limited intraspecific variability has been observed within the region regarding the above mentioned aspects, there seems to be no indication that *Z. cucurbitae* represents a species complex. A checklist of all of the species included in *Zeugodacus* as recently proposed by Virgilio et al. (2015) is provided.

Keywords

Melon fly, Cucurbitaceae, Afrotropical, pest species

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Introduction

The melon fly, *Zeugodacus cucurbitae* (Coquillett) is a major agricultural pest of Asian origin. Despite the vernacular English name and the species-group name, it is reported from a series of unrelated host families in addition to the vast host range within Cucurbitaceae (White and Elson-Harris 1994). The fact that a number of populations of *Z. cucurbitae* differ in their reported host plants, morphology, etc. from region to region, resulted in the species being included in the Coordinated Research Project on "Resolution of cryptic species complexes of tephritid pests to overcome constraints to SIT application and international trade", initiated by the Joint FAO/IAEA Programme in 2010. This paper reviews the taxonomic position and history of the species within the Tephritidae, provides information on its worldwide distribution and genetic diversity, summarizes the current knowledge regarding the species in Africa, and provides a checklist of all of the species included in *Zeugodacus* as recently proposed by Virgilio et al. (2015).

Classification and taxonomic history

Zeugodacus cucurbitae (Figure 1) was originally described as *Dacus cucurbitae* by Coquillett (1899) from two males and two females bred from larvae found in green cucumbers in Honolulu, Hawaii (USA). *Bactrocera* was considered a subgenus of *Dacus* until Drew (1989) proposed a classification recognizing both taxa as genera, based upon the abdominal tergites being fused, (in *Dacus*), or not (in *Bactrocera*). Drew placed *Z. cucurbitae* in the subgenus *Zeugodacus*, first under *Dacus* following previous authors (Drew 1973), and later under *Bactrocera* (Drew 1989). The subgenus *Zeugodacus* belongs to a group of subgenera, characterized by the posterior lobe of the male lateral surstylus being long and the male abdominal sternite 5 being slightly concave along the posterior margin (rather than having a deep V shaped indentation) (Drew and Hancock 1999). At least 50% of the species included in the *Zeugodacus* group, for which host plant records are available, are cucurbit feeders. Recently the systematic position of *Zeugodacus* was revised as *Bactrocera*, *Dacus* and the subgenera of the *Zeugodacus* group have different evolutionary histories (Krosch et al. 2012, Virgilio et al. 2015). The molecular data provided support the hypothesis of White (2006) who suggested a common ancestry for *Zeugodacus* and *Dacus* (but see Hancock and Drew 2015 for a different hypothesis). Here we refer to the classification proposed by Virgilio et al. (2015) by using the new generic combination *Zeugodacus (Zeugodacus) cucurbitae* for the melon fly, although most existing literature refer to it under the former combination, *Bactrocera (Zeugodacus) cucurbitae*.

The genus *Zeugodacus* currently includes 192 species (see list in Supplementary material 1). Most species within this genus are restricted to the Oriental and Australasian Regions, with a few species reaching into the eastern Palearctic in China and Japan, except for *Z. cucurbitae* which was introduced into other parts of the world.

Figure 1. Habitus image of *Zeugodacus cucurbitae* (copyright R.S. Copeland).

Zeugodacus cucurbitae is rather distinctive in adult morphology and can be differentiated from other related species by the following combination of characters: scutum red-brown, with medial and lateral yellow postsutural vittae; large apical spot on the wing with posterior margin reaching about halfway between vein R_{4+5} and vein M; infuscation present over crossvein dm-cu and usually also crossvein r-m, wing cells bc and c hyaline, abdomen with a narrow transverse black band across basal margin of tergite 3 and a medial longitudinal black stripe over tergites 3-5 (White 2006, Drew and Romig 2013).

Contrary to other species like the *Bactrocera dorsalis* (Hendel) populations found in Africa (see Drew et al. 2005, White 2006), there is little intraspecific variability observed in adult *Z. cucurbitae* specimens with regard to scutal and abdominal patterns. Drew and Romig (2013) only mention that the fuscous marking on the scutum can be absent or present. White (2006) indicates that the anterior supra-alar and prescutellar acrostical setae can be rarely absent (the latter being one of the main differentiating characters between *Dacus* and most *Bactrocera* species), while the basal scutellar seta can be rarely present (hence, four setae in total rather than the usual two which are situated apically on the scutellum). The crossband on r-m is not always distinct. However, these differences do not seem to reflect any particular pattern linked to cryptic

speciation but rather phenotypic plasticity. *Zeugodacus cucurbitae* was not included in the list of the Asian species complexes defined by Drew and Romig (2013). No key is available to differentiate it from all other *Zeugodacus* species. Drew (1989) provides a general key for *Bactrocera* of the Australasian and Oceanian regions, including *Z. cucurbitae* and 19 other *Zeugodacus* species, while Drew and Romig (2013) provide descriptions and some diagnostic features for 101 species from South-East Asia, but no key. White (2006) and Virgilio et al. (2014) provide a key for African Dacina including *Z. cucurbitae*.

DNA barcoding shows remarkably low intraspecific variability. A pilot study including COI barcodes of 44 specimens originating from 11 countries along the entire distribution range (Virgilio and De Meyer, unpublished data) revealed an average K2P genetic distance (Kimura 1980) of only 0.02% (Figure 2). Similarly, the concatenation of mitochondrial DNA sequences (COI and ND6 gene fragments) from 100 specimens from Asia, Hawaii, African continent and islands of the Indian Ocean resulted in 22 haplotypes with 21 polymorphic sites and an average p-distance of only 0.003% (Jacquard et al. 2013). Minimum Spanning Network indicated the occurrence of two main haplotype groups corresponding to specimens from (a) Asia and Hawaii, and (b) the African continent including also Reunion Island.

Distribution, origin and population structure

Although *Z. cucurbitae* was originally described from the Hawaiian Islands, its presence there was the result of accidental human-mediated introduction (Bess 1961). About a decade later the first record from India was published (Froggatt 1909). Since then, it has been reported from multiple countries in the Asian and Australian-Oceanian Regions (Dhillon et al. 2005, Drew 1982, 1989, Drew and Romig 2013). It is abundant throughout Central and East Asia (including Pakistan, India, Bangladesh, Nepal, China, Indonesia and the Philippines) and Oceania (including New Guinea and the Mariana Islands). In some of these regions, it has been the subject of a number of introductions, eradication attempts and subsequent re-introductions. This is in particular the case in parts of the Pacific like the Northern Mariana Islands and Nauru (Dhillon et al. 2005), although it has also been successfully eradicated (Suckling et al. 2014) from regions in which it was well established, such as southern Japan in the 1990ies, using Sterile Insect Technique (Koyama et al. 2004). Since 1956 *Z. cucurbitae* has been detected a number of times in California (Papadopoulos et al. 2013), but its permanent establishment on the North American mainland is not confirmed.

In Africa, the first record dates back to 1936 from Tanzania (based upon a male specimen in the collection of the Natural History Museum in London, collected at Tanga on January 10th, 1936 by N. Krauss. See Bianchi and Krauss (1937) for report on this expedition, although this record is not specifically mentioned). No other species that are closely related to *Z. cucurbitae* are found in Africa, and its occurrence on the continent is also attributed to introduction. However, it is unclear whether it was

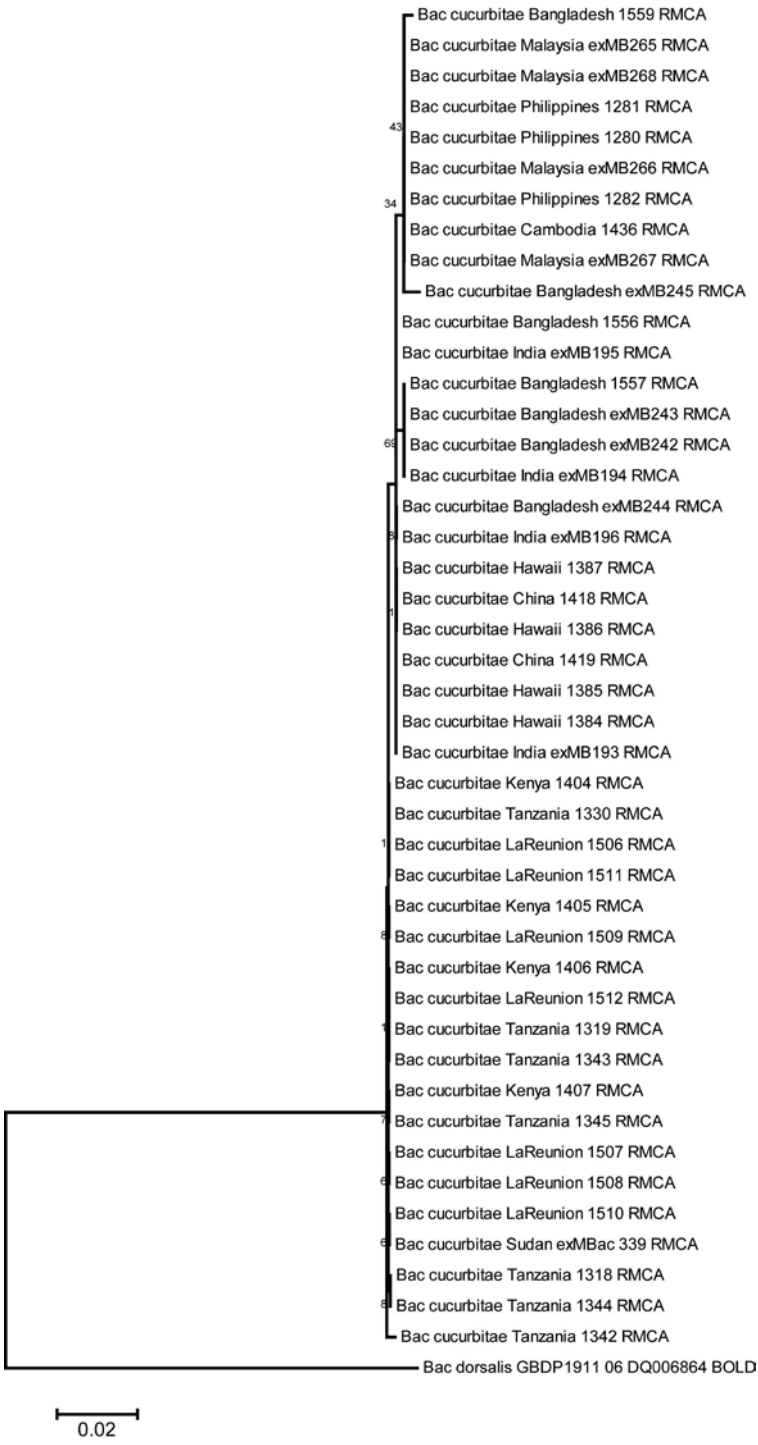

Figure 2. NJ tree (K2P distance, Kimura 1980) including 44 COI DNA barcodes of *Zeugodacus cucurbitae* from 11 countries (Virgilio and De Meyer, unpublished data).

Table 1: First records of *Zeugodacus cucurbitae* in African countries (based upon records in Orian and Moutia 1960, Vayssières et al. 2007 and De Meyer and White 2007).

Country	Locality	Year
Tanzania	Tanga	1936
Kenya	Rabai	1937
Mauritius	N/S	1942
Réunion	N/S	1972
Gambia	Brikama	1999
Ivory Coast	Korhogo	1999
Seychelles	Mahé	1999
Mali	Bamako	2000
Burkina Faso	Orodara	2000
Guinea	Foulaya	2000
Nigeria	Moruwa	2001
Cameroon	Garoua	2002
Senegal	Dakar	2003
Ghana	Sagyimase	2003
Benin	Cotonou	2004
Niger	Dosso	2004
DRCongo	Kinshasa	2006
Togo	Agou-Logopé	2006
Sudan	Singa	2006
Sierra Leone	Freetown	2006
Uganda	Jinja	2009
Burundi	Kigwena	2010
Ethiopia	Arba Minch	2010
Malawi	Kumbali	2010
Mozambique	Mocimboa da Praia	2013

introduced at that time (1936) or whether it was already present for a much longer time. There are historical ties between the eastern coastal area of Africa (dominated by the so-called Swahili culture) and the near East and Indian subcontinent that date back to 100 AD (Gilbert 2004), with movements and shipments of commodities between both regions. The first records from the African mainland were restricted to coastal Tanzania and Kenya (first record 1937) (Table 1).

Zeugodacus cucurbitae has also been introduced to several islands in the western Indian Ocean, with the first record in Mauritius in 1942 (Orian and Moutia 1960) and in La Réunion in 1972 (Vayssières 1999, White et al. 2001). More recently (since 1999) it was reported from the island Mahé of the Seychelles (White et al. 2001), where it is now also considered established. Its presence on the Comoro Archipelago is questionable (De Meyer et al. 2012) and so far no records are reported from Madagascar. Despite its longtime occurrence in eastern Africa and the Indian Ocean, *Z. cucurbitae* apparently did not spread rapidly to other parts of Africa. The first record from Central Africa was a mention in Fontem et al. (1999), where it is reported (as

Dacus cucurbitae) as the most prevalent insect pest observed by farmers on tomatoes in Cameroon. No voucher specimens could be traced to any collections in order to confirm this record, and there is the possibility that it was based on a misidentification of another dacine attacking tomatoes. For example, *Dacus punctatifrons* Karsch has been reported as a major pest of tomato in Cameroon (Okolle and Ntonifor 2005). The first voucher specimens from West Africa that could be confirmed to belong to *Z. cucurbitae* are from Ivory Coast and the Gambia and were collected in 1999 at Korhogo and Brikama, respectively, while in 2000 one of the authors (JFV) discovered it in Mali in cuelure traps and emerging from young pumpkins. Since the beginning of the 21st Century, several records of *Z. cucurbitae* from West and Central Africa became known (Table 1) and it is now well established in most parts of the region (Vayssières et al. 2007; Figure 3a).

In eastern Africa, *Z. cucurbitae* has been reported from a much larger range than just Kenya and Tanzania and it is now found from Ethiopia and the Sudan to Malawi and northern Mozambique (Table 1). It is unclear whether these 21st century records are a true reflection of a further recent expansion of its geographical range or that they are due to incomplete sampling in preceding decades. However, the currently observed dispersal of this species has also increased the awareness of its economic significance. *Zeugodacus cucurbitae* has been considered a major pest species of commercially grown cucurbits in large parts of Asia (Kapoor 1989, Koyama 1989) and Hawaii (Harris 1989) for a long time. However, in the Afrotropical region, not much research was devoted to this species in comparison to other cucurbit infesting dacines, except for La Réunion (White and Elson-Harris 1994, Vayssières 1999, Ryckewaert et al. 2010) and Mauritius (Sookar et al. 2012, 2013). This is currently changing due to the recent observations on its distribution and dominance in particular crops (see below under 'host range and interspecific competition').

Given the current geographic distribution of other *Zeugodacus* species (all restricted to the Oriental, Australasian and eastern Palearctic Regions) and the historical data of its occurrences in Africa and Hawaii, it is generally assumed that *Z. cucurbitae* originated in the Oriental Region and that its current distribution in Africa and in other parts of the world is the result of several invasion events (see Virgilio et al. 2010). The analyses by Jacquard et al. (2013) of sequences obtained from samples from throughout the known distribution range of *Z. cucurbitae* revealed a main genetic split between samples from (a) Asia and Hawaii, and (b) Sub Saharan Africa and La Réunion Island. The main differences between the African and all other samples suggested a bottleneck(s) following introduction, yet this model was not supported by the studies of Virgilio et al. (2010). Relationships between populations from different geographic areas were further resolved through a macrogeographic population structure analysis based on 25 populations genotyped at 12 microsatellite loci (Virgilio et al. 2010). Populations could be subdivided into five main geographic groups (African continent, Western Indian Ocean islands, Indian Subcontinent, South-East Asia, and Hawaii; Fig 4).

Levels of genetic diversity and individual Bayesian assignments (Virgilio et al. 2010) seem to suggest that *Z. cucurbitae* originated on the Indian Subcontinent and

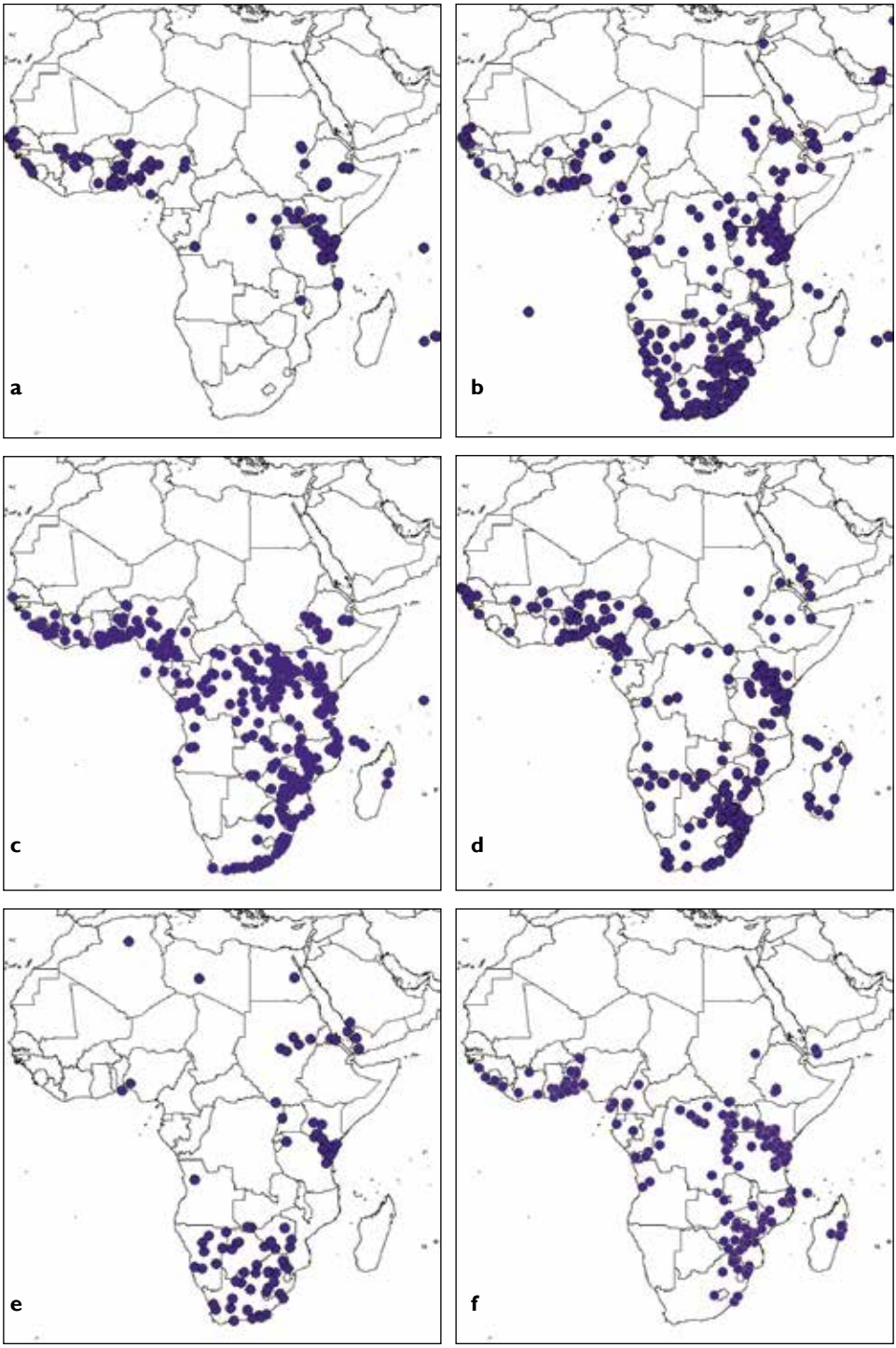

Figure 3. Distribution patterns for African tephritids: **a** *Zeugodacus cucurbitae* **b** *Dacus ciliatus* **c** *D. bivittatus* **d** *D. vertebratus* **e** *D. frontalis* **f** *D. punctatifrons* (source of data: http://projects.bebif.be/fruitfly/index.html).

might have expanded its range to South-East Asia and Hawaii on one hand and to Africa and the Indian Ocean islands on the other (although recent anthropogenic transport might have contributed to inter-regional gene flow). Sookar et al. (2013) looked at the mating compatibility between populations of Mauritius, the Seychelles and Hawaii but only found random, non-assortative mating between the populations. Within La Réunion, Jacquard et al. (2013) also described the occurrence of local genetic clusters with distinct distributions across the eastern and western coast of the island. These clusters have possible African origin and are interconnected by high levels of gene flow both within La Réunion and between La Réunion and the African mainland.

Host range and interspecific competition

Dhillon et al. (2005) list 81 plant species, including several non-cucurbits, as possible hosts for *Z. cucurbitae*. However, several of these hosts are considered doubtful because they were either based on casual observations (White and Elson-Harris 1994) or they are a result of induced oviposition under laboratory conditions. The latter approach provides unreliable data regarding the true natural host range of any fruit fly and should be considered with caution when determining host status (Aluja and Mangan 2008). De Meyer et al. (2007) list 45 plant species, belonging to 9 different families, that are considered hosts of *Z. cucurbitae* in Africa (including Indian Ocean islands) (Table 2).

The majority of these records are based on rearing of infested fruits collected in the wild. Twenty-nine of them are Cucurbitaceae. *Cucumis* spp. (in particular cucumber (*C. sativus* L.) and melon (*C. melo* L.)) and *Momordica* spp. (in particular *M. cf trifoliata* Hook. f. and bitter gourd (*M. charantia* L.)) were the preferential hosts both in West and East African studies (western Africa: Vayssières et al. 2007; Tanzania: Mwatawala et al. 2010). These studies have shown that in general cucurbit hosts are preferred over non-cucurbit hosts, with very low infestation rates and incidences in the latter. However, Vayssières et al. (2007) indicated that there are geographical differences with *Z. cucurbitae* being more oligophagous on La Réunion Island (with no genetic differences between flies infesting wild and cultivated hosts, see Jacquard et al. 2013), while having a broader host range in western Africa. Also, infestations rates can differ according to the region. For example *Cucumis melo* yielded 26-50 specimens/kg of fruits in West Africa, compared to 51–75 in Réunion, and more than 100 in Tanzania. *Lagenaria siceraria* (Molina) Standl. yielded very low numbers in West and East Africa but more than 100 specimens/kg in Réunion. These examples are, however, based on too limited a number of samples to draw definite conclusions, and it is not clear what are all of contributing causes of these differences in infestation rates. Seasonal differences (Mwatawala et al. 2009), weather variability, host availability, and interspecific competition could also be factors (Mwatawala et al. 2009, 2010, Vayssières et al. 2008). Although the low preference for non-cucurbit hosts has limited impact on actual crop loss, the mere presence in commercial hosts, such as mango (*Mangifera indica* L.),

Table 2. Host records for *Zeugodacus cucurbitae* from Africa.

Family	Scientific name	Country, Reference
Anacardiaceae	*Anacardium occidentale* L.	Benin, Burkina Faso: Vayssières et al. 2007
Anacardiaceae	*Mangifera indica* L.	Benin, Mali: Vayssières et al. 2008; Ivory Coast: Hala et al. 2008; Tanzania: Mwatawala et al. 2010; Mauritius: Quilici and Jeuffrault 2001
Annonaceae	*Annona senegalensis* Pers.	Western Africa: Vayssières et al. 2007
Cucurbitaceae	*Citrullus colocynthis* (L.) Schrader	Réunion: Vayssières 1999; Mauritius and Réunion: Quilici and Jeuffrault 2001
Cucurbitaceae	*Citrullus lanatus* (Thunb.) Matsum. and Nakai	Western Africa: Vayssières et al. 2007; Tanzania: Mwatawala et al. 2010; Réunion: Vayssières 1999; Mauritius and Réunion: Quilici and Jeuffrault 2001
Cucurbitaceae	*Coccinia grandis* (L.) Voigt	Kenya: White 2006; Copeland et al. 2009; Tanzania: Mwatawala et al. 2010; Réunion: Vayssières 1999; Quilici and Jeuffrault 2001
Cucurbitaceae	*Coccinia trilobata* (Cogn.) C. Jeffrey	Kenya: Copeland et al. 2009
Cucurbitaceae	*Cucumeropsis mannii* Naud.	Benin: Vayssières et al. 2007
Cucurbitaceae	*Cucumis anguria* L.	Réunion: Vayssières 1999; Quilici and Jeuffrault 2001
Cucurbitaceae	*Cucumis dipsaceus* Ehrenb. ex Spach	Kenya: White 2006; Copeland et al. 2009; Tanzania: Mwatawala et al. 2010
Cucurbitaceae	*Cucumis figarei* Naud.	Kenya: White 2006
Cucurbitaceae	*Cucumis ficifolius* A. Rich	Kenya: Copeland et al. 2009
Cucurbitaceae	*Cucumis melo* L.	Western Africa: Vayssières et al. 2007; Tanzania: Mwatawala et al. 2010; Réunion: Vayssières 1999; Mauritius and Réunion: Quilici and Jeuffrault 2001
Cucurbitaceae	*Cucumis sativus* L.	Kenya: White 2006; Copeland et al. 2009; Tanzania: White 2006; Mwatawala et al. 2010; Western Africa: Vayssières et al. 2007; Mauritius: Sookar et al. 2012; Réunion: Vayssières 1999
Cucurbitaceae	*Cucurbita maxima* Duchesne ex Lam.	Western Africa: Vayssières et al. 2007; Mauritius: Sookar et al. 2012; Réunion: Vayssières 1999
Cucurbitaceae	*Cucurbita moschata* Duchesne	Tanzania: Mwatawala et al. 2010
Cucurbitaceae	*Cucurbita pepo* L.	Western Africa: Vayssières et al. 2007; Mauritius: Sookar et al. 2012; Réunion: Vayssières 1999
Cucurbitaceae	*Cucurbita* sp.	Kenya: 1937; South African National Collections Pretoria (South Africa) data; Tanzania: Mwatawala et al. 2010
Cucurbitaceae	*Cyclanthera pedata* (L.) Schrader	Réunion: Vayssières 1999; Quilici and Jeuffrault 2001
Cucurbitaceae	*Diplocyclos palmatus* (L.) C.Jeffrey	Kenya: White 2006; Copeland et al. 2009
Cucurbitaceae	*Kedrostis leloja* (J.Gmel.) C.Jeffrey	Kenya: White 2006; Copeland et al. 2009
Cucurbitaceae	*Lagenaria leucaritha* (Dush) Pusby	Mauritius and Réunion: Quilici and Jeuffrault 2001
Cucurbitaceae	*Lagenaria sphaerica* (Sond.) Naudin	Mauritius and Réunion: Quilici and Jeuffrault 2001; Réunion: Vayssières 1999
Cucurbitaceae	*Lagenaria siceraria* (Molina) Standl.	Western Africa: Vayssières et al. 2007; Tanzania: Mwatawala et al. 2010; Réunion: Vayssières 1999
Cucurbitaceae	*Luffa acutangula* (L.) Roxb.	Tanzania: Mwatawala et al. 2010; Mauritius and Réunion: Quilici and Jeuffrault 2001; Réunion: Vayssières 1999
Cucurbitaceae	*Luffa cylindrica* M.Roem.	Western Africa: Vayssières et al. 2007; Mauritius and Réunion: Quilici and Jeuffrault 2001; Réunion: Vayssières 1999

Family	Scientific name	Country, Reference
Cucurbitaceae	*Momordica charantia* L.	Kenya: White 2006; Western Africa: Vayssières et al. 2007; Tanzania: Mwatawala et al. 2010; Mauritius and Réunion: Quilici and Jeuffrault 2001; Réunion: Vayssières 1999
Cucurbitaceae	*Momordica foetida* Schumach.	Kenya: White 2006; Copeland et al. 2009; Tanzania: Mwatawala et al. 2010
Cucurbitaceae	*Momordica rostrata* A. Zimm.	Kenya: Copeland et al. 2009; Tanzania: Mwatawala (pers. observations)
Cucurbitaceae	*Momordica trifoliata* Hook. f.	Kenya: White 2006; Copeland et al. 2009; Tanzania: Mwatawala et al. 2010
Cucurbitaceae	*Sechium edule* (Jacq.) Sw.	Mauritius and Réunion: Quilici and Jeuffrault 2001; Réunion: Vayssières 1999
Cucurbitaceae	*Trichosanthes cucumerina* L.	Mauritius and Réunion: Quilici and Jeuffrault 2001; Réunion: Vayssières 1999
Cucurbitaceae	*Telfairia occidentalis* Hook	Ivory Coast: Vayssières et al. 2007
Cannellaceae	*Warburgia ugandensis* Sprague	Kenya: Munro 1984
Caricaceae	*Carica papaya* L.	Tanzania: Mwatawala et al. 2010
Oxalidaceae	*Averrhoa carambola* L.	Benin, Ivory Coast: Vayssières et al. 2007
Passifloraceae	*Passiflora edulis* Sims	Réunion: Vayssières 1999; Quilici and Jeuffrault 2001
Rutaceae	*Citrus reticulata* Blanco	Benin: Vayssières et al. 2007
Rutaceae	*Citrus sinensis* Osbeck	Benin, Burkina Faso: Vayssières et al. 2007
Solanaceae	*Capsicum annuum* L. var. *longum* DC	Tanzania: Mwatawala et al. 2010.
Solanaceae	*Capsicum frutescens* L.	Western Africa: Vayssières et al. 2007
Solanaceae	*Solanum lycopersicum* L.	Réunion: Vayssières 1999; Tanzania: Mwatawala et al. 2010
Solanaceae	*Solanum aethiopicum* L.	Tanzania: Mwatawala et al. 2010
Solanaceae	*Solanum anguivi* Lam.	Tanzania: Mwatawala et al. 2010
Solanaceae	*Solanum macrocarpon* L.	Tanzania: Mwatawala et al. 2010
Solanaceae	*Solanum nigrum* L.	Tanzania: Mwatawala et al. 2010

citrus (*Citrus* spp.) or carambola (*Averrhoa carambola* L.), can have regulatory implications for export of particular commodities. On the other hand, other polyphagous fruit fly species in Africa, such as *Bactrocera dorsalis*, *Ceratitis capitata* (Wiedemann) or *Ceratitis rosa* Karsch, which attack these commercial non-cucurbit hosts, are rarely encountered in Cucurbitaceae (Mwatawala et al. 2009).

While no other *Zeugodacus* species occurs in Africa, various indigenous dacines belonging to the genus *Dacus* are known cucurbit pests, the most noteworthy and widespread being *Dacus ciliatus* Loew, *D. bivittatus* (Bigot), *D. vertebratus* Bezzi, *D. frontalis* Becker, and *D. punctatifrons*. All these species have a large geographic overlap with *Z. cucurbitae* (Figure 3b–f) and there is thus, interspecific competition for the same larval food source. Studies on the interspecific interactions between these cucurbit feeders in Africa are, however, scarce. Mwatawala et al. (2010) studied the host range and relative abundance of cucurbit feeders in central Tanzania. They concluded that *Z. cucurbitae* dominated most cucurbit hosts, in comparison to the indigenous *Dacus* species. Only *Dacus ciliatus* was predominant in some hosts like *Citrullus lanatus* (Thunb.) Matsum. and Nakai (and *Momordica charantia* to a lesser extent). A pilot study exploring genetic differentiation between 42 Tanzanian *Z. cucurbitae* specimens

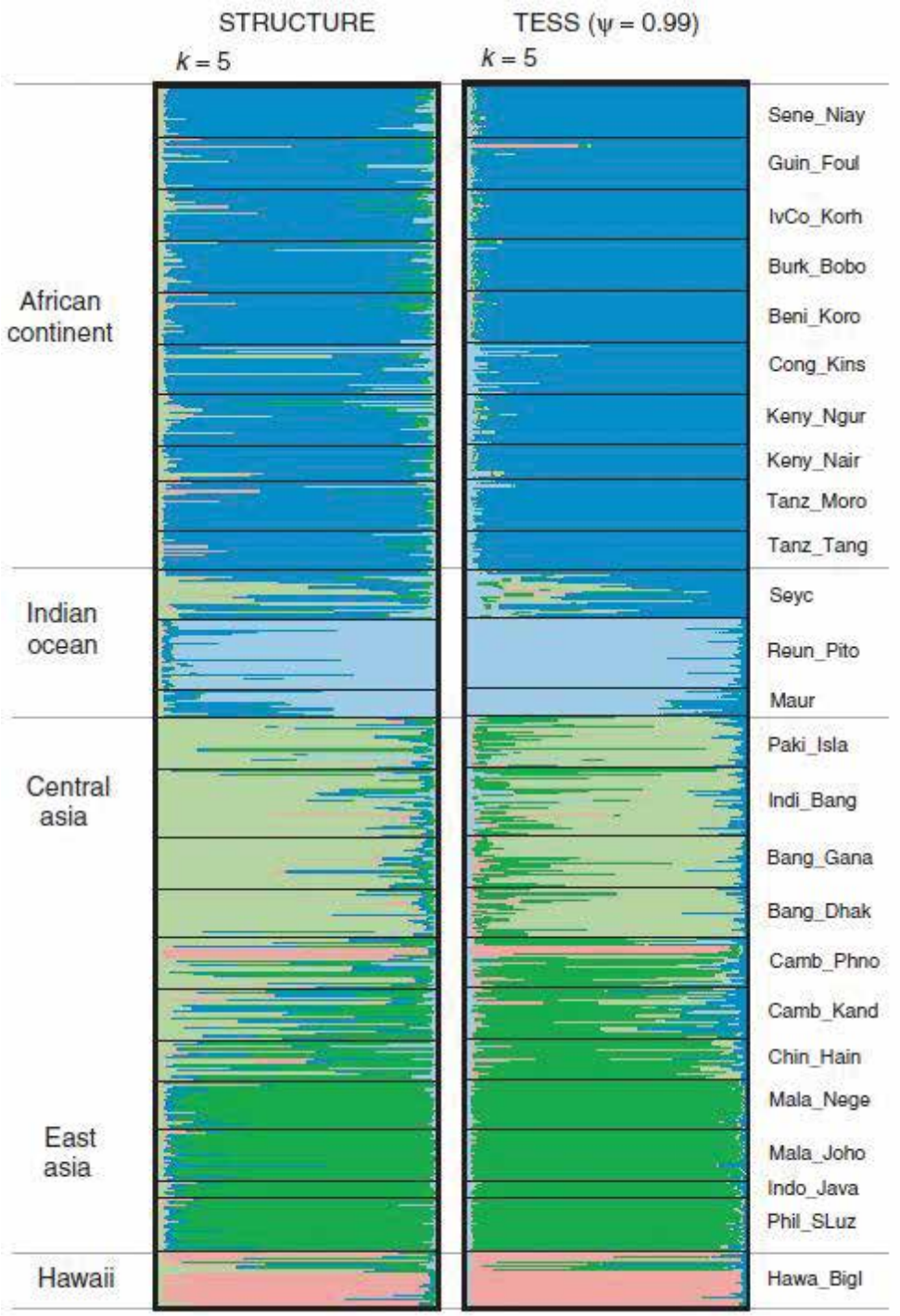

Figure 4. Individual admixture proportions (K=5) of 25 different populations of *Zeugodacus cucurbitae* (after Virgilio et al. 2010).

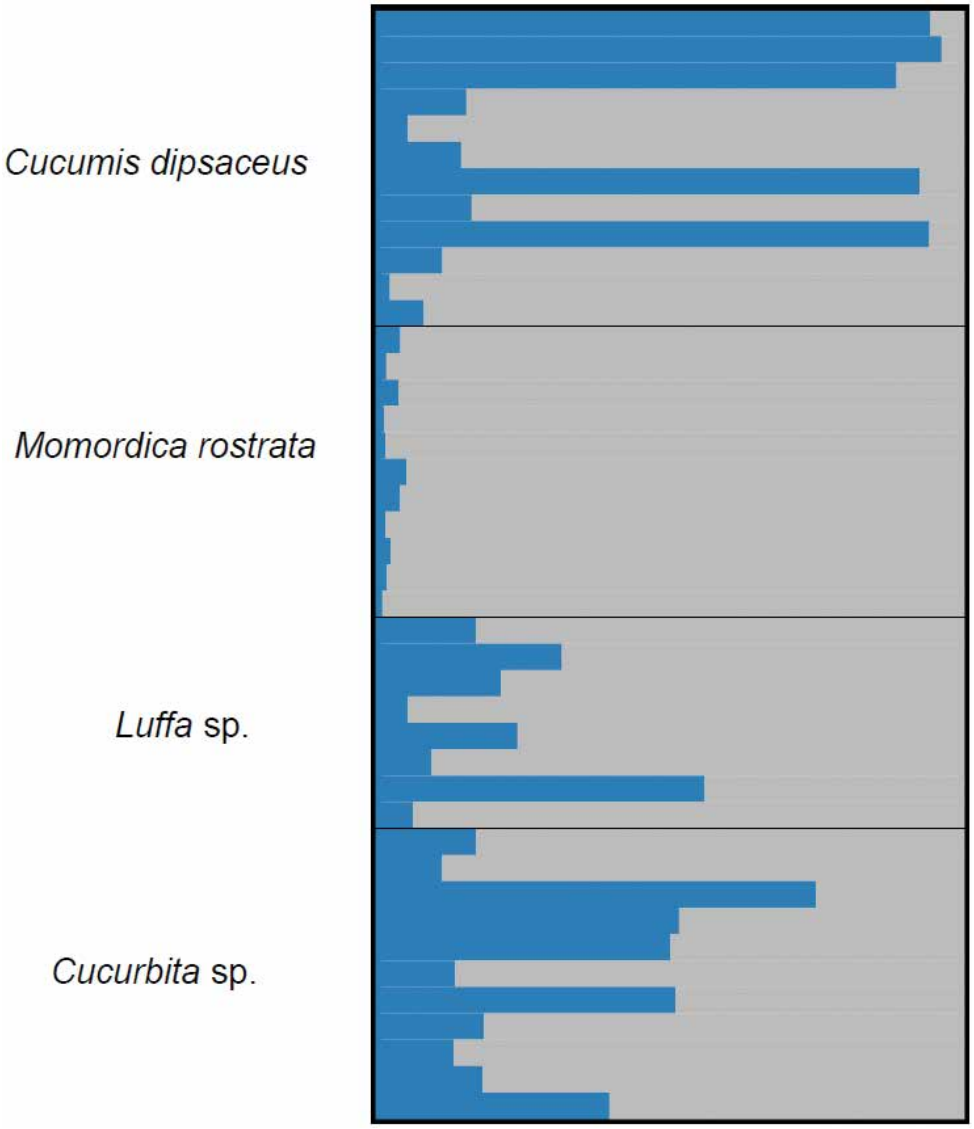

Figure 5. *Zeugodacus cucurbitae* specimens (n = 42) reared from four different hosts (*Cucumis dipsaceus*, *Cucurbita* sp., *Luffa* sp., *Momordica rostrata*) at the Sokoine University of Agriculture (Morogoro, Tanzania) and genotyped at 19 microsatellite loci (Mwatawala, Virgilio, De Meyer, unpublished data).

reared from different cucurbit hosts (*Cucumis dipsaceus* Ehrenb. ex Spach, *Cucurbita* sp., *Luffa* sp., *Momordica rostrata* A. Zimm.) and genotyped at 19 microsatellite loci did not suggest the occurrence of possible host races (Figure 5)

On La Réunion Island (1996-1999), three species (*Z. cucurbitae*, *Dacus ciliatus*, and *Dacus demmerezi* (Bezzi)) infested a range of 16 cucurbit species (Vayssières and Carel 1998; Vayssières 1999). The altitudinal limits of *Z. cucurbitae*, *D. ciliatus* and *D. dem-*

merezi were, respectively, 1200m, 1400m, and 1600m during the hot season. These three species have an overlap on all cucurbit crops up to 600m during the cold season and until 1200m during the hot season. At least one abiotic factor (altitude) and two biotic ones (host availability, interspecific competition) are the main screening factors for species-dominance in La Réunion. Among the 16 cucurbit hosts, *D. ciliatus* dominated in the cultivated hosts *Citrullus colocynthis* (L.) Schrader, *Cyclanthera pedata* (L.) Schrader, *Secchium edule* (Jacq.) Sw., and several cultivars of *Cucumis melo* and *Cucurbita pepo* L., which were cultivated above the altitudinal limit of *Z. cucurbitae* (600m during the cold season and up to 1200 meters during the hot season). *Zeugodacus cucurbitae* dominated on wild species such as *Coccinia grandis* (L.) Voigt., *Cucumis anguria* L., *Lagenaria sphaerica* (Sond.) Naudin, *Momordica charantia*, and also cultivated ones such as *Citrullus lanatus*, *Cucumis melo*, *Cucumis sativus*, *Curcubita pepo*, *Luffa acutangula* (L.) Roxb., *Luffa cylindrica* M. Roem., *Momordica* sp., and *Trichosanthes cucumerina* L. (Vayssières 1999). Vayssières et al. (2008) compared in detail the demography of *Z. cucurbitae* and *D. ciliatus* on La Réunion. They concluded that both species have a distinctly different life-history pattern with *Z. cucurbitae* being characterized by a later onset of reproduction, a longer oviposition time, longer life span and higher fecundity, while *D. ciliatus* has earlier reproduction, lower oviposition time, shorter life span and lower fecundity.

These differences in demography seem to lead to exploitative and interference competition between the two species (and most likely other cucurbit infesters as well), with *Z. cucurbitae* having an advantage over *Dacus ciliatus*. This predominance is suggested by the majority of infestations in wild cucurbit species in the field by *Z. cucurbitae*. Duyck et al. (2004) reviewed the invasion biology of (polyphagous) fruit flies and demonstrated that presence of several introduced species in areas already occupied by other tephritids, results in a decrease in number and niche shift of the pre-established species. This is largely governed by life-history strategies that species adopt for interactions in near-optimal conditions. Although the review focused on polyphagous species, a similar scenario should be considered for oligophagous pests like *Z. cucurbitae*. So far, all studies indicate that *Bactrocera* species are best adapted to exploit and to compete with other species in the same ecological niche (Duyck et al. 2006, Vayssières et al. 2008). It has also been suggested that host-range can allow niche differentiation (Duyck et al. 2008) and that this could be the reason for the different host ranges observed for *Z. cucurbitae* in La Réunion versus West Africa (Vayssières et al. 2007), with *Z. cucurbitae* being more polyphagous in West Africa. While only two indigenous cucurbit-feeding fruit flies are found on La Réunion (Vayssières and Carel 1998, De Meyer et al. 2012), at least nine are reported from West Africa (De Meyer et al. 2013). This could reflect higher interspecific competition in the latter case, with occasional shifts of *Z. cucurbitae* to non-cucurbits.

In addition to interspecific competition, the host availability and ecological niches will also affect the occurrence and impact of *Z. cucurbitae*. Earlier studies in Hawaii have shown that it is a species that is mainly found in warmer areas and that its abundance declines with increasing rainfall and increasing elevation (Vargas et al. 1989). This preference for warmer periods was confirmed in studies in La Réunion (Vayssières 1999). Studies in Tanzania showed that *Z. cucurbitae* was either absent or relatively

less abundant at higher elevations along a transect from approx. 600 masl to 1650 masl. However, the exact relationship between these biotic and abiotic factors that can have an impact on the host range in different African populations, is currently poorly known and requires further investigation.

Conclusion

Morphologically and genetically *Zeugodacus cucurbitae* shows mating compatibility among test populations and limited intraspecific genetic and morphological variability. It is still not clear if the relatively recent records for this species on the African mainland (1930s in East Africa, beginning of 21st century in West Africa) are the result of local expansions of already established African populations or of one or more introductions from non-African sources. Regardless differences in host range reported across African populations there is no evidence supporting the existence of genetically isolated host races with specific feeding preferences and the observed host range variability seems more to be related to factors such as interspecific competition, host availability, and ecological niche partitioning. Although our study focused on the African populations, there is no indication that the situation might differ across the distribution of *Z. cucurbitae*.

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Supplementary material 1

Genus *Zeugodacus* (Diptera, Tephritidae), list of valid species

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Data type: list of species

Explanation note: This list includes species listed under subgenera *Asiadacus*; *Austrodacus*; *Diplodacus*; *Hemigymnodacus*, comb. n.; *Heminotodacus*; *Hemiparatridacus*; *Nesodacus*; *Niuginidacus*; *Papuodacus*; *Paradacus*; *Parasinodacus*, comb. n.; *Sinodacus*; and *Zeugodacus*.

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