WORKING MATERIAL

RESOLUTION OF CRYPTIC SPECIES COMPLEXES OF TEPHRITID PESTS TO OVERCOME CONSTRAINTS TO SIT APPLICATION AND INTERNATIONAL TRADE

FIRST RESEARCH COORDINATION MEETING OF A FAO/IAEA COORDINATED RESEARCH PROJECT HELD IN VIENNA, AUSTRIA FROM 2 TO 6 AUGUST 2010

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A. Background Situation Analysis (Rationale/Problem Definition)

RESOLUTION OF CRYPTIC SPECIES COMPLEXES OF TEPHRITID PESTS TO OVERCOME CONSTRAINTS TO SIT APPLICATION AND INTERNATIONAL TRADE

Tephritids are among the worst pests in agriculture and are of major economic importance in nearly all tropical, subtropical and temperate countries worldwide. These pest species cause enormous devastation to both food production and international trade and are major targets of insecticide applications. They are among primary causes of poverty, malnutrition and poor production and trade in fresh horticultural commodities in large areas of tropical developing countries (Waterhouse 1993; Allwood & Leblanc 1996; Lomborg 2004), where climatic conditions are favourable for labour-intensive fruit and vegetable-based agroindustries.

The study of the biology and management of tephritids therefore requires significant international attention to overcome these hurdles and to assist Member States in developing and validating environment-friendly 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 Mediterranean fruit fly. Demands for R&D support in that respect are diminishing due to successful integration into control programmes. There is now an urgent need to focus on the increasing demands from Member States in Africa, Asia and Latin America, to address the issue of major tephritid species complexes of economic importance. These are groups of species where the morphology is very similar or identical but biologically they are distinct species. Some major pest fruit fly species occur within complexes, and the uncertainty related to their questionable technical status results in major constraints to SIT application and international trade.

Problem to be addressed:

Considerable concern has been expressed by many Member States that some major pest species complexes include taxonomically described species that are actually only geographical variants of the same species. Conversely, some insect populations grouped taxonomically within the same pest species display different biological and genetic traits and show reproductive isolation which suggest that they are different species. This uncertain taxonomic status is having important practical implications on the effective development and use of the SIT against such complexes with respect to the rearing of the correct species and significantly affects international movement of fruit and vegetables resulting in the establishment of trade barriers to important agricultural commodities which are hosts to pest tephritid species. Moreover, this problematic situation occurs in four of the most important pest genera within the Tephritidae family: *Anastrepha*, *Bactrocera*, *Ceratitis* and *Rhagoletis*.

Regional insect rearing facilities that would produce sterile males for use in different countries or regions are desirable as this would make the SIT more cost-effective. It is of prime importance therefore to the success of these regional area-wide integrated
pest management (AW-IPM) programmes that have an SIT component that the mass-reared flies from such species complexes as *Anastrepha* and *Bactrocera*, etc. are behaviourally compatible with the target native fruit fly pest populations in the various recipient regions. If species complexes remain unresolved then these desirable outcomes will be difficult or impossible to achieve. The resolution of some of the major cryptic species complexes (i.e. insects whose specific status is questionable) is therefore critical both for SIT application and to assist subtropical and tropical Member States to overcome non-tariff trade barriers in order to export their fresh fruit and vegetable commodities to international high value markets.

A ranking of the major pest complexes with respect to the economic damage and regional impacts indicates that a number of species complexes are of concern to Member States. A Consultants’ Meeting discussed the complexes noted above and related technical issues and prioritised them as to economic importance and potential for SIT application. Two complexes and two suspected complexes were identified to be of significant importance that needed to be resolved to facilitate world agricultural trade and SIT programmes. They are:

- **Known complexes**
  - *Bactrocera dorsalis*
  - *Ceratitis rosa*

- **Suspected complexes**
  - *Anastrepha fraterculus*
  - *Bactrocera cucurbitae*

A report on the current state of knowledge on the specific status of pest fruit flies in each of these known or suspected complexes and on the technologies and techniques that are, or can be, used for accurate species discrimination is presented in Section C of this report.

**B. The Co-ordinated Research Project (CRP)**

This new Co-ordinated Research Project (CRP) is based on a Consultants Meeting which was held from 6 to 10 July 2009 in Vienna, Austria (report available), to review progress made in this area, to assess the potential for conducting co-ordinated R&D and to formulate a proposal for a CRP on “Resolution of Cryptic Species Complexes of Tephritid Pests to Overcome Constraints to SIT Application and International Trade”.

The overall objective of this new **CRP D4.10.23**, approved for the **period 2010-2014**, is to assist Member States in achieving sustainable fruit and vegetable production and in facilitating international trade and the area-wide integrated application of the SIT as part of suppression/eradication programmes against fruit flies of economic importance in Africa, Asia & Pacific, and Latin America.

The specific objectives are to define the species limits within the target complexes, followed by the development of robust species-specific diagnostic tools.
The main targets will be the *Anastrepha fraterculus* (Latin America), *Bactrocera dorsalis* (Asia & Pacific, Africa), and *Ceratitis rosa* (Africa) species complexes and with lower priority the *Bactrocera cucurbitae* (Asia & Pacific, Africa) complex. In each of these groups there are questions concerning either the validity of some of the species or their capacity to be diagnosed.

This international network research project is operated as part of the IAEA Research Contract Programme. The new FAO/IAEA CRP D4.10.23 consists of 22 research teams of which 15 are research contract holders and 7 are agreement holders. In addition several other research teams are participating fully funded by their Institutions and Governments. The duration of the CRP is 5 years and Research Coordination Meetings (RCMs) will be held every 18 months. The first RCM for this project was held in Vienna from 2 to 6 August 2010. These CRPs and RCMs have the effect of encouraging close collaboration and provide a forum for information exchange between scientists and institutions involved, as well as providing a focused approach to the development and technology transfer of environment-friendly technologies.

### C. Objectives of the Meeting

The objectives of the first RCM were as follows:

a) To present and discuss the results of related additional research carried out independently by some participation researchers  
b) To co-ordinate the processing and analysis of the experimental results  
c) To discuss and agree on a protocol for Phase 2 of the CRP  
d) To prepare work plans for each participating institute for Phase 2 of the CRP  
e) To co-ordinate the needs and provisions of material over the next 18 months  
f) To set up logical and harmonised systems for collection and transport of live and preserved fruit fly specimens  
g) To harmonise research and administrative linkages between institutions.

### D. Report for the 1\(^{st}\) RCM (Vienna 2010)

The meeting was attended by 31 scientists and observers. The list of participants and observers is given in **Appendix 4**. The meeting was formally opened by Jorge Hendrichs, Head of the Joint FAO/IAEA Insect Pest Control Section, who welcomed the participants and explained the importance of resolving the issues that surround the existence of cryptic Tephritid species complexes in order to facilitate international trade and the use of the SIT in area-wide management programmes. Andrew Jessup, as Scientific Secretary of the CRP, gave the overview of the Co-ordinated Research Project, the objectives of the meeting, the agenda and organisational aspects.

The agenda that was prepared for the meeting (**Appendix 1**) was followed with only minor changes. The progress reports of the participants covered the results they obtained during Phase 1 of the CRP. The abstracts of these presentations are given in **Appendix 2**. During the 3\(^{rd}\) and 4\(^{th}\) days of the RCM participants were divided into three separate working groups (**Appendix 3**) to review recommendations from the
Consultants Meeting, prepare conclusions, general technical administrative recommendations, as well as work plans and recommendations for each participating institute. In addition participants were asked to detail scientific and administrative linkages between participating institutes.

On the 2\textsuperscript{nd} day participants discussed some of the common issues that exist between institutes. The first was a protocol for collection and shipment of live and dead insects for rearing, morphology, chemical biology and molecular assays (Appendices 6 and 8) and the second was a discussion and review of procedures for field cage tests (Appendix 7). Relevant references are presented in Appendix 5.

**Summary of the Working Groups**

Three working groups were established based on pest fruit fly genera. The groups were:

Group 1: *Anastrepha fraterculus* (possibly five to seven distinct pest species)

Group 2: *Bactrocera dorsalis* pest species complex (at least five pest species: *B. dorsalis* s.s., *B. carambolae*, *B. papayae*, *B. philippinensis* and *B. invadens*)

Group 3: *Bactrocera cucurbitae* (possibly different populations) and the *Ceratitis* FAR pest species complex (comprising three species: *C. fasciventris*, *C. anonae* and *C. rosa*)

Group participants firstly reviewed the current status and knowledge gaps in each thematic area being considered in the CRP. In addition to this, participants in each group also provided their Group’s generic activities for the 5 year course of the CRP and each participant’s specific work plans for the first 18 months of the CRP. See Table 1 for a summary of thematic areas with corresponding researchers. Given the real need to be collaborative, transparent and co-operative within this project they were asked to consider how each sub-project within the CRP may be linked to other sub-projects, researchers and institutions (see Table 2 for these project linkages). These are reported in Section E. Groups then reviewed their inputs into the Logistic Framework to ensure that targets previously set were reasonable and feasible both technically and with respect to the duration of the CRP. This is reported in Section F.

After these tasks were completed all Groups rejoined to discuss any changes and new inputs and all agreed on the format and content on a draft Working Material for the 1\textsuperscript{st} RCM of this CRP.
Table 1. Thematic areas in relation to Tephritid species complexes and suspected complexes to be addressed by researchers

<table>
<thead>
<tr>
<th>Complex</th>
<th>Molecular</th>
<th>Behavior</th>
<th>Chemical Ecology</th>
<th>Taxonomy / Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anastrepha fraterculus</td>
<td>Canal, Malacrida, Silva</td>
<td>Joachim-Bravo, Vera</td>
<td>Nascimento, Kalinova, Vera</td>
<td>Canal, Hernández-Ortiz, Steck</td>
</tr>
<tr>
<td>Bactrocera dorsalis</td>
<td>Aketarawong, Armstrong, Malacrida, Zacharopoulou</td>
<td>Chinvinijkul, Clarke, Hee, Tan</td>
<td>Chinvinijkul, Hee, Tan</td>
<td>Clarke, Drew, Ji</td>
</tr>
<tr>
<td>Ceratitis FAR complex</td>
<td>Delatte</td>
<td>de Meyer,</td>
<td>Kalinova</td>
<td>de Meyer, Steck</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bactrocera cucurbitae</td>
<td>Delatte, Malacrida, Zacharopoulou</td>
<td>Delatte, Mwatawala</td>
<td>-</td>
<td>de Meyer</td>
</tr>
</tbody>
</table>

E. Conclusions on Current Status and Recommended Future Activities for the CRP Participants

**Group 1: Anastrepha fraterculus**

**Background Situation Analysis**

The South American fruit fly, *Anastrepha fraterculus* (Wiedemann) *s.l.* is present in most countries of the Americas from the USA to Argentina (Hernandez & Aluja 1993, Steck 1999, Zucchi 2000, 2007). On the South American subcontinent, this species is found in two broad, 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, there is recent evidence of its presence in parts of the Brazilian Amazon basin (pers. comm. Ronchi-Teles). Its centre of diversity is South America and it has been reported to infest about 80 host plants including major fruit crops (Norrbom & Kim 1988, Norrbom 2004, Zucchi 2007). This highly destructive pest imposes 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* has led to the conclusion that it probably is a complex of cryptic species rather than a single biological entity (Stone 1942,

Reproductive incompatibility has been reported both at pre- and post-zygotic levels (Selivon 1996, Vera et al. 2006, Cáceres et al. 2009) among some populations. At the pre-zygotic level, mating compatibility was evaluated among six different populations from Mexico, 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 sexually isolated. Flies were sexually active at different times of the day suggesting different sexual behaviour. Post-zygotic studies between two populations from Brazil (Selivon et al. 1999) and between one Argentine 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 (Selivon 1996, Malavasi & Morgante 1982, Selivon et al. 2005, Goday et al. 2006); while for the latter case, the same was shown with pheromone and cytological studies (Cáceres et al. 2009).

The combined results of these studies appear to suggest the existence of at least seven different biological entities which in this document are referred to as: A. sp. 1 aff. fraterculus (Yamada & Selivon 2001), A. sp. 2 aff. fraterculus (Yamada & Selivon 2001), A. sp. 3 aff. fraterculus (Selivon et al. 2004), A. sp. 4 aff. fraterculus (Selivon et al. 2004), A. fraterculus Mexican morphotype (Hernandez-Ortiz et al. 2004) and A. fraterculus from Andean highlands and Venezuela coastal lowlands (Steck 1991).

Despite previous studies, which have provided strong evidence supporting the existence of several species, there are still major knowledge gaps and the described studies have used different methodologies. In order to be able to formally describe and name some of these putative species, it is necessary to apply a complete set of methodologies to all of them in a comprehensive study involving populations from the entire geographic distribution range. This will allow the characterization of each putative species and will 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 critical for importing authorities in determining which of them may or may not be quarantine pests. This will 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.
Baseline Knowledge on *Anastrepha fraterculus*

**DNA**
- Current knowledge:
  - Only three gene regions have been studied for representatives of *A. fraterculus s.l.* populations. These are the COI, 16S, and period genes. COI and period genes are most useful in terms of inter-population variation. Data is available for only 24 specimens from only 16 populations representing five of the above mentioned seven morphotypes (see Table 3.).
- Gaps identified:
  - There are no data yet for two of the morphotypes.
  - The total number of specimens analyzed so far is small.
  - Microsatellite data are not yet available.
  - Other molecular markers have not been applied.

**CYTOLOGY**
- Current knowledge:
  - Karyotypes have been described for at least 24 species of *Anastrepha*.
  - These include the following morphotypes of *A. fraterculus s.l.*: Mexican, *A. sp. 1 aff fraterculus* (Argentina, Brazil), *A. sp. 2 aff fraterculus* (Brazil), *A. sp. 3 aff fraterculus* (Brazil), and *A. sp. 4 aff fraterculus* (Peruvian morphotype from Peru and Ecuador).
  - Polytene chromosomes were used to compare Argentine and Peruvian populations.
- Gaps identified:
  - Polytene chromosome banding patterns not yet described.
  - No karyotype data available for Andean and Venezuela lowland morphotypes.

**ALLOZYMES**
- Current knowledge:
  - Extensive data on allozyme variation are available for various populations of the following morphotypes: Mexican, Andean, Peruvian, Venezuelan lowland, *A. sp. 1 aff fraterculus* (Argentina, Brazil) and *A. sp. 2 aff fraterculus* (Brazil).
- Gaps identified:
  - As data are not directly comparable between the various studies performed in different laboratories, it is not a high priority to gather new isozyme data.

**MORPHOLOGY**
- Current knowledge:
  - Published morphometric data indicate that at least three morphotypes can be recognized in continental America (“Mexican”, “Andean” and “*A. sp. 1 aff fraterculus*”), based on discriminant function analysis of particular characters from the aculeus, wing and mesonotum (Hernández-Ortiz *et al.* 2004).
  - Unpublished data (Hernandez-Ortiz *et al.* in prep.) suggest the presence of four additional morphotypes called “Venezuelan”, “Peruvian” “*A. sp. 2 aff fraterculus*” (Brazil) and “*A. sp. 3 aff fraterculus*”(Brazil).
• Additional morphometric data using aculeus and wing characters show differences between the types *A. sp. 1* *aff fraterculus* (Brazil) and *A. sp. 2* *aff fraterculus* (Brazil).
• Egg morphological differences have been described between the three Brazilian morphotypes (Selivon & Perondini 1998, Selivon et al. 2004).
• Only preliminary descriptions of larval stages are available (Steck *et al.* 1990, Frias *et al.* 2006)

- Gaps identified:
  • Formal taxonomic descriptions of Mexican, Andean, and “*A. sp. 1* *aff fraterculus*” (Brazil) morphotypes are not yet published.
  • None of the larval stages has been thoroughly described and compared among morphotypes.
  • Egg morphology is unknown for four morphotypes.

**MALE LURE RESPONSE**
• Not applicable to the *Anastrepha fraterculus* complex

**PHEROMONE AND CUTICULAR COMPONENTS / CHEMICAL ECOLOGY**

- **Current knowledge:**
  • Pheromone composition has been described so far for only three populations (probably two morphotypes) with different methodologies.
  • Chemicals released by calling males were first investigated by Lima *et al.* (2001) using a single population from Pelotas, Rio Grande do Sul, Brazil. These chemicals were identified as alcohols, mono- and sesquiterpenes and three isomeric lactones. Later, Caceres *et al.* (2009) showed that a population from Argentina differed in its composition from a population from Peru.
  • Preliminary studies carried out on the response elicited by the pheromone produced by *A. fraterculus* males from an Argentine laboratory population have shown that female antennae respond to six out of thirteen compounds identified as volatiles released by calling males of this population, and these chemicals were identified as: (Z)-3-nonenol, (E,Z)-3,6-nonadienol, geranyl acetone, alfa-farnesene and (S,S)-epianastrephin.
  • There are few studies concerning hydrocarbon composition in pupae and adults of *A. fraterculus*.
  • Preliminary studies performed by Vanickova *et al.* (unpublished results) have shown that there are differences in the cuticular hydrocarbon composition between virgin males and females of one Argentine laboratory population.
  • Qualitative and quantitative differences were observed in the chemical composition of cuticular hydrocarbons according to the age of the fly.

- **Gaps identified:**
  • The composition of the pheromone is not known to all morphotypes.
  • The function of the identified chemicals either alone or combined in eliciting attraction of conspecific females towards males is still unknown.
  • Antennal response has been studied in only one Argentine population
  • Cuticular hydrocarbon composition is known only from virgin male and females from one Argentine population and one southern Brazil population.
BEHAVIOR
- Current knowledge:
  • *A. fraterculus s.l.* has a lek mating system.
  • Mating occurs primarily in the early morning, with some populations showing different mating times (midday for Peruvian population and late afternoon for Colombian population).
  • Pre-zygotic isolation was detected among populations at the continental level.
  • Male courtship has been described for one Argentine population.
  • There is some evidence suggesting that populations confined together in field cage trials form segregated leks.
  • For one Argentine population, it has been shown that female post-copulatory behavior depends on attributes of the first male suggesting that female decision on remating depends on the conditions of the first male.
- Gaps identified:
  • Mating compatibility has not been evaluated among all morphotypes.
  • Mating behavior has been described for only 3 morphotypes.
  • Factors involved in pre-zygotic isolation are unknown (temporal, ecological or sexual isolation, role of pheromones in mate recognition/acceptance).
  • Male courtship, male-male interactions and female responses within and among each putative species is not completely known.
  • The factors that determine lek location should be determined (environmental vs. avoidance of mixed leks).
  • Female postcopulatory behavior after mating with males from other populations is unknown.

DISTRIBUTION
- Current knowledge:
  • *A. fraterculus s.l.* is widely distributed from southern Texas to northern Argentina in South America, but the detailed regional patterns for the 7 morphotypes are not well understood.
  • Current unpublished information reveals that the Mexican type extends from Mexico to Central America; the Venezuelan type occurs only in one area of the Caribbean lowlands of Venezuela; the Andean type is found in the Venezuelan and Colombian highlands; while the Peruvian type has been found in at least three localities from Ecuador and Peru.
  • The distribution of the three Brazilian types is not well known and some of them occur in sympathy.
- Gaps identified:
  • The detailed distributions of all morphotypes in South America are unknown.
  • Elevational transects in Andean countries are lacking and needed to determine limits of highland and lowland morphotypes

HOST RANGE
- Current knowledge:
  • There are numerous host records available for *A. fraterculus s.l.*, but these have not been associated with the various morphotypes.
  • Host lists have been published for several countries, e.g., Venezuela (Hernandez & Morales, 2004), Brazil (Zucchi 2007, Zucchi 2008)
- Gaps identified:
  - Host ranges for most morphotypes are unknown.
  - Update and revision of available host lists to correct errors is needed.

**POST-ZYGOTIC COMPATIBILITY**

- Current knowledge:
  - Crosses between *A. sp. 1 aff fraterculus* and *A. sp 2 aff fraterculus* resulted in a reduction of egg hatch and larval viability and also a distorted sex ratio.
  - The same has been observed in crosses between populations from Peru and Argentina.
  - It is possible to maintain hybrid lines, including those with their initial sex ratio distortion maintained, under artificial (laboratory) rearing conditions.
  - The occurrence of *Wolbachia* was confirmed for three populations (Brazil, Argentina, Peru).

- Gaps identified:
  - The occurrence of hybrid sterility has not been evaluated.
  - The role of *Wolbachia* in post-zygotic incompatibility has not been determined.

Number of specimens examined for each morphotype and gene region

<table>
<thead>
<tr>
<th>Morphotype</th>
<th>COI</th>
<th>16S</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>sp. 1, 2, 3 (all Brazil) (unfortunately, it is unknown which of the 3 morphotypes tested correspond to the specimens examined)</td>
<td>SC - 2</td>
<td>-</td>
<td>SP – 1</td>
</tr>
<tr>
<td></td>
<td>ES – 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BA -1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SP - 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MG -1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ARG - 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peruvian (= “sp. 4” of Selivon)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mexican</td>
<td>3</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Andean</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Venezuelan lowland</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

Specific Project Plans: *Anastrepha fraterculus* Complex

**DNA**

Anna Malacrida

- 5 year plan:
  - Identification of SSR markers in different populations possibly representing specific entities across the *A. fraterculus* complex range and evaluating their degree of differentiation.
  - Development of a database for population variability/ differentiation within the *A. fraterculus* complex.
  - Assessing the presence of postzygotic isolation within *A. fraterculus* entities.
  - Development of species-specific molecular markers as diagnostic tools for *A. fraterculus* entities.
- For the next 18 months:
  - Development of SSR markers in A. fraterculus for one morphotype.
  - Assess if it’s transferable to other morphotypes.

Janisete Silva  
- 5 year plan:
  - Obtain samples from A. fraterculus populations in Brazil, including colonies already established.
  - Evaluate DNA variation using mitochondrial and nuclear gene sequences.
  - Compare populations for evidence of population structure and potentially distinct gene pools.
  - Test the monophyly of the A. fraterculus complex using multiple specimens from each putative cryptic species.
  - Elucidate the number of putative species in the A. fraterculus complex in Brazil.
  - Map haplotype distribution in Brazil to guide future control programs.

- For the next 18 months:
  - Obtain collections of different populations of A. fraterculus in Brazil as well as some other related Anastrepha species for the phylogenetic analysis.
  - Screen populations and already established colonies for one mitochondrial DNA marker (COI).

Nelson Canal  
- 5 year plan:
  - Analyze ITS-1 from different populations of A. fraterculus and A. obliqua from Colombia.

- For the next 18 months:
  - Obtain samples and optimize ITS amplification.

CYTOLOGY  
Nelson Canal  
- 5 year plan:
  - Examine and compare karyotypes from different populations of A. fraterculus and A. obliqua from Colombia with previously described karyotypes.

- For the next 18 months:
  - Perform karyotype analysis of the Andean population.

MORPHOLOGY
Vicente Hernández-Ortiz / J. García Gonzalez / E. Arévalo / G. Palma / J. Martínez / J. Trigrero / N. Nolazco / C. Rivera
- 5 year plan:
  - Perform a comparative morphometric analysis of populations of the nominal species Anastrepha fraterculus (Wiedemann) throughout Mexico and Central America in order to describe the involved species.
  - Study the morphological variability of populations from Colombia, Ecuador and Peru, comparing samples from lowland Pacific coastal plain with those samples from highland Andean areas from those countries.
  - Describe the inter-population variability and morphotypes established in each geographic area and identify their distribution patterns.
- Determine the usefulness of morphological characters to recognize and describe the species of the complex.
- For the next 18 months:
  - Field sample collection.
  - Preservation and mounting structures.
  - Digital imaging.
  - Measurement of structures in 12 populations.

**Nelson Canal**
- 5 year plan:
  - Describe egg and larval external morphology of the two species.
  - Study adult morphology of different populations from *A. fraterculus* and *A. obliqua* from Colombia.
- For the next 18 months:
  - Population collection
  - Establish laboratory colonies
  - Perform morphological studies on immature and adult stages.

**Gary Steck**
- 5 year plan:
  - To develop a strategy for sampling, acquiring host plant and geographic distribution data, rearing and breeding specimens for diverse studies of adults and immature stages, preservation, and vouchering of specimens.
  - To assist in the acquisition of field collections, as needed, based on participation of various agencies throughout the Americas.
  - To examine and describe immature stages of various populations of the *A. fraterculus* complex in search of diagnostic characters to separate discrete species, as provided by cooperating teams.
- For the next 18 months:
  - Design a population sampling strategy for the *A. fraterculus* complex throughout its geographic range that will maximize the types of data that can be used for description of discrete species, and determining their geographic distributions, and associations with host plants.
  - Describe immature stages of various populations of the *A. fraterculus* complex (contingent on provision of specimens by cooperating teams of the CRP).

**BEHAVIOR**
**Iara Joachim-Bravo**
- 5 year plan:
  - To evaluate the level of reproductive isolation among populations from three Brazilian morphotypes through demographic parameters (fertility, sex ratio, and larva and pupa viability), sexual compatibility and competitiveness.
  - To describe the pre-mating behavior pattern from the three Brazilian morphotypes.
  - To analyze comparatively the acoustical signals emitted during the courtship in each morphotype.
- For the next 18 months
  - Collect infested fruit in the field from different regions in Brazil.
  - Establish laboratory colonies of two Brazilian morphotypes (*A. sp 1 aff. fraterculus* and *A. sp 2 aff. fraterculus*) from the flies collected in the field.
  - Initiate compatibility tests between the two established colonies of the two Brazilian morphotypes.
  - Initiate laboratory video recordings in order to characterize the courtship behavior of the two established colonies of the two Brazilian morphotypes.
  - Perform a comparative analysis of the sounds emitted by the flies during the courtship behavior from each of the two established colonies of the two Brazilian morphotypes.

**Teresa Vera / Diego Segura / Solana Abraham / Juan Rull**
- 5 year plan:
  - To perform mating compatibility tests from as many putative species as possible.
  - To characterize aspects of the sexual behavior of each population.
  - To determine the occurrence of post-zygotic isolation among populations.
  - To study the sexual behavior of the hybrids.
- For the next 18 months
  - Establishment/renew colonies from the putative species at Seibersdorf.
  - Perform mating compatibility tests with the colonies.
  - Perform post-copulatory behavior studies.
  - Perform post/zygotic isolation studies among the different colonies.

**CHEMICAL ECOLOGY**

**Ruth do Nascimento**
- 5 year plan:
  - To determine the exact composition of the volatile pheromone profile released by *A. fraterculus* males from different morphotypes.
  - To determine the exact composition of the cuticular hydrocarbons (CHCs) profile of *A. fraterculus* males from different morphotypes.
  - To identify the differences between CHCs composition depending on age and sex of flies within and between different populations.
  - To perform behavioural assays to identify the biological relevance of pheromone differences between populations.
- For the next 18 months:
  - Collection and establishment of *A. fraterculus* populations under laboratory conditions from two morphotypes (*A. sp 1 aff. fraterculus* and *A. sp 2 aff. fraterculus*).
  - Collection and characterisation of volatiles from calling males of the two morphotypes.
  - Extraction and characterisation of cuticular hydrocarbons from males and females of the two morphotypes.
  - To perform Gas chromatography-Electroantennography (GC-EAG) analyses of male extracts.
Blanka Kalinova
- 5 year plan:
  • To determine the exact composition of the cuticular hydrocarbons (CHCs) of *A. fraterculus* males from different morphotypes.
  • Study the CHCs and cuticular esthers differences between males and females to improve our understanding of pre-zygotic reproduction isolation in different populations of these species.
- For the next 18 months:
  • Extraction and characterisation of cuticular hydrocarbons from males and females of the already established colonies at Seibersdorf and some of its hybrids.

Teresa Vera / Diego Segura / Solana Abraham / Juan Rull
- 5 year plan:
  • To characterize the male sexual pheromone profile of each morphotype.
- For the next 18 months:
  • Male sexual pheromone will be collected from colonies established at Seibersdorf, Austria.

Group 2: *Bactrocera dorsalis*

Background Situation Analysis

Across Asia and the Pacific the fruit fly subfamily Dacinae contains some 48 recognised pest species (Drew, pers. comm.). Of these 48 pest species, eight are currently recognized within the *Bactrocera dorsalis* complex with some being the worst of all pest species within the subfamily (Drew and Hancock, 1994). Known collectively as the Oriental fruit fly complex, they are also categorized as the fourth worst of all global insect pests. While it is difficult to put a precise value on the economic losses, these have been estimated by the Griffith University International Fruit Fly Centre in Australia to be approximately US$1 billion per annum for the Asian region. These losses 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.

Despite background research carried out on this particular group of species over the last two decades, particularly in Malaysia, Thailand, Vietnam, and Indonesia (Tan and Nishida, 1996, 1998; Muraji and Nakahara, 2002; Naeole and Haymer, 2003; Smith *et al*., 2003; Tan, 2003a; Zimowska and Handler, 2005). This background research has generated data on diagnostics, field surveillance including quarantine strategies, field pest control, and some export trade. However this background knowledge has also identified key gaps in resolving the *B. dorsalis* complex; there is no consensus on species limits of the major *Bactrocera dorsalis* complex pest species, particularly *B. dorsalis* s.s., *B. papayae*, *B. philippinensis*, *B. carambolae* and potentially *B. invadens* (Clarke *et al*., 2005; Drew *et al*., 2008; Wee and Tan, 2005; Ebina and Ohto, 2006). Failure to resolve this complex prevents further development towards SIT implementation in Area-Wide Integrated Pest Management (AW-IPM) programmes against these pest insects and limits international trade.
The Asian region has been a primary source of invasions of species of the \textit{B. dorsalis} complex into many other regions of the world, including Africa, Middle East, northern and southern Pacific Islands (including Papua New Guinea and Australia), Indian Ocean, and South America. Currently some of these introduced species are causing devastation in Africa, PNG, and eastern Pacific Islands. Background research into the taxonomy of the \textit{B. dorsalis} complex has been unable to provide definitive identification of some species (Clarke \textit{et al}., 2005). This confounds the collection of associated host plant records and geographic distributions. It is absolutely essential that the species be accurately identified in order to be able to apply AW-IPM field programmes that include an SIT component.

Because trade implications and response systems to detections and/or incursions are not the same for all species in the complex, "near-enough" identification is not, unfortunately, good-enough. Consequently Member States have difficulty overcoming the phytosanitary barriers to export trade that satisfy major importers such as Japan, USA, Australia, and New Zealand. Not least is the severe problem arising if one member of the complex is detected or becomes established in a Member State but is unable to be differentiated from others in the complex. In this case that State would then be forced to indicate that any and all members of the complex may in fact be present, which would result in trade embargoes.

In this component of the CRP, a comprehensive biological, morphological, chemooecological and molecular study on the pest species of the \textit{B. dorsalis} complex will be undertaken so as to:

(i) Resolve species limits by seeking a consensus result from different tests including:
   (a) Identifying the levels of inter- and intra-specific genetic (\textit{e.g.} cytology) and molecular variation between and within the species,
   (b) Carrying out morphological and morphometric analysis,
   (c) Carrying out biological tests including cross-mating and host use studies,
   (d) Pheromone analyses to compare component ratios across putative species
(ii) Examine congruence between data from above mentioned studies, (a) to (d) which may support taxonomic revision or retain existing species, and
(iii) Develop robust diagnostic tools for the above listed five species.

**Baseline Knowledge on Five Priority Species in the \textit{Bactrocera dorsalis} Complex**

**DNA**
- Current knowledge:
  - Mitochondrial DNA
    o COI ‘barcode’ gene, and other mtDNA markers, show no clear distinction between currently defined species but was considered that data from more populations may refine this technique for species differentiation
  - Nuclear DNA
    o ITS1+2 diagnostic for separating \textit{B. carambolae} from remaining four species
  - Microsatellites
Sequences from *B. dorsalis* s.s., *B. invadens*, *B. oleae* and *B. papayae* are available and may be useful for population genetic structure but not sure if they will work with the *B. dorsalis* complex for species separation.

- Nuclear coding gene data for individual species within the complex are also known but species differentiation has not been assessed.
- Exon primed intron crossing from *actin* gene – for *B. papayae* and *B. carambolae* and *B. dorsalis* s.s. showed distinction but without population level analyses.
- It was noted that not attaining markers may indicate that markers suitable for the purpose of this CRP have not been discovered. But how many negative tests do we conduct before giving up?
- The inferences for all the above are based on standard phylogenetic and phylogeographic analyses which may be a limitation to accurate interpretation.

**Gaps identified:**
- Adequate and consistent sample coverage for the five target species

### CYTOLOGY

- **Current knowledge:**
  - Baimai studies on mitotic karyotypes identified several forms within the *B. dorsalis* complex but did not distinguish between putative species. This needs further testing.
  - Polytene maps are available for *B. dorsalis* s.s.
  - It is possible that polytene mapping for these species could be linked with genomic data.
- **Gaps identified:**
  - Tests not done on distinguishing between putative species
  - No polytene maps for species other than *B. dorsalis* s.s.

### ALLOZYMES

- **Current knowledge:**
  - Not applicable as a means to discriminate between species as it is too dependant on climatic factors
  - Thai team tried allozymes several years ago but data are not published
- **Gaps identified:**
  - Considered not a useful technique for this CRP

### GENOMICS

- **Current knowledge:**
  - *piggyBac* and other mobile elements (in particular the *Hopper* element) have been documented to be in some *B. dorsalis* species populations and may be used for species differentiation. Noted as difficult to apply for defining species limits
  - We have unpublished Hawaiian *B. dorsalis* genome
  - Also an unpublished *C. capitata* genome which could be used as a reference
  - Transcriptomics – under way for *B. dorsalis* s.s., *B. philippinensis* and *B. carambolae*. There are potentially some markers for these species within that data set
- Gaps identified:
  - Current unavailability of the genome information

MORPHOLOGY
- Current knowledge:
  - A revision of the SE Asian Dacini is under way but is not expected to impact on the four nominated SEA pest species in this CRP (Drew, pers. comm.)
- Gaps identified:
  - Egg and immature morphology – not tested for *B. dorsalis* complex species
  - Explicit intra-specific population-level variation in both external and internal morphological characters
  - and relative environmental/genetic influences on morphological phenotype

MORPHOMETRICS
- Current knowledge:
  - A relatively large number of papers on morphometrics for *B. dorsalis* complex species exists. In some cases resolution occurs but not always. Often also with overlap between morphometric traits and often impossible to determine what might be within and between population variation
  - Geometric morphometrics (shape analysis) shows some promise but needs further research
  - Morphometric approaches have rarely been linked adequately with other morphological or genetic approaches
- Gaps identified:
  - Explicit intra-specific population-level variation in both external and internal morphological characters
  - and relative environmental/genetic influences on morphological phenotype

MALE LURE RESPONSE (PARAPHEROMONES)
- Current knowledge:
  - Each species nominated for this CRP respond to methyl eugenol (ME)
  - However some species are more sensitive to ME at low concentrations e.g. *B. dorsalis* is more sensitive to low concentrations of ME than *B. carambola* (Wee et al., 2002)
  - The metabolites derived from being fed ME can be used to discriminate *B. carambola*. To be further tested in *B. philippinensis*.
- Gaps identified:
  - Lack of specific or detailed knowledge of the response of *B. philippinensis* and *B. invadens*.

DEVELOPMENTAL PHYSIOLOGY
- Current knowledge:
  - There are differences between species e.g. in attaining sexual maturity and in age-related development of response to parapheromones – e.g. work done at Seibersdorf and by Dr Tan on *B. carambola* (Wee & Tan 2001)
  - There are differences in ovariole numbers between some *Bactrocera* species but has not been tested on *B. dorsalis* complex species
- Gaps identified:
  - Baseline developmental data for each of the species, *e.g.* thermal thresholds and day-degree requirements
  - It is noted that there is a huge gap in knowledge on the application of this technique to species discrimination
  - Unknown how this knowledge can be used on its own for species discrimination

**BEHAVIOUR**
- Current knowledge: Host preference and performance: individual knowledge for individual species from isolated studies
- Mating compatibility: individual knowledge for individual species crosses from isolated studies. Evidence for mating compatibility between a mix of wild/long-term cultures: *B. papayae/B. carambolae; B. dorsalis/B. invadens; B. dorsalis/B. carambolae; B. philippinensis/B. dorsalis*

- Gaps identified:
  - Host preference and performance: comparative knowledge across species
  - Mating compatibility: comparative knowledge across species acquired under equivalent/simultaneous conditions

**PEROMONE COMPONENTS, CONCENTRATIONS**
- Current knowledge:
  - Pheromone components and their concentrations are exactly the same in laboratory cultures of each of 3 complex species: *B. dorsalis s.s.* , *B. papayae* and *B. invadens.*

- Gaps identified:
  - Wild populations have not been tested to confirm identical pheromone systems between some species.

**HOST RANGE**
- Current knowledge:
  - Well documented for most species being tested in this CRP but not yet fully for *B. philippinensis & B. invadens*
  - Host range data are constantly being updated in all regions

- Gaps identified:
  - Host range data not fully documented for *B. philippinensis & B. invadens.*

**DISTRIBUTION**
- Current knowledge:
  - Original species descriptions relied heavily on distribution data. This has proven to be an unreliable character
  - It is used for *B. philippinensis*, also lists state that, for example, *B. dorsalis* is restricted to northern Thailand and *B. papayae* to southern Thailand and new data from Seibersdorf suggests this is not correct
  - Distributions may change over time
  - New information obtained from this CRP may contribute to revised distribution lists
- Gaps identified:
  - Detailed fruit trade and movement between and among countries inadequately correlated with potential for unintentional fly movement and distribution

PROTEOMICS
- Current knowledge:
  - It was considered that proteomics may be a good approach to use for species discrimination
  - Potentially useful if both proteomics and genomics are combined
  - There are some proteins that may be species specific in Tephritidae (e.g. proteins in the male accessory gland
- Gaps identified:
  - No proteomic descriptions exist for B. dorsalis complex species except in studies on proteins used as carriers for pheromone components
  - We have no knowledge of this technique being used for species discrimination
  - No potential species specific proteins identified for target species

METABOLOMICS
- Current knowledge:
  - None
- Gaps identified:
  - Nothing has been done on Tephritids but may have potential for species discrimination

Specific Project Plans: Bactrocera dorsalis complex

TAXONOMY AND MORPHOLOGY
Qinge Ji
- 5 year plan:
  - Establish colonies of B. dorsalis complex from different regions for other work (e.g. behavioural) with other members of the CRP as appropriate – Finally confirm the species of B. dorsalis complex in mainland China, based on morphological characters or with supporting evidence where available (e.g. corresponding pheromone, molecular or behavioural information)
- For the next 18 months:
  - Survey the populations of B. dorsalis complex in different regions in mainland China
  - Collecting specimens of B. dorsalis complex by ME trapping and collecting damaged hosts. Specimens dry pinned for morphological screening with corresponding material alcohol preserved for future genetic screening
  - Describe and illustrate the morphological variations if they occur in collaboration with R.A.I. Drew

R.A.I Drew
- 5 year plan:
  - Extensive morphometric analyses of aedeagus (and aculeus where available) measurements of five target species from specimens already housed in existing
collections in Brisbane, in addition to material obtained through the CRP. This data will be correlated with geographic origin of samples.
- Review existing host fruit database from fruit fly surveys across SE Asia
- For the next 18 months:
  - Collaborate with Qinge Ji on morphological work from mainland China and prepare illustrations
  - Provide diagnosis, under current taxonomic species definitions, on fly cultures established for behavioural experiments as undertaken by other members of the CRP: Seibersdorf, Thailand, and Malaysia research groups. *N.b.* minimum of 30 whole specimens of both sexes dry pinned required with 2-3 week turnaround on identification.

**Tony Clarke, Mark Schutze**
- 5 year plan:
  - Morphological and morphometric analyses of adults from all five target species in the *Bactrocera dorsalis* complex
  - Morphological and morphometric analyses of juvenile stages
- For the next 18 months:
  - Continued development of geometric morphometrics as a potential discriminatory and diagnostic tool
  - Collection of material for dispersal to molecular workers in CRP
  - Collaboration with R.A.I. Drew on morphometric data collation and analyses

**Sunday Ekesi**
- 5 year plan:
  - Collect and share biological material of *B. invadens* with other CRP participants as required
  - Conduct morphometric analyses involving African, Sri Lankan, and Indian populations of *B. invadens* in relation to populations of the *B. dorsalis* complex;
- For the next 18 months:
  - Obtain collections of different populations of *B. invadens* from Africa, India and Brazil for morphometric analysis and sharing with workers in CRP.
  - Conduct morphometric analyses involving African, Sri Lankan, and Indian populations of *B. invadens*.

**BEHAVIOUR**
**Tony Clarke/Mark Schutze**
- 5 year plan:
  - Specimens from each established culture confirmed by R.A.I. Drew.
  - Post-zygotic compatibility measured to egg-hatch of F1 from crosses
  - Where possible, characterise additional elements of the mating systems of the target species (*e.g.* acoustic communication, pheromone capture)
- For the next 18 months:
  - Carry out inter-specific cross-mating compatibility between at least 4 species (*B. papayae* (Serdang*, Malaysia), *B. dorsalis* s.s. (Saraburi / Nakhon Sri Thammarat, Thailand), *B. philippinensis* (Guimaras Is., Philippines), and *B. carambolae* (Paramaribo, Suriname)). Collect data as per FAO/IAEA protocols (*e.g.* compatibility indices).
• Possible inclusion of Todd Shelly in carrying out remaining cross-mating trials at Seibersdorf.
• Possible R&D for Todd Shelly on studies on ±ME effects in cross-mating trials.
• Collaboration with Norman Barr in genetic studies.

Suksom Chinvinijkul
- 5 year plan:
  • Include *B. carambola* as per other species.
  • Specimens from each established culture confirmed by R.A.I. Drew.
  • Post-zygotic compatibility measured from egg-hatch of F1 to pupation and sex-ratios from crosses.
- For the next 18 months:
  • Confirm that *B. dorsalis s.s.* is represented by one species in Thailand, by carrying out intra-specific cross-mating compatibility among six geographic populations of *B. dorsalis s.s.* Populations sourced from different hosts.
  • Confirm that *B. papayae* is represented by one species in Thailand, by carrying out intra-specific cross-mating compatibility among three geographic populations of *B. papayae*. Populations sourced from different hosts.
  • Based on objectives 1+2, carry out inter-specific cross-mating compatibility tests between *B. dorsalis s.s.* and *B. papayae*.

K.H. Tan, S.L. Wee, A. Hee
- 5 year plan:
  • Intra- and interspecific mating compatibility studies between *B. dorsalis s.s.* (Thailand; population selected based on Thai study – see above), *B. papayae* (Malaysia), *B. carambola* (Malaysia), and possibly: *B. invadens* and *B. philippinensis*.
  • Post-zygotic compatibility measured to F3 generation: female fertility; pupation; adult sex-ratios.
  • Specimens from mating studies kept for other work (*e.g.* morphometrics and molecular)
  • Molecular analysis outsourced to Thailand group
- For the next 18 months:
  • Intra- and interspecific mating compatibility studies between *B. papayae* (Malaysia), *B. carambola* (Malaysia) and post-zygotic compatibility, Establishment of wild/wildish fruit fly culture of *B. carambola* and *B. papayae*. Specimens from each established culture confirmed by R.A.I. Drew.

Sunday Ekesi
- 5 year plan:
  • Investigate whether heritable life history differences exist between African and Asian populations of *B. invadens*.
- For the next 18 months:
  • Collection and establishment of colonies of African and Asian population.
CHEMICAL ECOLOGY
Suksom Chinvintjikul
- 5 year plan:
  - Rectal-gland pheromone collection and analysis for *B. dorsalis* s.s. (from all six regions) and *B. papayae* (from all three regions) – from year 3 of CRP. Analysis outsourced to Malaysian group

K.H. Tan, S.L. Wee, A. Hee, Ritzuo Nishida (Malaysia)
- 5 year plan:
  - Rectal gland analysis on material collected and supplied by Thai group (see above)
  - Pheromone collection (pheromone gland (+/-ME fed) and headspace) and analysis for wild/wildish individuals of all five target species.
- For the next 18 months:
  - Pheromone collection (pheromone gland (+/-ME fed) and headspace) and analysis for wild/wildish individuals of *B. carambolae* and *B. papayae* Components and ratios measured.

MOLECULAR AND GENETIC
Karen Armstrong
- 5 year plan:
  - Undertake phylogenetic and other genetic analyses of the sequence data.
  - Based on the inferred species delineations from molecular and behavioural data, retrospective identifications will be sought from R.A.I. Drew.
  - Based on the inferred species delineations from molecular and behavioural data, molecular diagnostic markers will be developed.
- For the next 18 months:
  - Screening mtDNA and nDNA genes for geographic populations of at least the following four species: *B. dorsalis* s.s. (Thailand, Taiwan), *B. papayae* (Malaysia, Indonesia) *B. carambolae* (Suriname, Malaysia, Indonesia) and *B. philippinensis* (Philippines). Specimens provided by TC/MS through Australia

Anna Malacrida
- 5 year plan:
  - Microsatellite analysis of *B. dorsalis* s.s. (Thailand, China, and other SE Asian locations) and *B. invadens* (Sub-Saharan Africa and Sri Lanka)
  - For at least one species, potential EST library development for specific relevant tissues associated with behavioural or reproductive biology (from year 3).
- For the next 18 months:
  - Extension of microsatellite analysis to specimens provided by Karen Armstrong and Nidchaya Aketarawong

Antigone Zacharopoulou
- 5 year plan:
  - colonies in Seibersdorf - from the five target species (as listed previously).
  - Same for F1 progeny or subsequent generations if available.
  - Upon variability of the strains assess the need to analyse more fly strains at the chromosome level (beyond 18mths)
- Depending on the chromosomal maps obtained, diagnostic characters for each species will be developed accordingly.
- Given the opportunity, genomic information developed elsewhere (from within or outside from the CRP) will be mapped onto the chromosomes

- For the next 18 months:
  - Screening using mitotic polytene chromosomes from established
  - Chromosome distribution of the two elements: piggyBac and Hopper elements in the same materials from point 1 through in situ hybridisation.

**Nidchaya Aketarawong**

- 5 year plan:
  - Complete the genetic analysis of *B. dorsalis* species complex and examine congruence with other types of evidence and diagnostic characters.

- For the next 18 months:
  - Complete screening, start applying microsatellite DNA typing and carry out genetic analysis of *B. dorsalis* species population samples from the following theme:
    - *B. dorsalis* species complex colonies from Seibersdorf
    - Wild *B. dorsalis* species complex population samples across geographical range
    - Wild *B. dorsalis* species complex population samples from Malaysian Peninsula
  - Begin providing genotyping and genetic analyses for the other group sample
    - Behaviour: samples from Suksom *et al*.
    - Chemical ecology: samples from Suk Ling Wee
    - Taxonomy
  - Increase potential to develop and evaluate novel molecular markers, diagnostic tools

**Sunday Ekesi**

- 5 year plan:
  - Obtain samples of *B. invadens* populations from Africa, India and Sri Lanka including colonies already established and contribute to the development of a population genotype database (in collaboration with A. Malacrida).
  - Evaluate DNA variation using mitochondrial and nuclear gene sequences and compare populations for evidence of population structure and potentially distinct gene pools.
  - Collect and share biological material of *B. invadens* with other CRP participants as required.

- For the next 18 months:
  - Obtain collections of different populations of *B. invadens* from Africa, India and Brazil for DNA variation analysis, population genotype database and sharing with workers in CRP.

Screen populations for one mitochondrial (COI) and nuclear (period) gene.
Group 3: *Bactrocera cucurbitae & Ceratitis FAR Complex*

*Bactrocera cucurbitae*

**Background Situation Analysis**

*Bactrocera cucurbitae* is a major pest of cucurbit crops and 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 in poor village communities and restrictions to trade in some cucurbit crops.

Some populations have been identified in Africa, Indian Ocean and South East Asia that indicate that very closely related species may be present. This problem needs to be resolved before species-specific treatments such as the SIT can be effectively applied in all situations and regions. There are a number of single species that differ in host, morphology, etc. from region to region and *B. cucurbitae* may be one of these. Population genetic studies can resolve five major groups worldwide (with microsats): African mainland + Seychelles, Réunion+Mauritius, Central Asia, SE Asia, Hawaii (Virgilio *et al.*, 2010). However phylogeographic patterns could not be discerned (using mitochondrial and nuclear gene fragments (total of 2764 bp) Virgilio, unpubl.). Field cage compatibility tests were conducted between populations of Mauritius, Seychelles and genetic sexing strain of Hawaii. No incompatibility was found (Sookar *et al.*, in press). Further mating tests could help to resolve their species status.

**Baseline Knowledge on Bactrocera cucurbitae**

**DNA**
- Current knowledge:
  - We have general information on phylogeography/population genetics on a worldwide basis. We have in depth information on population genetics with regard to African mainland and Réunion.
- Gaps identified:
  - We need to have more data on host races.

**CYTOLOGY**
- Current knowledge:
  - Work has been carried on two populations (GSS Hawaii & Bangladesh wild type) regarding karyotyping and polytene maps (Zacharapoulou *et al.*, subm.)
- Gaps identified:
  - Need to have same information from other populations

**ALLOZYMES**
- Current knowledge:
  - Previous work done by Malacrida *et al.* (1996)
- Gaps identified:
  - No further studies planned; covered by DNA work
GENOMICS
- Current knowledge:
  • No studies conducted
- Gaps identified:
  • Malacrida will conduct genomic analysis

MORPHOLOGY
- Current knowledge:
  • *B. cucurbitae* can be morphologically differentiated from other species within the subgenus Zeugodacus.
- Gaps identified:
  • Check with Dick on possible confusion with other valid taxa

MORPHOMETRICS
- Current knowledge:
  • Unexplored so far.
- Gaps identified:
  • Could provide additional information for recognition of geographic or host races. Only preliminary work done at Seibersdorf for testing methodology.

MALE LURE RESPONSE
- Current knowledge:
  • Attraction by cue lure, not by methyl eugenol.
- Gaps identified:
  • No need for further study with regard to CRP

DEVELOPMENTAL PHYSIOLOGY
- Current knowledge:
  • Seem to lack information but unsure whether it is going to provide additional information that is useful for CRP objectives
- Gaps identified:

BEHAVIOR
- Current knowledge:
  • Good knowledge through studies in Hawaii and recently in Seibersdorf
- Gaps identified:
  • No need for further work with regard to CRP in the immediate future. Mating compatibility in future if need arises.

PHEROMONE COMPONENTS AND CONCENTRATIONS
- Current knowledge:
  • Pheromone analysis and profile done in GSS: related to age and affected by diet (Haq et al., in prep). Previous work done by Japanese researchers.
- Gaps identified:
  • No need for further work planned in the immediate future; perhaps at later stage to relate pheromones with host races
HOST RANGE
- Current knowledge:
  - Well documented as a whole. Work by Sookar on host preference. Host range specifically in Tanzania studied (Mwatawala et al. 2010)
- Gaps identified:
  - See comment under DNA: possibility of host races

DISTRIBUTION
- Current knowledge:
  - Well documented
- Gaps identified:
  - No need for further work with regard to CRP.

PROTEOMICS
- Current knowledge:
  - No study conducted.
- Gaps identified:
  - Not considered for this CRP

Cuticular hydrocarbons
- Current knowledge:
  - Not studied
- Gaps identified:

Specific Project Plans: Bactrocera cucurbitae

TAXONOMY
Marc de Meyer
- 5 year plan:
  - Provide morphometric diagnostic markers to differentiate geographic and/or host races
- For the next 18 months:
  - In collaboration with Fellow from Seibersdorf: conduct morphometric study on wing landmarks in adults from different geographic regions.

Sunday Ekesi
- 5 year plan:
  - Collect and share biological material of B. cucurbitae with other CRP participants as required.
- For the next 18 months:
  - Collection and establishment of colonies of B. cucurbitae.

BEHAVIOR
Hélène Delatte and Serge Quilici
- 5 year plan:
  - Define the host range of La Réunion populations through laboratory experiments (choice and non-choice on mango and Solanaceous fruits) including interspecific competition with other fruit flies
• If required (based on results from Hélène Delatte, Serge Quilici and Maulid Mwatawala): test mating incompatibility between geographic and/or host races at Seibersdorf

**Maulid Mwatawala**
- 5 year plan:
  • Host plant preferences, incidence and infestation rates of *B. cucurbitae* in Tanzania. Verification of host choice and the relationship with intraspecific variation.
  - For the next 18 months:
    • Larval and adult survey in different parts of Tanzania. Data on host preferences

**CHEMICAL ECOLOGY**
• Not considered within framework of CRP

**MOLECULAR/CYTOGENETICS**
**Antigine Zacharopoulou**
- 5 year plan:
  • Confirmation karyotype and polytene similarity among the different geographic populations
  - For the next 18 months:
    • Study additional populations from Mauritius/La Réunion

**Massimiliano Virgilio and Hélène Delatte**
- 5 year plan:
  • Analysis of the population structure according to host races in Tanzania and La Réunion
  - For the next 18 months:
    • Genotyping of samples obtained through larval surveys in Tanzania and La Réunion

**Anna Malacruda**
- 5 year plan:
  • To be decided after feedback from her
  - For the next 18 months:
    • To be decided after feedback from her

General remark 1: activities within this CRP are currently focusing on African mainland (i.e. Tanzania) and La Réunion. There is the need to involve other partners in order to supplement the data with records from the native range and/or regions where *B. cucurbitae* has been introduced. Such extension is desirable.

Participation from Australia, Thailand, Malaysia, China

General remark 2: should there be indication of existence of host races, based upon the activities listed above, the research could be extended into the fields of pheromone and cuticular hydrocarbons analyses.
**Ceratitis FAR Complex**

**Background Situation Analysis**

The Afro-tropical group of fruit flies, *Ceratitis rosa*, *C. fasciventris* and *C. anonae*, together with *C. capitata*, are considered to be among the major horticultural pests of that region (White & Elson-Harris 1992; De Meyer 2001a) and these species are of quarantine significance (EPPO/CABI 1997). They are highly polyphagous and damage a wide range of unrelated wild and cultivated crops (De Meyer *et al.* 2002a) resulting in enormous economic losses wherever they occur (Barnes 2000; Lux 2000; De Meyer 2001b).

*Ceratitis rosa*, *C. fasciventris* and *C. anonae* are considered the three members of the FAR species complex (Virgilio *et al.* 2007a, 2007b). From the taxonomic point of view, *C. fasciventris* was considered to be a variety of *C. rosa* (Bezzi 1920). Only recently has it been recognized as a different entity with species status (De Meyer 2001a). Unlike *C. capitata*, which has spread from its home range in East Africa and attained an almost world-wide distribution over the last century (Fletcher 1989; White & Elson-Harris 1992), *C. rosa*, *C. fasciventris* and *C. anonae* have so far not been reported outside the African continent (except La Réunion and Mauritius) but are potentially invasive. Due to the difficulty in distinguishing some members of the complex morphologically, a number of molecular approaches for species recognition were used (Balliraine *et al.*, 2004; Barr *et al.*, 2006; Virgilio *et al.*, 2008). However these remain inadequate for quarantine purposes and much more robust molecular markers are needed.

**Baseline Knowledge on the Ceratitis FAR Complex**

**DNA**
- Current knowledge:
  - Attempts to develop specific diagnostic markers were made but they are ineffective
- Gaps identified:
  - There is the need for further exploration, especially regarding microsats.

**CYTOLOGY**
- Current knowledge:
  - No data available.
- Gaps identified:
  - Has the potential to provide a diagnostic tool. Need for researcher to conduct this study.

**ALLOZYMES**
- Current knowledge:
  - Previous work conducted by Malacrida *et al.* (1996) only on *C. rosa* (perhaps also including *C. fasciventris*)
- Gaps identified:
  - No further work considered, covered by DNA work

**GENOMICS**
- Current knowledge:
  - Current focus on *C. capitata*
- Gaps identified:
  - No further work considered, covered by DNA work

**MORPHOLOGY**
- Current knowledge:
  - Adults can be distinguished to some extent.
- Gaps identified:
  - Separation of females difficult. Larval morphology not studied.

**MORPHOMETRICS**
- Current knowledge:
  - No data available
- Gaps identified:
  - Morphometric study on females might have potential for separation of females

**MALE LURE RESPONSE**
- Current knowledge:
  - All attracted to trimedlure
- Gaps identified:
  - No need for further study with regard to the CRP

**DEVELOPMENTAL PHYSIOLOGY**
- Current knowledge:
  - Has not been done for diagnostic purposes

**BEHAVIOR**
- Current knowledge:
  - Not studied, except for some studies on *C. rosa.*
- Gaps identified:
  - Mating compatibility studies can contribute to resolving the specific status of the taxa within the complex

**PHEROMONE COMPONENTS**
- Current knowledge:
  - Not studied.
- Gaps identified:
  - Pheromone component studies can contribute to resolving the specific status of the taxa within the complex
HOST RANGE
- Current knowledge:
  • Host range is well known for representatives of the complex
- Gaps identified:
  • No need for further study with regard to the CRP

DISTRIBUTION
- Current knowledge:
  • Distribution patterns are well known for representatives of the complex
- Gaps identified:
  • No need for further study with regard to the CRP

PROTEOMICS
- Current knowledge:
  • Not studied.
- Gaps identified:
  • Not applicable for this CRP.

CUTICULAR HYDROCARBONS
- Current knowledge:
  • Not studied.
- Gaps identified:
  • Cuticular hydrocarbon studies can contribute to resolving the specific status of the taxa within the complex

Specific Project Plans: Ceratitis FAR Complex

TAXONOMY
Marc de Meyer
- 5 year plan:
  • Provide diagnostic markers to differentiate adults of both sexes for species within the complex (except below).
  • In collaboration with Fellow from Seibersdorf laboratory: provide morphometric diagnostic markers to differentiate females within the complex
- For the next 18 months:
  • Marc: Study additional morphological characters that can be used to differentiate adults
  • Conduct morphometric study on wing landmarks in adult females

Sunday Ekesi
- 5 year plan:
  • Collect and share biological material of the FAR group with other CRP participants as required.
- For the next 18 months:
  • Collection and establishment of colonies of the FAR group.
Gary Steck
- 5 year plan:
  • To provide indication whether morphological characters of immature stages (larvae) provide diagnostic character for differentiation
- For the next 18 months:
  • Commence assessment of larval morphology as a diagnostic tool for differentiation

BEHAVIOR
Marc de Meyer
- 5 year plan:
  • If necessary: to provide additional information on the specific status in case of conflicting data from other work packages using mating compatibility tests.
- For the next 18 months:
  • No activities planned in the first 18 months

CHEMICAL ECOLOGY
Lucie Vanickova
- 5 year plan:
  • To improve the efficiency of new and sustainable control methods by means of use the sex pheromone and cuticular hydrocarbon profile of Ceratitis spp. of the FAR complex
- For the next 18 months:
  • To identify the differences between cuticular hydrocarbons (CHCs) composition depending on age of flies and different composition of CHCs between males and females within and between different populations using mass-spectrometry techniques Lucie: pheromone: sexually mature male specimens of each species within the FAR complex to collect the pheromone volatiles

MOLECULAR
Hélène Delatte and Massimiliano Virgilio (and possibly Anna Malacrida – after discussion)
- 5 year plan:
  • Provide discriminant markers that can be used as a diagnostic tool to differentiate the three species of the complex
- For the next 18 months:
  • Development of microsat bank; screening markers to differentiate three species
## Project Linkages

Table 2. Project linkages

<table>
<thead>
<tr>
<th>Participant</th>
<th>Country</th>
<th>DNA</th>
<th>Cytology</th>
<th>Genomics</th>
<th>Taxonomy, Morphology/metrics</th>
<th>Behaviour (pre, post zygotic)</th>
<th>Pheromones, chemical ecology</th>
<th>Host range</th>
<th>Distribution</th>
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<tbody>
<tr>
<td>Aketarawong</td>
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</table>

- **Bactrocera cucurbitae**
- **Ceratitis FAR complex**
- **Anastrepha fraterculus**
- **Bactrocera dorsalis complex**
### F. Logical Framework

Table 3.

<table>
<thead>
<tr>
<th>NARRATIVE SUMMARY</th>
<th>OBJECTIVE VERIFIABLE INDICATORS</th>
<th>MEANS OF VERIFICATION</th>
<th>IMPORTANT ASSUMPTIONS</th>
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</thead>
<tbody>
<tr>
<td>OVERALL OBJECTIVE</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>To assist Member States in achieving sustainable fruit and vegetable production and in facilitating international trade and the area-wide integrated application of the SIT as part of suppression/eradication programmes against fruit flies of economic importance in Africa, Asia &amp; Pacific, and Latin America</td>
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<td>Fruit and vegetable production in Member States continue to suffer major losses due to endemic and introduced fruit fly pests</td>
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<td>International trade in fruit and vegetable commodities continue to increase and be disrupted by fruit fly pests, requiring expensive pre- and post-harvest treatments and quarantine measures</td>
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<td>International trade continues to suffer from unnecessary restrictions due to confusion about the biological identity, geographic distribution and lack of diagnostic tools for pest fruit fly species</td>
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<td>In contrast to pesticide applications, a more environmentally friendly pest fruit fly control requires precise applications based on the knowledge of the biology of species</td>
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<td>An area-wide integrated pest management</td>
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<td>NARRATIVE SUMMARY</td>
<td>OBJECTIVE VERIFIABLE INDICATORS</td>
<td>MEANS OF VERIFICATION</td>
<td>IMPORTANT ASSUMPTIONS</td>
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<tr>
<td>SPECIFIC RESEARCH OBJECTIVE</td>
<td>Identify and define the biological limits of closely related pest species within the South American fruit fly (<em>A. fraterculus</em>) complex. Facilitate the taxonomic revision and develop corresponding diagnostic tools to distinguish these species</td>
<td>Basic and applied research that defines biological species limits of species within the <em>A. fraterculus</em> complex and develops tools to distinguish them so as to facilitate trade and SIT implementation as part of AW-IPM</td>
<td>Sampling protocols, diagnostic tools, species descriptions and geographic distributions, trapping and ecological information, presented in reports and publications</td>
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<tr>
<td></td>
<td>Identify and redefine the biological limits and species status of closely related pest species within the Oriental fruit fly (<em>B. dorsalis</em>) complex. Develop corresponding diagnostic tools to distinguish these species</td>
<td>Basic and applied research that defines biological species limits within the <em>B. dorsalis</em> complex and develops tools to distinguish them so as to facilitate trade and SIT implementation as part of AW-</td>
<td>Sampling protocols, diagnostic tools, species descriptions and geographic distributions, trapping and ecological information, presented in reports and publications</td>
</tr>
</tbody>
</table>

approach to fruit fly pests, including where appropriate SIT as the non-polluting suppression/eradication component, will increasingly be a cost-effective and sustainable method to deal with fruit fly pests.
<table>
<thead>
<tr>
<th>NARRATIVE SUMMARY</th>
<th>OBJECTIVE VERIFIABLE INDICATORS</th>
<th>MEANS OF VERIFICATION</th>
<th>IMPORTANT ASSUMPTIONS</th>
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</thead>
<tbody>
<tr>
<td>Determine the variability within the melon fly (<em>B. cucurbitae</em>) regarding geographic and/or host races among populations from Africa and Asia &amp; Pacific</td>
<td>Mating compatibility, morphological and genetic variability assessed so as to allow efficient application of the SIT to <em>B. cucurbitae</em> in these regions</td>
<td>Sampling protocols, diagnostic tools, geographic distributions and ecological information, presented in reports and publications</td>
<td>There are concerns that there may be intra-specific differences which would interfere with efficient SIT applications and trade</td>
</tr>
<tr>
<td>Develop more robust species markers for the identification of the African pest species complex: <em>C. rosa</em>, <em>C. fasciventris</em> and <em>C. anonae</em></td>
<td>New robust species markers developed and validated so as to facilitate trade and protection of agriculture and biological diversity through improved quarantine services</td>
<td>Diagnostic tools, reports and publications</td>
<td>Existing diagnostic tools are not suitably robust for quarantine use</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OUTPUTS</th>
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<tbody>
<tr>
<td><em>A. fraterculus</em></td>
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<tr>
<td>Establish a network for targeted, structured sampling of the complex together with collection of ecological data. Develop protocol for collecting, handling and preserving, transport, recording, etc of specimens.</td>
<td>Protocols developed and network established for structured collecting, handling and preserving, transport, recording, vouchering of specimens.</td>
<td>Protocol developed and available to investigators.</td>
<td>Established protocols can be adopted for use in collectionsInvestigators apply protocols.</td>
</tr>
<tr>
<td>NARRATIVE SUMMARY</td>
<td>OBJECTIVE VERIFIABLE INDICATORS</td>
<td>MEANS OF VERIFICATION</td>
<td>IMPORTANT ASSUMPTIONS</td>
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<tr>
<td>Establish colonies of all putative species to allow behavioural, chemical, cytological, larval morphology, etc studies. Carry out reproductive compatibility analysis as required between selected populations of named species – under semi-natural, choice situations. Record behavioural aspects. Cross-matings and behaviour studies carried out between putative species of the <em>A. fraterculus</em> complex under semi-natural, choice situations using wild / wildish flies.</td>
<td>Colonies of the seven morphotypes established and available for studies that required live material. Cross-matings and behaviour studies carried out between putative species of the <em>A. fraterculus</em> complex under semi-natural, choice situations. Post-zygotic studies carried out when necessary to confirm/evaluate presence of reproductive isolation.</td>
<td>Reports of behaviour and mating compatibility tests. International peer reviewed publications</td>
<td>The procedures to establish species limits are scientifically acceptable.</td>
</tr>
<tr>
<td>Perform genetic, cytological morphological and other relevant biological and chemical studies to help characterize/discriminate among morphotypes.</td>
<td>Sex pheromone and cuticular hydrocarbons profiles determined for each morphotypes. Cytological studies performed and karyotypes described for</td>
<td>Reports of pheromones studies and analyses. Karyotype and polytene chromosome data published.</td>
<td>The samples are representative of the true population and are processed according to the established protocol. The samples are representative of the true population and are processed according to the established protocol.</td>
</tr>
<tr>
<td>NARRATIVE SUMMARY</td>
<td>OBJECTIVE VERIFIABLE INDICATORS</td>
<td>MEANS OF VERIFICATION</td>
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<tr>
<td></td>
<td>each morphotypes.</td>
<td>Reports and published genetic analyses.</td>
<td>New gene regions can be accessed and used to screen populations</td>
</tr>
<tr>
<td></td>
<td>Molecular genetic analyses carried out on material collected from across the geographical range of <em>A. fraterculus s. l.</em></td>
<td>Reports and published morphological studies.</td>
<td>Sufficient representative samples available to conduct appropriate analysis.</td>
</tr>
<tr>
<td></td>
<td>Morphological studies carried out on material collected from across the geographical range of <em>A. fraterculus s. l.</em></td>
<td>Reported protocols made publicly available</td>
<td>The samples are representative of the true population and that they are processed according to the established protocol.</td>
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<tr>
<td></td>
<td>Other potentially discriminating biological attributes investigated for all morphotypes.</td>
<td>Published data on biology of flies with particular reference to assisting discrimination between populations/species.</td>
<td>Experimental staff capable of carrying out standardised behavioural protocols.</td>
</tr>
<tr>
<td>Describe and diagnose putative species by collating, analysing and interpreting new and existing data.</td>
<td>Putative species described and diagnosed based on the information collected using different approaches.</td>
<td>Published results on the taxonomy of the <em>A. fraterculus</em> complex focussing on pest species of economic significance.</td>
<td>Independent avenues of conducted research will suggest a consensus outcome.</td>
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<td>The previous research as part of CRP (mating, pheromones, behaviour) is completed and made available.</td>
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<tr>
<td>NARRATIVE SUMMARY</td>
<td>OBJECTIVE VERIFIABLE INDICATORS</td>
<td>MEANS OF VERIFICATION</td>
<td>IMPORTANT ASSUMPTIONS</td>
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<tr>
<td>Clarify the nomenclature for <em>A. fraterculus</em> s. l.</td>
<td>Holotypes examined. Synonyms resurrected as appropriate. If name changes are required the International Code of Zoological Nomenclature Commission will be petitioned. Consultant meeting of taxonomists held to review all evidence collected to name/rename species. Species named/renamed.</td>
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<tr>
<td>Develop improved diagnostic tools for each newly described/renamed species within the complex.</td>
<td>Diagnostic tools developed based on morphological, molecular and other evidence collected.</td>
<td>Published diagnostic protocols.</td>
<td>The previous objective (defining species limits in <em>A. fraterculus</em>) has been achieved. Having determined species limits, diagnostic markers can be found.</td>
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<tr>
<td><em>B. dorsalis</em></td>
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<tr>
<td>Quantify field cage mating behaviour of two or more geographically distinct <em>B. dorsalis</em> s.s. populations as a basis for subsequent comparative studies within the complex.</td>
<td>Within species variation in mating behaviour documented for <em>B. dorsalis</em> s.s.</td>
<td>Results published/reported.</td>
<td>Confirming that <em>B. dorsalis</em> s.s. is a single species allows the establishment of centralised rearing facilities. Individuals collected from targeted collection localities are <em>B. dorsalis</em> s.s. based on initial morphological identification.</td>
</tr>
<tr>
<td>NARRATIVE SUMMARY</td>
<td>OBJECTIVE VERIFIABLE INDICATORS</td>
<td>MEANS OF VERIFICATION</td>
<td>IMPORTANT ASSUMPTIONS</td>
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<tr>
<td>Carry out targeted sampling for species listed in 4.2.2. Establish a laboratory network for structured sampling (for rearing, morphology, pheromone and genetic work).</td>
<td>Protocols developed and network established for structured collecting, handling and preserving, transport, recording, etc of specimens</td>
<td>Published protocol checklist publicly available. Central repository of collection material curated and live cultures established and maintained.</td>
<td>Collaborators can be found to undertake collections.</td>
</tr>
<tr>
<td>Carry out cross-species matings under semi-natural, choice situations using wild / wildish flies as required. Record behavioural aspects as per QC Manual. Multiple generation crosses to record post zygotic characteristics will be carried out on at least two species.</td>
<td>Cross-matings and behaviour studies carried out between species of the <em>B. dorsalis</em> complex to compute and record mating isolation indices. Multiple generation crosses carried out between at least two species of the <em>B. dorsalis</em> complex which detail and record any post zygotic mating effects.</td>
<td>Published results on cross-mating studies</td>
<td>Flies can be maintained under wildish conditions over minimum generations to produce meaningful results. There is staff available to undertake experiments and maintain cultures. The timing between field collections, cultures, and available staff will coincide.</td>
</tr>
<tr>
<td>NARRATIVE SUMMARY</td>
<td>OBJECTIVE VERIFIABLE INDICATORS</td>
<td>MEANS OF VERIFICATION</td>
<td>IMPORTANT ASSUMPTIONS</td>
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<tr>
<td>Establish relative attraction of males of different species to methyl eugenol.</td>
<td>Relative taxon sensitivity to methyl eugenol determined</td>
<td>Published results on pheromone profiles</td>
<td>Suitable fly specimens and specialists (including specialist equipment) are available to analyse and compare information</td>
</tr>
<tr>
<td>Determine sex pheromone profiles for each species with and without prior methyl eugenol consumption.</td>
<td>Sex pheromone profiles determined for each species.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Investigate other potentially informative biological attributes (as listed in Table 1) for discriminating between species</td>
<td>Other potentially discriminating biological attributes investigated for species.</td>
<td>Reported protocols made publicly available. Published data on biology of flies with particular reference to assisting discrimination between populations/species.</td>
<td>Experimental staff capable of carrying out standardised behavioural protocols Quality of outcomes highly dependent on essential co-ordination between groups</td>
</tr>
<tr>
<td>Carry out genetic analyses on material collected from across the geographical range of pest species.</td>
<td>Measures of intra- and inter-species variation for three populations of each of at least <em>B. dorsalis</em>, <em>B. papayae</em> and <em>B. philippinensis</em>.</td>
<td>Published results of genetic analyses. Reports with specific statement on whether genetic data supports separate/single species for <em>B. dorsalis s.l.</em></td>
<td>New gene regions can be accessed and used to screen populations. Sufficient representative samples available to conduct appropriate analysis.</td>
</tr>
<tr>
<td>NARRATIVE SUMMARY</td>
<td>OBJECTIVE VERIFIABLE INDICATORS</td>
<td>MEANS OF VERIFICATION</td>
<td>IMPORTANT ASSUMPTIONS</td>
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</tr>
<tr>
<td>Species status for pest putative species newly defined based on outputs 1 to 6.</td>
<td>Species status defined for at least <em>B. dorsalis</em>, <em>B. papayae</em>, <em>B. philippinensis</em> and <em>B. invadens</em>.</td>
<td>Published results on the taxonomy of the <em>B. dorsalis</em> complex focusing on pest species of economic significance.</td>
<td>Independent avenues of conducted research will suggest a consensus outcome. The previous research as part of CRP (mating, pheromones, behaviour) is completed and made available.</td>
</tr>
</tbody>
</table>

Develop improved morphological and molecular diagnostic tools based on the acquired knowledge (for adults and immatures).  

At least one diagnostic tool developed for each species defined in output 7. | Diagnostic protocols published. | The previous objective (defining species limits in *B. dorsalis* s.l.) has been achieved. Having determined species limits, diagnostic markers can be found. |

**Bactrocera cucurbitae**

Develop protocol for collecting, handling, preserving, transport, recording, etc of specimens from Africa, Hawaii, Asia. Establish a network for targeted, structured sampling of species together with ecological data.  

Characterization of potential host races through ecological, morphometric and genetic studies. If there are positive differences then proceed to other studies. | Clarification on existence or non-existence of host races within *B. cucurbitae* among populations from Africa and Indian Ocean. | Published results on mating compatibility. | Flies can be built up in culture to sufficient numbers over minimum generations to produce meaningful results. There is staff available to undertake experiments and maintain cultures. The timing between field collections, cultures, and available staff will coincide. |
<table>
<thead>
<tr>
<th>NARRATIVE SUMMARY</th>
<th>OBJECTIVE VERIFIABLE INDICATORS</th>
<th>MEANS OF VERIFICATION</th>
<th>IMPORTANT ASSUMPTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carry out cross-matings as required between selected populations under semi-natural, choice situations using wild / wildish flies. Record behavioural aspects.</td>
<td>Mating compatibility assessments and behaviour studies carried out among populations from Africa, Indian Ocean, and Asia &amp; Pacific under semi-natural, choice situations using wild / wildish flies</td>
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<tr>
<td><strong>C. rosa, C. fasciventris and C. anonae.</strong></td>
<td></td>
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</tr>
<tr>
<td>Develop more robust morphological and molecular diagnostics to be able to distinguish each species.</td>
<td>Robust discriminating markers developed and made available for the three species.</td>
<td>Markers to analyse extensive field samples.</td>
<td>More robust markers can be identified and utilized.</td>
</tr>
<tr>
<td>Confirmation of specific status of taxa within the complex (e.g. molecular, pheromone, cuticular hydrocarbon, mating compatibility, etc.)</td>
<td>Additional support to the specific status of taxa within the FAR complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ACTIVITIES</strong></td>
<td><strong>Consultants Meeting.</strong></td>
<td><strong>CRP proposal.</strong></td>
<td><strong>CRP approved by committee Proposals submitted/accepted.</strong></td>
</tr>
<tr>
<td></td>
<td>Consultants meeting held 6-10 July 2009.</td>
<td></td>
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</tr>
<tr>
<td>NARRATIVE SUMMARY</td>
<td>OBJECTIVE VERIFIABLE INDICATORS</td>
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<td>IMPORTANT ASSUMPTIONS</td>
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<tr>
<td>Award research agreements and contracts to fruit fly researchers, taxonomists and fruit fly control programmes and establish CRP.</td>
<td>Research contracts agreements.</td>
<td>Signed agreements and contracts.</td>
<td>Agreements and contracts approved and funded.</td>
</tr>
<tr>
<td>Organise 1st RCM to plan, coordinate and review research activities.</td>
<td>1st RCM held in August 2010.</td>
<td>Working material of 1st RCM printed and distributed.</td>
<td>Consultants’ report refined by participants</td>
</tr>
<tr>
<td>Carry out R and D</td>
<td>R and D findings</td>
<td>Research reports.</td>
<td>Continued institutional support, including support for establishment of colonies</td>
</tr>
<tr>
<td>Hold 2nd RCM to begin analysis of collection data, draft technical protocols as required, and plan future activities.</td>
<td>2nd RCM held in late 2011 / early 2012.</td>
<td>Working material printed and distributed for 2nd RCM, research published in scientific literature and disseminated to Member States and Scientific community.</td>
<td>Analytical tools appropriate and verifiable.</td>
</tr>
<tr>
<td>Continue R and D.</td>
<td>R and D findings.</td>
<td>Research reports.</td>
<td>Continued institutional support.</td>
</tr>
<tr>
<td>Hold 3rd RCM to continue analysis of collections and colony material and to plan future activities.</td>
<td>3rd RCM held in June 2013.</td>
<td>Working material printed and distributed for 3rd RCM, research published in scientific literature and Reports published and distributed following each RCM.</td>
<td>Mid-year review of CRP approved.</td>
</tr>
<tr>
<td>NARRATIVE SUMMARY</td>
<td>OBJECTIVE VERIFIABLE INDICATORS</td>
<td>MEANS OF VERIFICATION</td>
<td>IMPORTANT ASSUMPTIONS</td>
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<tr>
<td>Carry out final R and D.</td>
<td>R and D findings.</td>
<td>Research reports.</td>
<td>Continued institutional support.</td>
</tr>
<tr>
<td>Hold Consultants Meeting of taxonomists to review all evidence collected to name/renamed species.</td>
<td>Species named/renamed.</td>
<td>Consultants Meeting report.</td>
<td>Funding and participation of relevant taxonomists.</td>
</tr>
<tr>
<td>Hold final RCM to review data, reach consensus and prepare final report.</td>
<td>Final RCM held in January 2015</td>
<td>Final CRP report distributed and research published in scientific literature</td>
<td>Funding available to complete CRP and to publish proceedings.</td>
</tr>
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</table>
G. Appendices
<table>
<thead>
<tr>
<th>Time</th>
<th>Session/Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>08:00 - 08:45</td>
<td>Identification and registration at VIC Gate</td>
</tr>
<tr>
<td>08:45 - 09:00</td>
<td><strong>Jorge HENDRICHIS</strong>: Opening and welcome statements.</td>
</tr>
<tr>
<td>09:00 - 09:30</td>
<td><strong>Andrew JESSUP</strong>: Overview and status of CRP, goals of the meeting, agenda, administrative and other issues.</td>
</tr>
<tr>
<td>09:30 - 10:00</td>
<td><strong>Marc de MEYER</strong>: Current research on molecular and morphological recognition of African fruit fly pests and their congeners</td>
</tr>
<tr>
<td>10:00 - 10:30</td>
<td><strong>Antigone ZACHAROPOULOU</strong>: Cytogenetic studies of the oriental fruit fly, <em>Bactrocera dorsalis</em> s.s. (Hendel) and the melon fly, <em>Bactrocera cucurbitae</em> (Coquillett): A comparative analysis</td>
</tr>
<tr>
<td>10:30 – 11:00</td>
<td>COFFEE BREAK</td>
</tr>
<tr>
<td>11:00 - 11:30</td>
<td><strong>Giuliano GASPERI</strong>: Development and use of population genotype databases for species resolution within <em>Bactrocera dorsalis</em> and <em>Anastrepha fraterculus</em> complexes</td>
</tr>
<tr>
<td>11:30 - 12:00</td>
<td><strong>Janisete G. SILVA</strong>: Use of MtDNA and nuclear markers to delimit and diagnose Brazilian species within the <em>Anastrepha fraterculus</em> complex</td>
</tr>
<tr>
<td>12:00 - 12:30</td>
<td><strong>Hélène DELATTE</strong>: Population genetics of two fruit flies damaging cucurbits in La Réunion: <em>Bactrocera cucurbitae</em> and <em>Dacus ciliatus</em> and future prospects</td>
</tr>
<tr>
<td>12:30 - 13:00</td>
<td><strong>Nidchaya AKETARAWONG</strong>: Screening microsatellite DNA markers for population genetic study of <em>Bactrocera dorsalis</em> species complex</td>
</tr>
<tr>
<td>13:00 - 14:00</td>
<td>LUNCH</td>
</tr>
<tr>
<td>14:00 - 14:30</td>
<td><strong>Karen ARMSTRONG</strong>: Molecular genetic resolution of pest species within the <em>Bactrocera dorsalis</em> complex</td>
</tr>
<tr>
<td>15:00 - 15:30</td>
<td><strong>Lucie VANÍČKOVÁ</strong>: Analysis of epicuticular composition in genera <em>Anastrepha</em></td>
</tr>
<tr>
<td>15:30 - 16:00</td>
<td><strong>Nelson CANAL</strong>: Cryptic species of <em>Anastrepha fraterculus</em> and <em>A. obliqua</em> in Colombia: Methodological purpose</td>
</tr>
<tr>
<td>16:00 – 16:30</td>
<td>COFFEE BREAK</td>
</tr>
</tbody>
</table>
16:30 - 17:00  **Suk Ling WEE**  
Male rectal gland volatile constituents of five economically important cryptic species within the Oriental fruit fly, *Bactrocera dorsalis* complex (Diptera: Tephritidae)

17:00 - 17:30  **Ruth do NASCIMENTO**  
Pheromone analysis of *Anastrepha fraterculus*

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**TUESDAY, 3 AUGUST 2010**

**SESSION III : BEHAVIORAL** - Chairperson: Gary STECK

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>08:30</td>
<td>Iara. S. JOACHIM-BRAVO</td>
<td>Analysis of ecological speciation among <em>Anastrepha fraterculus</em> populations from Brazil through behavioral and demographic parameters</td>
</tr>
<tr>
<td>09:00</td>
<td>Keng Hong TAN</td>
<td>Mating compatibility of economically important cryptic species in the <em>Bactrocera dorsalis</em> complex (Diptera: Tephritidae)</td>
</tr>
<tr>
<td>09:30</td>
<td>Teresa VERA</td>
<td>Reproductive compatibility: What did <em>Anastrepha fraterculus</em> tell us so far?</td>
</tr>
<tr>
<td>10:00</td>
<td>Qinge JI</td>
<td>Overview of the study on <em>Bactrocera dorsalis</em> s.s in China</td>
</tr>
</tbody>
</table>

**SESSION IV : TAXONOMIC** - Chairperson: Antigone ZACHAROPOULOU

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
<th>Title</th>
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</thead>
<tbody>
<tr>
<td>11:00</td>
<td>Maulid MWATAWALA</td>
<td>Host utilization and genetic divergence among populations of <em>Bactrocera invadens</em> Drew, Tsuruta and White and <em>Bactrocera cucurbitae</em> (Coquillett) in Tanzania</td>
</tr>
<tr>
<td>11:30</td>
<td>Gary STECK</td>
<td>A multi-faceted approach to resolving the South American fruit fly complex</td>
</tr>
<tr>
<td>12:00</td>
<td>Dick DREW</td>
<td>The <em>Bactrocera dorsalis</em> complex</td>
</tr>
<tr>
<td>12:30</td>
<td>Tony CLARKE</td>
<td>Current work in defining species limits in the <em>Bactrocera dorsalis</em> complex</td>
</tr>
</tbody>
</table>

**13:00 – 14:00 LUNCH**

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
<th>Title</th>
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</thead>
<tbody>
<tr>
<td>14:00</td>
<td>Suksom CHINVINIJKUL</td>
<td>Thailand Resolution of <em>Bactrocera dorsalis</em> Complex</td>
</tr>
<tr>
<td>14:30</td>
<td>Vicente HERNÁNDEZ-ORTIZ</td>
<td>Morphology, distribution and taxonomic characterization of the species complex <em>Anastrepha fraterculus</em> from Mesoamerica and Northwestern of South America</td>
</tr>
<tr>
<td>15:00</td>
<td>Sunday EKESI (UNABLE TO ATTEND)</td>
<td>Morphometric analysis, genetic characterization and mating compatibility studies among populations of <em>Bactrocera invadens</em> from different origins</td>
</tr>
</tbody>
</table>

**15:30 – 16:00 COFFEE BREAK**

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
<th>Title</th>
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<tbody>
<tr>
<td>16:00</td>
<td>Protocol for Collection and Shipment of Live and Dead Insects for rearing, morphology, biochemical and molecular assays.</td>
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<tr>
<td>17:30</td>
<td>Discussion and Review of Procedures for Field Cage Tests</td>
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</table>
### WEDNESDAY, 4 AUGUST 2010

**SESSION V - Chairperson: Andrew JESSUP**

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
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<tbody>
<tr>
<td>08:30 - 10:00</td>
<td><strong>Working Groups</strong>: Establishment of Working Groups. Review of all potential techniques, techniques not being addressed by CRP participants and possibilities of coordinated research. Drafting of scientific/technical conclusions and recommendations for the different genera and techniques. Review of research needs that should be addressed.</td>
</tr>
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<tr>
<th>Time</th>
<th>Event</th>
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<tbody>
<tr>
<td>10:30 – 11:00</td>
<td><strong>COFFEE BREAK</strong></td>
</tr>
<tr>
<td>10:30 - 13:00</td>
<td><strong>Working Groups - continued</strong></td>
</tr>
<tr>
<td>13:00 - 14:00</td>
<td><strong>LUNCH</strong></td>
</tr>
<tr>
<td>14:00 - 16:00</td>
<td><strong>Working Groups - continued</strong></td>
</tr>
<tr>
<td>16:00 – 16:30</td>
<td><strong>COFFEE BREAK</strong></td>
</tr>
<tr>
<td>16:30 - 18:00</td>
<td><strong>Working Groups - continued</strong></td>
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<th>Time</th>
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<tbody>
<tr>
<td>19:00</td>
<td><strong>GROUP DINNER</strong></td>
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</table>

### THURSDAY, 5 AUGUST 2010

**SESSION VI - Chairperson: Andrew JESSUP**

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
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<tbody>
<tr>
<td>08:30 - 10:00</td>
<td><strong>Working Group Presentations</strong>: Review of conclusions and recommendations for the different outputs.</td>
</tr>
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</table>

<table>
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<tr>
<th>Time</th>
<th>Event</th>
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<tbody>
<tr>
<td>10:30 – 11:00</td>
<td><strong>COFFEE BREAK</strong></td>
</tr>
<tr>
<td>10:30 - 13:00</td>
<td><strong>Working Groups</strong>: Discussion and drafting of individual research proposals for the different genera and techniques, taking into account the proposed R&amp;D of each research group.</td>
</tr>
<tr>
<td>13:00 - 14:00</td>
<td><strong>LUNCH</strong></td>
</tr>
<tr>
<td>14:00 - 16:00</td>
<td><strong>Working Groups - continued</strong>: Continued drafting of individual research proposals for the different genera and techniques for each research team.</td>
</tr>
<tr>
<td>16:00 – 16:30</td>
<td><strong>COFFEE BREAK</strong></td>
</tr>
<tr>
<td>16:30 - 18:00</td>
<td><strong>Working Group Presentations</strong>: Review of conclusions and recommendations for the individual research proposals for the different applications of each research team.</td>
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### FRIDAY, 6 AUGUST 2010

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
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<tbody>
<tr>
<td>08:30 - 10:30</td>
<td><strong>General Discussion</strong>: Review and amend the logical framework.</td>
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<table>
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<tr>
<th>Time</th>
<th>Event</th>
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<tbody>
<tr>
<td>10:30 – 11:00</td>
<td><strong>COFFEE BREAK</strong></td>
</tr>
<tr>
<td>11:00 – 13:00</td>
<td><strong>Working Groups</strong>: Drafting and compiling of RCM report.</td>
</tr>
<tr>
<td>13:00 – 14:00</td>
<td><strong>LUNCH</strong></td>
</tr>
<tr>
<td>14:00 – 15:00</td>
<td><strong>Working Groups</strong>: Finalization of draft RCM report.</td>
</tr>
<tr>
<td>15:00 – 15:30</td>
<td><strong>COFFEE BREAK</strong></td>
</tr>
<tr>
<td>15:30 - 17:00</td>
<td><strong>General Discussion</strong>: Agreement on content of RCM report, on information exchange mechanisms, on location of 2\textsuperscript{nd} RCM, and closure of the RCM.</td>
</tr>
</tbody>
</table>
Appendix 2: Abstracts
Screening microsatellite DNA markers for population genetic studies of *Bactrocera dorsalis* species complex

Nidchaya Aketarawong\textsuperscript{1,2}, Siriwan Isasawin\textsuperscript{2}, Watchreeporn Orankanok\textsuperscript{3}, Grenda Obra\textsuperscript{4}, and Sujinda Thanaphum\textsuperscript{2}

\textsuperscript{1}Agricultural Science Division, Kanchanaburi Campus, Mahidol University, THAILAND
\textsuperscript{2}Department of Biotechnology, Faculty of Science, Mahidol University, THAILAND
\textsuperscript{3}Department of Agricultural Extension, Ministry of Agriculture, THAILAND
\textsuperscript{4}Philippine Nuclear Research Institute, PHILIPPINES

Abstract:

The *Bactrocera dorsalis* species complex is known as a large group of tropical fruit flies inhabiting the Asia-Pacific region. A few members, consisting of *B. dorsalis sensu stricto*, *B. papayae*, *B. carambolae*, and *B. philippinensis*, are recorded as key agricultural pests. Each of these species may have distinctly different geographical and host ranges but sometimes these overlap making identification based on these aspects difficult. Also some species have been assessed as the same biological unit based on evidences from cross-species mating tests. It is difficult to discriminate this species complex, in particular when they are sympatric and/or of unknown origin, by using only adult morphological data. The consequence of this species complexity may hamper the effectiveness of Sterile Insect Technique (SIT) application, especially when this approach has been launched in an area-wide manner. The concept of population genetics could be used to study these fruit fly populations in terms of genetic variation, population structure, and gene flow. Recently, microsatellite DNA markers have been isolated and characterized from *B. dorsalis s.s.* and *B. papayae*. These microsatellite markers were used to study genetic variation within and between the two species and showed high polymorphism between different fruit fly populations. Several population samples of the *B. dorsalis s.s.* were collected from reference laboratories and subsequently screened with twelve established microsatellite markers. Preliminary data suggest that these microsatellite markers could be applied for cross-species amplification leading to further population genetic study of the *Bactrocera dorsalis* species complex in both sympatric and allopatric areas.
Cytogenetic studies of the Oriental fruit fly, *Bactrocera dorsalis* s.s. (Hendel) and the melon fly, *Bactrocera cucurbitae* (Coquillett): A comparative analysis.

Zacharopoulou, Antigone\(^1\,\(^2\); Augustinos, A. Antonios\(^2\); Sayed, AA Waheed\(^1\); Yesmin, Farzana\(^1\); Robinson, S Alan\(^1\); Franz, Gerald\(^1\).

\(^1\)Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Agency’s Laboratories, Seibersdorf, Austria.

\(^2\)Department of Biology, University of Patras, Patras 26500, Greece.

**Abstract**

We report here the construction of polytene chromosome maps for the melon fly and the Oriental fruit fly and show that they can be used for cytogenetic studies within each species, as well as for comparative studies among Tephritids. Two laboratory colonies from each species maintained in the FAO/IAEA Insect Pest Control Laboratory, Seibersdorf were used in this study. Mitotic and polytene chromosome preparations were made from brain ganglia and salivary glands of late third instar larvae, respectively. The polytene maps were constructed using the Adobe Photoshop CS2 software. The mitotic karyotype of both species consists of six pairs of chromosomes including one pair of heteromorphic sex (XX/XY) chromosomes. Five long polytene elements (10 polytene arms) corresponding to the five autosomes were observed in each species. The characteristic features and the most prominent landmarks of each chromosome are shown. Chromosomal homology among *B. dorsalis* s.s., *B. cucurbitae* and *C. capitata* was determined by comparing chromosome banding patterns. This comparative analysis reveals the presence of mainly intra-chromosomal rearrangements in the two *Bactrocera* species, relative to *C. capitata*. Numerous heterozygous chromosome inversions were detected in *B. dorsalis* s.s. strains and their possible implication in the speciation process within the *B. dorsalis* complex is discussed. The salivary gland polytene chromosomes of *B. dorsalis* s.s. and *B. cucurbitae* are suitable for cytogenetic analysis, for comparative studies among species of the Tephritidae family and can support the development of genetic control methods of these species. They also provide a genetic tool that could accelerate species identification within the *B. dorsalis* complex and clarify the phylogenetic status of *B. cucurbitae*. 
Pheromone Analysis of *Anastrepha fraterculus*


*Universidade Federal de Alagoas*

**Abstract:**

The fruit fly species *Anastrepha fraterculus* (Diptera: Tephritidae) is one of the most invasive world pest species with a large range of species speciation. The aim of this study is to understand the complex relationship among different populations of *A. fraterculus* by means of modern analytical techniques. The proposed project aims to a) identify the differences between compositions of cuticular hydrocarbons (CHCs) depending on fly age and between males and females within and between different populations (laboratory populations and wild populations) using mass-spectrometry techniques, b) determine the exact composition of the volatile pheromone mixture released by males and c) perform behavioural assays to identify the biological relevance of pheromone differences between populations. Chemical analysis of volatiles released by *A. fraterculus* calling males from a laboratory population (originated from Argentina and provided by FAO/IAEA, Seibersdorf, Austria) by Gas Chromatography-Mass Spectrometry (GC x GC-TOF-MS) demonstrated that it is a mixture of alcohols, mono- and sesquiterpenoids and three isomeric lactones named suspensolide, anastrephin and epianastrephin. Electrophysiological analyses coupled to Gas Chromatography (GC-EAD) revealed that six of the compounds released by *A. fraterculus* males elicit female antennae response, they are: \((Z)-3\)-nonenol, \((Z, Z)-3,6\)-nonadienol, geranylacetone, \(\alpha\)-farnesene, suspensolide and \((S,S)-epianastrephin\). Male antennae respond to \((S,S)-epianastrephin\) and \(\alpha\)-farnesene. These findings suggest that there are sex specific differences in perception of male sex pheromone of *A. fraterculus*. Further studies are being conducted in order to collect the volatiles from *A. fraterculus* males (two wild populations/Brazil) for chemical and electrophysiological analyses.
Use of MtDNA and nuclear markers to delimit and diagnose Brazilian species within the *Anastrepha fraterculus* complex.

Janisete G. Silva

*Universidade Estadual de Santa Cruz*

**Abstract:**

The South American fruit fly, *Anastrepha fraterculus* (Wiedemann), is among the most serious agricultural pests in South America. In Brazil, *A. fraterculus* has been reported to infest 76 host species in 20 plant families. A series of morphological and genetic studies has revealed that *A. fraterculus* actually comprises a complex of multiple species. The actual number of putative species within the *A. fraterculus* complex and their associated biogeography is uncertain. Based on mating studies, at least four putative species are recognized. All available information indicates that Brazilian populations of this nominal species most likely represent at least three distinct biological entities. This has been determined based on few/limited previous genetic studies, which focused mainly on populations collected in the state of São Paulo and very few populations from other states. Due to its economic importance, *A. fraterculus* is one of the targeted species for the Medfly program in Brazil. Therefore, it is of paramount importance to study populations from a wide geographic range in Brazil to identify genetically isolated populations. Results of such analyses can direct behavioral studies to verify mating compatibility prior to the implementation of area-wide management programs.

Our general objective is to conduct genetic analyses of *A. fraterculus* populations throughout Brazil. These analyses will assist in better establishing the genetic make-up of the Brazilian populations of *A. fraterculus* and add important information to the international base of knowledge regarding *A. fraterculus*. We expect to provide an accurate estimation of the number of putative species in the *fraterculus* complex in Brazil. We will collect samples throughout Brazil from representative populations of the various putative species of *A. fraterculus* that will be suitable for genetic studies. We will also acquire and analyze DNA sequence data that can be integrated with data from other studies (e.g., mating compatibility, karyotype, host plants, etc.).

Initially, we will obtain collections of different populations of *A. fraterculus* in Brazil as well as some other related *Anastrepha* species for the phylogenetic analysis. We will screen populations for one mitochondrial DNA marker (COI) and also characterize laboratory colonies. Within the next 5 years, these results will allow us to build a platform upon which we will obtain sequences from several populations of *A. fraterculus* and other *Anastrepha* species in Brazil using additional mitochondrial and nuclear markers, carry out phylogenetic reconstruction and phylogeographic analyses and also interpopulational analyses using nuclear markers to verify gene flow.
Analysis of ecological speciation among *Anastrepha fraterculus* populations from Brazil through behavioral and demographic parameters

I. S. Joachim-Bravo

*Federal University of Bahia, Brazil*

**Abstract:**

*Anastrepha fraterculus*, known as South American fruit fly, is a polyphagous species, attacking over 67 plant hosts and is considered as the most important pest of native fruits in South America (Alberti *et al.* 2008, Dutra *et al.* 2007). It is a major pest in apple orchards in south of Brazil.

Despite its economic importance and substantial interest in environmentally friendly control methods, such as SIT, there is a lack of information on biology, taxonomy and behavior (Sciurano *et al.* 2007). The high level of variability among natural population of *A. fraterculus* suggests that it is a complex of sibling species and not a single biological entity (Stone 1942, Malavasi & Morgante 1982, Steck 1991, Selivon 1996, Vera *et al.* 2006).

In Brazil genetic and behavioral studies on mating choice suggest an hypothesis that there are at least three different species groups in the country from the southeast (mainly Sao Paulo) and northeast (mainly Bahia and Rio Grande do Norte) (Morgante *et al.* 1980, Selivon 2005). Concerning the population from the south region, there are no data to confirm if it belongs to the same group as those from Sao Paulo or is already a distinct species. Some studies indicate that there is morphological and genetic diversity in the *fraterculus* complex associated with reproductive barriers which should result in reproductive isolation and hence an incipient speciation (McPheron *et al.* 1999, Silva 2000, Selivon 2005). The assessment of pre- and post zygotic isolation mechanisms present among the natural populations, based on behavioral and demographic aspects, is crucial to define their taxonomic status and will support the implementation of a SIT program for *A. fraterculus* in Brazil.

The study would bring the selection of *A. fraterculus* populations to be mass reared at Medfly Rearing Facility, Brazil, establishment of colonies, applied irradiation dose for insect sterilization and tests of competitiveness and sterility between selected strains and wild flies.
Current work in defining species limits in the *Bactrocera dorsalis* complex

Karen Armstrong, Laura Boykin, Stephen Cameron, Toni Chapman, Anthony Clarke, Deborah Hailstones, Andrew Jessup & Mark Schutze

*Queensland University of Technology*

**Abstract:**

Funded through the [Australian] Cooperative Research Centre for National Plant Biosecurity, a project is currently underway to develop robust molecular diagnostics for *B. dorsalis s.s.*, *B. papayae*, *B. carambolae* and *B. philippinensis*. Some work is also being done on *B. invadens*. Current members of the project are the Queensland University of Technology, NSW Industry & Investment, CSIRO Entomology [all Australia], Lincoln University [New Zealand] and FAO/IAEA [Austria].

The project is using multiple lines of evidence (molecular, morphological, and behavioural) to determine species boundaries of the target taxa, an essential first step towards developing definitive diagnostics. Approximately half-way through its three-year life, the major achievements of the project to date are the following:

Collection of fresh, preserved material for morphological and genetic screening (over 1,200 specimens from Taiwan, Thailand, Malaysia, the Philippines, PNG, Indonesia, Suriname and Australia). That material is currently being screened for three mitochondrial and three nuclear genes.

The establishment of new fly colonies at the FAO/IAEA Seibersdorf facility for comparative studies on the target species’ mating systems (including mating compatibility, pheromone analysis, and bioacoustics) of which preliminary mating compatibility results will be presented at the meeting.

Geometric morphometric analysis of wing-shape variation has been completed on museum specimens collected and named by R.A.I. Drew. The technique shows discriminatory potential.
Analysis of epicuticular composition in genera Anatrephe

Lucie Vaníčková, Vladimír Vrkošlav, Blanka Kalinová, Radka Břízová, Michal Hoskovec

Institute of Organic Chemistry and Biochemistry AS CR, v.v.i.

Abstract:

The insect cuticle has unique properties and functions. Primarily, it prevents desiccation. Secondarily, the uppermost cuticle layer (epicuticle) contains compounds that serve in many species as identification and communication signals (pheromones, kairomones) (Blomquist 1979, Cvačka 2006). Cuticular hydrocarbons (CHCs) and cuticular esters of fatty acids (CE) are major components of the insect epicuticle. Recent studies suggest that epicuticle composition is species specific, sex specific and even may differ between different populations of the same species (Rundle et al., 2009). Thus, the epicuticle serve as a highly specific identification characteristic (“fingerprint”). The aim of the proposed work is to study epicuticular composition, mainly hydrocarbons (CHCs) and fatty acid esters (CHCs), of different populations of the genera Anatrepha and Ceratitis (Diptera: Tephritidae) by means of two analytical techniques: gas-chromatography coupled with mass spectrometry (GC/MS) and matrix-assisted laser desorption/ionization analysis (MALDI). The results of the research will contribute to the international effort to clarify and map cryptic speciation in these species.
Thailand: Resolution of the *Bactrocera dorsalis* Complex

Suksam Chinvinijkul\(^1\), Watchreern Orankanok\(^1\), Sunyanee Srikachar\(^2\), Wipada Plodkornburee\(^2\), Weerawan Amornsak\(^3\) and Supaap Pinkaew\(^1\)

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Abstract:

The Oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) is the most serious insect pest in fruit production and is distributed throughout every geographic region of Thailand. Two other tephritid pests that have been identified as part of the *B. dorsalis* complex, and found in different parts of Thailand, are *Bactrocera papayae* and *B. carambolae*. *Bactrocera papayae* is found in the southern part while *B. dorsalis* s.s. is restricted to northern Thailand. *B. carambolae* has been reported (in 2001) infesting some fruits in southern Thailand other than carambola. The ultimate goal of the research is to assess if the *B. dorsalis* complex species found in each geographic region of Thailand constitute only the one species. Furthermore the relationships between these three *B. dorsalis* complex species will be assessed.

A research project on trapping tendency for fruit fly surveillance in Thailand was carried out between April 2008 and September 2009. The influence of trapping density was analyzed from 31,928 data sets collected from 307 modified steiner traps charged with methyl eugenol covering 160 km\(^2\) in four selected areas over the country. Trap density was one trap per 1, 2, 4, 8 and 10 km\(^2\). Traps were inspected weekly for 52 weeks continuously. Data showed that the average flies per trap per day (F/T/D) of *B. dorsalis* complex in the four areas was 18.51. In areas with no fruit fly control in the south there was significantly higher F/T/D than others with an average of 51.56 while in the area with fruit fly control in the north there was a significantly lower F/T/D than others at 4.19. Trapping density of one trap per 1 km\(^2\) caught significantly higher numbers of *B. dorsalis* complex species than a density of one trap per 2 km\(^2\) with F/T/D of 19.79 and 16.85 respectively but was not significantly different from a trap density of one trap per 4 km\(^2\) density. Only 40 specimens of *Bactrocera papayae* were trapped (0.0161% of a total of 15,964 flies trapped in the southern selected area) and most of them were trapped with a trap density of one trap per 1 km\(^2\). There were no *B. carambolae* trapped in any selected area. Species identification in this research was visible morphological differences.

Studies on morphology and morphometric analysis, biological tests, cross-mating, host use studies and pheromone analyses may resolve the *B. dorsalis* complex species limits, reduce problems with phytosanitary barriers, enhance the SIT program, and may be of great benefit to Thailand.
Morphology, distribution and taxonomic characterization of the species complex *Anastrepha fraterculus* from Mesoamerica and Northwestern of South America.

Vicente Hernández-Ortiz, Juan O. Tigrero, Emilio Arévalo, Gloria Palma, Javier Martínez, Norma Nolazco & Carlos Rivera.

*Instituto de Ecología A.C. (México), Escuela Politécnica del Ejército (Ecuador),*  
*Instituto Colombiano Agropecuario (Colombia), and*  
*Servicio Nacional de Sanidad Agraria (Perú).*

Abstract:

The nominal species *Anastrepha fraterculus* (Wiedemann), known as “South American fruit fly”, indeed represents a cryptic species complex. This has been evidenced by morphological variability (Stone 1942), differences in host use patterns and pest status (Baker *et al.* 1944, Baker 1945, Zucchi 2000, Aluja *et al.* 2003, Hernández-Ortiz & Morales-Valles 2004); karyotype differences (Mendes 1958, Morgante *et al.* 1980, Solferini and Morgante 1987); mitochondrial DNA divergence (Steck and Sheppard 1993) or genetic differences recognizing three distinct taxonomic entities in Brazil (Selivon *et al.* 2004, 2005). Previous results of our research (funded in part by the IAEA RC: 12644/2004-2006), using multivariate morphometric techniques provided conclusive evidence that the AF complex would be composed of 7 morphotypes. This research also established that biogeographical factors are implicated in the origin of these natural groups (Hernandez-Ortiz *et al.* in prep.).

During this project, the main problem to be addressed is to analyze the morphological variability to describe the most important morphological features for the taxonomic recognition of the natural groups, making a characterization of three morphotypes of the AF complex, as well as to determine the current biogeographical distribution of those inhabit the Mesoamerican dominion, and the Northwestern South American sub-region (*sensu* Morrone 2006).

We will analyze 25 population samples from 6 Latin American countries (Mexico, Central America, Colombia, Ecuador and Perú) which are representing three morphotypes. Most of them will be collected in several eco-geographical regions from Mesoamerica and the Andean countries. Samples will be analyzed using 15 females by locality. On the basis of structures as the aculeus, mesonotum and wing, 19 morphometric traits will be assessed for each specimen following methods described by Hernández-Ortiz *et al.* (2004). We expected the publication of scientific papers related to the population variability from Mesoamerican and the Andean countries, describing their species involved. A reference collection will be mounted and preserved for further research.
Morphometric analysis, genetic characterization and mating compatibility studies among populations of *Bactrocera invadens* from different origins

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*International Centre of Insect Physiology and Ecology (icipe), Nairobi, Kenya*

**Abstract:**

Among the Tephritidae (Diptera), dacine fruit flies are highly destructive, frequent invaders and colonizers of new ecologies, and rank high on quarantine lists worldwide. *Bactrocera invadens* was first detected in Kenya in 2003 and is now reported from over 28 African countries (with South Africa recently added to the list) attacking over 30 host plants, mostly of high value fruits and vegetables. Despite its recent description and importance, the taxonomic status of *B. invadens* has remained rather controversial and there is no consensus on its species limit. This has serious implications on trade given its high quarantine status. Taxonomic uncertainties can adversely affect the development and implementation of area-wide IPM that include sterile insect technique (SIT) component, as well as limiting the chance of identifying promising biological control agent for the pest. Preliminary joint research activities between icipe, University of Pavia and the Royal Museum for Central Africa, Tervuren have revealed differences in *B. invadens* populations across Africa at the molecular level. Variations in response to attractants, entomopathogens and parasitoids from different populations have also been observed. Given this new information, there is a need to test for possible evidence of reproductive isolation through mating compatibility studies among populations from different origins both at the local and the continental level. Although initially thought to be a low-land resident pest, *B. invadens* have also been trapped and reared from host fruits at elevations > 1645 m above sea level, occupying heterogenous habitats across the altitudinal gradients. Vast landscape and geographical barriers along altitudinal gradients across Africa may limit gene flow and enhance local adaptation leading to reproductive isolation among populations of *B. invadens* further compounding taxonomic confusion. In order to allow for estimation of the degree of genetic relatedness among the populations across the geographical distributional area, documenting the ancestral home range with respect to adventive populations, infer the demographic history and the degree of differentiation of the adventive populations of each species and perform fly traceability surveys to identify the source of invasion we also propose to collect *B. invadens* and ship to University of Pavia for molecular studies and the establishment of a population genotype database. The following objectives are proposed for the CRP in close collaboration with University of Pavia, Italy: (1) Conduct morphometric analysis involving several African, Sri Lankan and Indian populations of *B. invadens* (2) Assess mating compatibility between African vs. African populations of *B. invadens* and whenever possible between African vs. Sri Lanka and Indian populations as well as between high- and low-altitude African populations (3) Contribute to the development of population genotype database for *B. invadens* (4) Investigate whether heritable life history and genetic differences exist between high- and low-altitude populations of *B. invadens* (5) Study the behaviour of different populations in field-caged host tree with regard to mating, within tree distribution, and daily patterns of activity.
Mating Compatibility of Economically Important Cryptic Species in the *Bactrocera dorsalis* Complex (Diptera: Tephritidae)

Alvin Kah-Wei Hee¹, Suk-Ling Wee² & Keng-Hong Tan³

¹Universiti Putra Malaysia,

²Universiti Kebangsaan Malaysia,

³Phi Biotech Sdn Bhd

Abstract:

Whilst the *Bactrocera dorsalis* complex of tropical fruit flies is largely endemic to the South East Asian region, this complex contains some of the world’s most damaging insect pests such as the Oriental fruit fly, *B. dorsalis* Hendel and its sibling species that are also highly polyphagous - *B. papayae* Drew & Hancock and *B. philippinensis* Drew & Hancock. The recent incursion and rapid establishment of another sibling species, *B. invadens* Drew, Tsuruta & White in Africa are already threatening the economies of many poor African nations that are heavily dependent on agriculture. It has been suggested that the use of an appropriate pest control strategy such as the Sterile Insect Technique (SIT), implemented as part of Area-Wide Integrated Pest Management (AW-IPM) programmes can effectively be used to achieve acceptable economic thresholds. However, pivotal to success of this approach is the correct identification of the target pest species. Unfortunately, these economically important species in the *B. dorsalis* complex are morphologically very similar, possess overlapping wide host ranges and are able to interbreed resulting in viable offspring. In complementing numerous techniques that have been employed (e.g. morphometrics, DNA sequencing/molecular and pheromone analyses) in resolving this issue; and to be a part of the international efforts led by FAO/IAEA in resolving the species limits of the *B. dorsalis* complex, this proposed research aims to evaluate the mating compatibility of those economically important cryptic species between *B. dorsalis* s.s., *B. papayae*, *B. philippinensis* and *B. invadens*. Pre- and post-zygotic reproductive isolation level in the interspecific mating between those sibling species will be also investigated. An outcome of this proposed research will be the clarification on the status of the sibling species in relation to *B. dorsalis* s.s. based on cross-mating and associated reproductive isolation evaluation.
Current research on molecular and morphological recognition of African fruit fly pests and their congener

Virgilio, M.; Backeljau, T. & De Meyer, M.

Royal Museum for Central Africa (Tervuren, Belgium) &

Royal Belgian Institute of Natural Sciences (Brussels, Belgium)

Abstract:

Over the last five years, the Royal Museum for Central Africa and the Royal Belgian Institute of Natural Sciences have been involved in international collaboration with several partners from other institutions and in developing molecular and morphological diagnostic systems for recognition of the main African frugivorous fruit fly pests and their congeneric taxa. The main activities that are directly linked to the main themes of this Coordinated Research Project, are briefly summarized here.

Recognition of species within the Ceratitis FAR complex (Ceratitis fasciventris, C. anonae and C. rosa): A taxonomic revision of the representatives of the genus Ceratitis, upon morphological grounds, has shown that all three taxa included in the FAR complex can be considered as separate taxonomic entities and were given species status. Secondary sexual characters in the males provide adequate characteristics to differentiate between them. Females can only be differentiated between C. anonae on one hand and C. fasciventris/C. rosa on the other. Molecular studies, in collaboration with APHIS-USDA (Texas, USA) tested several mitochondrial and nuclear markers but did not define any marker that can be used to differentiate between the three taxa. Specimens belonging to C. fasciventris can be separated in two clusters: West-Central African versus East African. Both morphological and molecular characteristics shows signals to this effect.

The macrogeographic population structuring of Bactrocera cucurbitae was studied, in collaboration with CIRAD, La Réunion. Twenty-five worldwide distributions were genotyped at 13 microsatellite loci. Five main geographical groups can be differentiated: African continent, Western Indian Ocean, Central Asia, East Asia, and Hawaii. The study suggests that the species originated in Central Asia and expanded its range along separate pathways to East Asia and Hawaii on one hand, and to Africa and Western Indian Oceaan on the other. Currently a more extensive study is ongoing with relation to the population structuring within the Afrotropical continent. There is no indication on morphological or molecular grounds that the populations of different geographic regions belong to different taxonomic entities with specific status, nor that populations within a particular region are composed of several different taxa.
Reproductive compatibility: What did *Anastrepha fraterculus* tell us so far

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2Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

3Instituto de Genética, INTA Castelar, Argentina.

4Instituto de Ecología A.C, Xalapa, México.

Abstract:

It has long been proposed that the nominal species *Anastrepha fraterculus* is a species complex. This review summarizes studies on reproductive compatibility among populations from different regions. Mating compatibility was evaluated among lowland (Peru) and highland (Colombia) areas from the Andean region and the south-eastern part of South America (Brazil and Argentina). Most of the populations were sexually incompatible with each other, except for Argentina and Brazil which showed a lower degree of isolation. Full mating compatibility was detected only within Argentina and Peru. Flies were sexually active at different times of the day suggesting temporal isolation. Preliminary results including a population from Mexico also showed behavioral isolation between this population and those from Argentina, Brazil and Peru. The populations from Argentina and Peru were further analyzed in experiments involving the two strains and their hybrids. Mating time and location of mating couples in the tree depended on the way the hybrids were obtained. There were quantitative and qualitative differences in the sex pheromone between Argentina and Peru, while hybrids produced a mixture of the two parental blends. Hybrid males showed no isolation with either parental females, whilst hybrid females preferred to mate with hybrid males. The pre-zygotic isolation barriers were complemented by high levels of post-zygotic inviability and sex ratio distortion. Karyotypic differences between the parental strains were detected and hybrids evidenced extensive asynapsis in salivary glands polytene chromosomes. More recently, studies in Brazil between a laboratory strain from Piracicaba, Sao Paulo, and a wild population from Pelotas, Rio Grande do Sul, revealed some degree of sexual isolation between the two origins that was not extended to the post-zygotic level. This suggests that even within what was considered to be a single entity within the complex (*A. sp.1 aff fraterculus*) there are some incompatibilities among populations from different regions. The combined results reveal the presence of reproductive incompatibility and provide insight in the possible number of different biological entities within the complex. The practical implication is that colonies of this pest to be used in any sterile insect technique approach should be derived from the target population or from a compatible one. Our aim in this CRP is to extend previous studies on reproductive compatibility as to clearly identify the putative species included within this species complex and the degree of isolation among all of them.
Molecular genetic resolution of pest species within the *Bactrocera dorsalis* complex

Karen Armstrong, Laura Boykin, Stephen Cameron, Toni Chapman, Anthony Clarke, Deborah Hailstones, Andrew Jessup & Mark Schutze

*Bio-Protection Research Centre, Lincoln University* (1 Presenting author)

**Abstract:**

This project is contributing molecular data to a larger project, funded by the Australian Cooperative Research Centre (CRC) for National Plant Biosecurity, aiming to gather multiple lines of evidence to establish the species boundaries for key pest groups within the *Bactrocera dorsalis* complex. Compared to any previous studies, a greater depth of analysis is possible in this project through the use of specimens of several species originating from sympatric, allopatric and type localities. These specimens will also be classified morphologically, morphometrically and behaviourally. Once species boundaries are confirmed, the goal will be to develop species-diagnostic molecular markers.

Focus in this project is on the described Asian *B. dorsalis* ss, *B. carambolae*, *B. papaya* and *B. philippinensis*, plus the Australian dorsalis species, *B. cacuminata* and *B. opiliae*, and the very closely related *B. musae* (currently outside the complex). Given the very recent evolutionary time frame of speciation within this group, and absence of knowledge of speciating gene(s), several regions of the genome will be analysed to accumulate potentially species-diagnostic DNA polymorphisms.

Nuclear and mitochondrial gene regions typically used for phylogenetic and diagnostic analysis of closely related species are being considered. A population subset has been used to screen 11 standard loci to date, by either direct sequencing or a simplified ecoTILLING method; the latter will be used later to screen larger numbers of specimens. In addition, generation of the transcriptome sequence for flies of *B. dorsalis* ss, *B. carambolae*, *B. papaya* and *B. philippinensis* is underway using the Illumina (Roche) next generation sequencing platform. Appropriate novel gene regions identified from that comparative data will be also be screened for potential species-level polymorphisms.

Bioinformatic methods to analyse the data for evidence of species-level groupings and statistically supported correlations with biological evidence are being developed elsewhere in the project.
Male rectal gland volatile constituents of five economically important cryptic species within the Oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae)

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Abstract:

The Oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae) is a noxious insect pests causing serious direct and indirect losses worldwide. Despite the fact that much research background has been generated through years, such as diagnostics, filed surveillance, field pest control and some export trade, the emergence of the idea that it is a complex comprises at least 52 morpho-species with different geographical/regional distribution has further threaten already weakened fruit industries and export trades. The ambiguity of the taxonomy status of these flies further complicated the situation which leads to more trade barriers for fresh fruit produce into potential importing countries, particularly with the increase of international awareness on biosecurity threat posed by invasive and exotic pests, and more stringent biosecurity measures taken by potential importing countries. A centralized and concerted effort in providing comprehensive biological, morphological, chemo-ecological and molecular data systematically is urgently needed to resolve the putative species in the *B. dorsalis* complex in order to prevent future food/trade crises as well as to enhance further development towards SIT implementation as part of area-wide IPM programmes against these pest insects. We propose to employ chemotaxonomic technique through chemical analyses which will be performed on the male rectal gland, a putative pheromone gland in the tephritid fruit fly, to analyze and compare the pheromone composition of five cryptic species of great economic importance identified by the present CRP, namely *B. dorsalis* s.s., *B. papayae*, *B. carambolae*, *B. philippinensis* and *B. invadens*. In addition, this research also intends to investigate and compare the relative sensitivity of each species towards the parapheromone, methyl eugenol (ME), as well as their rectal gland chemistry after consuming ME. It is hope that this research, in compliment with other research techniques, will help to resolve the current dilemma facing the putative species as well as shed light on the species speciation within the *B. dorsalis* complex.
Population genetics of two fruit flies damaging cucurbit crops in La Réunion: 
*Bactrocera cucurbitae* and *Dacus ciliatus* and future prospects

Delatte, Hélène*; Jacquard, Cathy; Simiand, Christophe; & Quilici, Serge.

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Abstract:

*Bactrocera cucurbitae* and *Dacus ciliatus* are two fruit fly species damaging various

cucurbit crops. Of Asiatic origin, *B. cucurbitae* has become an invasive pest in many
countries especially on the African continent. Conversely, *D. ciliatus*, which
originates in Africa has increased its distribution during the last years and is now
becoming a pest in different Asiatic countries. Both species rank among invasive pests
and has been introduced in La Réunion, a French island in the South-Western Indian
Ocean over the last 50 years. They now damage the whole range of cucurbit
cultivated in the island and cause important yield losses. La Reunion is a subtropical
island, with high altitudinal gradient (up to 3000 m) and two seasons (hot and rainy
summer, milder and drier winter). Nevertheless, those two fruit fly species are found
in most of the areas, regardless of the season and the altitude. However, they show
some altitudinal preferences: *B. cucurbitae* is more abundant in the lowlands and *D.
ciliatus* at medium altitudes.

In order to study the population structure of both species, we developed sets of
microsatellite markers for both species, and sampled various sites (at high, medium
and low altitudes) during winter and summer on different host plants. A complete
genetic study has been done on the data set.

Future prospects will be discussed on the use of the newly built markers and results
obtained on the genetic studies to solve problems raised with regard to proper
characterization of fruit fly pests belonging to the genera *Bactrocera* and *Ceratitis*.
Host utilization and genetic divergence among populations of *Bactrocera invadens* Drew, Tsuruta and White and *Bactrocera cucurbitae* (Coquillett) in Tanzania

1Mwatawala, M.W., 1Rwegasira, G. and 2De Meyer, M.

1Sokoine University of Agriculture, Morogoro, Tanzania

2Royal Museum for Central Africa, Tervuren, Belgium

Abstract:

Two invasive species, *Bactrocera invadens* and *Bactrocera cucurbitae* have been recorded in Tanzania and continue to inflict heavy losses to the agricultural sector. There is an information gap in, among other things, host range and preference of *B. cucurbitae* in East Africa, is limited. Furthermore, there is an indication of existence of different morpho types or genotypes within each of the two species. However, relationship between these different types and their host preferences is unknown. The proposed studies aim at: Investigating host plant preferences, incidences and infestation rates of Tanzanian populations of *B. cucurbitae* and *B. invadens*; To verify if host plant choice can be related to intraspecific differentiation observed in *B. cucurbitae* and *B. invadens*; Quantification of levels of genetic divergence among populations and testing the occurrence of host races will be conducted. Fruits suspected to be hosts of *B. cucurbitae* and by *B. invadens* will be collected from a number of locations within different agro-ecological zones of Tanzania. Additionally, colonies of the two species will be established in the laboratory and different fruits will be simultaneously introduced into the rearing cages. Specimens of *B. cucurbitae* and *B. invadens* obtained from the rearing experiments will be genotyped using the available microsatellite markers to compare the genetic structures of samples 1) collected from different locations and 2) infesting different hosts. The generality of patterns observed will be compared with populations from other parts of Africa.
Cryptic species of *Anastrepha fraterculus* and *A. obliqua* in Colombia: Methodological purpose

Nelson A. Canal

*Universidad del Tolima*

**Abstract:**

Species of the genus *Anastrepha* are the principal fruit flies attacking fruits in Colombia. *Anastrepha fraterculus* and *A. obliqua* are the most important species, widespread in the country; the first one is hosted by fruits at medium and high altitude and the second at lowlands. *A. fraterculus* is an important pest over 1,800 m, but not from 1,000 to 1,800 m. *A. obliqua* is an important pest until it reaches over 1,000 m. To know if these species populations belong to more than one cryptic species, morphological, karyotypic, molecular and reproductive studies will be done. Morphological studies will carried out on adults, larvae and eggs. Populations will be obtained from the host fruits, and then we will rearing the F1 on an artificial diet or fruits (guava, banana passionfruit or papaya). Initial morphological testes were held with adults and larvae of three populations of *A. obliqua* and two of *A. fraterculus* and some differences between them were found.
The *Bactrocera dorsalis* complex

Richard A.I. Drew

*Griffith University*

**Abstract:**

The *Bactrocera dorsalis* complex is one of the most confusing groups of tephritid species that we know. This group of species is based primarily in South-East Asia and contains species of major economic importance with worldwide impacts. These pest species have significant economic impacts on quarantine, international trade and SIT agendas.

Our research team at Griffith University in Brisbane has undertaken extensive field surveillance programs across South-East Asia over the last 25 years. These surveillance programs have included male lure trapping where we have set Steiner type traps with cue lure and methyl eugenol at the same location for one to two years and across different ecosystems. In addition, we have collected some 30,000 fruit samples from which we have compiled host records. As a result of this long term surveillance, we have in Brisbane the best collection of tephritid species and specimens worldwide and we are currently bringing all of this information together in a major revision of South-East Asian Dacinae, to be published in 2011.

In order to define species within the *B. dorsalis* complex, we must consider the current biological species definition accepted by zoologists worldwide, “individuals of a natural population which interbreed in the wild to produce fertile offspring”. This definition specifically excludes artificial matings in captivity.

For practical purposes, definitions of *Bactrocera* species must consider characteristics that include morphology, reproductive isolation in the wild, ecological and behavioural characteristics, genetic differences and chemistry of male pheromones. In our taxonomic research programs on the Dacinae, we have always endeavoured to include as many of these characteristics as possible.

As a contribution to the CRP Project, at Griffith University, we will provide support to other CRP researchers through the provision of specimens from our extensive collections, review morphological and host data, and undertake a new morphometric research project to assess if geographic variation occurs in the genitalia characters.

*Bactrocera invadens* in its original concept, is not considered a member of the *dorsalis* complex. The definition was based on a population in Sri Lanka that is red-brown in colour and probably what Fabricius defined as *Musca ferruginea* in 1794. It appears that there have been two separate species invasions in Africa which have confused the picture.
Development and use of population genotype databases for species resolution within *Bactrocera dorsalis* and *Anastrepha fraterculus* complexes


*Department of Animal Biology, University of Pavia, Via Ferrata 1, 27100 Pavia, Italy*

**Abstract:**

The molecular markers applied to tephritid populations and species represent suitable tools for recognizing cryptic species within species complexes. Natural populations of *Bactrocera dorsalis ss* from Asia and *B. invadens* from Africa have already been approached using neutral genetic markers such as Simple Sequence Repeats (SSRs). The degree of intra/inter-population variability and differentiation across the supposed geographic ranges of the species have been assessed and the migration routes of the populations have been inferred. The genetic dataset of these *Bactrocera* species represent specific databases of markers useful to record allele frequencies at different times in these two species. A similar methodological approach is to be applied to *Anastrepha fraterculus* from Argentina.

A novel approach to recognize species within a complex can be derived from the genomic study of the species in question. The search for barrier genes to gene flow may represent a suitable biological tool for cryptic species discrimination.
Overview of the study on *Bactrocera dorsalis* s.s in China

Qinge Ji

_Beneficial Insects Laboratory, College of Plant Protection, Fujian Agriculture and Forestry University, Fuzhou 350002, China_

Abstract:

Fruit flies of economic importance in China belong to the genus *Bactrocera* Macquart mainly, in which *Bactrocera dorsalis* is the dominant species.

Biology study shows that *B. dorsalis* has a large number of host plants in China, and it can devastate almost all fruits, gourds and many kinds of wild plants in tropical, subtropical and temperate areas in China. *B. dorsalis* occurs year-round in southern China, such as in the provinces Fujian, Guangdong and Yunnan. The population dynamics depends on the abundance of host plants, climatic conditions and geographical conditions. It can move with the trade of fruits and gourds and with human transport over long distances, but there are no reports of it establishing populations during winter in northern China.

In order to rapidly identify different fruit fly larvae such as *B. dorsalis, B. cucurbitae, B. tau* and others, some molecular techniques were used for quarantine purposes, but there is a lack of study on the cryptic species of the *B. dorsalis* complex.

The complete mitochondrial genome of the *B. dorsalis* from the Chinese population was determined and analyzed in 2007 by sequencing and cloning the target DNA. The results indicated that the complete mitochondrial genome of *B. dorsalis* is a circular molecule of 15,915 nucleotides (GenBank accession no. DQ845759).

Studies on different geographic populations showed that the main reason for the observed genetic differentiation is related to geographic isolation.

Heat treatment, cold treatment, irradiation, and chemical treatment are mainly used for quarantine.

There have more studies on control measurements in the fields such as monitoring, MAT, protein bait, SIT and parasitoids.

Normally in China, the *B. dorsalis* species complex is treated as a single species *B. dorsalis* s.s., but there is a lack of knowledge and study of *B. dorsalis* complex in China.
A multi-faceted approach to resolving the South American fruit fly complex

Gary Steck

Florida Department of Agriculture & Consumer Services, Division of Plant Industry

Abstract:

Development of SIT for one or more members of a cryptic species complex will involve research that is on the cusp of population genetics and alpha taxonomy and break new ground in fruit fly systematics by integrating non-morphological characteristics into the taxonomic process.

This paper addresses issues of population sampling, specimen rearing, establishment of breeding colonies, and preservation and vouchering of specimens for diverse studies by multiple laboratories. Sampling along broad geographical transects must be carefully implemented and specimen banks established for access to multiple researchers. Specimens derived from broad and thorough sampling will form the foundation for a successful resolution of the complex to be based on multiple techniques. Multi-faceted study of a common pool of specimens will ensure that the derived data can be integrated into sets that meet the requirements of taxonomic description. Important techniques will likely include morphology (adult and immature stages), DNA sequencing, karyotyping, and cross-mating studies. Results from these analyses can be mapped onto geographic distributions and help to establish fly/host plant associations, seasonal phenologies, and biologies.

The foregoing issues will be discussed in the context of the South American fruit fly, *Anastrepha fraterculus* s.l, which has long been recognized as a complex of closely related species, for which much remains to be done in diagnosing species, delimiting geographic distributions, and determining biologies of the constituent taxa. A summary of *A. fraterculus* species concepts from the time of Stone’s 1942 revision of the genus to the author’s isozyme and mtDNA studies of these and related taxa plus results of more recent studies are provided.

Additionally, the utility of broad geographic sampling and establishment of a specimen bank in facilitating research on cryptic species will be demonstrated by an example from research on genetic “fingerprinting” of Mediterranean fruit fly populations. Results have proven useful in determining pest pathways into the U.S. and in evaluating the success/failure of eradication programs.

By necessity, development of SIT will have to resolve issues related to species identities and boundaries. In turn, this will assist in many of the regulatory processes involved in identification, pest management, and pest risk and pathways analyses.
Appendix 3: Working Groups

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<tr>
<th><strong>Anastrepha fraterculus</strong></th>
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<td>M. Teresa Vera (Leader)</td>
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## Appendix 4: Participants and Observers

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Appendix 5: List of References


Haymer DS, Tanaka T, Teramae C. 1994. DNA probes can be used to discriminate between tephritid species at all stages of the life cycle (Diptera: Tephritidae). *J. Econ. Entomol.* 87: 741–46


Selivon D, Perondini ALP, Morgante JS. 1999. Haldane’s rule and other aspects of reproductive isolation observed in the Anastrepha fraterculus Complex (Diptera: Tephritidae). Genetics and Molecular Biology 22:(4) 507-510.


Appendix 6: Draft Template - Protocols for Collection and Shipment of Live and Dead Insects for Vouchering, Rearing, Morphology, Morphometrics, Chemical Ecology and Molecular Assays

1. **Labelling**
   1.1. Place of origin (GPS / latitude:longitude, town, country)
   1.2. Host fruit, what plant insect was collected from
   1.3. Date and time of collection
   1.4. Person who collected the insect

   Note: strict rules of how to refer to the relationship between the fly and the plants e.g. trapped in tree, found as larvae in fruit, clearly differentiate between on fruit, in fruit, ex fruit, etc.

   Consider pre-designed forms that should be attached to any specimen, forms attached as an appendix

2. **Live insects**
   2.1. **Collection**
   2.1.1. From wild infested fruit
   2.1.1.1. Fruit type described, photo
   2.1.1.2. Number of fruit needed to collect
   2.1.1.3. How many trees, orchards, regions were collections made from? How wide a population should the sample be collected from?
   2.1.1.4. Base number of insects required for R&D
   2.1.2. From harvested fruit placed in the field
   2.1.3. From non-toxic traps
   2.1.4. From netting, aspiration, etc
   2.1.5. From laboratory cultures
   2.1.6. Shipment of wild-infested fruit from the field to local laboratory – packaging to reduce package-induced mortality, temperature management, etc

2.2. **Shipment**
   2.2.1. Common considerations
   2.2.1.1. Shipping by:
   2.2.1.1.1. Hand luggage for air travel
   2.2.1.1.2. Booked-on package
   2.2.1.1.3. Via courier
   2.2.1.1.4. Via Post
   2.2.1.1.5. Personally by car
   2.2.1.1.6. Packaging type, size, weight
   2.2.1.2. Quarantine security – FORMS!!!
   2.2.1.2.1. Packaging type, size, weight
   2.2.1.2.2. Description of contents
   2.2.1.2.3. Liquids present
   2.2.1.2.4. Contact names, addresses, phone numbers, e-mail addresses – both sender and receiver
   2.2.1.2.5. Exporting country requirements
   2.2.1.2.6. Importing country requirements
   2.2.1.2.7. Courier company requirements
   2.2.1.2.8. Airline company requirements
2.2.2. As pupae
   2.2.2.1. Strong packaging material
   2.2.2.2. Reduce desiccation
   2.2.2.3. Vibration, movement of pupae causing physical damage
   2.2.2.4. Access of pupae to air
   2.2.2.5. Permissible transit temperature range
   2.2.2.6. Quarantine security, escape of pupae, adults

2.2.3. As eggs
   2.2.3.1. Transport of liquids
   2.2.3.2. Reduce leakage, maintain package integrity
   2.2.3.3. Eggs in water, or in agar agar solution

2.2.4. As infested fruit
   2.2.4.1. Distance to travel
   2.2.4.2. Time to travel
   2.2.4.3. Temperature management to reduce adult eclosion

2.3. Receipt of live insects
   2.3.1. Customs clearance – FORMS!!!
   2.3.2. Retrieving eggs, pupae, adults from package
   2.3.3. Identification and separation of species in a mixed culture, other insects, parasitoids, etc
   2.3.4. Setting up laboratory culture
   2.3.5. Minimum quantity of insects required to be a valid sized culture – justification of sample size, recording

3. Dead insects
   3.1. Collection
      3.1.1. Wild flies reared from fruit, collected from traps, nets, etc
      3.1.2. Part/s of insect required for shipment
      3.1.3. Retaining remains of insect for trace-back or for study in another collaborating laboratory
      3.1.4. Labelling requirements for receiving agency / laboratory, etc
   3.2. Shipment
      3.2.1. Shipment of insect extracts e.g. DNA extracts, pheromones
      3.2.1.1. Specialist handling and shipment procedures
      3.2.2. Quarantine considerations as above
      3.2.3. Preserved as a dried specimen or in alcohol or alternative preservatives
      3.2.3.1. Alcohol concentration
      3.2.4. Shipment in alcohol and importer, airline, courier requirements
      3.2.4.1. Amount of alcohol
      3.2.4.2. Alcohol concentration
      3.2.5. Alternatives to shipment in alcohol e.g. PEG, etc
   3.3. Packaging of specimens
   3.4. Receipt of specimens
      3.4.1. Customs clearance

4. Post receipt handling
   4.1. Quarantine security requirements of the receiving institutes
   4.2. Quarantine security facilities at the receiving institutes
   4.3. Quarantine security requirements of the importing country
5. Trapping
   5.1. Type of trap, type of specimen required
      5.1.1. For dry specimen
      5.1.2. Trapped in water
      5.1.3. Trapped in a preserving solution (e.g. PEG)
      5.1.4. With or without toxin

6. Collection of rectal glands and/or volatiles from live insects – See paper by K.H. Tan and work by Peter Teal
   6.1. Guarding against contamination
   6.2. Storage and transport in 100% alcohol
   6.3. Other issues described in the paper
   6.4. Collection of pheromones (e.g. work done by Peter Teal)

7. Validation of species by recognised expert
   7.1. Reference material
      7.1.1. Validation before experiment start
      7.1.2. Validation after experiment
      7.1.3. Keep Voucher Specimens
         7.1.3.1. How many to keep?
         7.1.3.2. In alcohol and / or
         7.1.3.3. As a dried (pinned?) specimen
         7.1.3.4. Preservation and vouchering these specimens for future
      7.1.4. Sharing these specimens for later molecular or morphology

8. Other issues to be addressed
   8.1. Feedback on how culture is progressing, return information, courtesy call / e-mail on receipt of goods, etc
   8.2. Agreement reached on terms, agreements and structures for payment requirements, if necessary, for suppliers, for analysers, etc
   8.3. Consider the possibility of assessing the same individual insects for mating studies, then morphological studies, then pheromone analysis, then molecular, etc. What logistics are required if each assessment is to be done in geographically distant facilities?
   8.4. Share progeny from captured wild females for study
   8.5. Detailed descriptions and documentation of collection methods, dates, times, vouchering, personnel, experimental designs, etc
   8.6. Collaboration and co-operation
   8.7. Potential / possibility of on-line collaboration, questions, FAQs, problems and solutions
   8.8. Publication considerations, intellectual property, data sharing rights, legal requirements

9. Other reports, manuals, etc that are already published / used? E.g. ICIPE, etc.
   9.1. List as many other publications that are already available.
Appendix 7: Guidelines for performing Mating Compatibility Field Cage Test


Overall Objective

The overall objective of the mating compatibility field-caged test is to determine the degree of sexual compatibility between two populations/strains under semi-controlled field conditions by releasing in a confined semi-natural environment (i.e. field cages with trees inside) sexually mature fertile but yet virgin flies from these two populations to observe their mating behaviour and interactions during the time of sexual activity.

Specific Objectives

1. To determine the number of mating couples obtained in each of the possible mating combinations between the two populations.
2. To characterise male calling behaviour (time, location, aggregation in leks, number and type of males in the lek, aggressive behaviour, etc.).
3. To characterise male – female interactions once the females arrive at the lek.
4. To determine mating duration and any possible correlation with the mating combination or type of cross (homotypic vs. heterotypic).
5. To determine possible changes in female postcopulatory behaviour.
6. To determine the impact on the above mentioned variables of the origin of the other population released in the field cage (presence of plasticity in the mating behaviour).
7. Determine sperm transfer.
8. Obtain couples to perform studies of postzygotic compatibility.

Discussion

Mating behaviour field cage tests aimed either to evaluate mating compatibility of male performance, are the best compromise between laboratory conditions and costly and/or impractical field observations to assess tephritid fly mating behaviour under semi-natural conditions.

Sexual compatibility is the degree to which two groups of animals tend to mate randomly without regard to their group of origin rather than mating selectively with members of their own group. For tephritid SIT programmes, the sexual compatibility between the target wild population and the candidate strain or population aimed at being transferred to mass rearing intended to be used for release should be measured before initiating any large-scale operations. In some tephritid species, sexual incompatibility could reveal the presence of sexually isolated populations and, to some further extent, the presence of cryptic species previously undetected.

The FAO/IAEA/USDA QC Manual offers a standard design for field-cage tests and it is recommend that within the CRP people follows this protocol with the particular modifications presented here. Data from these tests can be used to generate simple, reproducible, meaningful indices of sexual compatibility that can be used for making comparisons between populations. Given it was concluded in previous CRPs that the nutritional status, sex ratio and density of flies in relation to available canopy surface in the field cage influences test results, efforts should be made to strictly follow the standards described thereafter in order to minimize the impact of these variables on the overall results and thus permit comparisons among different research groups.
Sources and Handling of Flies

Biological material can come from already established laboratory strains or from wild populations. In both cases pupae should be placed in proper conditions to allow adult emergence. Within a few hours of adult emergence, select only flying adults and separate the sexes. Sexing has to be 100% effective. Female cages in which even one male is detected cannot be used for the tests and will have to be discarded, the same is recommended for male cages in which one female has been mislplaced. Then, hold the flies according to the day of emergence in laboratory cages (screen or Plexiglas) containing water and a food until sexually mature. Age of sexual maturity will vary with species or strain so in case no data is available, preliminary tests may be required to determine the appropriate testing age. The type of food to be given should be equal to all origins and should resemble the one the flies consume in nature. Moistened dry fruits such as figs is a good worldwide available option; however a source of protein is required. Preferably, the sexes should be placed into two separate rooms at a maximum density of ±40 flies per litre volume for the case of medfly and less for bigger flies such as some *Anastrepha* or *Bactrocera* spp.

Equipment

- All necessary equipment to maintain the flies in the laboratory from emergence until release in the field cage: Plexiglas cages with “Flight Ability” devices, aspirators to sex the flies, ≈1 l containers, water and food containers, etc.

- All necessary equipment to mark the flies according to the chosen technique (see below): (1) fluorescent powder; (2) water-based paint, thin soft camel hair brushes and a meshed bag; (3) cake colorants to dye the diet.

- Outdoor field cage, 2 m tall by 2.9 m in diameter, set up over a plant that fills a large portion of the volume of the cage (Error! Reference source not found.). The plant should be a local host plant of the fly species to be tested (citrus, guavas, etc.). Ideally, the plant should be rooted in the ground, but potted plants may suffice if ground-rooted plants are unavailable. Artificial trees may also be an option and have the advantage that flies are not exposed to plant volatiles that alter flies behaviour. Care must be taken in setting up the cages. The available foliage must provide an abundant substrate for mating behaviour, but could be lightly pruned (if necessary) so that flies will be visible to the observer. An average of 20 medium-sized leaves should be available per fly released in the field cage. If adequate foliage and light are available within the cages and if not more than 150-200 flies are released per cage (regardless of the type of field cage test done), little if any mating activity should take place on the screen of the cage.

- Plastic pill vials, scintillation vials, or similar containers of about 10-20 ml preferably clear, to collect the mating couples (depending on the amount of flies released 50 – 80 per cage).

- Grease pencils and/or masking tape and pens for marking vials.

- Long-wave ultra-violet lamp (should flies not be marked with paint and identifiable only by the presence of fluorescent dye) or fluorescent microscope.

- A minimum of 4 dental cotton wicks impregnated with water should be placed per tree as source of water for the flies.

- Data recording forms with pencil.

- Hydro-thermometer and luxmeter.
**Procedure**

Before releasing the flies in the cages, it would be necessary to mark them in order to distinguish at which strain/population they belong. Three methodologies are widely used and can be applied in conjunction:

1. dyeing with fluorescent powder as in operational programmes
2. painting the flies in the thorax with water based paint, or
3. providing the flies with diets dyed with non-toxic food colouring.

Each technique is applied at different times. In the case of fluorescent powder, dying occurs at the pupal stage before adult emergence with approximately 1.5 gr per kilo of pupae. In the case of painting the thorax, at least 24 to 48 hours before the test, flies are marked individually by applying a small dot of paint on the dorsal surface of the thorax. Flies can be immobilise by chilling them at approximately 5° C for a few minutes or by placing them in a bag made of mosquito net (18 mesh), placing the bag on a table, and holding the mesh down gently around each fly, one at a time. Use a thin, soft camel hairbrush to apply a small drop of paint to the fly. This procedure is illustrated in **Insert Figure 1**. For the case of adding the colorant to the diet, it is advisable to do it since adult emergence. A small drop of non-toxic food colouring is added to the adult food in order to obtain the same food with different colours. Flies that ingest the food with have their abdomen coloured with the provided colour as illustrated in **Figure 3**. Even though marking of flies with water-based paint or cake colorants appears to have no effect on mating performance field cage test results, colours used for marking strains should be randomised among replicates and brands of paints or colorants could be evaluated ahead of time to ensure that the mark does not affect fly behaviour or survival.

Immediately after marking, flies are transferred to containers suitable for releasing them into the field cages with water and food in groups of 25 – 50 flies, depending on the species, per 1 litre container (see **Insert Figure 24**).

**Insert Figure 1: Procedure for marking tephritid adult individuals with water-based paint.** A. Adults are gently blown from the aspirator into a bag; B. The bag is stretched to immobilize the fly and a drop of paint is made on the notum; C. The result: a marked Anastrepha fraterculus male calling in a field cage.

**Insert Figure 2: Flies dyed with non-toxic food colouring.**

**Insert Figure 4: Container used to hold the flies after individual marking and before releasing into the field cage.** A. Food (protein+sugar); B. Water; C. Label mentioning the strain, sex and number of adults.

On the day of the test, males are released into a screen cage and given a period of time (e.g., 15-30 min) to disperse and establish territories. The number of flies to be released depends on the species; for the case of medfly normally 50 flies of each strain and sex are released which totals 200 flies per cage while for other species it is recommended to release 25-30 flies of each sex and origin. Time of release should precede the time of peak mating for that species. Flies should be able to fly out of the container by themselves and should not be forced to do so by shaking the container or pushing flies out. Flies that are left inside the container, dead, deformed, or apparently incapable of flying should be replaced. Then, females are released.

Starting time of mating performance field cage test should be adjusted according to environmental conditions and fly activity (i.e. early start on hot days) and test duration should cover most of the known
sexual activity period for the species under the local conditions. In the absence of such information, the
test should cover most of the day. Ideally, tests should be run “blind”; i.e., technicians running the test
should not be told which colour of marking corresponds to wild or sterile. In addition colors should be
alternated randomly among replicates.

**Insert Figure 5: Collection of an *Anastrepha fraterculus* mating pair during mating performance
field cage test**

**Data gathering**

**Collection of mating couples**
After release of females, observers should screen the tree for mating couples. This can be done
continuously or a census of mating couples can be done under a certain frequency. Census frequency
should be at least every 10-15 minutes, or even shorter for species that have shorter mating durations.
Capture mating couples individually in the vials, taking care to get only one pair (and only 2 flies) per
vial. Look at the pair carefully ([Insert Figure 5]) in order to confirm that copulation is taking place;
males sometimes mount the female without but real genital contact. If flies are not marked with paint but
with fluorescent dye, keep them well identified (number each vial in accordance to the record is
followed in the form) for a later recognition under the UV lamp or the fluorescent microscope.

**Location of couples**
Write time the position on the tree (height, substrate, quadrant, and any information considered relevant)
or any other place on the cage from where the couple is collected. Record all pertinent data, including
time and type of male and female for each vial on the form. Label the vial accordingly, indicating the
number assigned to the field cage in which the mating took place (if more than one cage is run on a
given day), day of test and the number of the mating pair (couples should be numbered in the order they
are collected in a given cage, starting from 1, for the first mating pair). This is extremely critical and
attention should be paid in order not to mix the mating pairs.

**Mating duration**
Write time when pairs were captured on the form. Once the couple is in the vial, care must be taken to
ensure that the vials are kept in the shade and otherwise handled to minimize thermal or other stresses to
the flies. Observe mating couples in vials on a regular basis (every 5 min or less) and note the time when
uncoupling occurred.

Continue the test until the natural period of peak mating for that species under local conditions is well
over. Once the test is over, with the aid of an aspirator remove the flies that did not mate from the cage.
It is advisable to wash the tree to help removing any chemical signal left by the flies if the cage is to be
used on the following or consecutive days.

In addition to the standard procedure described in the previous paragraphs, it is recommended to collect
the following information:

**Male calling time:**
To determine the extent and timing of the males’ participation in pre-mating behaviours, a census
may be taken at regular intervals (e.g., every 10-15 min or half hour). During the census, the number,
location, and colour of mark are recorded for each male that is “calling”, or releasing pheromone within
the cage or involved in courtship with female. Calling males are typically (but not always) on the
underside of leaves. In medfly and in some *Anastrepha* species, they can be identified by the presence of
what appears to be a drop of liquid on the tip of the abdomen (in reality, a sac is extended from the anus) and males also may inflate pouches on the pleura (Figure 6). Calling males may intermittently vibrate or “fan” their wings while standing in place; during fanning, anal sac is partially retracted and held under the abdomen, and thus is more difficult to observe. These data can also be used to check diel periodicity of sexual behaviour among sterile males in comparison with wild males.

**Insert Figure 6: Anastrepha fraterculus calling male.**

**Incidence of remating**
This requires continuation of the test for a second or even more days. It is recommended then that this part is held under laboratory conditions to avoid dependency on weather conditions. Here again, care should be taken when collecting mating pairs during the first day to ensure that they are not disturbed to the point where they uncouple prematurely. Given that female behaviour is the core of this part, it is recommended that females are held singly in vials and two virgin males are offered every certain established period until females remate or the observation period ends. Remating rate and refractory period are the variables to be analyzed. The test should be run at least twice the time of the mean refractory period (if known) and virgin males of the same population of the females should be offered at regular intervals (i.e. every day, every two days or three times a week). If possible, an ovisposition substrate can be provided to the females and the number of eggs laid recorded to be used as a covariable in the analysis. If female receptivity is very sensitive to fly density, then it is recommended to run the test under relaxed conditions in order to allow the females reject the males. For meaningful data, 25 females of each category should be analyzed.

**Interpretation**

**Measures of sexual activity:**
For meaningful data, the basic mating performance field cage test (one-day observation) should be replicated ca. 6 - 10 times. Test replicates with more than ca. 25% of wild flies on the cage screen during calling periods may reflect inadequate environmental conditions (such as inadequate light, lack of water, leaves, etc.) and should therefore be repeated. In general it is suggested that fly activities occurring away from the host tree should not be included in the data analysis. This statement, however, should be taken with caution in compatibility tests given that the occurrence of *leks* and matings in the screen of the cage may also reflect some kind of spatial (i.e. ecological) isolation. For this reason it is recommended that any information should be recorded during the test and discarded if considered properly at the moment of data analysis.

**Proportion of mating (PM).** The proportion of flies mating measures the suitability of the flies and the environment for mating and is defined as

\[ PM = \frac{\text{No. of pairs collected}}{\text{No. of females released}} \]

Mean percentage of mating provides a useful indication of mating propensity. With *C. capitata*, mating propensity is considered adequate when 50% of flies from all combinations of strain and sex participate in mating. In practice, flies from some wild strains (especially the females) are more reluctant than sterile females to mate in field cages. At any rate, data from a cage should be discarded if less than 20% of both males and females from any strain participate in mating.

**Indices of strain sexual compatibility:**
Several indices have been developed to quantify the sexual compatibility between strains/populations. The indices should be computed separately for each cage. Analysis of test results
should involve the use of all of the main indices available (RII, ISI, FRPI, MRPI, see below for a
detailed description). It is advisable to include $\chi^2$ Goodness of Fit analysis to assess statistically
significant departure from random mating.

**Relative Isolation Index (RII).** The $RII$ is a measure of mating compatibility between two strains.

$$RII = \frac{AA \times BB}{AB \times BA}$$

Values of 1 indicates random mating between strains which is desirable in terms of SIT control; values
greater than one indicate positive assortative mating, i.e., males from population A tends to mate with
females from population A and vice versa (see Figure 7). The $RII$ has some advantages over other
indices of compatibility being the most relevant to this type of tests the fact that it is not affected by the
overall level of participation of the different types of flies, but only by whom they chose to mate.

![Figure 7: Graphic representation of the Relative Isolation Index (RII).](image)

*The value shown represents the mean value obtained when comparing wild and sterile *Ceratitis capitata* flies in field cages.*

However, $RII$ also has some disadvantages. First, it is undefined if one of the two heterotypic crosses is
zero. Second, when the number of matings in any one category is small, adding or subtracting a single
mating in that category will cause a large change in the value of the index. Third, it can be difficult to
normalize if the data are to be analysed statistically. Values of $RII$ larger than 1 indicate that there is
some difference in mating behaviour (in a broad sense) between the two populations, and that one or
both strains are tending to mate assortatively (i.e., like with like). Values of $RII$ that trigger corrective
action will probably be found to vary from species to species, and wild flies from some areas seem to
consistently produce higher $RII$’s than flies from other areas.

**Isolation Index (ISI).** The $ISI$ is a measure of mating compatibility.

$$ISI = \frac{(AA + BB) - (AB + BA)}{AA + BB + AB + BA}$$

Its values range from -1 (complete negative assortative mating; i.e., all matings are with members of the
opposite strain) through 0 (random mating) to +1 (complete positive assortative mating; total mating
isolation of the two strains) (see Figure 8). The main advantage of this index is that, by ranging from $-1$
to +1, it is easier to assess the deviation from the expected value of 0 than it is with index ranging from 0
to infinity. Compared to the $RII$, $ISI$ is not as sensitive to a change in a single mating and can always be
defined, whatever are the values of the 4 types of mating. In general, values of $ISI$ consistently larger
than 0.5 suggest that some positive assortative mating took place (which should be explained by analysing the values of MRPI and FRPI).

Figure 8: Graphic representation of the Isolation Index (ISI) and of the Male and Female Relative Performance indices (MRPI and FRPI). These indices should only be considered together for a better understanding. The value shown was obtained when comparing wild and sterile *Anastrepha ludens* flies in field cages (after Hernandez et al. 2003).

**Male Relative Performance Index (MRPI).** The MRPI is a relative measure of mating propensity of males of one population versus males from the other.

\[
MRPI = \frac{(AA + AB) - (BA + BB)}{AA + AB + BA + BB}
\]

A value of 1 indicates all matings in the cage were done by sterile males, and -1 indicates all mating was done by wild males. Zero indicates that wild and sterile males participated equally in mating (see Figure 8). This index must be used in addition to the mean percentages of different types of flies participating in mating, and complements the ISI and FRPI (below).

**Female Relative Performance Index (FRPI).** The FRPI is the counterpart of the MRPI and serves as a measure of mating propensity for female flies (see Figure 8).

\[
FRPI = \frac{(AA + BA) - (AB + BB)}{AA + AB + BA + BB}
\]
The joint analysis of ISI, MRPI and FRPI, should provide a complete and reliable picture of the sexual compatibility between strains and, should a deviation from the expected standard be encountered, the reasons why it occurred.

**Mating time:**
The time at which matings take place is species and/or strain specific, as such in this type of test it is important to evaluate of this trait is affected or not by the origin of the other population present.

**Copulation duration:**
Length of time spent *in copula* can be an indication of laboratory adaptation of a strain and can be related to transfer of sperm and accessory gland fluid to the female fly or the occurrence of some kind of guard posture. Duration of copulation can be compared among crosses by means of ANOVA or with non parametric statistics. In *C. capitata*, it has been noted that an exceptionally long duration of mating (>3 hours) very often resulted in no sperm being transferred to the female. However, short duration of mating for sterile males, relative to those of males mating with the same type of female, may be indicative of some kind of isolation, and may correlate with other related aspects, such as those on the incidence of remating.

**Male calling time:**
Calling, or releasing pheromone to attract female flies, is an early and critical step in a male’s effort to secure a mate. The incidence of calling is a component of mating propensity, and a low incidence of calling could be indicative of low fly quality or vitality. In addition, location of calling males is relevant in this type of tests given that they may reflect the occurrence or not of mixed leks.

**Female acceptance:**
Estimating the rate at which females accept males from their own origin or from the other requires more detailed observational studies, quantifying the number of female visits to the different types of calling and courting males, and recording rejection or acceptance of males by females. However, these data is of outmost importance in the case of incipient isolation between two origins.

**Incidence of remating:**
Remating in wild tephritid females is more common in nature than previously believed. This behaviour can be interpreted as the outcome from what the female received during the first mating and Postcopulatory decision affected by her reproductive potential. Failings in the first election may be reverted in a second mating and in the case of compatibility studies it may reflect a rejection of the first male.

**Modification to the mating performance field cage test**
Other variations of the mating performance field cage test may be conducted if deemed necessary. Examples of possible alternatives include: different ratios; use of various potted host plants; use of higher male:female ratio by releasing females slowly over time (as would be expected in natural leks in the field); replacement of individuals as the couples are formed; etc. In the specific case of this cryptic species complexes CRP it is desirable to agree to which extent changes are appropriate or may impede comparisons between investigations. Use of tests with alternate designs may be particularly valuable in diagnosing causes of less-than-desirable levels of sexual compatibility but it is recommended to perform first the agreed protocol.
Literature


M. Teresa Vera / July, 2010 for IAEA CRP
Appendix 8: Male Sex Pheromonal Components in *Bactrocera* Species – Extraction of Rectal (Pheromonal) Gland for Chemical Analyses

Jacentkovski in 1932 first demonstrated the existence of insect sex pheromone by using traps containing virgin gypsy moth females, which attracted and trapped many males, placed in gypsy moth infested woods. Only till 1959, Butenandt and coworkers after extracting from about half a million female silkworms, *Bombyx mori*, reported the first sex pheromone 'bombykol' was identified using traditional chemistry. We have come a long way since then, with the help of highly sensitive instrumentation and recording techniques, such as the gas chromatography-mass spectrophotometry (GC-MS) coupled to an electro-antennograph (EAG), a scientist can now extract and identify an insect's sex pheromone from a few individuals. In the case of fruit flies, particularly those from the genus *Bactrocera*, (unlike the lepidopterans) males produce sex pheromone at sexual maturity. For many species, males make use of external sources of chemical (attractant) to produce sex pheromonal component(s). Therefore, a single male may be sufficient to confirm identity of the sex pheromonal component(s) after feeding on the specific attractant, provided proper procedure is followed.

All *Bactrocera* species may be categorized into three groups based on their non-response (28 species confirmed and 258 species listed under “lures unknown”) or response to two very potent attractants – cue-lure (195 species) and methyl eugenol (ME) (ca 84 species) (IAEA 2000, 2005). Approximately a dozen of the ME responsive/sensitive species of *Bactrocera* including several putative sibling species of the *Bactrocera dorsalis* complex, such as *B. carambolae* Drew & Hancock, *B. dorsalis* (Hendel), *B. invadens* Drew, Tsuruta & White, *B. occipitalis* (Bezzi), *B. papayae* Drew & Hancock, and *B. philippinensis* Drew & Hancock form a group of serious polyphagous pests that currently cause high economic losses in the production of fruits. They are also very important quarantine pests that may interfere or disrupt international free and fair trade of fresh fruits and vegetables between exporting and importing countries globally.

Credit must be given to Howlett who first demonstrated that male tephritid flies (Dacus spp.) were attracted to citronella oil in 1912. He subsequently showed that the flies were actually attracted to ME in 1915. Since then, there were much speculations as to the actual role of ME in tephritid flies.

Males of *B. dorsalis* and *B. papayae* are strongly attracted to and compulsively feed on the ME which acts as a sex pheromone precursor that is converted to two major components - *E*-coniferyl alcohol and 2-allyl-4,5-dimethoxyphenol (Nishida et al. 1988; Tan and Nishida 1996, 1998). Nonetheless, the ME can also act as a booster component to complement endogenously produced sex pheromone as in *B. carambolae* in which it is converted only to *E*-coniferyl alcohol (Tan and Nishida 1996; Wee and Tan 2007). Table 1 shows the male sex pheromone components of several *Bactrocera* species after feeding on an attractant ME or raspberry ketone in the case of the melon fly *B. cucurbitae*. In the male *B. dorsalis/papayae*, the consumed ME is biotransformed in the crop and its metabolites are then transported via the haemolymph to the rectal gland (Hee and Tan 2006), where they are sequestered via rectal papillae and temporarily stored before being released as sex pheromone (Fig. 1) (Khoo and Tan 2005).

Native males seek and ingest ME from natural sources (Tan 2009; Tan et al. 2002, 2006). There are ca 350 plant species belonging to 61 families that possess ME, in varying quantities from trace amounts to >90% of essential oils, as a constituent component and/or release as a component of floral fragrance (Tan, compiled list unpublished). Consumption of the ME has been shown to improve significantly male mating performance and competitiveness of *B. dorsalis* (Shelly and Dewire 1994, 2000; Tan and

**Table 1. Sex pheromone after feeding on methyl eugenol or raspberry ketone**

<table>
<thead>
<tr>
<th>Species</th>
<th>Pheromone Components</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. carambolae</em></td>
<td>- E-coniferyl alcohol (CF) (endogenously produced - 6-oxo-1-nonanol + N-3 methylbutyl acetamide + 1,6-nonanediol + ethyl benzoate)</td>
</tr>
<tr>
<td><em>B. dorsalis</em></td>
<td>- 2-allyl-4,5-dimethoxyphenol (DMP) + CF + 3,4-dimethoxy-cinnamyl alcohol (DCA)</td>
</tr>
<tr>
<td><em>B. papayae</em></td>
<td>- DMP + CF + DCA</td>
</tr>
<tr>
<td><em>B. umbrosa</em></td>
<td>- DMP + DCA + 3,4-dimethoxy-hydroxyallyl-benzene</td>
</tr>
<tr>
<td><em>B. cucurbitae</em></td>
<td>- Raspberry ketone (endogenously produced - 1,3-nonanediol + methyl, ethyl &amp; propyl 4-hydroxybenzoate + N-3-methylbutyl methoxyacetamide)</td>
</tr>
</tbody>
</table>

**Figure 1.** Male rectal gland of *B. dorsalis* showing the presence of auto-fluorescent compounds during sequestration of sex pheromonal chemicals from the haemolymph at 10 to 120 minutes after feeding on methyl eugenol. (Adapted from Khoo and Tan (2005). White arrows – the chemicals detected within 1 hour.)
Figure 2. Male rectal glands extracted from sexually mature males (15 days old) – deprived of ME (left) and fed with ME (right - one day post-feeding)

With highly sensitive instrumentation such as the GC-MS that uses fine capillary column to analyze sex pheromonal components, it is very important that samples injected into the column must not contain contaminants (especially non-volatile compounds and macromolecules) that will clog up the column. It is also important to note that sex pheromonal compounds are usually volatile and exist in microgram or submicrogram levels. Therefore, it is pertinent to follow proper rectal gland extraction procedure, before preserving it for future chemical analyses.

1. Solvent for extraction preferably pure ethanol – redistilled (N.B. This solvent is very hygroscopic - so do not expose to atmospheric air for long periods, especially in the humid tropics). If this solvent is not available, then, absolute alcohol (ethanol) is suitable (N.B. Please provide a 1 ml sample for solvent check for possible impurities).
2. Prepare glass vials (1 or 2 ml with teflon lined screw-caps) for preservation of gland – fill each vial with 250 microlitre (0.25 ml) of the solvent and tighten the screw cap until use. (N.B. One vial for preservation of one rectal gland).
3. Fine pointed forceps – Firstly, wash and clean with a detergent; and secondly, rinse the tips with a few drops of the solvent just before use.
4. Immobilize a male fly, preferably without the use of carbon dioxide, by transferring the fly into a small clean plastic bag or cleaned container, making sure that the fly cannot escape, and then place it in either a refrigerator for 20-30 minutes at < 15° C or a freezer < - 5° C for 1-2 minutes.
5. Rectal gland removal - Place the immobilized fly ventral side up on the stage of a dissecting microscope (Fig. 3). One may hold the fly in place either with a pair of forceps or with one's fore finger and thumb. Then, with a pair of cleaned fine pointed forceps grip firmly the base of the aedaegus (Fig. 3A), and simultaneously pull it gently away from the fly's abdomen until the rectal gland is completely exposed (Fig. 3B & C) – due care should be taken, by avoiding sudden jerks, to ensure that the rectal sac does not burst especially when it is bloated. Then cut the hind gut at approximately 1 mm from the rectal gland (Fig 3 D). The freshly removed gland (Fig. 4) on the forceps tip is immediately introduced into a vial containing pure alcohol. After that, the teflon lined screw-cap is replaced and tightened (finger tight) to the vial. To avoid possible leakage during transportation (especially drastic climatic changes), the narrow gap between rim circumference of the screw-cap and the glass vial is tightly sealed by simultaneously stretching
and wrapping a parafilm strip (ca 1.5 cm x 5 cm) several times around it. After labeling the vial, store the extracted rectal gland in a refrigerator until dispatch for chemical analyses.

6. For comparison of sexually mature flies fed with and deprived of an attractant, e.g. methyl eugenol (ME) – item 5 needs to be repeated for rectal gland removal from the ME-fed and the ME-deprived (as controls) flies.

**Figure 3. Removal of rectal (pheromone) gland under magnification. Steps A-D.**

![Figure 3 A](image1.png) ![Figure 3 B](image2.png) ![Figure 3 C](image3.png) ![Figure 3 D](image4.png)

**Figure 4. Freshly removed male rectal gland and associated aedeagal apparatus.**

![Figure 4](image5.png)
References


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