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Use of mtDNA and nuclear markers for examining the genetic variation among Brazilian collections of the species complex *Anastrepha fraterculus*

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Introduction

The South American fruit fly, *Anastrepha fraterculus* (Wiedemann), ranges from northern Mexico to northern Argentina and is among the most serious agricultural pests in South America. Its impact to the economy is felt not only through loses from the direct damage to domestic production but also by the losses in revenue as a result of trade restrictions. In Brazil, *A. fraterculus* has been reported to infest 76 host species in 20 plant families (Zucchi 2008). Thus, while Brazil's annual production of fresh fruit is estimated at 43 million tons, only a little over 2% may be eligible for export due to the presence of agricultural pests in cultivated areas, among which fruit flies from the genus *Anastrepha* are one of the most economically important (Aluja 1994; Aluja *et al.* 1996; Norrbom & Foote 1989). This represents an annual potential loss of around 170 million USD in export revenues to Brazilian producers due solely to damage by fruit

flies. Further elucidation of the complex biology of *A. fraterculus* may yield molecular diagnostic methods that distinguish among the species. Also, since management strategies vary according to species, any substantial genetic variation observed could translate into subtle behavioral or morphological differences. This then provides an essential component to ongoing and future pest monitor and control efforts and the overall improvement of phytosanitary conditions in fruit production for Brazil and throughout its range.

A series of morphological and genetic studies have revealed that *A. fraterculus* actually comprises a complex of multiple species (Steck 1991, Silva 2000, Selivon *et al*. 2005, Silva & Barr 2008, for review). These studies, however, focused on detecting genetic differences between *A. fraterculus* populations based on small sample sizes and/or restricted geographic distributions of the species complex (e.g., Morgante *et al.* 1980, Steck 1991, Steck & Sheppard 1993, Santos 1994, Santos & Matioli 1997, Alberti *et al*. 1996, Alberti *et al*. 2002, Basso *et al*. 2003, Goday *et al*. 2006, Alberti *et al*. 2008) and often did not directly address the systematic relationships among the putative cryptic species. A few exceptions did compare the evolutionary relationships of the genetic differences (e.g., Morgante *et al*. 1980, McPheron *et al*. 1999, Barr *et al*. 2005) but these studies often included very few *A. fraterculus* samples. A synthesis of molecular data sets from existing literature is precluded because the original authors applied different methodologies (e.g., isozymes, karyotypes, RFLP, RAPD, and DNA sequences) or genetic loci (e.g., 16S rDNA, COI, *period*) to analyze their *A. fraterculus* collections. Consequently, most our understanding of the *A. fraterculus* complex is not based on phylogenetic models or evolutionary theory but rather a process of alpha-taxonomy using genetic and morphometric characters to document differences among populations.

The actual number of putative species within the *A. fraterculus* complex and their associated biogeography is yet uncertain. Based on mating studies at least four putative species are recognized: *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). Silva and Barr (2008) provide a summary of these and other putative species based on geography; this list includes an Andean type (Steck 1991), a Mexican type (also found in Costa Rica and Venezuela) (Steck 1991, Hernandez-Ortiz *et al*. 2004), a Guatemalan type (Smith-Caldas *et al*. 2001), a Venezuelan type (Smith-Caldas *et al*. 2001), and a Peruvian type (Steck 1991). The identification of these geographic lineages is based on several studies that

each applied unique DNA markers. Consequently, a single population could be described multiple times as several unique lineages if analysed using different DNA markers in the different studies. Based on all available information, Brazilian populations of the nominal species *A. fraterculus* most likely represent at least three species. However, previous genetic studies carried out in Brazil focused mainly on populations collected in the state of São Paulo and very few populations from other states.

The study of species limits within the *A. fraterculus* complex requires application of appropriate species concepts to describe species or identify putative species. Previous work by Selivon *et al*. (2004, 2005) has applied the biological species concept (Dobzhansky 1937) to lineages by testing mating incompatibility under lab conditions. Although an important approach, this method requires that the putative species be identified prior to mating studies and does not consider possible mating isolation mechanisms that are not present in the lab setting. Other concepts such as the phylogenetic species concept (Cracraft 1983) can provide an alternative source of information to the taxonomic problem. Application of the phylogenetic species concept using DNA sequences derived from multiple loci is a practical way to develop a common database of *Anastrepha* species and populations, identify unique lineages, and quantitatively compare variation within and among lineages. Based on these estimates it is possible to optimize subsequent crossing experiments to confirm mating incompatibility. Funk and Omland (2003) described this approach to the study of species-limits as "congeneric phylogeography" for mitochondrial studies of closely related species. The analysis of the COI gene by Smith-Caldas *et al*. (2001) is an example of this approach. That study included 16 specimens of *A. fraterculus* plus representatives of other species and the analysis identified several cryptic lineages. An expansion of this approach to include more populations of the *A. fraterculus* complex should facilitate discovery of cryptic lineages and provide biogeographic information regarding putative species. Analysis of the species using both mitochiondrial and nuclear DNA sequence markers enables hypothesis testing of species-level status (Barr & McPheron 2006, Virgilio *et al*. 2008, Barr & Wiegmann 2009).

Understanding both the species-limits and population-level variation of species are crucial to the development and maintenance of SIT programs. By developing robust and informative molecular markers and associated databases that represent geographic variation of each species, it is possible to target the appropriate putative species for rearing, target diverse population(s) of a species for initiating and out-

crossing of lab colonies, and monitor genetic diversity of SIT lab colonies for inbreeding or contamination. Due to its economic importance, *A. fraterculus* is one of the targeted species for the Medfly program in Brazil. Therefore, it is paramount to study populations from a wide geographic range in Brazil to identify genetically isolated populations. Results of such analysis can direct behavioral studies to verify mating compatibility prior to the implementation of area-wide management programs.

The major goal of this project is to collect samples from representative populations of the various putative species of *A. fraterculus* throughout Brazil that will be suitable for genetic, morphological, and behavioral studies; to acquire and analyze DNA sequence data that can be integrated with data from other studies with the ultimate goal of naming presently undescribed species in the *fraterculus* complex in Brazil.

This report describes our results regarding collections of different *A.fraterculus* populations in Brazil as well as other related *Anastrepha* species for the phylogenetic analysis and the screening of populations for one mitochondrial DNA marker (COI).

Materials and methods

1. Collection of *A. fraterculus* populations

We collected *A*. *fraterculus* samples from several locations in the state of Bahia. Collections were also carried out in the states of São Paulo and Rio Grande do Sul through collaborations with other colleagues. Entire bodies from adult specimens are maintained in 100% ethanol at –20°C at Centro de Biotecnologia e Genética, Universidade Estadual de Santa Cruz, Ilhéus, Bahia, Brazil prior to analysis.

2. DNA extraction

DNA was isolated using the DNeasy® Blood and Tissue Kit (Qiagen) following standard DNeasy guidelines for animal tissues or the protocol by Han & McPhron (1997). DNA was extracted from one or two legs from each specimen per collection and the body was kept as voucher. Extractions are stored at –20°C.

3. DNA amplification and sequencing

COI region

Samples were amplified first for the COI region of the mitochondrial genome using primers and amplification conditions that have already been tested for *A. ludens*, *A. obliqua, A. fraterculus,* and *A. sororcula* from several locations and are likely to be useful at the level of cryptic species (Smith-Caldas *et al*., 2001; Barr *et al*. 2006; Ruiz, 2010). Amplifications were performed in an Eppendorf Mastercycler®. PCR products were stained with ethidium bromide and visualized on 1.5% electrophoresis agarose gels. Documentation of these gels was via a GE Image Quant 350 digital imaging system. Amplification products were purified with ExoSAP-IT (USB Corp.) prior to sequencing. PCR products were sequenced on an ABI 3730XL DNA Analyzer with the ABI Data Collection Program (v 2.0) at the Centro de Estudos do Genoma Humano, Universidade de São Paulo, São Paulo, Brazil.

16S, ITS1 and ND6 regions

We also carried out tests with primers 16S (McPheron et al. 1999), ITS1 (Prezotto, 2008), and ND6 (Ruiz 2009) using samples of several *Anastrepha* species.

Results and Discussion

1. Collection of *A. fraterculus* populations

We obtained samples of *A. fraterculus* from 22 localities in the state of Bahia, one locality on the state of São Paulo, one locality in the state of Rio Grande do Sul in Brazil. We also obtained samples from one locality in Mexico and one locality in Argentina with the help of Juan Rull and Teresa Vera. We also obtained samples of *Anastrepha obliqua, Anastrepha bahiensis, Anastrepha coronilli, Anastrepha distincta*, and *Anastrepha turpiniae* in the *fraterculus* group and *Anastrepha curitis, Anastrepha pickeli, Anastrepha serpentina, Anastrepha pulchra, Anastrepha striata* and *Anastrepha grandis* from different localities in Brazil that will be used in the phylogenetic analysis (Table 1).

2. DNA extraction, amplification and sequencing

We extracted DNA from a total of 185 *A. fraterculus* specimens and 59 specimens of other *Anastrepha* species, totalling 244 specimens extracted (Table 1). The methods used worked well for the specimens analyzed so far (Figure 1).

Table 1 – Collection data and information on *Anastrepha* species extracted.

Country	Locality	State	Species	Nº samples extracted
Brazil	Manaus	Amazonas	*A. obliqua*	5
Brazil	Rio Preto	Amazonas	*A. obliqua*	5
Brazil	Vitória da Conquista	Bahia	*A. obliqua*	5
Brazil	Itapé	Bahia	*A.fraterculus*	10
Brazil	Ipiaú	Bahia	*A.fraterculus*	9
Brazil	Wenceslau Guimarães	Bahia	*A.fraterculus*	5
Brazil	Taperoá	Bahia	*A.fraterculus*	8
Brazil	Valença	Bahia	*A.fraterculus*	7
Brazil	Ibirataia	Bahia	*A. fraterculus*	10
Brazil	Porto Seguro	Bahia	*A. fraterculus*	9
Brazil	Una	Bahia	*A.fraterculus*	4
Brazil	Nova Ibiá	Bahia	*A. fraterculus*	4
Brazil	Itacaré	Bahia	*A. fraterculus*	5
Brazil	Camamu	Bahia	*A. fraterculus*	9
Brazil	Itabuna	Bahia	*A. fraterculus*	4
Brazil	Gandu	Bahia	*A. fraterculus*	10
Brazil	Jitaúna	Bahia	*A. fraterculus*	9
Brazil	Ilhéus	Bahia	*A. fraterculus*	7
Brazil	Ituberá	Bahia	*A. fraterculus*	5
Brazil	Livramento de Nossa Senhora	Bahia	*A. fraterculus*	6
Brazil	São Matheus	Espiríto Santo	*A. fraterculus*	10
Brazil	Laranjal Paulista	São Paulo	*A. fraterculus*	5
Brazil	Itiruçu	Bahia	*A. fraterculus*	3
Brazil	Eunapólis	Bahia	*A. fraterculus*	2
Brazil	Itaberaba	Bahia	*A. fraterculus*	1
Brazil	Santa Maria da Vitória	Bahia	*A. fraterculus*	1
Brazil	Anagé	Bahia	*A. fraterculus*	2
ARGENTINA	Tucuman	Argentina	*A. fraterculus*	14
Brazil	Itaguaquecetuba	São Paulo	*A. fraterculus*	5
Mexico	-	México	*A. fraterculus*	11
Brazil	Vacaria	Rio Grande do Sul	*A. fraterculus*	10
Brazil	Manaus	Amazonas	*A. curitis*	4
Brazil	Manaus	Amazonas	*A. bahiensis*	4
Brazil	Manaus	Amazonas	*A. coronilli*	2
Brazil	-	Roraima	*A. pickeli*	2
Brazil	Maracás	Bahia	*A. pickeli*	2
Brazil	Manaus	Amazonas	*A.coronilli*	2
Brazil	Manaus	Amazonas	*A. turpiniae*	4
Brazil	Maués	Amazonas	*A. distincta*	2
Brazil	Manaus	Amazonas	*A. serpentina*	1
Brazil	Manaus	Amazonas	*A. pulchra*	1
Brazil	Manaus	Amazonas	*A. striata*	1
Brazil	-	São Paulo	*A. grandis*	19
			Total	**244**

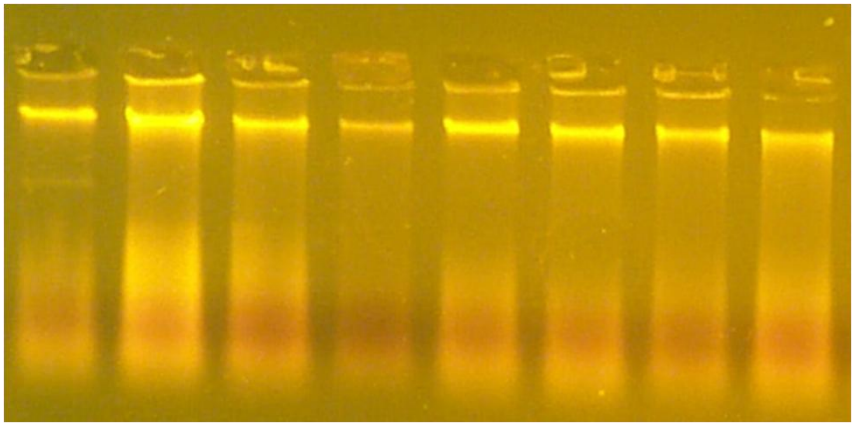

Figure 1 – Total nucleic acid extractions of *A. fraterculus* on 1% agarose gel

COI region

We amplified and sequenced 145 samples of *A. fraterculus* from different localities for the COI region and 59 samples of other *Anastrepha* species totalling 204 sequences (Figure 2, Table 2). We obtained sequences that ranged from 803 bp to 1056 bp for this region. We are aligning the sequences and will be analyzing them soon.

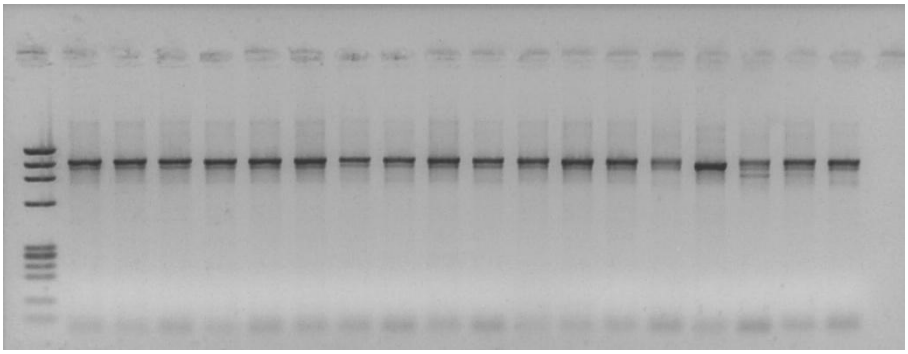

Figure 2 – PCR products of *A. fraterculus* for the COI region

We also have sequences for the COI region for 95 *Anastrepha* spp. totalling 463 sequences through collaboration between our team (Janisete Silva ,Bruce McPheron, Norman Barr, Raul Ruiz, Roberto Zucchi) and Allen Norrbom, Peter Kerr, Don Thomas, and Beatriz Ronchi-Teles.

Table 2 – Collection data and information on *Anastrepha* species sequenced for the COI region.

Country	Locality	State	Species	Nº samples sequenced
Brazil	Manaus	Amazonas	*A. obliqua*	5
Brazil	Rio Preto	Amazonas	*A. obliqua*	5
Brazil	Vitória da Conquista	Bahia	*A. obliqua*	5
Brazil	Itapé	Bahia	*A.fraterculus*	10
Brazil	Ipiaú	Bahia	*A.fraterculus*	9
Brazil	Wenceslau Guimarães	Bahia	*A.fraterculus*	5
Brazil	Taperoá	Bahia	*A.fraterculus*	8
Brazil	Valença	Bahia	*A.fraterculus*	7
Brazil	Ibirataia	Bahia	*A. fraterculus*	10
Brazil	Porto Seguro	Bahia	*A. fraterculus*	9
Brazil	Una	Bahia	*A.fraterculus*	4
Brazil	Nova Ibiá	Bahia	*A. fraterculus*	4
Brazil	Itacaré	Bahia	*A. fraterculus*	5
Brazil	Camamu	Bahia	*A. fraterculus*	9
Brazil	Itabuna	Bahia	*A. fraterculus*	4
Brazil	Gandu	Bahia	*A. fraterculus*	10
Brazil	Jitaúna	Bahia	*A. fraterculus*	9
Brazil	Ilhéus	Bahia	*A. fraterculus*	7
Brazil	Ituberá	Bahia	*A. fraterculus*	5
Brazil	Livramento de Nossa Senhora	Bahia	*A. fraterculus*	6
Brazil	São Matheus	Espiríto Santo	*A. fraterculus*	10
Brazil	Laranjal Paulista	São Paulo	*A. fraterculus*	5
Brazil	Itiruçu	Bahia	*A. fraterculus*	3
Brazil	Eunapólis	Bahia	*A. fraterculus*	2
Brazil	Itaberaba	Bahia	*A. fraterculus*	1
Brazil	Santa Maria da Vitória	Bahia	*A. fraterculus*	1
Brazil	Anagé	Bahia	*A. fraterculus*	2
Brazil	Manaus	Amazonas	*A. curitis*	4
Brazil	Manaus	Amazonas	*A. bahiensis*	4
Brazil	Manaus	Amazonas	*A. coronilli*	2
Brazil	-	Roraima	*A. pickeli*	2
Brazil	Maracás	Bahia	*A. pickeli*	2
Brazil	Manaus	Amazonas	*A.coronilli*	2
Brazil	Manaus	Amazonas	*A. turpiniae*	4
Brazil	Maués	Amazonas	*A. distincta*	2
Brazil	Manaus	Amazonas	*A. serpentina*	1
Brazil	Manaus	Amazonas	*A. pulchra*	1
Brazil	Manaus	Amazonas	*A. striata*	1
Brazil	-	São Paulo	*A. grandis*	19
			TOTAL	**204**

16S, ND6 and ITS1 regions

We carried out some tests with the 16S, ITS1 and ND6 primers and obtained good amplifications for samples of *A. fraterculus* as well as other *Anastrepha* species (Figures 3 -5).

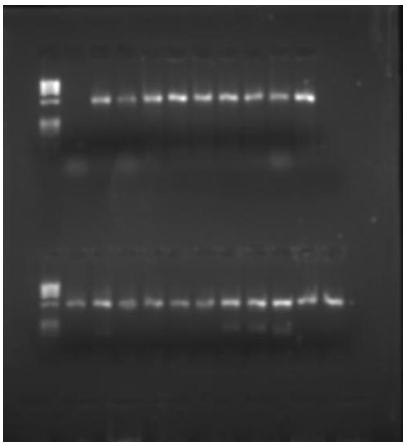

Figure 3 – PCR products of *Anastrepha* spp. for the 16S region

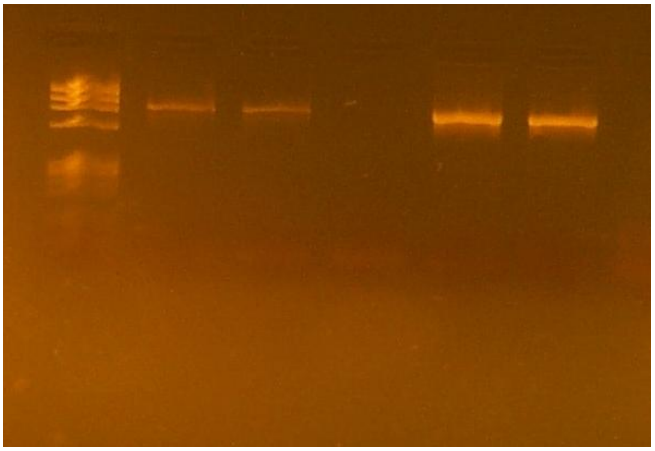

Figure 4 – PCR products of *Anastrepha* spp. for the ITS1 region

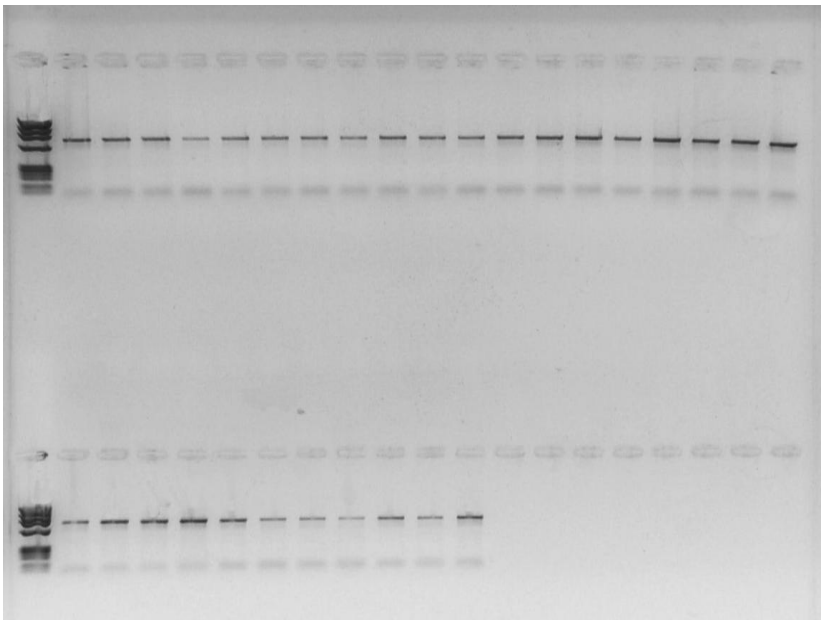

Figure 5 – PCR products of *A. fraterculus* for the ND6 region

Priorities for future work:

1.Continue to obtain samples from *A. fraterculus* populations in Brazil through collaborations with colleagues at Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) in different states, Instituto Biológico de São Paulo in the state of São Paulo, Universidade Federal Rural do Semi-Árido in the state of Rio Grande do Norte, Universidade Federal de Alagoas in the state of Alagoas and other collaborations that we are trying to establish.

2.Continue assessment of DNA variation within and among populations of *A. fraterculus and* other related *Anastrepha* species for the phylogenetic analysis and the screening of populations for other mitochondrial and nuclear DNA markers.

3. Begin phylogenetic tree reconstruction using DNA sequences will be estimated using Bayesian, maximum parsimony (MP), and maximum-likelihood (ML) approaches. Sequences of *A. fraterculus* populations and other *Anastrepha* species (e.g. *A. obliqua, A. ludens, A. sororcula, A. pickeli,* among others) will be included to provide proper analysis of species-limits.

4. Begin assessment of DNA variation using microsatellites.

References

ALBERTI, A. C., G. CALCAGNO, B. O. SAIDMAN, J. C. VILLARDI. Genetic structure of a natural population of *Anastrepha fraterculus* (Wied.) (Diptera: Tephritidae). In: 2nd Meeting of the working group on fruit flies of the western hemisphere, Viña del Mar, Chile, 1996. Abstracts. Viña del Mar, Chile, p. 40.

ALBERTI, A. C., M.S. RODRIGUERO, P. G. CENDRA, B. O. SAIDMAN, J. C. VILLARDI. Annals of the Entomological Society of America 95(4): 505-512, 2002.

ALBERTI, A.C.; CONFALONIERI, V.A.; ZANDOMENI, R.O.; VILARDI, J.C. Phylogeographic studies on natural populations of the South American fruit fly, *Anastrepha fraterculus* (Diptera : Tephritidae). Genetica 132: 2008 1-8.

ALUJA, M. Bionomics and management of *Anastrepha*. Annual Review of Entomology 39: 155-178, 1994.

ALUJA, M.; CELEDONIO-HURTADO, H.; LIEDO, P.; CABRERA, M.; CASTILLO, F.; GUILL, J.; RIOS, E. Seasonal population fluctuations and ecological implications for management of *Anastrepha* fruit flies (Diptera: Tephritidae) in commercial mango orchards in southern Mexico. Journal of Economic Entomology 89: 654-667, 1996.

ARMSTRONG, K.F., C.M. CAMERON, E.R. FRAMPTON. Fruit fly (Diptera: Tephritidae) species identification: a rapid molecular diagnostic techinique for quaratine application. Bull.Ent.Res. 87: 111-118, 1997.

BARR, N. B., B.M. WIEGMANN. Phylogenetic relationships of *Ceratitis* fruit flies inferred from nuclear CAD and *tango*/ARNT gene fragments: Testing monophyly of the subgenera *Ceratitis* (*Ceratitis*) and *C*. (*Pterandrus*). Molecular Phylogenetics and Evolution 53: 412-424, 2009.

BARR, N. B.; B. A. MCPHERON. Molecular phylogenetics of the genus *Ceratitis* (Diptera: Tephritidae). Molecular Phylogenetics and Evolution 38: 216-230, 2006.

BARR, N.B.; COPELAND, R.S.; DE MEYER, M.; MASIGA, D.; KIBOGO, H.G.; BILLAH, M.K.; OSIR, E.; WHARTON, R.A.; MCPHERON, B.A. Molecular diagnostics of economically important *Ceratitis* fruit fly species (Diptera : Tephritidae) in Africa using PCR and RFLP analyses. Bulletin of Entomological Research 96: 505-521, 2006.

BARR, N.B.; LIWANG, C.; MCPHERON, B.A. Molecular systematics of nuclear *period* in genus *Anastrepha* (Tephritidae). Annals of the Entomological Society of America, v. 98, n. 173-180, 2005.

BASSO, A. C., A. SONVICO, L. A. QUESADA-ALLUE, F. MANSO. Karyotipic and molecular identification of laboratory stocks of the South American fruti fly *Anastrepha fraterculus* (Wied.) (Diptera: Tephritidae). Journal of Economic Entomology 96: 1237-1244, 2003.

CRACRAFT, J, Species concepts and speciation analysis, Current Ornithology. 1: 159–187, 1983.

DOBZHANSKY, T. 1937. Genetics and the Origin of Species. Columbia University Press, New York.

FUNK D.J., K.E. OMLAND.Species-level paraphyly and polyphyly: Frequency, causes and consequences, with insights from animal mitochondrial DNA. Annual Review Ecology, Evolution and Systematics 34: 397–423, 2003.

GODAY, C., D. SELIVON, A. L. P. PERONDINI, P. G. GRACIANO, M. F. RUIZ. Cytological characterization of sex chromosomes and ribosomal DNA location in *Anastrepha* species (Diptera: Tephritidae). Cytogenetic and Genome Research 114: 70-76, 2006.

GOUDET, J. FSTAT (Version 1.2): A computer program to calculate F-statistics. Journal of Heredity 86: 485-486, 1995.

HERNANDEZ-ORTIZ V, GOMEZ-ANAYA JA, SANCHEZ A, MCPHERON BA, ALUJA M. Morphometric analysis of Mexican and South American populations of the *Anastrepha fraterculus* complex (Diptera : Tephritidae) and recognition of a distinct Mexican morphotype. Bulletin of Entomological Research 94, 487-499, 2004.

INSTITUTO BRASILEIRO DE FRUTAS. Soluções fruta a fruta. São Paulo. Disponível em: www.ibraf.com.br, 2005. Acessado em fevereiro/2005.

MCPHERON, B.A.; HAN, H.Y.; SILVA, J.G.; NORRBOM, A.L. Phylogeny of the genus *Anastrepha* and *Toxotrypana* (Trypetinae: Toxotrypanini) based upon 16SrRNA mitochondrial DNA sequences, p.343-361. *In*: Aluja, M.; Norrbom, A.L. (eds.), Fruit flies (Tephritidae): Phylogeny and evolution behavior. Boca Raton, CRC Press, 944p, 1999.

MORGANTE, J. S.; A. MALAVASI; G. L. BUSH. Biochemical systematics and evolutionary relationships of Neotropical *Anastrepha*. Annals of the Entomological Society of America 73: 622-630, 1980.

NORRBOM, A. L., R. H. FOOTE. The taxonomy and zoogeography of the genus Anastrepha (Diptera: Tephritidae), pp. 15-26 in A.S. Robinson and G. Hooper, [eds.], World Crop Pests. Fruit Flies, their biology, natural enemies and control. Amsterdam, Elsevier Science. v. 3A, 1989.

ROZAS, J.; SANCHEZ-DELBARRIO, J.C.; MESSEGUER, X.; ROZAS, R. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496-2497, 2003.

RUIZ-ARCE, R. Phylogeography of *Anastrepha ludens* and *Anastrepha obliqua*. 105p. Tese (Doutorado em Entomologia) – Department of Entomology, The Pennsylvania State University, University Park, PA, E.U.A., 2009.

SANTOS, P. Variabilidade do DNA mitocondrial em populações de três espécies de *Anastrepha* (Diptera: Tephritidae). São Paulo, SP, 73p. (MSc. Thesis USP), 1994.

SANTOS, P., S. R. MATIOLI. Mitochondrial and ribosomal DNA variation in Brazilian populations of three *Anastrepha* species (Diptera: Tephritidae). p. 47. In 2nd Meeting of the working group on fruit flies of the western hemisphere, Viña del Mar, Chile, 1997. Abstracts. Viña del Mar, Chile, 1997.

SELIVON, D., A. L. P. PERONDINI, J. S. MORGANTE. A genetic-morphological characterization of two cryptic species of the *Anastrepha fraterculus* complex (Diptera: Tephritidae). Annals of the Entomological Society of America 98(3): 367-381, 2005.

SILVA, J. G. Estudos moleculares. *In*: Malavasi, A., Zucchi, R.A. (eds.). Moscas-das-frutas de importância econômica no Brasil. Conhecimento básico e aplicado. Ribeirão Preto: Holos, Editora, p. 29-39, 2000.

SILVA, J.G.; BARR, N.B. Recent advances in molecular systematics of *Anastrepha* Schiner, pp. 13-18. *In* Sugayama, R.L., R.A. Zucchi, S.M. Ovruski, and J. Sivinski (eds.). Proceedings of the 7th International Symposium on Fruit Flies of Economic Importance. Color Press, Salvador, Bahia, Brazil.

SMITH-CALDAS, M.R.B.; MCPHERON, B.A.; SILVA; J.G.; ZUCCHI, R.A. Phylogenetic relationships among species of the *fraterculus* group *(Anastrepha*: Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase I. Neotropical Entomology v.30, p.565-573, 2001.

STECK, G. J. Biochemical systematics and population genetic structure of *Anastrepha fraterculus* and related species (Diptera: Tephritidae). Annals of the Entomological Society of America 84: 10-28, 1991.

STECK, G. J., W. S. SHEPPARD. Mitochondrial DNA Variation in *Anastrepha fraterculus*. pp. 9-14. In M. Aluja and P. Liedo (eds.) Fruit flies: biology and management. Springer-Verlag, New York, NY, USA, 1993.

TAMURA, K.; DUDLEY, J.; NEI, M.; KUMAR, S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599, 2007.

THOMPSON, J.D.; HIGGINS, D.G.; GIBSON, T.J. Clustal-w - improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673-4680, 1994.

URAMOTO, K. Biodiversidade de moscas-das-frutas do gênero *Anastrepha* (Diptera, Tephritidae) no Campus Luiz de Queiroz, Piracicaba, São Paulo. 85p. Dissertação (Mestrado) - Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo, 2002.

VIRGILIO, M. T. BACKELJAU, N. BARR, M. DE MEYER. Molecular evaluation of nominal species in the *Ceratitis fasciventris*, *C. anonae*, *C. rosa* complex (Diptera: Tephritidae). Molecular Phylogenetics and Evolution 48: 270–280, 2008.

YAMADA, S, SELIVON, D. Rose, an eye color mutation in a species of the Anastrepha fraterculus complex (Diptera: Tephritidae). Annals of the Entomological Society of America, v. 94, n. 4, p. 592-595, 2001.

ZUCCHI, R.A. Diversidad, distribución y hospederos del género *Anastrepha* en Brasil. *In:* V. Henández-Ortiz (Ed.), Moscas de la Fruta en Latinoamérica (Diptera: Tephritidae): Diversidad, biologia y manejo. S y G editores, Distrito Federal, México.pp. 77-100, 2007.