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), geographic distribution range is 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 losses 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 2007). 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* is needed and 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 monitoring and pest management 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 *A. fraterculus* collections. Consequently, our understanding of the *A. fraterculus* complex is largely based on alpha-taxonomy using genetic and morphometric characters to document differences among populations. Incorporating a phylogenetic approach to these systematic questions will enable comparative studies between both populations and species and provide in a greater understanding of the significance of observed differences.

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). A careful analysis of genetic results is required to avoid overestimating the
number of putative species. The development of a common DNA marker that is available to all researchers should facilitate comparison of genotypes across 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 mitochondrial 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 the 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

In this third year, samples of *A. fraterculus* and other *Anastrepha* species were collected from the Amazon region in Brazil through collaborative efforts. Our collaborators at the USDA and Penn State University also contributed with specimens of *A. fraterculus* and other *Anastrepha* species from their collections. Identification of the specimens was based on the female morphology and carried out by experts. Entire bodies from adult females were maintained in 100% ethanol at –20°C at Centro de Biotecnologia e Genética, Universidade Estadual de Santa Cruz, Ilhéus, Bahia, Brazil and USDA, Texas, USA, prior to analysis.

2. DNA extraction

DNA was isolated using the DNeasy<sup>®</sup> Blood and Tissue Kit (Qiagen) following standard DNeasy guidelines for animal tissues. DNA was extracted from one or two legs from each specimen per collection and the body was kept as voucher. Extractions were 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-Arce et al., 2012). 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.

4. Microsatellites
We carried out amplification tests using 16 non-labeled primers selected among the primers developed for *A. fraterculus* by Dr. Silvia Lanzavecchia (INTA – Argentina) and Dr. Anna Malacrida (Università di Pavia, Italy) (Lanzavecchia et al. unpublished data) as part of a collaborative effort that was initiated during this CRP.

Reaction components consisted of 1µL of 10X buffer, 0.6 µL of MgCl₂ (25mM), 1 µL of dNTP mix (2mM), 0.5µL of primer F (10µM), 0.5 µL of primer R (10µM), 0.08µL of Taq (5U/ µL), 2.23µL of MiliQ H₂O, and 4µL of DNA at 30ng/ µL to complete the final volume of 10 µL. Amplifications were performed in a MultiGene Thermal Cycler TC9600-G (Labnet International Inc.). Cycling conditions for amplification of fragments were 5 min at 94°C followed by 29 cycles of 30 sec at 94°C, 30 sec at 58°C (primer set dependent, see Table 1), 30 sec at 72°C, and a final extension of 5 min at 72°C. PCR amplicons were verified electrophoresis agarose gels prior to fragment analysis.

A total of 5 specimens per population for four populations (Table 1) were tested with the selected primers that would be used to screen all specimens in the four populations. We selected 10 primers (Table 2) that amplified well within the expected size and without unspecific bands. These amplification reactions followed the same conditions described above with the forward primers 5’ end-labeled with fluorescent dye for laser beam detection of DNA fragments with the ABI.
3130 XL (Applied Biosystems) for fragment separation. Allele size was scored against the internal-lane 500 ROX standard (Bioventures) using GeneMarker HID version 2.4.0 (Soft Genetics, State College, PA).

Table 1 – Primers with their respective sizes and annealing temperatures.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Size (bp)</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>A10</td>
<td>130-150pb</td>
<td>58°C</td>
</tr>
<tr>
<td>D4</td>
<td>140-165pb</td>
<td>58°C</td>
</tr>
<tr>
<td>D105</td>
<td>140-165pb</td>
<td>58°C</td>
</tr>
<tr>
<td>A7</td>
<td>120-160pb</td>
<td>58°C</td>
</tr>
<tr>
<td>A112</td>
<td>125-160pb</td>
<td>55°C</td>
</tr>
<tr>
<td>A115</td>
<td>125-150pb</td>
<td>55°C</td>
</tr>
<tr>
<td>A117</td>
<td>145-180pb</td>
<td>58°C</td>
</tr>
<tr>
<td>A120</td>
<td>150-200pb</td>
<td>58°C</td>
</tr>
<tr>
<td>A122</td>
<td>125-150pb</td>
<td>58°C</td>
</tr>
<tr>
<td>C103</td>
<td>150-170pb</td>
<td>60°C</td>
</tr>
</tbody>
</table>

Table 2 – Samples of A. fraterculus from four populations in Brazil used in the microsatellite analysis

<table>
<thead>
<tr>
<th>Species</th>
<th>State</th>
<th>Locality</th>
<th>Specimens (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. fraterculus</td>
<td>Bahia</td>
<td>Una</td>
<td>30</td>
</tr>
<tr>
<td>A. fraterculus</td>
<td>Bahia</td>
<td>Porto Seguro</td>
<td>27</td>
</tr>
<tr>
<td>A. fraterculus</td>
<td>Espírito Santo</td>
<td>São Mateus</td>
<td>23</td>
</tr>
<tr>
<td>A. fraterculus</td>
<td>Rio Grande do Norte</td>
<td>Monte Alegre</td>
<td>30</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>110</strong></td>
</tr>
</tbody>
</table>

5. Morphometric analysis

Members of our research team participated in the training course “Micromorphometric analysis of South American Anastrepha fraterculus, a cryptic species of fruit fly for Central America” taught by Dr. Vicente Hernandez-Ortiz in Brazil in November 2013 and financed by the IAEA. We started to analyze populations from distinct geographic localities in Brazil using morphometrics in collaboration with Drs. Vicente Hernandez-
Results and Discussion

1. Collection of *A. fraterculus* populations

We obtained samples of *A. fraterculus* from two localities in the Amazon region, one locality in the southeastern region and one locality in the southern region in Brazil. We also obtained samples of *A. obliqua* from 10 localities in the state of Amapá in the Amazon region in Brazil. These samples were in addition to the samples reported last year (Table 3 and data not shown but available upon request).

**Table 3** – Samples of *Anastrepha fraterculus* collected and sequenced

<table>
<thead>
<tr>
<th>Country</th>
<th>Localities (N)</th>
<th>Number of sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Brazil</td>
<td>35</td>
<td>216</td>
</tr>
<tr>
<td>Belize</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Bolivia</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ecuador</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Guatemala</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Mexico</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>Peru</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Venezuela</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td><strong>266</strong></td>
</tr>
</tbody>
</table>
2. DNA extraction, amplification and sequencing

We extracted DNA from a total of 105 samples of *Anastrepha* (30 specimens of *A. fraterculus* and 75 specimens of *A. obliqua*) from Brazil. We screened from 5-10 specimens from each population for one mitochondrial DNA marker (COI) totalling 75 samples for sequencing of the COI region. The 20 samples of *A. fraterculus* from the Amazon region failed to amplify and thus could not be sequenced.

The samples from the four *A. fraterculus* populations from Brazil selected for the microsatellite analyses amplified well with the 10 primers selected (Figure 1). Figure 2 shows an electropherogram obtained from individuals from the Una population for the loci D105 (blue) and A122 (green) to verify polymorphisms.

A preliminary analysis of 110 flies from the four populations revealed 118 alleles from 10 loci (11.8/locus) with observed heterozygosity (*H₀*) ranging from 0.066 to 0.999, with an average of 0.627.

![Figure 1](image-url) – A representative gel (1.5% agarose) showing PCR products for the microsatellite primers D4, D105, A10, and A120. The plus signal refers to the positive control and the minus signal refers to the negative control.
Figure 2 – Electropherogram for loci D105 (blue) and A122 (green) for specimens of A. fraterculus from the Una population.

3. Manuscripts

We have been working on a manuscript with data on behavior, sexual pheromones and DNA sequencing for five populations of Anastrepha fraterculus from Brazil with Dr. Iara Bravo, Dr. Ruth Nascimento, Dr. Blanka Kalinova and their respective research teams.

Priorities for future work:

1. Obtain additional samples from A. fraterculus populations in Brazil and other countries through collaborations with colleagues at several research institutions.

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 that are being developed by Dr. Norman B. Barr and Dr. Raul Ruiz (USDA).

3. Perform phylogenetic analyses that include tree reconstruction with DNA sequences using Bayesian, maximum parsimony (MP), and maximum-likelihood (ML) approaches. Sequences of additional A. fraterculus populations and other Anastrepha species (e.g.
A. obliqua, A. ludens, A. sororcula, and A. pickeli, among others) will be included to provide proper analysis of species-limits other mitochondrial and nuclear DNA markers that are being developed by Dr. Norman B. Barr and Dr. Raul Ruiz (USDA).

4. Continue microsatellite analysis for other populations in Brazil as well as data analyses in collaboration with Dr. Silvia Lanzavecchia (INTA – Argentina) and Dr. Anna Malacrida (Università di Pavia, Italy).

5. Continue morphometric analysis of populations from distinct geographic localities in Brazil in collaboration with Dr. Vicente Hernandez-Ortiz, Dr. Roberto Antonio Zucchi and Dr. Keiko Uramoto.

6. Submit the manuscript in collaboration with Dr. Iara Bravo, Dr. Ruth Nascimento, Dr. Blanka Kalinova and their respective research teams.

7. Have a meeting at USDA Texas, US with Dr. Norman B. Barr and Dr. Raul Ruiz to analyze and discuss sequencing data.

8. Have a meeting at INTA, Castellar, Argentina with Dr. Silvia Lanzavecchia to analyze and discuss microsatellite data.

References


