

Recent Advances in Molecular Systematics of *Anastrepha* Schiner

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INTRODUCTION

The field of insect molecular systematics is experiencing dramatic growth regarding the number of taxa being studied and amount of data being generated. This is evident in the large number of molecular publications in entomological journals and insect DNA sequence submissions to databases such as GenBank. This growth is largely due to the development of DNA-based markers applicable to many insect groups (e.g., Hillis & Dixon 1991; Brower & DeSalle 1994, Simon *et al.* 1994) and advances in phylogenetic theory and computational technology to facilitate their analysis (e.g., Hillis *et al.* 1996, Huelsenbeck & Rannala 1997, Clement *et al.* 2000, Huelsenbeck & Ronquist 2001, Swoford 2002, Nylander *et al.* 2004).

Like behavioral physiology or ecology, a molecular approach to systematic and taxonomic investigation is a useful way to complement traditional morphological methods of character analysis. Although molecular and non-molecular data sets can produce conflicting results or phylogenies, this should not be regarded as evidence that the "methodologies" are incompatible. Indeed, conflict should not be surprising because a gene tree (or morphological tree) is not necessarily the species tree (Avise 2004) and how char-

acters in data sets are treated can affect the systematic analysis. For example, different interpretations of morphological characters within Brachycera (Diptera) have generated debate over the phylogeny of the group (see Wiegmann *et al.* 2003). Despite issues regarding how and when to combine multiple data sets, we believe systematic studies will benefit from comparing multiple markers (e.g., Giribret *et al.* 2001, Winterton *et al.* 2001, Damgaard *et al.* 2005, Selivon *et al.* 2005a). In fact, the use of molecular data in insect systematics has complemented and enhanced the value of morphological and ecological data, by helping to address questions such as species identification, speciation, geographic variation, structured genetic variation and phylogeny (Hillis *et al.* 1996, Caterino *et al.* 2000, McPheron 2000, Pilgrim *et al.* 2002, Kryzywinski & Besansky 2003, Thanaphum & Thaenkham 2003; Clarke *et al.* 2005, Nardi *et al.* 2005, Schwarz *et al.* 2005).

Many molecular studies of tephritid taxa have been published in recent years (e.g., Smith *et al.* 2002, Feder *et al.* 2003, Jamnongluk *et al.* 2003, An *et al.* 2004, Gilchrist *et al.* 2004, Armstrong & Ball 2005, Augustinos *et al.* 2005, Han & Ro 2005, Barr & McPheron 2006, Velez *et al.* 2006). Most have focused on the genera *Anastrepha* Schiner, *Bactrocera* Macquart, *Ceratitis* MacLeay and *Rhagoletis* Loew, and the evolution of fruit fly species (i.e. the phylogeny, population structure, and modes of speciation or isolation). Since these studies

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contribute to the understanding of tephritid biology and evolution, they also provide essential information for pest management and control programs. The correct delimitation and identification of a species, or a complex of species (White 1996), is essential for basic and applied research and has far-reaching practical consequences (McPheron 2000, Armstrong & Ball 2005, Scheffer 2005). For example, the choice of management approaches such as the use of the sterile insect techniques, which involves mass production of a species for release programs, is strongly affected by the correct identification of the target pest species (Morgante *et al.* 1980, Malavasi & Morgante 1982, McPheron 1993, Robinson & Zacharoupoulou 1996, Roderick 1996a, Roderick 1996b, Caterino *et al.* 2000, Kryzyski & Besansky 2003, Clarke *et al.* 2005, Brelsfoard *et al.* 2006).

The genus *Anastrepha* is the largest of the three genera in the tribe Toxotrypanini, subfamily Trypetinae, which also includes the genera *Toxotrypana* Gerstaecker and *Hexachaeta* Loew. This genus of more than 200 described species is primarily restricted to tropical and subtropical areas of the Neotropical region, although a few species occur in the southernmost part of the Nearctic Region (Norrbon *et al.* 1999).

The geographical distribution of the genus ranges from the southern U.S.A. (Rio Grande Valley of Texas, southern Florida) to northern Argentina, including most islands in the Caribbean. *Anastrepha* species have been the subject of many studies owing to their interesting behaviors and ecologies, as well as economic importance. Seven species, namely *A. fraterculus* (Wiedemann), *A. grandis* (Macquart), *A. ludens* (Loew), *A. obliqua* (Macquart), *A. serpentina* (Wiedemann) and *A. striata* Schiner, have been more thoroughly studied due to their status as major agricultural pests in several countries (Aluja 1994). However, the fullest evolutionary history of the genus can be clarified only through the

study of species of non-economic and economic importance alike. Except for thirteen species in the *A. daciformis* group (Norrbon 1998), there has been no rigorous phylogenetic analysis based on morphological characters for most of the genus, although some species groups have been proposed based on putative synapomorphies or unique, derived characters (Norrbon *et al.* 1999).

A revised intrageneric classification of *Anastrepha* spp. into seventeen species groups, many of which appear to be monophyletic, was proposed based on morphology and host plant use by Norrbom *et al.* (1999). Table 1 lists the recognized species groups (including the genus *Toxotrypana*), the number of species currently assigned to each, and preliminary morphological evidence of monophyly. Norrbom *et al.* (1999) present a preliminary cladogram with two major clades, a section exhibiting most of the derived character states and comprising the *grandis*, *doryphoros*, *spatulata*, *ramosa*, *pseudoparalela*, *serpentina*, *striata*, and *fraterculus* groups (referred to as section 1 in our paper; Table 1); and a second section (referred to as section 2; Table 1) including the *cryptostrepha*, *daciformis*, *dentata*, *benjamini*, *robusta*, *schausi*, *punctata*, *leptozona*, and *mucronota* groups. More recently, an eighteenth species group was recognized by Norrbom *et al.* (2003); called the *hastata* group, this putatively monophyletic lineage comprises *A. hastata* Stone and two recently described species. For the purposes of this review the Norrbom *et al.* (1999) classification is adopted, and *A. hastata* is included in the *mucronota* species group.

Relationships among species groups remain poorly resolved using morphology. Norrbom *et al.* (1999) discuss the difficulties faced when trying to reconstruct a phylogeny of *Anastrepha* based on morphology due to the relatively small number of useful characters, the intergradation of the most useful ones, and the presence of apomorphic states

in a relatively small number of species. They also suggest that additional morphological characters in the male genitalia, larvae, eggs, and molecular characters should be further explored to help answer important questions in the phylogeny of *Anastrepha*.

In addition to the study of higher-level systematics of *Anastrepha* (and its species groups), tephritid researchers are interested in species discovery (e.g., Norrbom & Caraballo 2003), species diagnosis (e.g., Armstrong *et al.* 1997) and population genetics (e.g., Alberti *et al.* 1996). A molecular approach to alpha taxonomy has generated several interesting papers on cryptic species in the nominal *A. fraterculus* (e.g., Steck 1991, Selivon *et al.* 2005a). Most *Anastrepha* species have not received as much attention as *A. fraterculus*, so it is uncertain how many species complexes there are in the genus. As mentioned, this has important implications for the quarantine and management of pests. Identification of cryptic species is also crucial for proper interpretation of higher-level systematic analyses.

Despite several efforts to investigate *Anastrepha* phylogeny using molecular methods, there are still many questions left unresolved (e.g., McPheron *et al.* 1999, Barr *et al.* 2005). Publications relevant to this goal appear in disparate sources (e.g., book chapters and various journals), analyze different levels of the phylogeny (e.g., species complex versus species group), use different tree building methods, and include different species. Consequently, the goal of this review is to describe our current understanding of the molecular systematics of the genus *Anastrepha*.

We begin by discussing the phylogeny of *Anastrepha* and its species groups. We focus our analysis of higher-level phylogenetics on those publications that used DNA sequences and included adequate taxonomic sampling to represent major lineages within the genus. Other studies that report phylogenies based on non-sequence data such as allozymes and restriction patterns (e.g., Morgante *et al.* 1980,

Steck 1991) or that were performed using a limited numbers of species (e.g., Segura *et al.* 2006) are also included in the review. To facilitate our discussion of phylogenetics, we present a hypothetical tree of species group evolution based on available morphological and molecular evidence (Figure 1). We continue our review by discussing molecular contributions to the study of species complexes and conclude with a section devoted to future needs, studies and approaches.

MOLECULAR PHYLOGENETICS OF *ANASTREPHA*

An important question in a phylogenetic analysis of *Anastrepha* is whether the genus is monophyletic (i.e., does it represent a real evolutionary lineage?). Morphological and immunological studies indicated that *Anastrepha* and *Toxotrypana* are very similar and clearly comprise a monophyletic group (reviewed in Norrbom *et al.* 1999, pg. 305, 310). The genus *Toxotrypana* has multiple autapomorphies in support of its monophyly. In contrast, a single character (apical curvature of the medial vein) was believed to define *Anastrepha*, but several species lack this diagnostic trait (Norrbom *et al.* 1999). The typical *Anastrepha* wing pattern (C-, S-, and V-bands) could be a synapomorphy with subsequent modification in some species, but the “wasp-mimic” pattern of *Toxotrypana* could be derived from the *Anastrepha* pattern, as occurs within the *daciformis* group (Norrbom *et al.* 1999).

Phylogenetic analyses using portions of the 16S (McPheron *et al.* 1999) and *period* (Barr *et al.* 2005) genes reject monophyly of *Anastrepha* (see Figure 1, branch 6). Barr *et al.* (2005) hypothesize that the *daciformis*, *robusta*, and *dentata* groups and particularly *A. cordata* Aldrich (*cryptostrepha* species group) are more closely related to *Toxotrypana* than to other species of *Anastrepha*. This is interest-

ing because *A. cordata* lacks the strong apical curvature of the medial vein that is common to most *Anastrepha* species and also has extensive mesonotal markings, a state rare among other *Anastrepha* species. In conclusion, representatives of *Toxotrypana* should be included in all systematic studies of the genus *Anastrepha*. Han & McPherson (1999) demonstrated that *Hexachaeta* is the sister taxon to the lineage comprising *Anastrepha* and *Toxotrypana*. In light of the paraphyletic placement of *Anastrepha*, future phylogenetic studies should include a second outgroup taxon to confirm relationships in the tribe Toxotrypanini.

As mentioned, Norrbom *et al.* (1999) recognized seventeen species groups and *Toxotrypana* is treated as the eighteenth. Note that several of these species groups were regarded as artificial by the authors (see Table 1 for groups lacking evidence of monophyly). Although the 16S and *period* analyses included representative species from fifteen species groups, six groups were represented by a single species, thereby precluding evaluation of monophyletic status. Monophyly of the other nine groups are reported in Table 2 based on gene and tree building methodology (NJ = Neighbor-Joining, MP = Maximum Parsimony, and BAY = Bayesian).

The groups considered questionably monophyletic based on morphology (Table 1) were not supported as monophyletic in the molecular analyses. However, two of the groups believed to be monophyletic based on morphology were not monophyletic in the gene trees. These groups are the *fraterculus* group and the *spatulata* group. For the *fraterculus* group, a single species (*A. barbiellini* Lima) failed to cluster in the clade (except in the 16S- MP tree). This species was tentatively placed in the species group and lacks one of the "diagnostic characters" of the group: lateral brown markings on the mediotergite or subscutellum. Analyses of species from the *fraterculus*, *striata*, and *serpentina* groups using the COI

gene (Smith-Caldas *et al.* 2001; Boykin *et al.* 2006) also support this placement of *A. barbiellini* outside of the *fraterculus* group (see Figure 1). The placement of *A. barbiellini* in the *fraterculus* group should be revised or the composition of the entire group reconsidered to include the *striata* group species. Regarding the *spatulata* group, monophyly is rejected because *A. spatulata* Stone fails to form a clade with the other analyzed species of the group.

Monophyly of the *fraterculus* group has also been tested in other studies but with a much more limited taxonomic sampling. For example, isozyme analysis of five *fraterculus* group species plus ten other *Anastrepha* species by Morgante *et al.* (1980) did not support its monophyly because *A. barnesi* Aldrich (*leptozona* group) clustered with *A. antunesi* Lima of the *fraterculus* group. Likewise, analyses by Ruiz *et al.* (2007a, 2007b) using DNA sequences of the *doublesex* and *transformer* genes did not support monophyly of the *fraterculus* group. These trees included seven *fraterculus* group species (i.e., *A. obliqua*, *A. sororcula* Zucchi, *A. amita* Zucchi, and four putative *A. fraterculus* complex species), two *striata* group species, and one representative from each the *grandis* and *serpentina* species groups. Segura *et al.* (2006) recovered a monophyletic *fraterculus* group using mitochondrial DNA sequences but the group is represented by only three species: *A. obliqua*, *A. ludens*, and *A. suspensa* (Loew).

The phylogenetic relationships among the species groups and the *Toxotrypana* clade within the taxon *Anastrepha* are not well resolved. The major findings from morphology and DNA sequence data are indicated in Figure 1. (Note that this tree was generated by reporting branches either supported with bootstrap in the original publication and/or recovered in multiple data sets. It is not a "supertree" as described in Bininda-Emonds *et al.* 2002.) Molecular data clearly supports the division of *Anastrepha* into a monophyletic

group (section 1) and a polyphyletic group (section 2); this is represented by branch number 3. The Barr *et al.* (2005) study identified an unplaced species (*A. sylvicola* Knab) as the sister lineage of the section 1 division (branch 4). However, its inclusion within section 1 or 2 needs to be further addressed.

The *striata* group is supported as the sister lineage of the *fraterculus* group (excluding *A. barbiellinii*) using the COI and *period* genes (Figure 1, branch 1). The COI data indicate that *A. barbiellinii* (*fraterculus* group) is the sister lineage to the rest of the *fraterculus* group + the *striata* group (branch 2). The *period* gene supports a shared common ancestor to the *daciformis*, *robusta*, *dentata*, *Toxotrypana*, and *cryptostrepha* (part) groups (branch 5). The 16S data also agrees with clustering of the *daciformis*, *robusta* and *dentata* groups. As mentioned, *A. cordata* is the sister lineage of *Toxotrypana* based on *period* and 16S (branch 6).

Since taxon sampling is not exhaustive for any of the larger groups, interpretation of phylogenetic relationships within monophyletic species groups is problematic for the aforementioned molecular studies. The greatest sampling was performed for the *fraterculus* group (ca. 35%). However, even for data sets with adequate taxon sampling, short divergence times (i.e., small genetic distance) within a relatively young, species-rich group such as the *fraterculus* group may

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Table 1. Description of the species groups within *Anastrepha* (including *Toxotrypana* and Unplaced species) based on Norrbom *et al.* (1999). Sections refer to either one of the two putative evolutionary lineages identified by the above authors. Several groups had few synapomorphies to support monophyly.

| Species groups | No. species | Section | Evidence of monophyly |
|------------------------|-------------|---------|-----------------------|
| <i>cryptostrepha</i> | 8 | 2 | No |
| <i>daciformis</i> | 13 | 2 | Yes |
| <i>dentata</i> | 10 | 2 | Yes |
| <i>benjamini</i> | 4 | 2 | No |
| <i>robusta</i> | 16 | 2 | Yes |
| <i>schausi</i> | 4 | 2 | Yes |
| <i>punctata</i> | 5 | 2 | Yes |
| <i>leptozona</i> | 5 | 2 | Yes |
| <i>mucronota</i> | 31 | 2 | No |
| <i>grandis</i> | 10 | 1 | No |
| <i>doryphoras</i> | 3 | 1 | Yes |
| <i>spatulata</i> | 13 | 1 | Yes |
| <i>ramosa</i> | 2 | 1 | Yes |
| <i>pseudoparallela</i> | 20 | 1 | No |
| <i>serpentina</i> | 7 | 1 | Yes |
| <i>striata</i> | 3 | 1 | Yes |
| <i>fraterculus</i> | 28 | 1 | Yes |
| <i>Toxotrypana</i> | 13 | 2 | Yes |
| unplaced species | 32 | NA | NA |

complicate phylogenetic reconstruction because of insufficient accumulation of character state changes and time for lineage sorting of genes (Nei & Kumar 2000).

The 16S and *period* trees did not produce highly supported relationships within the *fraterculus* group nor did they produce similar topologies. The COI study analyzed *fraterculus* group relationships but used different representative species than did the 16S and *period* studies, precluding a useful comparison of trees. One similarity that is consistent among the three gene trees is the place-

ment of *A. fraterculus* specimens from the Andean region of Venezuela and Colombia as distinct from other *A. fraterculus* specimens. The implications of this finding will be discussed in the following section on cryptic species.

The only other group with a proposed phylogeny is the *daciformis* group. The majority of its species are represented in the morphological analysis of Norrbom (1998). The molecular studies represent a more limited sampling ($\leq 30\%$) of the group than reported in Norrbom (1998).

Table 2. Evidence in support or against monophyly of species groups based on gene trees reported in McPheron et al. (2000) and Barr et al. (2005) from the 16S and *period* (*per*) data sets, respectively. The number of species representing a group in a data set is indicated by the value N. The “?” indicates a multifurcating branch precludes resolution.

| Taxon | N(16S) | N(per) | 16S - NJ | 16S - MP | per - BAY | per -MP |
|------------------------------|--------|--------|----------|----------|-----------|---------|
| <i>fraterculus</i> group | 10 | 9 | No* | Yes | No* | ? |
| <i>striata</i> group | 2 | 2 | Yes | Yes | Yes | Yes |
| <i>serpentina</i> group | 2 | 2 | Yes | Yes | Yes | Yes |
| <i>pseudoparallela</i> group | 4 | 4 | No | No | No | No |
| <i>spatulata</i> group | 5 | 5 | No | No | No | No |
| <i>cryptostrepha</i> group | 2 | 2 | No | No | No | No |
| <i>mucronota</i> group | 3 | 3 | No | No | No | No |
| <i>Toxotrypana</i> | 3 | 2 | Yes | Yes | Yes | Yes |

* Evidence against monophyly is caused by placement of a single species: *A. barbiellinii*

MOLECULAR ALPHA-TAXONOMY AND CRYPTIC SPECIES IDENTIFICATION

The description of species (alpha level taxonomy) is dependent on good diagnostic characters. Although technological advances in microscopy and computational tools for morphometric analysis will undoubtedly enhance efforts of traditional taxonomists (e.g., Selivon & Perondini 1999, Hernández-Ortiz et al. 2004, Frias 2005), genetic and molecular techniques have been shown to be extremely useful tools for species diagnosis (e.g., karyotype analysis by Selivon et al. 2005b and

DNA barcoding by Armstrong & Ball 2005 and Scheffer et al. 2006). Because genomes comprise regions of relatively high variation (in comparison to morphology), it is possible to detect differences between populations of a species and, if reproductively isolated, evidence of cryptic species (see Avise 2004, pp. 356-360 for a good review of cryptic species identification). It is important to stipulate that identification of a species complex by differences in DNA sequences does not reveal the phylogeny of those species. It is possible that the morphotype characteristic of the complex is homoplastic.

Figure 1. Hypothetical phylogeny of *Anastrepha* species groups based on evidence from morphology (Norrbon *et al.* 1999), 16S DNA (McPherson *et al.* 1999), COI gene (Smith-Caldas *et al.* 2001), and the *period* gene (Barr *et al.* 2005). The data sets that provide evidence for a branch are indicated. An asterisk (*) indicates that a species group was represented by ≤ 1 species in the molecular studies.

Support for Branch #

1: *period* + COI

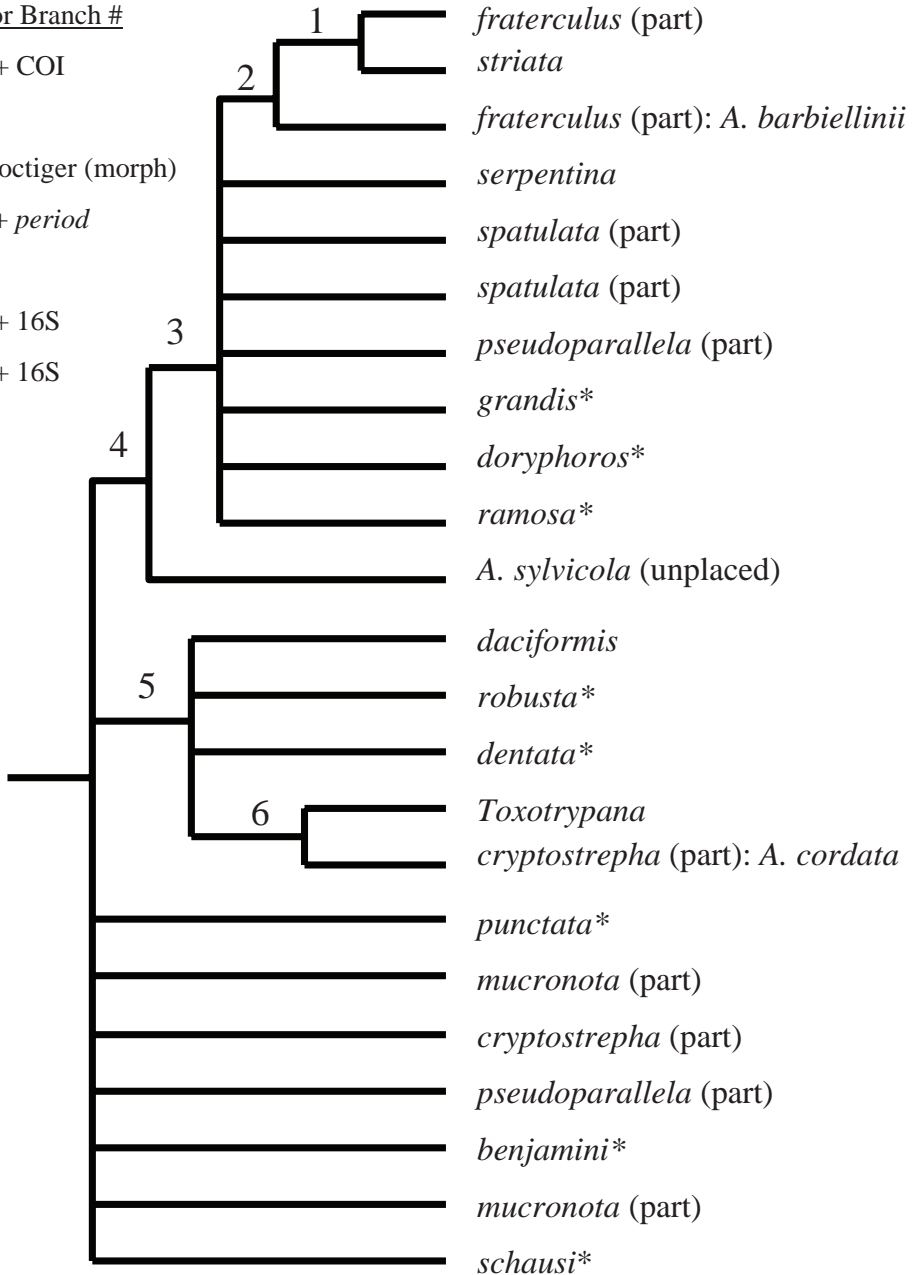
2: COI

3: male proctiger (morph)
+ 16S + *period*

4: *period*

5: *period* + 16S

6: *period* + 16S



ic variation (Berlocher 1984, Roderick 1996a, Kryzyski & Besansky 2003).

Morgante *et al.* (1980) were the first to report molecular evidence that *A. fraterculus* comprises a species complex. They analyzed thirteen *Anastrepha* species, most of which occur in Brazil, and sixteen populations of *A. fraterculus* from different hosts and locations in the southern, southeastern and northeastern regions of Brazil. Based on their isozyme data, they divided the complex into four groups, group A comprising populations from the northeastern (states of Bahia, Sergipe and Pernambuco) and southeastern regions (São Paulo state), group B with populations only from the southeastern region (São Paulo state), group C with two populations from the northeastern region (Bahia state), and group D with populations only from the southeast (states of São Paulo, Rio de Janeiro and Minas Gerais). The group C flies have since been determined to be *A. sororcula* populations (Morgante *et al.* 1993) and exhibit a karyotype (Solferini & Morgante 1987 – Karyotype 3, Selivon *et al.* 2005b) distinct from *A. fraterculus* karyotypes.

Steck (1991) studied four species of *Anastrepha* (*A. distincta* Greene, *A. fraterculus*, *A. obliqua* and *A. striata*) focusing on eight geographic populations of *A. fraterculus* from several hosts in Central and South America. His results demonstrated a high genetic variability (based on isozymes) within *A. fraterculus*. Steck divided the populations into two groups, the first including populations from northeastern Brazil (Bahia state), coastal Venezuela, Costa Rica and Mexico, and the second one comprising populations from southeastern Brazil (São Paulo state), Andean Venezuela and Peru. Populations within the second group were genetically distinct from the first group and possibly from each other as well. According to this study, the *A. fraterculus* complex is not monophyletic.

Another technique that has been used in the study of genetic variation of the *A. frater-*

culus complex is called restriction fragment length polymorphism (RFLP) analysis of DNA. This method has been especially useful when applied to mitochondrial DNA because of the relatively high rate of substitution in this genome (Simon *et al.* 1994; Roderick 1996a) Steck & Sheppard (1993) used the PCR-RFLP technique in an analysis of different populations of *A. fraterculus* from Brazil (states of São Paulo and Bahia) and Venezuela (Los Caracas, coastal area and Mérida, Andes). A high level of polymorphism was detected among the four geographic populations and little or no gene flow was observed.

Santos (1994) and Santos & Matioli (1997) also focused on geographic populations of *A. fraterculus* in Brazil in their analyses of RFLP of mitochondrial and nuclear DNA. Their results showed two geographic populations of *A. fraterculus*, one inland and the other coastal, to be distinct. According to the authors, these two groups appear to represent two distinct species within the nominal species *A. fraterculus*. They also state that this species complex is not monophyletic as the coastal populations are closer to *A. sororcula* than to the inland populations.

The phylogenetic studies of McPherson *et al.* (1999) and Barr *et al.* (2005) used DNA sequence data and included multiple specimens of *A. fraterculus*. In the case of the 16S data, a specimen collected from Mérida, Venezuela was shown to be distinct from a specimen collected in the state of São Paulo, Brazil. The *period* gene tree included four specimens from Mérida, Venezuela, one from Los Caracas, Venezuela (coastal region), one from the state of São Paulo, Brazil, and two from Mexico. The samples from Mérida formed a clade (distinct from the other specimens), the samples from Mexico and Los Caracas formed a clade (distinct from other specimens) and the São Paulo state specimen was distinct from the two clades. Both of these genes suggest that the species complex is not monophyletic.

The analysis of the COI gene by Smith-Caldas *et al.* (2001) included sixteen specimens of *A. fraterculus* and representatives of eleven other *Anastrepha* species. The specimens were from collections made in the Andean regions of Venezuela and Colombia, Caracas (Venezuela), Argentina, Mexico, Costa Rica, Guatemala, and several regions of Brazil. Their COI gene tree does not support monophyly of the species complex. In fact, the tree suggests that there may be over six cryptic species dispersed throughout the phylogeny. For example, COI supports a lineage including Mexican and Costa Rican *A. fraterculus* populations, an Andean lineage, a Guatemalan lineage, an independent Venezuelan lineage, and several Brazilian lineages.

A surprising result of the COI study was evidence that *A. obliqua* may be a species complex because populations formed two distinct clusters. Subsequent analysis of the COI data including 107 sequences of *A. suspensa* from Florida and Caribbean populations from different hosts supported monophyly of *A. suspensa* (Boykin *et al.* 2006).

A recent report by Selivon *et al.* (2005a) focused on ten populations of *A. fraterculus* from different localities in Brazil that underwent a joint analysis using isozymes, karyotypes, morphometry and crossings. The authors recognized two clusters within the populations analyzed, which represented two distinct biological species and were denominated *A. sp.1 aff. fraterculus* and *A. sp.2 aff. fraterculus*. This study is a good example of how multiple methodologies should be integrated to study cryptic species.

Goday *et al.* (2006) used molecular cytogenetics to characterize sex chromosomes and locate rDNA loci of *Anastrepha*, focusing mainly on four species of the *A. fraterculus* complex, three of these species are from two different localities in São Paulo state, Brazil and one species is from Guayaquil, Ecuador. They report that the heterochromatin orga-

nization on the sex chromosomes revealed by three different molecular cytogenetic techniques allows the precise karyotypic identification of the aforementioned species.

Several studies were performed to characterize population genetic variation of *A. fraterculus* within Argentina. However, these did not detect significant structure and supported the hypothesis that these populations are a single species. These studies were performed using protein electrophoresis (Alberti *et al.* 1996), PCR-RFLP of 16S DNA (Alberti *et al.* 2002), DNA sequences of COII (Alberti *et al.* 2008) and RAPD (randomly amplified polymorphic DNA) techniques coupled with cytogenetic analyses (Basso *et al.* 2003). The populations were similar to populations collected in the state of Rio Grande do Sul, Brazil.

To summarize, there appear to be over five cryptic species in the *A. fraterculus* complex. There is (1) an Andean species (supported by COI, 16S, and *period* sequences, PCR-RFLP, and isozyme data), (2) a lineage present in Mexico, Costa Rica, and possibly Venezuela and Brazil (based on Steck's [1991] isozyme work, the *period* and COI genes, and supported by Hernández-Ortiz *et al.* 2004 using morphometrics), (3) a lineage in Guatemala (based only on COI), (4) a second species in Venezuela (based only on COI), (5) a lineage in Peru (based on Steck's [1991] isozyme work), and over two additional species from Brazil (based on Morgante *et al.* [1980], the COI gene, Selivon *et al.* [2005a], and Ruiz *et al.* [2007a, 2007b]). Brazilian populations likely represent more than two species, but some of these may be conspecific with some of the above populations found in other countries. Without a way to compare results among studies, there will be a tendency to inflate the number of cryptic species in the complex.

It is important to note that the cryptic species identified by these studies are not monophyletic. Therefore, two possible hypotheses are either the *A. fraterculus* "morphotype" evolved independently multiple times or this

morphology is an ancestral form. If the latter is true, then this provides evidence regarding polarity of character evolution within the *fraterculus* group.

These two hypotheses for *A. fraterculus* evolution, however, assume that a gene tree equates with a species tree. Unfortunately, interpretation of the molecular phylogeny can be confounded by shared ancestral polymorphisms; i.e. if the variation present in *A. fraterculus* populations and other *Anastrepha* species predates speciation events within the species group. This implies that lineage sorting of variation rather than accumulation of new characters is represented in the molecular phylogeny (Figure 2). Although not our preferred hypothesis, it is possible that the *A. fraterculus* complex is the sister clade to the other *fraterculus* species. For example, speciation events within the "*A. fraterculus*" lineage could have generated daughter species that did not evolve novel morphological characteristics.

FUTURE NEEDS AND RECOMMENDATIONS

When systematists discuss the future needs of a research program, the two most common areas for improvement are the addition of characters and taxa to the study. In terms of characters, DNA sequence data should be regarded as an essential approach to phylogenetic study. Although methods such as protein electrophoresis (or other frequency-based methods) are useful, these methods require an *a priori* grouping of data points (usually by geography) before phylogenetic reconstruction. DNA sequences are preferable because they are more information-rich, they allow a clearer statement of character homology (as opposed to frequency data), and each sequence represents the individual in a phylogenetic analysis. The COI, 16S, and *period* sequences are useful tools but there will surely be a need for additional markers

for particular questions. For example, 16S and *period* gene trees did not provide supported branches in the study of the *fraterculus* group.

Fortunately, the development of protocols to sequence other genes is an active area of research. For example, Soto-Adames *et al.* (1994) reported sequence data of the *G₆pdh* gene (*glucose-6-phosphate dehydrogenase*) from *A. suspensa*, Fritz (2006) compares sequences of the internal transcribed spacer region 2 (ITS-2) from *A. suspensa* and *A. fraterculus*, Segura *et al.* (2006) analyzed a region of the mtDNA encompassing the 3' end of the cytochrome b gene (*cytb*), a serine transfer RNA gene (*tRNA^{ser}*) and the 3' end of subunit 1 of the NADH dehydrogenase gene (*ND1*) for five species (*A. ludens*, *A. obliqua*, *A. serpentina*, *A. striata* and *A. suspensa*), Ruiz *et al.* (2005, 2007a, 2007b) isolated and characterized the *doublesex* and *transformer* genes from twelve *Anastrepha* species. The phylogenetic utility of these genes will require further study. In addition, phylogenetic studies of other tephritids and flies have produced several useful markers (e.g., Barr & McPherson 2006, Moulton & Wiegmann 2004) that can be applied to *Anastrepha* systematics. It is important to realize that new characters can also be developed using morphological and ecological studies. Ideally, future systematic studies should incorporate an approach that integrates molecular data (from multiple genes) with non-molecular data sets.

The problem of taxon sampling is crucial to our understanding of *Anastrepha*. Thus far, molecular phylogenetic studies have included a minority of the species present in species groups. Although uncovering the systematic position of these groups in a tree is important, one needs to include sufficient representatives to assess the monophyletic status of species groups. When sampling species from a species group, researchers should first demonstrate that the selected species are in fact representative of the spe-

cies group. Based on our current data, few species groups possess strong evidence of monophyly.

The *fraterculus* group is an interesting taxonomic problem because it includes many economically important species and evidence of cryptic species. Despite serious efforts to sample the *A. fraterculus* complex and other species, the study of the group suffers from a lack of a common molecular benchmark; specimens analyzed by different researchers (or in different studies by the same group) are not scored using a common marker system. For example, the samples used in the Smith-Caldas *et al.* (2001) paper differed from the samples used in Steck's (1991) isozyme study. It is not possible to combine these data sets, estimate variation in a species or compare phylogenetic placement of cryptic species as the two *A. fraterculus* specimens might represent two distinct species of a species complex. As well, it is not possible to determine if the trees built using different data sets even disagree because it is possible that two specimens of the same species could actually represent two distinct species of a species complex.

The aforementioned limitations are of serious concern for interpretation of *A. fraterculus* complex studies. The COI data of Smith-Caldas *et al.* (2001) demonstrate a very large genetic diversity of haplotypes for *A. fraterculus*. It is not possible to correlate these putative cryptic species with other studies. This leaves the reader with only geographical data with which to assign nomenclature. This is an imperfect approach for some regions (such as the state of São Paulo, Brazil) where multiple species occur. As mentioned, the *A. fraterculus* complex could be the sister taxon to a lineage comprising multiple extant species in the *fraterculus* group (Figure 2). This can only be evaluated by reconstructing phylogenies with thorough taxon and population sampling. For example, the monophyly of the *A. fraterculus* complex

should be tested using multiple specimens from each putative cryptic species. This should also be done for the other *Anastrepha* species to determine the inter- and intra-specific variation in the tree.

In addition to selecting a standard molecular marker (such as COI) for comparison of putative cryptic species, it is also important to study the biological relevance of these putative species. Selivon *et al.* (2005a) performed basic biological studies of the effects of reproductive isolation on putative cryptic species. Genetic difference should not be equated with species description. Multiple explanations could account for these differences (including insufficient sampling) so biologists should be careful in interpretation of molecular taxonomic studies.

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