SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Phylogenetic Relationships Among Species of the *fraterculus* Group (*Anastrepha*: Diptera: Tephritidae) Inferred from DNA Sequences of Mitochondrial Cytochrome Oxidase I

MARTHA R.B. SMITH-CALDAS[1, 3], BRUCE A. MCPHERON[2], JANISETE G. SILVA[1,2] AND ROBERTO A. ZUCCHI[3]

[1]*Depto. Ciências Biológicas, Universidade Estadual de Santa Cruz, Rod. Ilhéus/Itabuna Km 16, 45650-000, Ilhéus, BA*
[2]*Dept. Entomology, The Pennsylvania State University, 501 A.S.I. Building,University Park, PA, USA, 16802*
[3]*Depto. Entomologia, Fitopatologia e Zoologia Agrícola, Escola Superior de Agricultura "Luiz de Queiroz"/USP, C.postal 9, 13418-900, Piracicaba, SP*

Neotropical Entomology 30(4): 565-573 (2001)

Relações Filogenéticas Entre Espécies de *Anastrepha* do Grupo *fraterculus* (Diptera; Tephritidae) Através do Sequenciamento do Gene Mitocondrial COI

RESUMO – Foi analisado um fragmento de 808 pares de base do gene mitocondrial citocromo oxidase I (COI) para 15 espécies de *Anastrepha*: 12 pertencentes ao grupo *fraterculus*, uma espécie sem grupo definido e duas como grupo externo. As relações filogenéticas entre os táxons incluídos foram inferidas pelos métodos de "neighbor-joining" e máxima parcimônia. A distância genética média (Jukes-Cantor) entre as espécies foi 0,033 ± 0,006, tendo o nível de divergência das seqüências variado de 0,0 a 0,083. Os resultados do estudo com o COI indicaram a inclusão de *A. acris* Stone, espécie sem grupo morfologicamente definido, no grupo *fraterculus*. A inclusão de *A. barbiellinii* Lima no grupo *fraterculus* e a monifilia do referido grupo são também discutidas. Além disso, a presença de múltiplos conjuntos gênicos na espécie nominal *A. fraterculus* (Wiedemann) e a não-monofilia de *A.fraterculus* são corroboradas pelos dados obtidos no presente estudo. As espécies *A. amita* Zucchi, *A. turpiniae* Stone e *A. zenildae* Zucchi foram analisadas geneticamente pela primeira vez.

PALAVRAS-CHAVE: Insecta, moscas-das-frutas, *Anastrepha fraterculus,* COI.

ABSTRACT – A fragment of 808 base pairs within the mtDNA gene cytochrome oxidase I (COI) was analyzed for 15 species of *Anastrepha*: 12 within the *fraterculus* group, one unplaced species and two outgroups. Phylogenetic relationships among the included taxa were inferred using neighbor-joining and maximum parsimony methods. The average Jukes-Cantor genetic distance among the species was 0.033±0.006 and the level of sequence divergence ranged from 0.0 to 0.083. Our results of COI indicate the placement of *A. acris* Stone, an unplaced species, in the *fraterculus* group. The membership of *A. barbiellinii* Lima in the *fraterculus* group and the monophyly of the aforementioned group are also discussed. Moreover, the presence of multiple gene pools in the nominal species *A. fraterculus* (Wiedemann) and the nonmonophyly of *A. fraterculus* are corroborated by data obtained in our study. The species *A. amita* Zucchi, *A. turpiniae* Stone and *A. zenildae* Zucchi were genetically studied for the first time.

KEY WORDS: Insecta, fruit flies, *Anastrepha fraterculus,* COI.

The genus *Anastrepha* Schiner contains 197 currently recognized species and nearly 50 new species yet to be described (Norrbom *et al*. 1999). The species in this genus are endemic to the Americas and restricted to tropical and subtropical areas, ranging from the southern United States to northern Argentina and also including most of the Caribbean Islands (Stone 1942, Aluja 1994). Several authors have proposed species groups within this genus. The most recent and comprehensive study, based on morphological characters and host plant use, placed *Anastrepha* species into 18 groups (Norrbom *et al.* 1999). The *fraterculus* group includes 29 species, 17 of which occur in Brazil (Norrbom *et al*. 1999, Zucchi 2000). This species group is as widespread as the genus *Anastrepha* itself, infests a very diverse group of hosts, and some of the species are economically important (Aluja 1994, Norrbom *et al.* 1999). Among the most serious agricultural pests is *A. fraterculus* (Wiedemann) itself, which infests 67 host species, and *A. obliqua* (Macquart), with a host list of 28 plant species in Brazil (Zucchi 2000).

Only minor morphological characters are used to

distinguish the species within the *fraterculus* group, making it difficult to determine species boundaries, especially when one considers that there is variation in the aculeus, a major diagnostic character (Zucchi 1997, Araújo 1997), due to genetic and environmental factors (Aluja 1994). Misidentifications at the species level can lead to serious problems in the implementation of quarantine restrictions, management and control programs (McPheron 2000). Additional morphological and molecular characters need to be explored to improve our knowledge of the relationships among the species within the various *Anastrepha* species groups (McPheron *et al.* 1999, Norrbom *et al.* 1999).

A. fraterculus is probably the most economically important species in South America. Because of this, it has been the object of several morphological and genetic studies, which have revealed that it is actually a complex of multiple species (Stone 1942, Morgante *et al*. 1980, Steck 1991, Steck & Sheppard 1993, Selivon 1996). Morgante *et al.* (1980) analyzed 13 species of *Anastrepha* using isozymes, including several populations of *A. fraterculus* from Brazil, but only four species in the *fraterculus* group were included in their study. Steck (1991) focused on geographic populations of *A. fraterculus* and two other species in the *fraterculus* group in his isozyme study. Steck & Sheppard (1993) also analyzed different populations of *A. fraterculus* from Brazil and Venezuela, using PCR-RFLP of mitochondrial DNA. Selivon (1996) also studied several Brazilian populations of *A. fraterculus* employing isozymes but included only four other species in the *fraterculus* group. These studies all focused on the *fraterculus* complex and were not designed to evaluate relationships within the entire *fraterculus* group.

The first study to use DNA sequences to look at relationships among different species groups within the genus *Anastrepha* was that of McPheron *et al.* (1999). They analyzed 40 species of *Anastrepha* belonging to 14 species groups, including 10 species within the *fraterculus* group, using sequences of the large subunit ribosomal DNA (16S rDNA) of the mitochondrial DNA (mtDNA). Their study did not satisfactorily resolve relationships within the *fraterculus* group due to insufficient variation in the 16S sequences.

We report the results of our investigation of the phylogenetic relationships and species boundaries among *Anastrepha* species in the *fraterculus* group using DNA sequences of subunit I of the mitochondrial cytochrome oxidase gene (COI). The COI gene was chosen for several reasons, among them: the presence of variable regions making it suitable for analysis of more closely related taxonomic groups (Lunt *et al*. 1996, Ståhls & Nyblom 2000); the availability of conserved primers for this entire gene for different insect groups (Simon *et al*. 1994), and the fact that COI sequences have been employed in a series of studies of phylogenetic relationships on insects (Brown *et al.* 1994, Brower 1994, Bernasconi *et al*. 1999, Mardulyn & Whitfield 1999, Ståhls & Nyblom 2000, Scarpassa *et al.* 2000).

Material and Methods

The taxa used in this study consisted of 15 species in the genus *Anastrepha*, representing 12 species within the *fraterculus* species group, one unplaced species and two outgroups. The species and populations sequenced, along with collection and preservation information, are listed in Table 1. Vouchers are deposited at the National Museum of Natural History, Smithsonian Institution, Washington, D.C. and at the insect collection at the Escola Superior de Agricultura "Luiz de Queiroz", USP, Piracicaba, SP, Brazil.

Further nomenclature in this paper will use *fraterculus* group to refer to the 29-species assemblage and *fraterculus* complex to refer to this set of cryptic species currently identified as *A. fraterculus* proper.

Total nucleic acid extractions of individual flies followed the protocol for pinned or alcohol-preserved specimens described in Han & McPheron (1997).

The polymerase chain reaction (PCR) was used to amplify 1,300 bp within the mitochondrial COI gene. The primers used for PCR are CI-J-2183 (5'CAACATTTATTTTGATTTTTTGG3') (Sperling & Hickey 1994) and TL2-N-3014 (5'TCCAATGCACTAATCTGCCATATTA3') (Simon *et al*. 1994). "Hot start" PCR reactions were performed in two steps and in 100 µl total reaction volumes. In the first step, 31.5 µl of the lower mix consisting of 1X Qiagen reaction buffer, 250 µM of each dATP, dCTP, dGTP and dTTP, 1.25 µM of each primer and sterile MQ water and one AmpliWax® PCR Gem 50 were added to each tube. The tubes were heated at 80°C for 5 min. In the second step, 66.5 µl of the upper mixture consisting of 1X Qiagen reaction buffer, 1.8 units of Qiagen Taq polymerase, sterile MQ water and 4 µl (20 – 150 ng/µl) of template DNA were added to each tube. The cycle program consisted of an initial denaturation step of 7 min. at 95°C followed by 35 cycles of 1 min. at 94°C, 1 min. at 45°C, 8 min. at 65°C with a final extension step of 15 min. at 65°C, using a Gene Amp PCR System 9700, Perkin Elmer.

PCR products were gel purified using the Qiagen QIAquick PCR Purification Kit or the Qiagen QIAquick Gel Extraction Kit.

DNA sequencing was carried out at the Penn State University Nucleic Acid Facility using cycle sequencing with dye terminator in a Perkin Elmer ABI 377 Automated DNA Sequencer. Sequences from both strands were obtained for each specimen.

The sequences obtained in this study were aligned using Omiga (Oxford Molecular), which uses Clustal W. Phylogenetic analyses were conducted using neighbor-joining (NJ) and maximum parsimony (MP) methods. The species *A. striata* Schiner and *A. serpentina* (Wiedemann) were used as the outgroup. PAUP*, version 4.0b1 (Swofford 1998) was used to perform MP analysis using the heuristic search procedure (tree-bisection-reconnection algorithm and the MULPARS option) to find the most parsimonious trees. All included characters were assigned equal weights in the input order. Bootstrapping (Felsenstein 1985) of the MP analysis (100 replicates) was performed under the heuristic search procedure, with a maxtree setting of 100 trees. NJ analysis was conducted using PAUP*, version 4.0b1 (Swofford 1998) and a NJ tree was generated using the Jukes-Cantor distance (chosen based upon criteria in

Kumar *et al.* 1993). Bootstrapping (100 replicates) was carried out to estimate the support for NJ topologies.

Results and Discussion

A fragment of 808 bp was sequenced from the 15 species (a total of 45 specimens) included in this study and the sequences were deposited in GenBank under Accession Numbers AF420611 - AF420655. This region encompasses positions 2,194-3,002 in the *Drosophila yakuba* Burla complete mtDNA sequence (Clary & Wolstenholme 1985). Average nucleotide composition across the taxa was 32.8% A, 37.3% T, 16.2% C, and 13.7% G, consistent with the A-T rich nature of this gene previously observed in other insect taxa (Simon *et al.* 1994). The nucleotide alignment was unambiguous for this region and no indels were present. Therefore, the alignment is not included (but is available from the senior author in nexus format). The average Jukes-Cantor distance among the 15 species analyzed was 0.033 ± 0.006; the level of sequence divergence ranged from a minimum of 0.0 among some conspecific samples to a maximum distance of 0.083 between the most distantly related species. This average distance value is higher than that observed for the *fraterculus* group using 16S mtDNA sequence data, where the average value was 0.018 ± 0.001 (McPheron *et al*. 1999).

Two most parsimonious trees were derived from MP analysis of the COI data, and the strict consensus tree is shown in Fig. 1. Of the 808 characters used in the analysis, 188 were variable and 110 were informative under parsimony. Of these parsimony informative characters, 40.6%, 5.6%, and 53.8% occupied first, second, and third codon positions, respectively. Bootstrap values higher than 50% are indicated above the appropriate branches.

The topology of the NJ tree is really quite similar to the strict MP consensus tree, although not identical in the relative placement of some of the species within the *fraterculus* group (Fig. 2). Bootstrap values higher than 50% are indicated in the figure.

This is the most extensive molecular study of the *fraterculus* group to date, both in number of included species and in replication of samples within species, even though we could not obtain specimens representing the entire *fraterculus* group. Of the 29 species included in the *fraterculus* group, we had exemplars of 12 species, *A. amita* Zucchi, *A. bahiensis* Lima, *A. barbiellinii* Lima, *A. coronilli* Carrejo & González, *A. distincta* Greene, *A. fraterculus*, *A. ludens* Loew, *A. obliqua*, *A. sororcula* Zucchi, *A. suspensa* Loew, *A.turpiniae* Stone, and *A. zenildae* Zucchi besides *A. acris* Stone (a species not included in the group based on morphological characters). We also had multiple exemplars from different populations for 16 of the species analyzed, most importantly for the nominal species *A. fraterculus*.

Norrbom *et al.* (1999) proposed a monophyletic *fraterculus* group based upon morphological characters. Our results using this mtDNA gene do not support this hypothesis. *A. striata* is clearly morphologically distinct from the *fraterculus* group, yet our results show strong support for a relationship of *A. striata* with all members of the *fraterculus* group tested here exclusive of *A. barbiellinii* (Figs. 1 and 2). This observation is consistent with results from the 16S mtDNA study of McPheron *et al.* (1999), who found an NJ topology with members of the *striata* group more closely associated with the remainder of the *fraterculus* group than was *A. barbiellinii*. Inclusion of *A. barbiellinii* in the *fraterculus* group was tentative (Norrbom *et al.* 1999), and perhaps further evaluation of this assignment is necessary. The remainder of the *fraterculus* group (from this point, we will use *fraterculus* group in the sense of all included species with the exception of *A. barbiellinii*) is resolved as a monophyletic group, but with only moderate statistical support.

A. acris, an unplaced species based on morphological characters (Norrbom *et al*. 1999), is included in the *fraterculus* group on the basis of our COI data, consistent with the results from 16S data (McPheron *et al.* 1999).

Previous analysis of some of these *fraterculus* group taxa did not clearly resolve relationships (McPheron *et al.* 1999), attributable to the recent divergence within the group and the low level of variation exhibited in the 16S gene. Our hope was that sufficient variability would be present at third positions in the protein-coding COI gene that we could resolve species relationships within the group and evaluate issues of species complexes at least within the nominal *A. fraterculus*. To some extent, that is possible, although our results reveal that complete understanding of evolutionary patterns in this group will require additional character sets.

The 16S mtDNA analysis and several isozyme analyses have clearly shown that *A. fraterculus* is not monophyletic (Steck 1991, Selivon 1996, McPheron *et al*. 1999). Steck (1991) found that samples from high elevation in the Andean region were distinct in their isozyme profiles from other *A. fraterculus* samples over the limits of the species' range. The 16S data also separated a sample of *A. fraterculus* from Merida, Venezuela (elevation 1,600 m) to the outside of the *fraterculus* group. Our COI data provide this same result - the three Andean populations included in our study form a strongly supported and highly divergent clade at the base of the remaining *fraterculus* group in the trees (Figs. 1 and 2). Given the strong molecular divergence (JC distance = 0.045) between geographically connected populations at low (Caracas, Venezuela) and high (Merida, Venezuela) elevations, renewed studies, including elevational sampling transects, are warranted to explore the boundaries of these species.

Both NJ and MP analysis identified an additional group of five *A. fraterculus* samples, and support for this assemblage was strong in the NJ tree. These samples included our single population from Argentina (Tucumán) and four Brazilian samples. The Brazilian samples in this cluster represent four of the five southernmost *A. fraterculus* populations from Brazil (only the sample from Chapecó, SC, in southern Brazil did not cluster with this grouping). We do not have a sufficient sample density through southern Brazil and Argentina to make a strong argument at this point, but the question of whether multiple gene pools exist within *A. fraterculus* in the southern portion of the continent is worthy of examination.

The remaining *A. fraterculus* samples were scattered throughout the tree. A specimen from Guatemala clustered with *A. bahiensis* and *A. distincta*, specimens from Costa Rica and Mexico were similar to each other and *A. suspensa*,

Table 1. Collection data and information on *Anastrepha* species sequenced in this study.

Species	Collection data	Collection sites	Location	Altitude (m)	Preservation	No. of sequenced samples
A. fraterculus	*Eugenia pyriformis* Myrtaceae	Monte Alegre do Sul, SP, Brazil	23º07'S; 46º33'W	744	Ethanol	2
	Psidium guavaja Myrtaceae	São José da Bela Vista, SP, Brazil	20º35'S; 47º38'W	730	Ethanol	1
	McPhail trap	Linhares, ES, Brazil	19º25'S; 40º02'W	33	Ethanol	2
	McPhail trap	Vacaria, RS, Brazil	28º30'S; 50º54'W	955	Ethanol	2
	McPhail trap	Janaúba, MG, Brazil	15º45'S; 43º25'W	533	Ethanol	2
	McPhail trap	Chapecó, SC, Brazil	26º06'S; 52º36'W	618	Ethanol	2
	Prunus persica Rosaceae	Caçador, SC, Brazil	26º47'S; 50º00'W	920	Ethanol	2
	Psidium guavaja Myrtaceae	Santo Amaro, BA, Brazil	12º33'S; 38º42'W	42	Ethanol	1
	Coffea arabica Rubiaceae	Mérida, Venezuela	08º35'N; 71º08'W	1700	Frozen	2
	McPhail trap	Caracas, Venezuela	10º22'N; 64º27'W	6	Frozen	2
	McPhail trap	Palin, Guatemala	14º24'N; 90º42'W	1148	Frozen	2
	McPhail trap	Tucumán, Argentina	26º49'S; 65º13'W	431	Frozen	1
	McPhail trap	La Mesa, Colombia	05º00'N; 74º37'W	1466	Frozen	1
	McPhail trap	Sevilla, Colombia	06º01'N; 73º37'W	1742	Frozen	2
	McPhail trap	Puntarenas, Costa Rica	09º22'N; 84º00'W	1	Frozen	1
	McPhail trap	Chiapas, Mexico	14º32'N; 90º23'W	920	Frozen	1
A. obliqua	*Spondias purpurea* Anacardiaceae	Narandiba, SP, Brazil	22º24'S; 51º31'W	420	Ethanol	1
	McPhail trap	Linhares, ES, Brazil	19º25'S; 40º02'W	33	Ethanol	1
	McPhail trap	Janaúba, MG, Brazil	15º45'S; 43º25'W	533	Ethanol	1
	McPhail trap	Natal, RN, Brazil	05º47'S; 35º13'W	1	Ethanol	1
	McPhail trap	Conceição do Almeida,BA, Brazil	12º45'S; 39º15'W	216	Ethanol	1
	McPhail trap	Sevilla, Colombia	06º01'N;73º37'W	1742	Frozen	1
	McPhail trap	Los Tuxtlas, Mexico	18º26'N; 95º11'W	1553	Frozen	1
	McPhail trap	Actopan, Mexico	20º16'N; 98º56'W	2005	Frozen	1
A. sororcula	*Psidium guavaja* Myrtaceae	Rosana, SP, Brazil	22º37'S; 53º03'W	280	Ethanol	1
	McPhail trap	Mossoró, RN, Brazil	05º11'S; 37º20'W	9	Ethanol	1
A. zenildae	*Psidium guavaja* Myrtaceae	Mossoró, RN, Brazil	05º11'S; 37º20'W	9	Ethanol	1
	McPhail trap	Lagoinha, PI, Brazil	05º49'S; 42º37'W	240	Ethanol	1
	McPhail trap	Santa Inês, MA, Brazil	03º40'S; 45º22'W	24	Ethanol	1
A. turpiniae	McPhail trap	Santa Inês, MA, Brazil	03º40'S; 45º22'W	24	Ethanol	1
A. amita	McPhail trap	Piracicaba, SP, Brazil	22º43'S; 47º37'W	541	Ethanol	1
	McPhail trap	Santa Inês, MA, Brazil	03º40'S; 45º22'W	24	Ethanol	1
	McPhail trap	Victoria Parish ,Trinidad and Tobago	10º15'N; 61º27'W	16	Frozen	1
A. distincta	McPhail trap	Santa Inês, MA, Brazil	03º40'S; 45º22'W	24	Ethanol	1
	Inga sp. Mimosaceae	Cruz das Almas, BA, Brazil	12º40'S; 39º10'W	220	Ethanol	1
	McPhail trap	Trujillo,Venezuela	09º22'N; 70º26'W	800	Frozen	1
A. striata	McPhail trap	Lagoinha,PI, Brazil	05º49'S; 42º37'W	240	Ethanol	1
A. acris	*Hippomanea mancinella* Anacardiaceae	Falcón,Venezuela	10º03'N; 69º18'W	694	Frozen	1
A. suspensa	Laboratory colony	Gainesville,USA	29º40'N; 82º20'W	560	Frozen	1
A. ludens	McPhail trap	Santa Engracia, Mexico	24º01'N; 99º12'W	220	Frozen	1
A. bahiensis	*Brazilosimum costaricum*	Taxisco, Guatemala	14º04'N; 90º28'W	214	Frozen	1
A. barbiellinii	*Pereskia aculeata* Cactaceae	Arceburgo, MG, Brazil	21º20'S; 46º50'W	693	Frozen	1
A. coronilli	McPhail trap	Palmichal, Venezuela	10º00'N; 66º43'W	914	Frozen	1
A. serpentina	McPhail trap	Lagoinha, PI, Brazil	05º49'S; 42º37'W	240	Ethanol	1
	Manilkara zapota Sapotaceae	Maracay, Venezuela	10º14'N; 67º35'W	552	Frozen	1

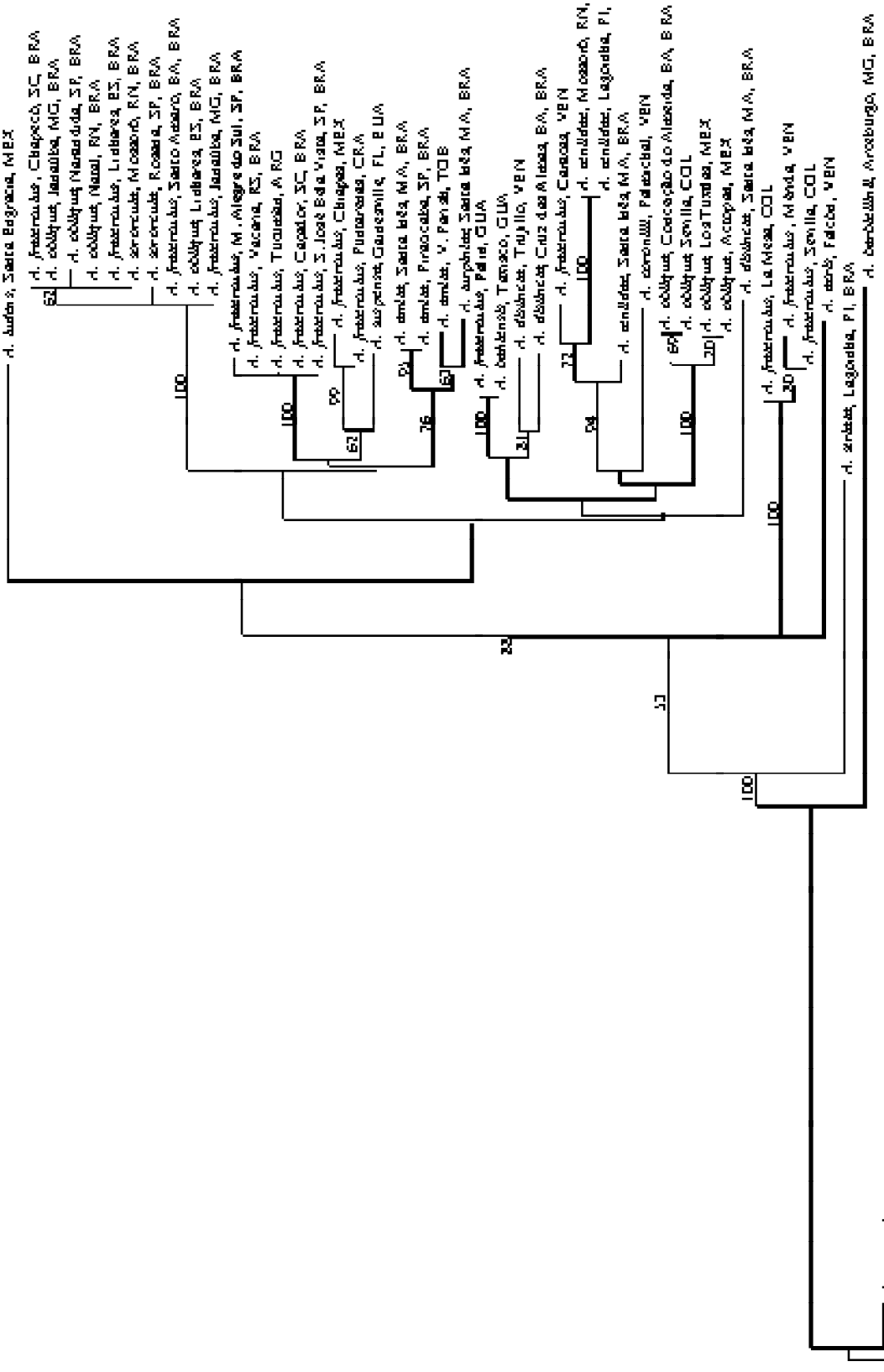

Figure 1. Phylogenetic relationships inferred from neighbor-joining tree based on Jukes-Cantor distances. Numbers on the branches indicate bootstrap values higher than 50% (100 replications).

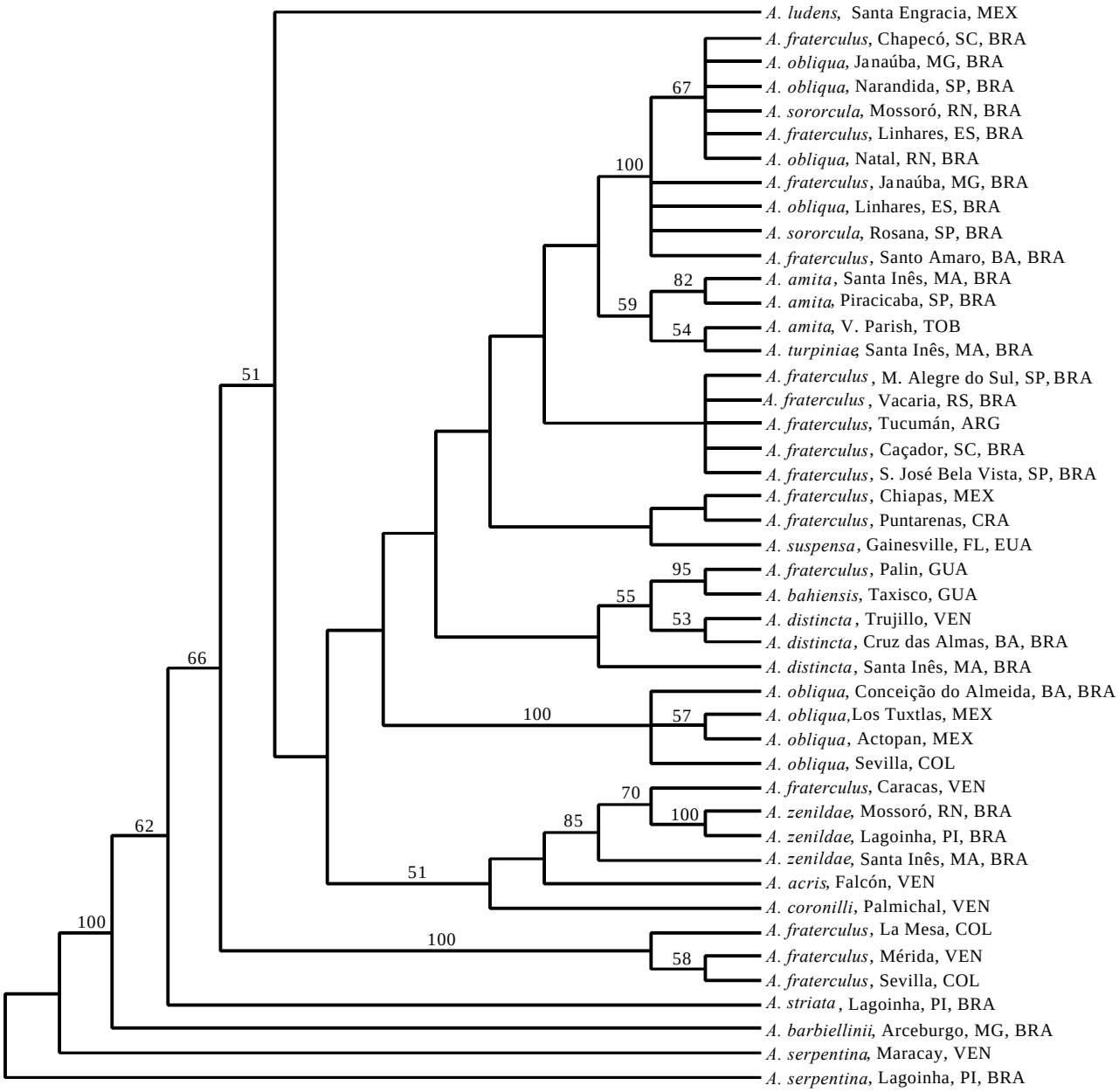

Figure 2. Phylogenetic relationships inferred from the strict consensus of two most parsimonius trees. Numbers in the branches indicate bootstrap confidence limits higher than 50% (100 replications).

and a sample from Caracas, Venezuela, was placed within a branch containing the morphologically similar *A. zenildae*. This sequence diversity is interesting - the analysis divides *A. fraterculus* much more finely among other species than has previous isozyme analysis (e.g., Steck 1991) but it is also puzzling. Is *A. fraterculus* that heterogeneous, or is there a substantial level of shared ancestral polymorphism among members of the *fraterculus* group, or is it possible that some of the *Anastrepha* species in this group have evolved independently from certain lineages within a relatively polymorphic *A. fraterculus*?

The eight populations of *A. obliqua* were not recovered as a monophyletic group in our study. Four samples form a well-supported clade, which includes specimens from Mexico, Colombia and Brazil (Bahia). The remaining *A. obliqua* samples were found in a second clade, in which our data also placed the two *A. sororcula* samples and four Brazilian *A. fraterculus* samples. Previous isozyme studies (Steck 1991, Selivon 1996) did not reveal significant differentiation among *A. obliqua* samples. Both of those studies included fewer samples of *A. obliqua* - in the former study only two populations from Brazil were included, and, in the latter, only populations from Brazil were analyzed. Further examination of population structure of this widely

distributed species should address this issue. The relationship between *A. fraterculus* and *A. sororcula* is also consistent with similarities in morphology, host use, karyotype and isozymes (Zucchi 1979, Malavasi *et al.* 1980, Solferini & Morgante 1987, Zucchi 1988, Morgante *et al.* 1993, Selivon 1996).

The variation in aculeus length for these three species shows some overlap, varying from 1.55 to 1.80 mm for *A. fraterculus*, from 1.55 to 1.80 mm for *A. obliqua* and from 1.45 to 1.60 mm for *A. sororcula* (Araújo 1997). Regarding geographical distribution, these species overlap widely – although *A. sororcula* is the one that has a more restricted distribution when compared to the other two species, occurring from southern to northern Brazil in a number of different states (Malavasi *et al.* 2000). Santos (1999) carried out experimental hybridization among *A. fraterculus*, *A. sororcula* and *A. obliqua*. Hybrid flies can be obtained in both directions in most crosses. This laboratory evidence of hybridization documents the possibility that some natural hybridization may still occur among these species. This would lead to shared mtDNA variants across species boundaries.

The species *A. amita* and *A. turpiniae* are included here for the first time in a molecular analysis. They are morphologically similar to each other and to *A. fraterculus* (Araújo 1997, Souza-Filho 1999). In our analysis, they comprise a single branch within branches leading to *A. fraterculus* and related species. Our three *A. amita* samples are paraphyletic with respect to our single sample of *A. turpiniae*, although with only modest statistical support. This relationship might also change if additional *A. turpiniae* samples were included.

A. ludens appeared at the base of the *fraterculus* group (only the clade of Andean *A. fraterculus* samples was more basal), consistent with previous mtDNA analysis (McPheron *et al*. 1999). The position of *A. distincta* close to *A. bahiensis* is also similar to the 16S topology (although our NJ results separate the three *A. distincta* samples somewhat). The position of *A. suspensa* is difficult to compare between the present study and that of McPheron *et al.* (1999), but it is relatively consistent in its position in the trees.

What questions are answered by this expanded analysis of the *fraterculus* group using COI sequence data? First, the integrity of the group as a whole is supported with the following cautions. The inclusion of *A. barbiellinii* in the *fraterculus* group should be reexamined. Also, adding samples of the remaining species in the group could affect the topologies recovered and the boundaries of the group relative to other species groups in the genus.

Second, we have added additional evidence to suggest the presence of multiple gene pools within the nominal *A. fraterculus*. There is now inescapable support for the existence of a new species in the high elevations of the Andes. The boundaries of this species relative to other members of the complex and its morphology, genetics, ecology, and behavior must all be approached through a well-designed study in that region. We suggest that a series of elevational transects are needed to clarify the overlap between the apparent species. Our study also suggests that further attention should be directed to an examination of *A. fraterculus* at the southern end of its geographic range. Although preliminary, our results indicate that flies from Argentina and southern Brazil may be genetically differentiated from populations elsewhere in the range of the species.

Third, further evaluation of *A. obliqua* may be required. The separation of *A. obliqua* into two clusters is not completely resolved. In other words, one group containing only *A. obliqua* is strongly supported, and a second group with *A. obliqua* plus *A. sororcula* and some *A. fraterculus* samples has high support. However, these two clusters are not necessarily widely separated from each other - resolution of this portion of our trees is not strong. It would be quite interesting to examine the population structure and species boundaries in that part of Brazil from which the *obliqua*/*sororcula*/*fraterculus* cluster is drawn.

Finally, relationships among many of the other members of the *fraterculus* group are beginning to take shape as more molecular analyses are completed. Morphology has not provided many testable hypotheses on which to design molecular tests. Perhaps we can reverse the situation and use some unexpected placements of *fraterculus* group members to inform our interpretation of morphological characters. In order to progress further with molecular approaches, a gene or genes with an even higher level of divergence will be required. The *fraterculus* group is clearly recently diverged in evolutionary time; a fast-evolving nuclear gene may be required to actually track patterns of evolutionary history within the group.

Acknowledgments

This study was conducted as partial requirement for the PhD degree of the senior author. Thanks are due to Allen Norrbom, Elton Araújo, Gary Steck, Jaime Piñero, Keiko Uramoto, Martin Aluja and Miguel F. de Souza-Filho for kindly providing specimens. We would also like to thank James Smith for his helpful comments regarding data analysis. The authors would like to thank CAPES for the fellowship to MRBSC, the International Atomic Energy Agency and the Pennsylvania Agricultural Experiment Station Project 3463 for funding.

Literature Cited

Aluja, M. 1994. Bionomics and management of *Anastrepha*. Annu. Rev. Entomol. 39: 155-178.

Araújo, E.L. 1997. Estudo morfométrico no acúleo de cinco espécies de *Anastrepha* Schiner, 1868 (Diptera: Tephritidae) do grupo *fraterculus*. M.S. thesis - Escola Superior de Agricultura "Luiz de Queiroz", USP, Piracicaba, 91p.

Bernasconi, M.V., C. Valsangiacomo, J.-C. Piffaretti & P.I. Ward. 1999. Phylogenetic relationship among Muscoidea (Diptera: Calyptratae) based on mitochondrial DNA sequences. Insect Mol. Biol. 9: 67-74.

Brower, A.V.Z. 1994. Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences

(Lepidoptera: Nymphalidae). Mol. Phylogenet. Evol. 3: 159-174.

Brown, J.M., O. Pellmyr, J.N. Thompson & R.G. Harrison. 1994. Phylogeny of *Greya* (Lepidoptera: Prodoxidae), based on nucleotide sequence variation in mitochondrial cytochrome oxidase I and II: congruence with morphological data. Mol. Biol. Evol. 11: 128-141.

Clary, D.O. & D.R. Wolstenholme. 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. J. Mol. Evol. 22: 252-271.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.

Han, H.Y. & B.A. McPheron. 1997. Molecular phylogenetic study of Tephritidae (Insecta: Diptera) using partial sequences of the mitochondrial 16S ribosomal DNA. Mol. Phylogenet. Evol. 7: 17-32.

Kumar, S., K. Tamura & M. Nei. 1993. MEGA: Molecular evolutionary genetics analysis, version 1.0. Institute of Molecular Evolutionary Genetics, Pennsylvania State University, University Park.

Lunt, D.H., D.X. Zhang, J.M. Szymura & G.M. Hewitt. 1996. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. Insect Mol. Biol. 5: 153-165.

Malavasi, A., J.S. Morgante & R.A. Zucchi. 1980. Biologia de "moscas-das-frutas" (Diptera: Tephritidae). I: lista de hospedeiros e ocorrência. Rev. Bras. Biol. 40: 9-16.

Malavasi, A., R.A. Zucchi & R.L. Sugayama. 2000. Biogeografia, p. 93-98. In A. Malavasi & R.A. Zucchi (ed.), Moscas-das-frutas de importância econômica no Brasil. Ribeirão Preto, Holos, 327p.

Mardulyn, P. & J.B. Whitfield. 1999. Phylogenetic signal in the CoI, 16S, and 28S genes for inferring relationships among genera of Microgastrinae (Hymenoptera; Braconidae): evidence of a high diversification rate in this group of parasitoids. Mol. Phylogenet. Evol. 12: 282-294.

McPheron, B.A. 2000. Population genetics and cryptic species, p. 483-490. In K. H. Tan (ed), Area-wide Control of Fruit Flies and Other Insect Pests. Penerbit Universiti Sains Malaysia, Penang, 782p.

McPheron, B.A., H-Y. Han, J.G. Silva & A.L. Norrbom. 1999. Phylogeny of the genus *Anastrepha* and *Toxotrypana* (Trypetinae: Toxotrypanini) based upon 16S rRNA mitochondrial DNA sequences, p. 343- 361. In M. Aluja & A. L. Norrbom (eds.), Fruit flies (Tephritidae): phylogeny and evolution of behavior. Boca Raton, Florida, CRC Press, 944p.

Morgante, J.S., A. Malavasi & G.L. Bush. 1980. Biochemical systematics and evolutionary relationships of Neotropical *Anastrepha*. Ann. Entomol. Soc. Am. 73: 622-630.

Morgante, J.S., D. Selivon, V.N. Solferini & S.R. Matioli. 1993. Evolutionary patterns in specialist and generalist species of *Anastrepha*, p. 15-20. In M. Aluja & P. Liedo (eds.), Fruit flies Biology and Management. Springer-Verlag, New York, NY, 492p.

Norrbom, A.L., R.A. Zucchi & V. Hernández-Ortiz. 1999. Phylogeny of the genera *Anastrepha* and *Toxotrypana* (Trypetinae: Toxotrypanini) based on morphology, p. 299-342. In M. Aluja & A.L. Norrbom (eds.), Fruit flies (Tephritidae): phylogeny and evolution of behavior. Boca Raton, Florida, CRC Press, 944p.

Santos, P. 1999. Variabilidade genética, isolamento reprodutivo e regra de Haldane em espécies de *Anastrepha* (Diptera: Tephritidae). Ph.D. thesis, Instituto de Biociências, USP. São Paulo, 102p.

Scarpassa, V.M., S. Geurgas, A.M.L. Azeredo-Espin & W.P. Tadei. 2000. Genetic divergence in mitochondrial DNA of *Anopheles nuneztovari* (Diptera: Culicidae) from Brazil and Colombia. Genet. Mol. Biol. 23: 71-78.

Selivon, D. 1996. Estudo sobre a diferenciação populacional em *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae). Ph.D. thesis, Instituto de Biociências, USP, São Paulo, 137p.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu & P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction "primers". Ann. Entomol. Soc. Am. 87: 651-701.

Solferini, V.N. & J.S. Morgante. 1987. Karyotype study of eight species of *Anastrepha* (Diptera; Tephritidae). Caryologia 40: 229-241.

Souza-Filho, M.F., A. Raga, N.A. Canal & R.A. Zucchi. 1999. *Anastrepha amita* Zucchi (Dip., Tephritidae): primeiro registro hospedeiro, nível de infestação e parasitóides associados. Arq. Inst. Biol. 66: 77-84.

Sperling, F.A.H. & D.A. Hickey. 1994. Mitochondrial DNA sequence variation in the Spruce Budworm species complex (*Choristoneura*: Lepidoptera). Mol. Bio. Evol. 11: 656-665.

Ståhls, G. & K. Nyblom. 2000. Phylogenetic analysis of the genus *Cheilosia* (Diptera, Syrphidae) using mitochondrial COI sequence data. Mol. Phylogen. Evol. 15: 235-241.

Steck, G.J. 1991. Biochemical systematics and population genetic structure of *Anastrepha fraterculus* and related

species (Diptera: Tephritidae). Ann. Entomol. Soc. Am. 84: 10-28.

Steck, G.J. & W.S. Sheppard. 1993. Mitochondrial DNA variation in *Anastrepha fraterculus*, p. 9-14. In M. Aluja & P. Liedo (eds.), Fruit flies Biology and Management. Springer-Verlag, New York, NY, 492p.

Stone, A. 1942. The fruit flies of the genus *Anastrepha*. Washington: USDA. 439p. (USDA. Misc. Publ. 439, 112p.).

Swofford, D.L. 1998. PAUP*: Phylogenetic analysis using parsimony, version 4.0b1. Sinauer Associates, Sunderland.

Zucchi, R.A. 1979. Novas espécies de *Anastrepha* Schiner, 1868 (Diptera: Tephritidae). Rev. Bras. Entomol. 23: 35-41.

Zucchi, R.A. 1988. Moscas-das-frutas (Dip., Tephritidae) no Brasil: taxonomia, distribuição geográfica e hospedeiros, p 1-10. In H.M.L. Souza (coord.), Moscas-das-frutas no Brasil. Campinas, SP, Fundação Cargill, 114p.

Zucchi, R.A. 2000. Lista das espécies de *Anastrepha*, sinonímias, plantas hospedeiras e parasitóides, p 41-48. In A. Malavasi & R.A. Zucchi (ed.), Moscas-das-frutas de importância econômica no Brasil. Ribeirão Preto, Holos, 327p.

Received 23/01/01. Accepted 30/09/01.