A Genetic–Morphological Characterization of Two Cryptic Species of the *Anastrepha fraterculus* Complex (Diptera: Tephritidae)

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ABSTRACT Biological characteristics of two entities of the *Anastrepha fraterculus* complex (Diptera: Tephritidae), referred to in previous publications as *A.* sp.1 *aff. fraterculus* and *A.* sp.2 *aff. fraterculus*, were further studied by a combined analysis of isozymes, karyotypes, morphometry, and crossings, in samples from 10 Brazilian populations. A survey of 16 enzymatic systems comprising 19 loci showed significant differences in the allele frequencies at four loci, FUM, ME, HEX, and LDH, allowing the recognition of two population clusters. These clusters also differ in their karyotypes, especially in the length of the sex chromosomes and in the size and location of heterochromatic regions. A morphometric analysis of wings and the aculeus in samples from five populations clearly showed a distinction between the two clusters but not between populations within each cluster. A phenetic analysis based on the Mahalanobis distance matrix also arranged the populations into the same two clusters. Crosses between populations of the same cluster showed no significant differences in egg hatching and in the adult sex ratio. However, a significant decrease in egg hatching was observed in the intercluster crosses. In crosses of cluster 1 males to cluster 2 females, a significant deviation in the sex ratio was observed according to Haldane's rule, but not in the reciprocal crosses, indicating that a certain degree of reproductive isolation occurs between populations of cluster 1 and cluster 2. The results indicate that the two population clusters actually represent two cryptic species of the nominal species *Anastrepha fraterculus*, corroborating previous studies on this complex. We propose that the denominations *A.* sp.1 *aff. fraterculus* and *A.* sp.2 *aff. fraterculus* should be maintained until an appropriate taxonomic revision is made.

KEY WORDS isozymes, chromosomes, wings, aculeus, Haldane's rule

THE FAMILY TEPHRITIDAE OF phytophagous insects comprises ≈4,000 species of worldwide distribution. The eggs of these insects are deposited in live vegetal tissue, from which the larvae feed and develop. Approximately 38% of the species infest fruit, many of which are commercial varieties, and thus the species assumes economic significance (White and Elson-Harris 1992).

The genus *Anastrepha* Schiner, recognized by its typical wing pattern, comprises ≈200 described species that infest a large number of host fruits. The species identification is based mainly on the morphology of the ovipositor aculeus (Norrbom et al. 1999). This genus is distributed throughout South and Central America, the southern United States, and the Caribbean islands, and ≈90 species occur in Brazil, infesting native as well as introduced fruits belonging to 31 families (Zucchi 2000, Norrbom 2004). In Brazil, some economically significant species belong to this genus, such as *Anastrepha fraterculus* (Wiedemann), *Anastrepha obliqua* (Macquart), and *Anastrepha sororcula* Zucchi (Malavasi and Morgante 1980).

Stone (1942) recognized in *A. fraterculus* an extensive variation in the wing pattern but attributed such variation to the existence of geographic races. Baker et al. (1944) suggested that a complex of cryptic species could be involved. Bush (1962) described in Mexican populations of *A. fraterculus* a different karyotype than the one described by Mendes (1958) for the populations from southeastern Brazil and suggested that they could represent two species. Afterwards, several analyses confirmed this hypothesis. Morgante et al. (1980), based on the analysis of 16 enzymatic loci in samples from 16 populations of *A. fraterculus* from southern, southeastern, and northeastern Brazilian regions, divided the *A. fraterculus* complex into four groups and showed that the populations from the northeastern differed from those collected in southern regions. Another isozymic survey was conducted by Steck (1991) in *A. fraterculus* populations. This author described significant divergences between two samples from Venezuela, one collected in lowland areas and the other in Andean regions, and suggested that two cryptic species could be involved, associated with altitudinal differences. He also reported divergences between populations collected in northeastern (Bahia) and southeastern (São Paulo) regions of Brazil, the former being more similar to the Central American,

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Table 1. Collecting sites and hosts of AF, AO, and SO samples

Code	Species	Host	Site	Altitude (m)
AF1	*fraterculus*	*Psidium guajava* L.	Baurú - SP 22° 17′ S, 49° 10′ W	526
AF2	*fraterculus*	*P. guajava*	Conceição do Almeida- BA 12° 30′ S, 39° 10′ W	190
AF3	*fraterculus*	*P. guajava*	Natal - RN 05° 48′ S, 35° 13′ W	30
AF4	*fraterculus*	*P. guajava*	Louveira - SP 23° 05′ S, 46° 50′ W	730
AF5	*fraterculus*	*Citrus sinensis* L. Osbec	Santa Isabel - SP 23° 18′ S, 46° 13′ W	554
AF6	*fraterculus*	*P. guajava*	Santa Isabel - SP 23° 18′ S, 46° 13′ W	554
AF7	*fraterculus*	*Terminalia catappa* L.	São Sebastião - SP 23° 40′ S, 45° 20′ W	1
AF8	*fraterculus*	*T. catappa*	São Sebastião - SP 23° 40′ S, 45° 20′ W	1
AF9	*fraterculus*	*Feijoa sellowiana* Berg.	Vacaria - RS 28° 27′ S, 50° 48′ W	971
AF10	*fraterculus*	*P. guajava*	Sete Lagoas - MG 19° 25′ S, 44° 12′ W	761
SO1	*sororcula*	*P. guajava*	Conceição do Almeida-BA 12° 30′ S, 39° 10′ W	190
SO2	*sororcula*	*P. guajava*	Natal - RN 05° 48′ S, 35° 13′ W	30
AO1	*obliqua*	*Spondias venulosa* Mart. ex Engl.	Ribeirão Preto - SP 21° 05′ S, 47° 50′ W	547
AO2	*obliqua*	*Spondias* sp.	São Luís - MA 02° 15′ S, 44° 10′ W	24

Mexican, and Venezuelan lowland populations. *A. fraterculus* samples from different regions were distinguished by the mitochondrial DNA (mtDNA) variability evidenced by restriction fragment-length polymorphism (Steck and Sheppard 1993, Santos 1994) and sequencing of 16S rRNA (McPheron et al. 1999) and COI region (Smith-Caldas et al. 2001). An isozyme survey conducted by Alberti et al. (1999, 2002) showed that in Argentina there was great genetic homogeneity among several samples of *A. fraterculus*, suggesting the existence of a single taxon that was similar to a sample from southern Brazil.

In addition to the genetic studies, karyotype analyses of the nominal *A. fraterculus* were made after the first descriptions of Mendes (1958) and Bush (1962). Solferini and Morgante (1987) described four karyotypes within the nominal species *A. fraterculus*, two of them in populations of southeastern (State of São Paulo) and two in populations of northeastern (State of Bahia) regions of Brazil. One of the karyotypes found in Bahia, however, was shown to actually belong to *A. sororcula* (Morgante et al. 1993). In Argentina and the southernmost areas of Brazil, only one karyotype, although polymorphic, was found in *A. fraterculus* samples (Selivon 1996, Basso and Manso 1998, Basso et al. 2003).

The result of these analyses was the conclusion that the nominal *A. fraterculus* indeed constitutes different biological entities. However, as in the studies cited above, different samples and/or different biological parameters were used, and the number of cryptic taxa could not be ascertained.

In 1998, Selivon and Perondini described differences in egg morphology between two entities of the *A. fraterculus* complex, which were called *A. fraterculus* sp.1 and *A. fraterculus* sp.2, later referred to as *Anastrepha* sp.1 *aff. fraterculus* and *A.* sp.2 *aff. fraterculus* (Yamada and Selivon 2001). Crosses among allopatric populations of these two species showed reduced egg hatching and deviation in the sex ratio of the adult progeny according to the Haldane's rule, indicating reproductive incompatibilities among them (Selivon et al. 1999). Differences also were found in bionomic parameters (Ometto 1997) and in some mechanisms of the embryonic development (Selivon and Perondini 1997a, Selivon et al. 1997). Later, Selivon et al. (2004), based on an analysis of the eggshell, mesoscutum morphology, and chromosomes, proposed the existence of two other entities in the *A. fraterculus* complex: *A.* sp.3 *aff. fraterculus*, that is common in coastal areas of the State of São Paulo, Brazil, and *A.* sp.4 *aff. fraterculus*, sampled in Guayaquil, Ecuador.

Recently, a morphometric analysis using the wings and the aculeus recognized that Mexican populations of *A. fraterculus* differ from specimens collected in Argentina, Brazil, and Colombia. However, no distinction was found between the samples from southern Brazil and Argentina that were studied (Hernández-Ortiz et al. 2004).

Herein, through a combined analysis of isozymes, karyotypes, morphometry, and compatibility crossings, we present a more detailed characterization of two species of the *fraterculus* complex, *A.* sp.1 *aff. fraterculus* and *A.* sp.2 *aff. fraterculus*, collected in a wide area of the geographic distribution of the complex in Brazil. In the isozyme analysis, samples of *A. sororcula* and *A. obliqua* also were included, to understand the relationship of the species of the *A. fraterculus* complex to other species of the *fraterculus* group.

Materials and Methods

Samples. Samples of *A. fraterculus* (sensu lato) (AF), *A. sororcula* (SO), and *A. obliqua* (AO) were collected in several sites in Brazil, as shown in Table 1. In this article, *A.* sp.1 *aff. fraterculus* and *A.* sp.2 *aff. fraterculus* will be abbreviated as *A.* sp.1 and *A.* sp.2. Samples AF2, AF5, AF6, and AF9 were taken from localities where previous studies on eggshell morphology and compatibility crosses indicated the occurrence of *A.* sp.1 (AF6 and AF9) and *A.* sp.2 (AF2 and AF5) (Selivon and Perondini 1998, Selivon et al. 1999). The AF5 and AF6 samples were collected in a single, noncommercial orchard but from different host fruit, oranges and guavas, respectively. A sample (AF8) of *A.* sp.3, previously characterized by Selivon et al. (2004), was included in the isozyme analysis. The other samples, AF1, AF3, AF4, AF7, and AF10, were

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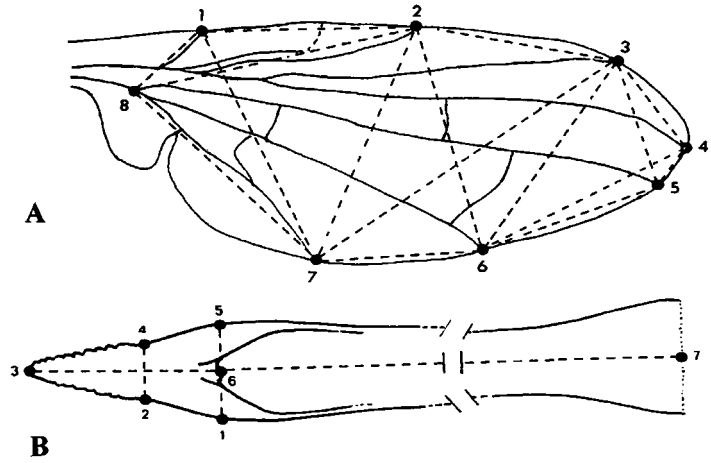

Fig. 1. Landmarks in the wing (A) and aculeus (B) and the segments (dashed lines) used for the morphometric analysis. (A) For the wings, the elected points were located at the junction of veins to the border of the wing or to other veins: 1, humeral cross vein; 2, R_1, sectoral branch of radial vein; 3, R_{2+3}, sectoral branch of radial vein; 4, R_{4+5}, sectoral branch of radial vein; 5, medial vein; 6, CuA_1, first branch of anterior cubital vein; 7, CuA_2, second branch of anterior cubital vein; 8, CuA_1 and medial vein junction (according to Foote et al. 1993). The variables (segments) for the discriminant analysis were D1 = 1-2; D2 = 2-3; D3 = 3-4; D4 = 4-5; D5 = 5-6; D6 = 6-7; D7 = 7-8; D8 = 8-1; D9 = 2-7; D10 = 3-6; D11 = 1-7; D12 = 2-8; D13 = 2-6; D14 = 3-7; D15 = 3-5; D16 = 4-6; D17 = 1-8-1. (B) For the aculeus, points 1 and 5, maximal inflection of the lateral curvature fixed by a transversal line passing by the oviduct end; points 2 and 4, maximal constriction below the serrate portion; 3, tip of the aculeus; 6, crossing point of imaginary lines connecting points 1–5 and 3–7; 7, point in mean position at the proximal end of aculeus. The defined variables were D1 = 1-5; D2 = 2-4; D3 = 3-6; and D4 = 3-7.

not previously characterized by any biological characteristic.

The infested fruit was brought to the laboratory, and emerged adults were recovered for the various analyses. Others (≈50) were used to establish laboratory colonies. The colonies were maintained in cages (30 by 30 by 60 cm) and fed water and a 3:1 mixture of sugar and corn protein hydrolysate, following the procedures described in Selivon and Perondini (1998). Except for *A. obliqua*, which was reared in mangoes, guavas were routinely furnished for oviposition, despite the fact that some of the colonies derived from individuals collected in different fruits. According to this protocol, the colonies were established and maintained at 25°C and 70 ± 5% RH.

Specimens from samples AF2, AF5, AF6, AF7, and AF9 were deposited in the dipteran collection of the Zoology Museum of the University of São Paulo.

Isozymes. Adult flies from each sample were recovered from infested fruit brought to the laboratory, and the isozymes were analyzed by standard starch gel electrophoresis, according to Malavasi and Morgante (1982), with minor modifications. Sixteen enzymatic systems comprising 19 loci were studied, by using staining solutions described by Williams (1980) and Malavasi and Morgante (1982): aconitase (Acon-1, Acon-2, 4.2.1.3.), alcohol dehydrogenase (Adh-1, 1.1.1.1), aspartate aminotransferase (Aat-1, Aat-2, 2.6.1.1.), fumarase (Fum, 4.2.1.2), α-glycerophosphate dehydrogenase (Gpdh, 1.1.1.8), hexokinase (Hk-1, 2.7.1.1), hydroxybutyric dehydrogenase (Hbdh, 1.1.1.30), isocitrate dehydrogenase (Idh, 1.1.1.42), lactate dehydrogenase (Ldh, 1.1.1.27), malate dehydrogenase (Mdh-1, Mdh-2, 1.1.1.37), malic enzyme (Me, 1.1.1.40), peptidase (Pep-2, 3.4.11–13), 6-phosphoglucose dehydrogenase (6Pgd, 1.1.1.44), phosphoglucose mutase (Pgm 2.7.5.1), phosphoglucose isomerase (Pgi, 5.3.1.9), and triose phosphate isomerase (Tpi, 5.3.1.1).

Detailed information about buffer systems and staining recipes is available upon request. As standards, electromorphs from sample AF1 were used, the value of 100 being arbitrarily attributed to the most common allele at each locus, and the mobility of other alleles measured relative to it. For each population, the allele frequency inferred from electromorphs was determined, and the Hardy–Weinberg conformance was tested locus by locus. F-statistics (F_{st}) also was calculated (Wright 1978), as well as genetic distance measures (Rogers 1972, Nei 1978). Using the genetic distance measures of Rogers (1972), a unweighted pair-group method with arithmetic average tree was generated (Sneath and Sokal 1973). These analyses were made using the BIOSYS-1 computer package (Swofford and Selander 1981).

Karyotypes. Chromosomes of larvae taken from infested fruit of samples AF2, AF3, AF4, AF5, AF6, AF7, and AF9 were analyzed. In this study, neural ganglia and adjacent imaginal discs of third instars were dissected, and the chromosomes were prepared by the standard C-banding method, as described by Selivon and Perondini (1997b). For each population, 20 males and 20 females were analyzed, and the best metaphase image from each one was digitalized using a DC100 Leica charge-coupled device coupled to the microscope and edited using Adobe Photoshop 7.0 software.

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Table 2. Genetic distance coefficients: above diagonal, Nei (1978) genetic distance; below diagonal, modified Rogers' distance (Wright 1978) for samples of AF, AO, and SO

Pop	AF1	AF2	AF3	AF4	AF5	AF6	AF7	AF8	AF9	AF10	SO1	SO2	AO1	AO2
AF1	—	0.233	0.195	0.001	0.243	0.004	0.218	0.091	0.001	0.000	0.145	0.142	0.440	0.440
AF2	0.439	—	0.008	0.233	0.001	0.225	0.004	0.186	0.240	0.230	0.187	0.182	0.606	0.606
AF3	0.405	0.096	—	0.192	0.010	0.184	0.010	0.195	0.196	0.191	0.155	0.150	0.508	0.580
AF4	0.048	0.441	0.404	—	0.243	0.002	0.219	0.083	0.002	0.000	0.142	0.140	0.444	0.444
AF5	0.449	0.049	0.102	0.451	—	0.234	0.006	0.192	0.249	0.242	0.194	0.188	0.616	0.614
AF6	0.075	0.430	0.393	0.058	0.439	—	0.210	0.082	0.004	0.004	0.138	0.136	0.417	0.416
AF7	0.425	0.081	0.107	0.427	0.086	0.415	—	0.179	0.225	0.218	0.176	0.167	0.609	0.608
AF8	0.289	0.398	0.406	0.277	0.405	0.273	0.390	—	0.102	0.090	0.204	0.192	0.534	0.532
AF9	0.048	0.448	0.409	0.052	0.457	0.072	0.434	0.305	—	0.001	0.141	0.140	0.436	0.433
AF10	0.041	0.439	0.404	0.034	0.451	0.069	0.428	0.288	0.043	—	0.141	0.140	0.440	0.440
SO1	0.357	0.398	0.365	0.356	0.406	0.347	0.386	0.418	0.355	0.355	—	0.000	0.333	0.336
SO2	0.354	0.393	0.359	0.353	0.400	0.345	0.377	0.406	0.354	0.353	0.039	—	0.354	0.356
AO1	0.578	0.647	0.636	0.582	0.654	0.563	0.645	0.623	0.580	0.581	0.516	0.527	—	0.000
AO2	0.579	0.649	0.637	0.583	0.654	0.563	0.646	0.623	0.579	0.582	0.518	0.530	0.047	—

The chromosomes from each population sampled were measured using the IPWIN32 software, and their length was represented as the percent value in relation to the total diploid complement length (Robertson 1957). The values for each population represent the average measurements of 10 metaphases. The karyotype measurement data for the different populations were compared by single-way analysis of variance (ANOVA), by using the GraphPad InStat computer package.

Morphometry. These measurements were performed on the right wings of males and females emerged from fruits of samples AF2, AF5, AF6, AF7, and AF9. For the females, the aculeus also was included in the analysis. A few days after emergence, the adults were killed by freezing, and the wings of each individual, as well as the aculeus, were excised and mounted (dorsal and ventral side up, respectively) in Permount under a coverslip. The morphometric variables for the wings were defined following the truss network method (Strauss and Bookstein 1982). As shown in Fig. 1A, eight landmarks were used for the wings, which allow the analysis of 17 variables (dotted lines). In the aculeus, no clear-cut landmarks could be ascertained, hence the exact truss network methodology could not be applied. Thus, seven reference points were selected, from which four variables (dotted lines) could be measured, as shown in Fig. 1B. The positions of the points for each structure of each individual were registered by a digitizing pad (SummaSketch 12"12") by using the Digitize program, and the distances were calculated by the Distance software.

Multivariate analysis was applied to the morphometric data, by using Statistica software (StatSoft, Tulsa, OK). The log-transformed data were submitted to a stepwise discriminant analysis. In the canonical analysis, four functions (roots) were extracted and the first two (greater discriminant power) used to calculate discriminant scores for each individual of each population, which were plotted in graphs. Additionally, based on the Mahalanobis distance matrix, an unweighted pair-group method with arithmetic average cluster analysis established the phenetic relationships among the populations.

Hybrid Progeny. Samples of populations AF2, AF5, AF6, and AF9 were used in these experiments. The methodology was the same as described in Selivon et al. (1999). To determine the rate of egg hatching, individual mating pairs were analyzed. Virgin, sexually mature adults were transferred from the laboratory colonies to smaller cages (15 by 15 by 25 cm). For egg collection, hemispheres made of 2% plain agar, stained with red aniline and wrapped in Parafilm M, were used (modification of Boller 1968). The hemispheres were replaced every 2–3 d. The eggs were taken from the artificial substrates and laid onto agar plates up to the time of hatching, ≈72 h later under laboratory conditions. After the first one to hatch, the eggs were examined for 6 d, after which the unhatched ones were considered nonfertile and discarded. In total, 16 types of crosses among individuals from the four samples were made, and the crosses within each sample were considered as controls. The length of time necessary for the females of intrapopulational (control) crosses to begin oviposition plus 1 wk was considered as the maximum period of time for the females of the interpopulation crosses to start laying eggs. The mating pairs whose females did not oviposit within this time interval were considered infertile and were replaced, to obtain a sufficient sample of eggs (100–120 eggs/cross). Statistical analysis was made with a GraphPad InStat computer package.

In another experiment, the frequency of emergence and the sex ratio of the adult progeny were analyzed. For each type of cross, five mating pairs were maintained in a single population cage, and guavas were furnished for oviposition. The pairs were maintained until 80–100 pupae were recovered from each cross. At emergence, the number of males and females was scored.

Results

Isozymes. The allele frequencies for 19 loci in the samples of *A. fraterculus* (sensu lato), *A. obliqua*, and *A. sororcula* are shown in the Appendix 1. Few cases of significant departures from Hardy–Weinberg expectations were observed in tetrameric enzymes and

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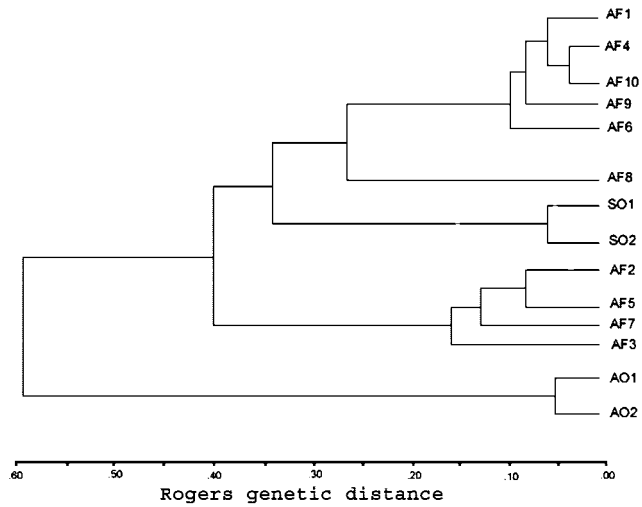

Fig. 2. Unweighted pair-group method with arithmetic average phenogram generated from a modified Rogers' genetic distances matrix (Wright 1978) for all analyzed populations of AF, AO, and SO.

could represent scoring imprecision, due to difficulties in making a precise identification of the electromorphs in starch gels.

Using allele frequency data, Nei (1978) and modified Rogers' (Wright 1978) distance matrices were obtained (Table 2). Rogers' modified distances were calculated by unweighted pair-group method with arithmetic average to produce the phenogram shown in Fig. 2. The major branching pattern of this phenogram was observed in all tree-building algorithms used and in phenograms produced by other distance measurements (data not shown).

From Fig. 2, it is possible to recognize two main clusters of nominal *A. fraterculus*, one comprising samples AF1, AF4, AF6, AF9, and AF10 (cluster 1) and the other encompassing samples AF2, AF3, AF5, and AF7 (cluster 2). Sample AF8 was found to be genetically distinct and to correspond to a different morph (*A.* sp.3), having different patterns of wing and mesoscutum morphology, chromosomes and eggshell morphology (Selivon et al. 2004). The *A. sororcula* samples showed genetic homogeneity, although they were collected in localities far apart (1,100 km), as were the *A. obliqua* samples (2,600 km).

The values of F_{st} presented in Table 3 show a high degree of differentiation among populations of nominal *A. fraterculus.* When all samples were included in the analysis, the average F_{st} was 0.57. However, when the two population clusters defined by genetic differences in the enzymatic loci were taken individually, the values of F_{st} decreased to 0.043 for cluster 1 (AF1, AF4, AF6, AF9, and AF10) and to 0.057 for cluster 2 (AF2, AF3, AF5, and AF7). Lower values were observed among samples of *A. sororcula* (0.010) and *A. obliqua* (0.017).

Table 3. Summary of F-statistics in 10 populations of *A. fraterculus* (sensu lato), five of *A. fraterculus* cluster 1, four of *A. fraterculus* cluster 2, and two of *A. sororcula* and two of *A. obliqua*

Species	F_{IS}	F_{ST}	F_{IT}
A. fraterculus (sensu lato)	0.207	0.570	0.659
A. fraterculus cluster 1	0.147	0.043	0.184
A. fraterculus cluster 2	0.207	0.057	0.253
A. sororcula	0.021	0.010	0.031
A. obliqua	0.112	0.017	0.127

The population AF8 is included in *A. fraterculus* (sensu lato) but not in the clusters analysis.

Karyotypes. Mitotic chromosomes were studied in samples from the two population clusters defined by isozyme analysis: AF4, AF6, and AF9 from cluster 1 and AF2, AF3, AF5, and AF7 from cluster 2. As described previously (Mendes 1958, Bush 1962, Solferini and Morgante 1987, Basso and Manso 1998, Basso et al. 2003, Selivon et al. 2004), the karyotype of nominal *A. fraterculus* exhibits 12 acrocentric chromosomes, an XX/XY sex chromosome system, and five pairs of autosomes of decreasing lengths. As shown in Fig. 3, these results were corroborated by the present analysis. Moreover, no significant differences in the relative sizes of corresponding autosomes in the seven populations were found, as shown by the ANOVA test (Table 4). Yet, the differences between cluster 1 and cluster 2 populations regarding the size of the X and Y chromosomes were found to be significant, with the individuals from cluster 1 populations having smaller sex chromosomes than those of cluster 2 (Table 4).

C-banding evidenced other differences among the chromosomes of the two clusters (Fig. 3A and B). The X chromosome of cluster 1 has two blocks of heterochromatin located at the tips, the distal one being larger than the proximal, centromeric one. In cluster 2, the X chromosome also shows two blocks of heterochromatin, one at the proximal end of the chromosome and the other at approximately two-thirds of the chromosome length. The Y chromosome of cluster 1 is almost entirely heterochromatic except at the

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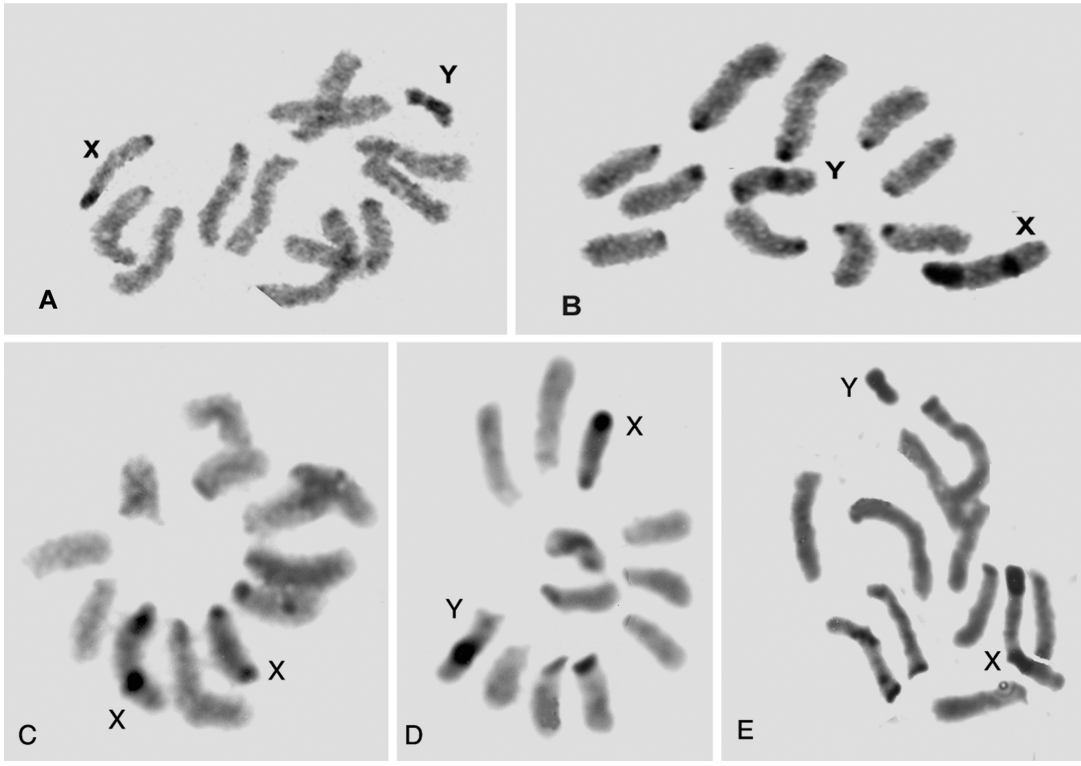

Fig. 3. Mitotic chromosomes of the *A. fraterculus* complex prepared by C-banding. (A) Male of cluster 1. (B) Male of cluster 2. (C–E) Mitotic chromosomes of F1 hybrids: female (C), male resulting from a cross of a cluster 1 female to a cluster 2 male (D), and male resulting from the reciprocal cross (E). Note the difference in size of the X and Y chromosomes, in the distribution of the heterochromatic blocks in the sex chromosomes and in the centromeric ends of autosomes of cluster 2 individuals.

proximal end, whereas the Y chromosome of cluster 2 has two blocks of heterochromatin, a smaller one at the proximal end and a larger one in the submedian region of the chromosome. All autosomes from cluster 2 individuals exhibit a clear, although small, block of heterochromatin at their proximal ends. These blocks are very faint in autosomes from cluster 1.

The differences between the chromosomes of these two clusters are clearly seen in F1 hybrids obtained in the laboratory, as shown in Fig. 3C–E.

Table 4. Mean length of the chromosomes of cluster 1 and cluster 2 and comparison among homologous chromosomes in the seven populations

Chromosome No.	Mean length[a] Cluster 1	Mean length[a] Cluster 2	ANOVA *F*	df	*P*	Obs.
II	10.1	10.6	0.475	6, 34	>0.50	ns
III	8.9	8.3	1.767	6, 34	>0.12	ns
IV	8.1	7.8	1.555	6, 34	>0.18	ns
V	7.4	7.5	1.539	6, 34	>0.20	ns
VI	7.1	7.0	2.433	6, 34	>0.05	ns
X	7.8	10.5	14.834	6, 34	<0.001	***
Y	4.1	8.2	23.510	6, 14	<0.001	***

[a] Length as percentage of total diploid complement.

Morphometry. Table 5 shows the average length of the variables elected by discriminant analysis to extract three functions (roots) for female and male wings, and two roots for the aculeus of individuals from samples AF6 and AF9 of cluster 1 and from AF2, AF5, and AF7 of cluster 2. Roots 1 and 2, which presented the highest eigenval values, were plotted in Fig. 4, and the individuals of each reference population were enclosed by an ellipsis encircling 95% of the points in the plane defined by the two discriminant functions. It is clear that function one of wings, from both males and females, allows the separation of the two population clusters of *A. fraterculus* (sensu lato). Measurements of the aculeus also point to the existence of two clusters, although not as clearly as the wing data.

The length of the elected variables indicates that the wings of cluster 1 individuals seem to be larger than in those of cluster 2. The three most powerful variables, although different for males and females, suggest that the difference in size should be more prominent in the proximal-distal axis of the wings. In other words, the average wing of cluster 2 individuals seems to be shorter than that of cluster 1 individuals. On the contrary, the aculeus of cluster 2 females is larger than that of cluster 1 individuals, both in width (variables D1

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Table 5. Mean length of the variables used in the stepwise discriminant analysis and the canonical correlation values (roots) for male and female wings and aculeus of cluster 1 and cluster 2 individuals of *A. fraterculus*

Variable	Length (mm) of variable		Root 1*	Root 2*	Root 3*	Root 4
	Cluster 1 Mean (SD)	Cluster 2 Mean (SD)				
Male wings						
D2	2.04 (0.09)	1.99 (0.04)	−1.0447	0.5910	0.2195	−1.0682
D14	3.96 (0.18)	3.64 (0.14)	1.0741	−0.6922	−1.4831	−0.3236
D4	0.65 (0.06)	0.64 (0.04)	−0.5620	0.5839	−0.6417	0.2426
D7	2.45 (0.09)	2.34 (0.14)	−0.6293	−0.5931	−0.0240	0.5959
D8	0.75 (0.04)	0.72 (0.04)	0.4588	0.7387	0.6089	1.2669
D17	13.41 (0.09)	12.34 (0.47)	−0.0554	−0.9858	1.3369	0.0339
Eigenval			5.7012	0.4113	0.2239	0.0220
Female wings						
D1	2.55 (0.12)	2.28 (0.15)	−1.6764	−0.2206	1.7754	−0.1479
D11	2.92 (0.15)	2.89 (0.17)	1.8784	−1.1692	0.0699	−0.5303
D6	1.89 (0.09)	1.71 (0.09)	−1.2700	−1.5908	−1.2148	−0.9486
D14	4.08 (0.19)	3.89 (0.19)	1.0048	4.5286	0.3632	1.1284
D5	2.19 (0.11)	2.08 (0.11)	−0.0205	−2.1027	−0.8371	−1.2751
D3	1.16 (0.07)	1.02 (0.07)	−0.3687	0.8226	−0.5386	1.3014
Eigenval			5.8512	0.3647	0.1966	0.1002
Aculeus						
D1	0.12 (0.006)	0.14 (0.006)	0.6575	0.5689	0.5351	
D4	4.65 (0.186)	4.98 (0.137)	0.7910	−0.6316	−0.6445	
D3	0.28 (0.017)	0.28 (0.016)	−0.5389	0.9616	−0.4405	
Eigenval			2.1592	0.3680	0.0411	

* Roots that were discriminant (ANOVA, $P < 0.05$).

and D3) and length (variable D4). A more detailed description of shape and size of these structures requires a specific analysis for this purpose.

A unweighted pair-group method with arithmetic average cluster analysis based on Mahalanobis distances (Fig. 4) showed the presence of the same two population clusters for both wings and aculeus, even though the relationships among the populations within each cluster differed according to the structure analyzed.

Hybrid Progeny. Table 6 summarizes the results obtained from crosses among individuals sampled from cluster 1 (AF6 and AF9) and cluster 2 (AF2 and AF5), two sympatric populations (AF5 and AF6) and two allopatric ones (AF2 and AF9). Of the intrapopulational (control) and intracluster crosses, on the average, 84.7% were fertile. However, for intercluster crosses, two sets of results were obtained. In crosses of cluster 1 females to cluster 2 males, only ≈42.5% of the females produced eggs, whereas in the reciprocal crosses 85% of the females were fertile, similarly to the controls.

Egg hatch rates in intracluster crosses did not present significant differences within nor between the two clusters, as shown by the ANOVA test ($F = 1.64$; df = 7, 48; $P = 0.1515$). A decrease in egg hatching was observed in the intercluster crosses (Table 6). When the egg hatching of these crosses was compared with their controls (for example, AF2 × AF6 compared with AF2 × AF2 and AF6 × AF6), significant differences were detected ($F = 7.037$; df = 11, 73; $P < 0.001$). Tukey's multiple comparison test showed that the differences in egg hatching of all intercluster crosses compared with their controls were significant (Table 6). Moreover, the differences in egg hatching in reciprocal crosses between population pairs (e.g., AF5 × AF2 and AF2 × AF5) were not significant.

The frequency of adults emerging from a known number of pupae was variable, and although it did not differ significantly among all types of crosses ($F = 3.383$; df = 2, 15; $P = 0.067$), it was close to the limit of significance (Table 6). When the data were analyzed by sex, significant differences were found. The emergence of adult females did not differ significantly among the crosses ($F = 0.1653$; df = 2, 15; $P = 0.849$), but highly significant differences were found regarding the emergence of adult males ($F = 11.042$; df = 2, 15; $P = 0.0016$). Tukey's test (Table 6) showed that males occurred with similar frequencies in the control, intracluster, and cluster 1 females × cluster 2 males crosses, but their frequency was significantly lower in the reciprocal intercluster crosses (cluster 2 females × cluster 1 males), resulting in a significant distortion of the sex ratio in favor of females, as shown in Table 6.

Discussion

Isozymes. The isozyme analysis showed that the Brazilian samples of *A. fraterculus* could be arranged into two clusters: cluster 1 comprising populations from higher altitudes (inland plateau areas) and cluster 2 from lowland (coastal) areas. The only exception was sample AF5 that is discussed below. Differentiation within the nominal species *A. fraterculus* was recognized previously by Morgante et al. (1980) and Steck (1991), and some of the groups they found fit well into the clusters discerned here. mtDNA variability pointed in the same direction, i.e., a divergence between individuals from populations of lowland and of inland plateau areas (Steck and Sheppard 1993,

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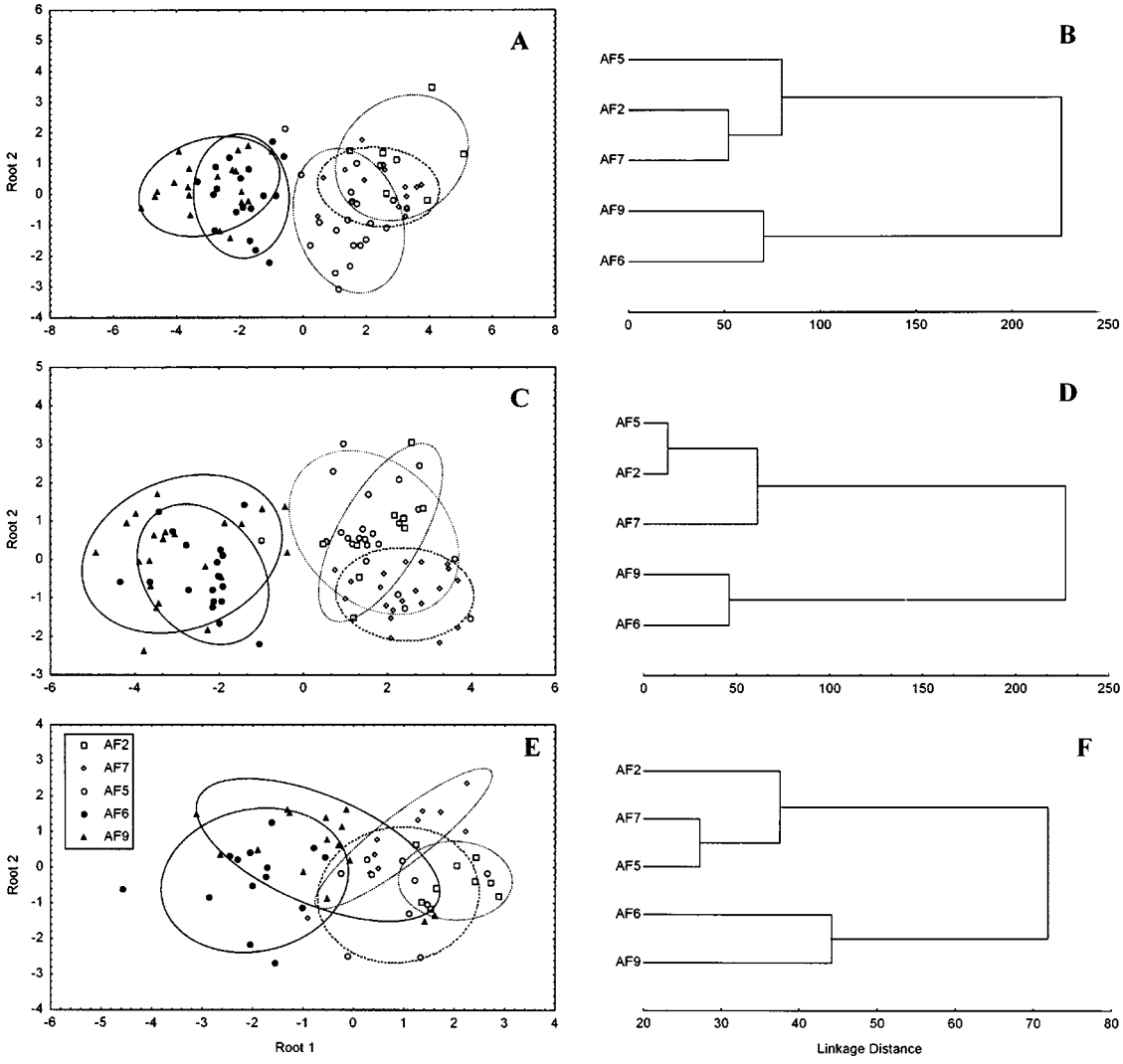

Fig. 4. Plots of discriminant functions (roots) 1 and 2 of cluster 1 (filled symbols) and cluster 2 (empty symbols) populations of *A. fraterculus*. The ellipses encircle 95% of the points for each population (left column). At right, phenograms from unweighted pair-group method with arithmetic average cluster analysis of Mahalanobis distances of five samples of *A. fraterculus* (codes in Table 1). (A and B) Female wings. (C and D) Male wings. (E and F) Aculeus.

Santos 1994, McPheron et al. 1999, Smith-Caldas et al. 2001). In the current study, with the exception of the samples from the coast of the state of São Paulo (AF7 and AF8), all the other samplings in lowland areas were from the northeastern Brazil, whereas the collections at higher altitudes were made in the southeastern and southern Brazil. Hence, it is still not clear whether the distribution of the two clusters is related to lower and higher altitudes or to a north/south differentiation.

The populations included in cluster 1 showed a higher genetic homogeneity than the populations of cluster 2, as shown in the dendrogram and by the F_{st} values. The mean genetic distance value (Nei 1978) among populations of cluster 1 and cluster 2, extracted from Table 2 (AF8 not included; see below), was 0.221, which is higher than that obtained by Berlocher et al. (1993) for cryptic species of *Rhagoletis zephyria* Snow and *Rhagoletis mendax* Curran (0.193) of the *pomonella* group. These authors considered the genetic distance values between these species as being low, compared with the data from other insect species.

Populations of clusters 1 and 2 showed significant differences in the allele frequencies at several loci, even for the two sympatric populations AF5 and AF6. Steck (1991) also reported differences at six loci between Bahia and São Paulo populations, which can be related to our AF2 and AF4. It should be noted that no hybrids were found in the sympatric samples AF5 and AF6. The fact that hybrids between the two clusters are produced in the laboratory (Selivon et al. 1999; this study) is suggestive of the fact that the absence or

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Table 6. Results of crosses within and among populations of *A. fraterculus* (sensu lato)

Cross (F × M)	*n*	Fertile crosses (%)	Mean % egg hatching ± SD[a]	No. pupae	Adult emergence *n* (%)			Sex ratio χ^2
					Total	Female	Male	
Intrapopulational (control)								
AF2 × AF2	8	6 (75.0)	79.3 ± 10.4a	100	54 (54.0)	26 (26.0)a	28 (28.0)a	0.018
AF5× AF5	7	6 (85.0)	76.2 ± 10.3a	114	65 (57.0)	32 (28.1)a	33 (28.9)a	0.000
AF6× AF6	7	6 (85.0)	82.8 ± 15.4a	100	59 (59.0)	32 (32.0)a	27 (27.0)a	0.424
AF9× AF9	7	6 (85.0)	77.2 ± 18.1a	110	57 (51.8)	28 (25.4)a	29 (26.4)a	0.000
Intraspecific								
AF2× AF5	7	7 (85.0)	77.4 ± 11.8a	120	62 (52.0)	30 (26.7)a	32 (26.7)a	0.016
AF5× AF2	8	6 (75.0)	69.2 ± 5.9a	110	50 (45.0)	26 (23.6)a	24 (21.8)a	0.033
AF6× AF9	6	6 (100)	72.7 ± 8.2a	120	71 (59.0)	37 (30.8)a	34 (28.3)a	0.056
AF9× AF6	7	6 (85.0)	56.5 ± 12.4a	144	86 (59.0)	43 (29.0)a	43 (29.0)a	0.000
Interspecific								
AF6× AF2	13	6 (46.1)	18.3 ± 19.5b	90	41 (45.5)	22 (24.4)a	19 (21.1)a	0.976
AF6× AF5	16	6 (43.7)	24.0 ± 19.3b	119	51 (43.3)	26 (21.8)a	25 (21.0)a	0.000
AF9× AF2	15	6 (40.0)	34.9 ± 22.8b	115	60 (52.0)	29 (25.2)a	31 (26.9)a	0.016
AF9× AF5	15	6 (40.0)	28.9 ± 19.8b	120	51 (43.0)	29 (24.2)a	22 (18.3)a	0.706
AF2× AF6	6	6 (100)	25.6 ± 23.6b	80	39 (48.7)	26 (32.5)a	13 (16.2)b	4.334*
AF2× AF9	6	6 (100)	33.5 ± 22.3b	83	47 (57.0)	33 (39.7)a	14 (16.3)b	7.681*
AF5× AF6	9	7 (67.0)	22.9 ± 24.6b	115	55 (48.0)	43 (37.4)a	12 (14.8)b	16.363*
AF5× AF9	8	6 (75.0)	37.7 ± 31.4b	92	45 (48.9)	31 (28.4)a	14 (12.8)b	5.689*

F, female; M, male.

[a] Same letters indicate nonsignificant differences.

rarity of hybrids in natural populations could be due to a low hybridization frequency; to a low viability of the hybrids; and/or, if they are viable and frequent, they may explore different host fruit, hence, they were not included in the samples. A more extensive study is necessary to elucidate this issue.

The analysis of the F_{st} values indicates the presence of different genetic pools in our *A. fraterculus* samples. Remarkably, the F_{st} mean of 0.570 (when all samples were included) was higher than the values obtained when the two clusters were considered apart (0.043 and 0.057 for clusters 1 and 2, respectively). Steck (1991) reported a mean F_{st} value of 0.428 for samples of *A. fraterculus* from Central and South America and considered such a high value as due to the existence of different species in the *A. fraterculus* complex. Lower F_{st} values were found by Alberti et al. (1999, 2002) for *A. fraterculus* samples from Argentina, which is in line with the proposition that there is just one taxon in that region.

It is worth noting that sample AF8 exhibited a genetic distinctiveness in relation to the samples of cluster 1. This finding corroborates previous data based on the analysis of other biological characteristics, which allowed the recognition of these individuals as a different entity in the *A. fraterculus* complex that was named *A.* sp.3 *aff. fraterculus* (Selivon et al. 2004).

The isozyme results are in agreement with those presented by Selivon and Perondini (1998), which, based on eggshell morphology, advocated the existence of two entities, *A.* sp.1 *aff. fraterculus* and *A.* sp.2 *aff. fraterculus*, in samples from the same locality here represented by AF6 and AF5, which were assigned to cluster 1 and cluster 2, respectively.

The hypothesis of nonmonophyly of the *A. fraterculus* complex, discussed by Steck (1991), McPheron et al. (1999), and Smith-Caldas et al. (2001), also is supported by our data showing that samples of cluster 1 are more closely related to *A. sororcula* than samples of cluster 2. The two samples of *A. sororcula* showed a genetic homogeneity not found by Smith-Caldas et al. (2001) in their samples analyzed for mtDNA.

It is relevant to note that McPheron et al. (1999) found great genetic similarity between *A. sororcula* and a sample of *A. fraterculus* collected in a coastal area of São Paulo, Brazil. It is possible that their *fraterculus* sample corresponds to biological entities here grouped to cluster 2, or even to the *A.* sp.3 *aff. fraterculus* described by Selivon et al. (2004), because this form occurs in sympatry with cluster 2 in coastal regions. In the report of McPheron et al. (1999), there is no other sample of *A. fraterculus* that, according to its geographic origin, could be related to cluster 1. So, the relationship between *A. sororcula* and the *A. fraterculus* complex remains to be elucidated.

A. obliqua occurred at the base in relation to other species of the *fraterculus* group analyzed here, similarly to the report of Steck (1991). We did not find any significant genetic distinction between the two samples of *A. obliqua* either, even though they were collected far apart from each other. However, genetic heterogeneity was described in *A. obliqua*, by using DNA sequences of mitochondrial cytochrome oxidase I (Smith-Caldas et al. 2001).

Karyotypes. The populations of cluster 1 and cluster 2, including the sympatric AF6 and AF5, also differed in their karyotypes, the main differences being related to the size of the sex chromosomes. No hybrid karyotypes were found in these samples of sympatric populations, which is in line with the results of the isozyme analysis.

Different karyotypes have been reported previously for *A. fraterculus*. Bush (1962) described a karyotype showing large sex chromosomes in a Mexican population, which should be similar to the karyotype

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of cluster 2 described here. These data are also in accordance with the observations of Steck (1991), who considered the population of Bahia (sample of Bahia, AF2, here included in cluster 2) similar to the Mexican populations of *A. fraterculus.* However, Mendes (1958) detected a heteromorphic pair in a sample from Campinas (state of São Paulo), similar to the karyotype of cluster 1 of this study. Solferini and Morgante (1987) found four karyotypes in *A. fraterculus* samples collected in São Paulo and Bahia, differing in their sex chromosomes. The karyotype they found in populations from São Paulo (karyotype 1) corresponds to the karyotype of cluster 1 described here; the karyotype they found in São Paulo samples (karyotype 2) was not found in the current study; karyotype 3 was later on recognized to be of *A. sororcula* (Morgante et al. 1993), and karyotype 4, found in Bahia, presented a Y chromosome that was larger than the X, thus being different from the karyotypes we found in the current study. This means that the karyotype of cluster 2 described here does not correspond to any of those found by Solferini and Morgante (1987). In Argentina, only one basic karyotype, although polymorphic, was found in several populations of nominal *A. fraterculus* (Basso and Manso 1998, Basso et al. 2003), having one heteromorphic pair of sex chromosomes, with a small heterochromatic Y and an X with heterochromatic blocks at its extremities. This karyotype seems to correspond to that of cluster 1 individuals described here, found in the inland plateau from São Paulo to southern Brazil. One X-chromosome variant having a constriction in the proximal heterochromatic block found by Basso and Manso (1998) and Basso et al. (2003) in Argentine populations, also was found by Selivon (1996) in a sample from southern Brazil (identified as AF9 here). These data are in line with those found by Smith-Caldas et al. (2001), who considered, based on DNA sequences of the mitochondrial COI gene, that flies from Argentina and southern Brazil were genetically similar but different from samples taken elsewhere in the range of the nominal species *A. fraterculus.*

Morphometry. The data on the morphometry of wings and aculeus are consistent with the findings of the isozyme and karyotype analyses, allowing the allocation of the samples into two clusters. It is relevant to note that distinct variables were elected by the discriminant analysis to extract the roots for male and female wings. This probably resulted from sexual dimorphism that may be present in this structure. Another interesting fact is that in the plots of Fig. 4, the data on male wings are less dispersed than the points for the female wings. One possible reason for this could be that, in males, the fanning of the wings plays an important role in the mating behavior, as was shown for several species of *Anastrepha,* including the nominal *A. fraterculus* (Aluja et al. 1999). It seems likely that restrictive selective forces are operating on the variation of shape and size of male wings.

The relative homogeneity within each cluster, even considering geographically distant samples (AF2 and AF5; AF6 and AF9), also should be noted. On the contrary, the differences between the two clusters added to the variation observed in other biological parameters strongly pointed to the existence of different entities within the *A. fraterculus* complex. A similar level of morphological distinctiveness was considered as indicative of the involvement of different entities in groups of species, as was shown for species of *Drosophila* Fallén and other organisms (Palmer and Wetton 1987, Cesaroni et al. 1989, Tidon-Sklorz and Sene 1995).

The morphometric analysis conducted by Hernández-Ortiz et al. (2004) showed no differences between two Brazilian samples, one collected in an inland plateau area, Caçador, state of Santa Catarina, and the other in São Paulo. If the sample from São Paulo also was taken from the inland plateau region, then their data are in line to ours, which revealed no distinction between samples AF9, collected in Vacaria (≈200 km apart from Caçador, both in plateau area) and Louveira (AF4), which is also located in a plateau region of the state of São Paulo. Moreover, one the variables of the aculeus (A4) measured by Hernández-Ortiz et al. (2004) is equivalent to variable D1 measured in the present analysis. They found a mean value of 0.12 for both samples (São Paulo and Caçador), and we obtained a mean value of 0.125 for our sample from Vacaria (AF9) and for another sample of cluster 1 individuals. The D1 variable of cluster 2 individuals, measured in the present analysis, shows the higher mean value of 0.137, distinct from cluster 1 individuals. These data suggest that the Brazilian specimens analyzed by Hernández-Ortiz et al. (2004) belong to morphotypes included here in cluster 1 populations.

Morphological differences in immature stages were first described for the eggshell of *A.* sp.1 *aff. fraterculus* and *A.* sp.2 *aff. fraterculus,* sampled from guavas and oranges from Santa Isabel, state of São Paulo (Selivon and Perondini 1998), which correspond to samples AF5 and AF6 of the present report. Differences in eggshell morphology were later described in two other entities of the *fraterculus* complex sampled in Brazil and Ecuador, which were respectively named *A.* sp.3 *aff. fraterculus* (corresponding to sample AF8 in the present report) and *A.* sp.4 *aff. fraterculus* (Selivon et al. 2004).

Hybrid Progeny. The present results and those reported previously (Selivon et al. 1999) indicate that reproductive incompatibilities are present among the entities of the *A. fraterculus* complex. It should be noted that no hybrids between cluster 1 and cluster 2 were detected in the samples of natural populations analyzed here. One of the best studied cases of reproductive isolation in fruit flies is that of *Rhagoletis* Loew from the *pomonella* group (reviewed in Bush 1992). Several pairs of sympatric species have been analyzed in which a low level of hybridization was detected in natural populations (Feder and Bush 1989, Smith et al. 1993). Studies carried out under laboratory conditions showed that postzygotic reproductive isolation varied from weak to almost complete between species belonging to this group (Smith et al. 1993).

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The decrease in egg hatching verified in laboratory crosses between cluster 1 and cluster 2 individuals was not surprising, because they differ considerably in several genetic parameters, as shown above. The reason for this decrease could not be determined conclusively, but some hypotheses can be raised. Besides possible problems in the development of the hybrid eggs due to failure in genomic adjustment, the decrease in the egg hatching rate also may be related to cytoplasmic incompatibility.

Selivon et al. (2002) reported the presence of *Wolbachia* bacteria in *A.* sp.2, and eggs of *A.* sp.1 also harbor a large population of these endosymbionts (D.S., unpublished data). Cytoplasmic incompatibility caused by different strains of *Wolbachia* is well documented in several insect species (Bourtzis and O'Neill 1998; Werren et al. 1995; Werren 1997, 1998) and is assumed to be involved in cytoplasmic incompatibilities in another fruit fly, *Rhagoletis cerasi* Loew (Riegler and Staufer 2002). Another possible explanation for such a decrease may be related to the presence of nonfertilized eggs, and in this case, we must consider that prezygotic isolation could not be totally broken. Breaking up such a premating barrier, even under laboratory conditions, may not be a simple task, and differences in the copulation behavior of *Anastrepha* species seems to be very frequent in prezygotic reproductive isolation (Aluja et al. 1999). Therefore, failure in mating or in the copulation process could explain the increased number of unfertile intercluster crosses observed.

The sex ratio distortions caused by the reduction in the number of males found in progeny of cluster 2 females crossed to cluster 1 males are in line with Haldane's rule. The explanation for this empirical rule is controversial, and none of the existent hypotheses is completely satisfactory, simply because Haldane's rule may not have a single explanation but may be the result of several different phenomena (Coyne and Orr 1989a, Coyne 1992, Wu and Hallocher 1998). Coyne and Orr (1989b) demonstrated that Haldane's rule applies when males become sterile or unviable before any effects arise in the females, in both reciprocal crosses between two taxa. In a few cases, however, sterility or lack of viability of both male and female offspring of the reciprocal crosses seems to occur early in the process of divergence. The authors concluded that total sterility or inviability of a hybrid progeny is nearly always preceded by sterility or inviability of males alone, indicating that Haldane's rule would represent a nearly obligatory first step in the evolution of postmating isolation in *Drosophila.*

The observation of Haldane's rule in the crosses of cluster 2 females to cluster 1 males could at first suggest that the speciation of these two forms is recent. However, the absence of sex ratio distortions in the reciprocal crosses, which nonetheless present a lower egg hatchability, indicate that, in these cases, both genders are affected. Hence, according to the hypothesis of Coyne and Orr (1989b), the data so far obtained on *A. fraterculus* would suggest that the divergence between cluster 1 and 2 is ancient enough to cause both genders to be affected. However, based on the scarce data existent so far, the hypothesis that male and female offspring could be unviable or sterile early in the process of speciation cannot be simply ruled out. Actually, the data set suggests that the *A. fraterculus* complex has a recent evolutionary origin.

Zone of Sympatry. As mentioned above, the association of the two clusters of *A. fraterculus* populations with different altitudes or latitudes is not clear. Regardless of these facts, it is surprising that the two forms are rarely found coexisting, because dispersal of the fruit flies is facilitated by anthropic action. McPheron (1990) stressed the importance of transportation of infested fruits in the maintenance of the genetic homogeneity of *Rhagoletis pomonella* (Walsh) in the northeastern United States. These kind of data are not available for fruit fly species in Brazil. The fact that samples from different host plants in several localities show no genetic differentiation (Morgante et al. 1980, Malavasi and Morgante 1982) indicates that populations of *A. fraterculus* clusters 1 and 2 are rarely found together. In the only region of sympatry detected so far, a valley region in the State of São Paulo (Vale do Paraíba), they infest different hosts, respectively, guavas and oranges.

Care also must be taken when distinctive host use in zones of sympatry is considered. One could interpret the fact of two different sympatric entities infesting alternative hosts (e.g., guavas and oranges infested, respectively, by *A.* sp.1 and *A.* sp.2) as representative of host-races, in a similar way as in *R. pomonella* (Diehl and Bush 1984). However, more extensive samplings in the range of the geographic distribution of the species indicate that, when in allopatry, both *A.* sp.1 and *A.* sp.2 preferentially infest guavas, meaning that they do not have any obligatory specificity in host use. If we consider that nominal *A. fraterculus* preferentially infest fruit of the family Myrtaceae (Malavasi and Morgante 1980) and that Santa Isabel is located on the inland plateau of São Paulo, we may consider that the population of *A.* sp.1 infesting guavas is the local one, and *A.* sp.2 is an introduced form infesting an available, exotic host, oranges. Regardless of the way in which this introduction was accomplished, the fact is that the individual characteristics of the two forms are being preserved, as demonstrated in the current study.

It is worth noting that the distinctive patterns of the biological characteristics analyzed are more pronounced when *A.* sp.1 and *A.* sp.2 are in sympatry. Likewise, more expressive distortions in the sex ratio and lower egg hatchability were observed in this zone. If these different levels of distinctiveness are indicative of peculiar evolutionary dynamics in allopatric and sympatric zones is an issue that remains to be clarified.

In conclusion, the data presented here provide the necessary support to suggest that cluster 1 and cluster 2 flies represent two different and distinct entities in the *fraterculus* complex, as pointed out by Selivon and Perondini (1998), based on an analysis of eggshell morphology.

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As mentioned above, in studies comprising samples from Argentina and southernmost Brazil (Basso and Manso 1998; Alberti et al. 1999, 2002; Basso et al. 2003), only one entity of the *fraterculus* complex was found, leading the authors to raise doubts about the existence of the *A. fraterculus* complex. This could be true for that region, but the nominal *A. fraterculus* do occur in a continent-wide distribution, and it is necessary to have samples from extensive and distant areas to accurately characterize this complex of cryptic species.

The present analysis of Brazilian nominal *A. fraterculus* populations has evidenced two clusters into which the samples can be arranged based on several biological features, such as genetic differentiation, karyotypes, morphology, and reproductive compatibilities. These data, combined to previous ones such as bionomic parameters, eggshell morphology, and some phenomena of early embryogenesis (Ometto 1997; Selivon and Perondini 1997a, 1998; Selivon et al. 1997), give the necessary support to consider *A.* sp.1 *aff. fraterculus* (cluster 1) and *A.* sp.2 *aff. fraterculus* (cluster 2) as truly distinct biological entities in the *fraterculus* complex. These two species differ from those characterized by Selivon et al. (2004) in samples from Brazil and Ecuador, named *A.* sp.3 *aff. fraterculus* and *A.* sp.4 *aff. fraterculus*, respectively. We propose that these names should be maintained until a proper taxonomic review can be carried out.

Therefore, at present, we have characterized four entities of the *fraterculus* complex, three of which occur in Brazil (*A.* sp.1, *A.* sp.2, and *A.* sp.3) and one in Ecuador (*A.* sp.4). The possible occurrence of other species in the *fraterculus* complex (Hernández-Ortiz et al. 2004), as well as the precise geographic distribution of the species characterized so far, are issues that remain to be solved. Integrative cooperation among different research groups is needed to deal with these tasks.

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Appendix. Allele frequencies for 19 loci in *A. fraterculus* (sensu lato) *A. obliqua* and *A. sororcula* populations

		Populations													
Locus	Allele	AF1	AF2	AF3	AF4	AF5	AF6	AF7	AF8	AF9	AF10	AS1	AS2	AO1	AO2
	(*n*)	20	25	31	23	45	42	23	13	25	20	25	19	14	24
Pgm	70	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	86	—	—	0.016	—	—	0.036	—	—	0.020	—	—	—	0.071	0.021
	90	0.100	0.020	0.016	0.065	0.022	0.071	0.022	—	0.080	0.075	0.020	—	0.750	0.854
	100	0.800	0.960	0.952	0.935	0.900	0.821	0.826	1.000	0.820	0.925	0.920	0.895	0.179	0.125
	110	0.100	0.020	0.016	—	0.078	0.071	0.152	—	0.080	—	0.060	0.105	—	—
	(*n*)	16	25	31	24	23	42	21	15	25	10	19	20	19	25
Pgi	100	1.000	1.000	1.000	1.000	1.000	0.988	1.000	1.000	0.960	1.000	1.000	0.975	1.000	1.000
	113	—	—	—	—	—	0.012	—	—	0.040	—	—	0.025	—	—
	(*n*)	17	25	29	15	38	39	18	11	17	11	25	20	19	13
Fum	70	—	—	—	—	0.039	—	—	—	—	—	—	—	—	—
	80	—	0.880	0.931	—	0.961	—	0.667	—	—	—	—	—	—	—
	90	—	0.120	0.069	—	—	—	0.333	—	—	—	—	—	—	—
	100	1.000	—	—	1.000	—	1.000	—	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	(*n*)	20	25	31	20	34	42	21	9	25	20	22	20	22	25
Aat-1	70	—	—	0.032	—	—	—	—	—	—	—	—	—	—	—
	100	1.000	1.000	0.968	0.975	0.971	1.000	1.000	1.000	0.940	1.000	1.000	1.000	1.000	0.900
	200	—	—	—	0.025	0.029	—	—	—	0.060	—	—	—	—	0.100
	(*n*)	16	18	19	7	37	31	16	9	19	20	19	20	14	16
Aat-2	−100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.969
	−60	—	—	—	—	—	—	—	—	—	—	—	—	—	0.031
	(*n*)	20	25	21	24	45	42	23	12	25	20	19	20	20	11
Mdh-1	65	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	78	0.200	—	—	0.083	0.033	0.024	—	—	0.080	0.075	—	0.050	—	—
	100	0.800	0.940	1.000	0.917	0.867	0.952	0.826	1.000	0.920	0.925	1.000	0.950	1.000	1.000
	118	—	0.060	—	—	0.100	0.024	0.174	—	—	—	—	—	—	—
	(*n*)	15	26	31	21	35	37	19	11	25	17	17	15	9	12
Mdh-2	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	(*n*)	11	25	18	19	41	32	21	11	16	13	15	11	20	23
Me	92	—	—	—	—	—	—	—	0.273	—	—	1.000	1.000	1.000	1.000
	100	1.000	0.120	0.167	1.000	0.049	0.859	0.095	0.727	1.000	1.000	—	—	—	—
	108	—	0.880	0.833	—	0.951	0.141	0.905	—	—	—	—	—	—	—
	117	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	(*n*)	20	25	31	22	28	42	20	15	21	20	17	15	25	14
Gpdh	92	—	—	—	—	—	0.012	—	0.067	—	—				
	100	1.000	1.000	1.000	1.000	1.000	0.976	1.000	0.933	1.000	1.000	1.000	1.000	1.000	1.000
	106	—	—	—	—	—	0.012	—	—	—	—	—	—	—	—
	(*n*)	8	5	5	5	29	11	14	6	7	5	12	8	25	18
Ldh	100	1.000	—	—	1.000	—	1.000	—	1.000	1.000	1.000	—	—	—	—
	110	—	1.000	1.000	—	1.000	—	1.000	—	—	—	1.000	1.000	—	—
	125	—	—	—	—	—	—	—	—	—	—	—	—	1.000	1.000
	(*n*)	16	22	31	24	38	42	22	15	21	11	25	20	28	25
ldh	70	—	—	—	—	—	—	—	—	—	—	—	—	0.036	0.020
	92	—	—	0.081	—	—	0.048	—	0.067	—	—	—	—	—	—
	100	1.000	0.818	0.903	1.000	0.829	0.845	0.932	0.800	1.000	1.000	0.940	1.000	—	—
	110	—	0.182	0.016	—	0.171	0.107	0.068	0.133	—	—	0.060	—	0.964	0.980
	(*n*)	20	25	31	24	45	42	23	15	16	20	25	20	27	22

Appendix. Continued

		Populations													
Locus	Allele	AF1	AF2	AF3	AF4	AF5	AF6	AF7	AF8	AF9	AF10	AS1	AS2	AO1	AO2
Hbdh	57	—	0.040	—	—	—	—	—	—	—	—	—	—	—	—
	84	—	0.120	0.097	—	0.056	0.048	0.022	—	—	—	0.100	0.025	0.019	—
	100	0.900	0.840	0.903	0.896	0.944	0.929	0.913	1.000	1.000	0.925	0.900	0.950	0.722	0.864
	128	0.100	—	—	0.104	—	0.024	0.065	—	—	0.075	—	0.025	0.259	0.136
	(*n*)	8	18	22	6	10	8	8	6	13	8	12	6	5	13
	93	—	—	—	—	—	—	—	—	—	—	—	—	1.000	1.000
6Pgd	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	—	—
	(*n*)	13	17	17	12	15	26	6	7	12	20	9	7	15	12
Hk-1	96	0.077	1.000	0.647	0.042	1.000	—	0.917	1.000	—	0.050	0.111	0.143	—	—
	100	0.923	—	0.353	0.958	—	1.000	0.083	—	1.000	0.850	0.889	0.857	1.000	1.000
	(*n*)	8	11	31	16	14	9	8	8	8	12	7	9	9	6
Pep-2	46	—	—	0.016	—	—	—	—	—	—	—	—	—	—	—
	70	—	—	0.048	—	—	—	—	—	—	—	—	—	0.056	—
	100	1.000	1.000	0.935	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.944	1.000
	(*n*)	20	25	31	24	17	42	17	8	19	12	18	9	16	11
Tpi	95	—	0.020	0.016	—	—	—	—	—	—	—	—	—	—	—
	100	1.000	0.980	0.984	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	(*n*)	16	24	21	24	35	29	23	12	24	20	22	20	10	9
Aco-1	60	—	0.021	—	—	—	—	—	—	—	—	—	—	—	—
	70	0.063	0.104	—	—	—	—	—	—	—	0.100	—	—	0.050	0.056
	85	—	0.208	0.333	—	0.200	0.034	0.283	—	0.042	—	0.023	0.100	—	—
	100	0.875	0.667	0.667	0.813	0.700	0.724	0.609	0.167	0.958	0.850	0.955	0.825	0.900	0.889
	116	0.031	—	—	0.167	0.100	0.207	0.109	0.792	—	—	0.023	0.075	—	—
	133	0.031	—	—	0.021	—	0.034	—	0.042	—	0.050	—	—	0.050	0.056
	(*n*)	8	14	25	17	35	31	13	7	12	11	11	20	9	5
	−110	—	—	—	—	—	0.016	—	—	—	—	0.591	0.575	—	—
Aco-2	−100	1.000	1.000	1.000	1.000	1.000	0.984	1.000	1.000	1.000	1.000	0.409	0.425	0.056	0.100
	−80	—	—	—	—	—	—	—	—	—	—	—	—	0.944	0.900
	(*n*)	11	25	31	10	41	36	23	8	14	10	20	19	9	13
Adh-1	−100	1.000	0.980	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.950	1.000	—	—
	−66	—	0.020	—	—	—	—	—	—	—	—	0.050	—	1.000	1.000

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