Neotropical Entomology

SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

A Morphometric and Molecular Study of *Anastrepha pickeli* Lima (Diptera: Tephritidae)

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Abstract

This study investigated the level of morphometric and genetic variability among populations of *Anastrepha pickeli* Lima from several localities in Brazil, one locality in Bolivia and one in Paraguay. Traditional and geometric morphometric analyses were used, as well as sequencing of a fragment of the cytochrome oxidase gene (COI). Six variables were measured from the aculeus for traditional morphometric analysis and 14 landmarks from the right wing were used for geometric analysis, using 10 specimes/population. The aculeus tip length, aculeus width at the end of the cloaca opening, and the serrate part length contributed with 62.7% for grouping. According to the results from traditional morphometry, there was no significant difference, but the multivariate tests showed that the canonical variables were statistically significant, indicating a difference in the wing conformation among populations. Molecular phylogenetic analysis indicated that the populations clustered into three clades and revealed a high level of genetic variation within *A. pickeli* populations from various geographic regions. *Anastrepha pickeli* populations differed among them according to the methods used in this study, showing incongruence among the methods used.

Introduction

Species identification is highly problematic in some *Anastrepha* species groups and misidentifications can pose serious problems for the implementation of quarantine restrictions, integrated pest management, and other control programs (McPheron 2000). Therefore, there is an ongoing need to explore additional morphological and molecular characters so as to more accurately elucidate species identification. Utilization of combined morphological and molecular methodologies will also improve our basic knowledge of the relationships among the species within the various *Anastrepha* species groups (McPheron *et al* 1999, Norrbom *et al* 1999, Smith-Caldas *et al* 2001).

Morphometric methods based on the size (traditional morphometry) and on the shape (geometric morphometry) variation of several structures have been used in the identification of some insect species by establishing landmarks (Strauss & Bookstein 1982, Sklorz 1992). In *Anastrepha*, morphometry has been used, for example, to analyze populations of the *Anastrepha fraterculus* complex (Hernández-Ortiz *et al* 2004, Selivon *et al* 2005).

Besides morphometric analysis, molecular methods have been used in *Anastrepha* systematics to complement morphological and ecological data to address questions such as species identification, speciation, geographic variation, structured genetic variation, and phylogeny (Silva 2000, Silva & Barr 2008). Sequencing of fragments of mitochondrial and nuclear genes has been used to

make inferences about relationships among specimens, populations, and species (Avise 1994).

It is known that some of what were considered widespread species such as *A. fraterculus* are in fact cryptic species complexes and *Anastrepha pickeli* Lima could be an example of such a complex based on the morphological study by Canal (1997) (Norrbom *et al* 1999). *Anastrepha pickeli* belongs to the *spatulata* group (Norrbom *et al* 1999), and is reported from Costa Rica to Guyana and Tobago, Venezuela to Peru, Brazil, and Argentina (Norrbom 2004). In Brazil, it is recorded in 15 states comprising all five Brazilian geographic regions (Zucchi 2007), where it has received attention as it infests the fruit of cassava (*Manihot esculenta*, Euphorbiaceae), consuming the pulp and the seeds, causing losses of up to 30% in fruit production (Lozano *et al* 1983, Morgante *et al* 1996). Although cassava is most important as a root crop, the effect of seed predation negatively affects cassava breeding programs (Farias & Bellotti 2006).

In this study, we investigated the morphometric and genetic variation among *A. pickeli* populations using traditional and geometric morphometry and sequencing of a fragment of the *COI* gene to improve identification of this cassava pest. We aimed at verifying if *A. pickeli*, a specialist species, shows cryptic speciation based on specimens collected in Bolivia, Brazil and Paraguay.

Material and Methods

Anastrepha pickeli females were collected in McPhail traps in Brazil, in the states of Bahia, Espírito Santo, Rio Grande do Norte and Amazonas, and also in Bolivia and Paraguay and preserved in frozen ethanol (Table 1). Voucher specimens were deposited at the insect collection at the Escola Superior de Agricultura "Luiz de Queiroz", USP, Piracicaba, SP, Brazil. The same specimens were used for both morphometric studies (the aculeus for traditional morphometry, the wing for geometric morphometry, and the thorax and legs for the molecular analysis).

Traditional morphometry

Ten females from each locality were used. The aculeus of each female was dissected and treated with 10% sodium hydroxide for 24h and then mounted on a microscope slide with glycerine. Aculei were placed ventral side up and carefully oriented in a consistent level position. The aculei were photographed with a camera attached to a stereomicroscope and the Motic Images Advanced 3.2® software was used for image analysis. Five variables: L1 (aculeus tip length), L2 (width of the aculeus apex at base of serrate part), L3 (distance between the cloaca opening and base of serrate part), L4 (aculeus length at the cloacal opening), and L5 (serrate part length) were measured (Fig 1). The aculeus length (variable L6) was measured under a
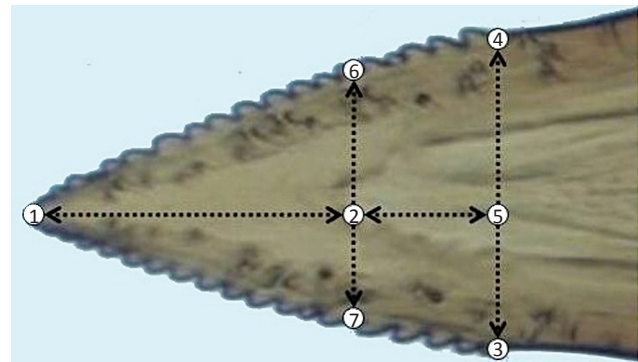

Fig 1 Aculeus apex of *Anastrepha pickeli* from Montes Claros, MG with the landmarks. 1 = aculeus apex; 2 = end of the cloaca opening; 3-4 = base of the serrate part; 5 = line showing the base of the serrate part; 6-7 = line showing the end of the cloaca opening. L1 = 1-2, aculeus apex length; L2 = 3-4, width of the aculeus apex at base of serrate part; L3 = 2-5, distance between the cloaca opening and base of serrate part; L4 = 6-7, aculeus apex width at the end of the cloaca opening and; L5 = 1-5, serrate part length.

Table 1 Colletion data and information on *Anastrepha* species used in this study.

Species	Collection sites	Locations	Altitudes (m)	N[1]	N[2]	Codes (morphometry)
A. pickeli	Teixeira de Freitas, BA, Brazil	18º06'S, 39º53'W	186	13	10	APBA
	Linhares, ES, Brazil	19º23'S, 40º04'W	33	10	10	APES
	Natal, RN, Brazil	05º47'S, 35º12'W	30	10	10	APRN
	Manaus, AM, Brazil	03º06'S, 60º01'W	92	10	10	APAM/APMA
	Cochabamba, Bolivia	17º38'S, 66º15'W	2574	15	10	APBO
	Asuncíon, Paraguai	25º26'S, 57º66'W	55	15	10	APPA
A. serpentina	Manaus, AM, Brazil	03º06'S, 60º01'W	92	02	00	-
A. striata	Manaus, AM, Brazil	03º06'S, 60º01'W	92	02	00	-

N[1] - number of specimens by locality
N[2] - number of specimens for morphometric analyses

stereomicroscope attached to a digital micrometric ocular. The average length (arithmetic mean) was calculated for each population and used for the UPGMA cluster analysis, followed by a principal component analysis to verify which variable most contributed to clustering. Differences among populations were assessed by the Wilk's Lambda test (Statistica 9.0®).

Geometric morphometry

Ten females from each locality were used. The right wing of each female was dissected and mounted on a microscope slide with glycerine and photographed with a camera attached to a stereomicroscope and the analySIS getIT® software was used. A TPS file was created from the image file in the software tpsUtil version 1.4® following Rohlf (2009a). Fourteen homologous landmarks were manually plotted at the designated vein intersections (Fig 2) for each specimen using the software tpsDig version 2.12® following Rohlf (2009b), based on previous morphometric studies on *Anastrepha* (Nascimento 2005, Selivon *et al* 2005). The centroid size was calculated for all anatomic landmark configurations for each wing, as well as the canonical variables and matrix of relative warps, using the software tpsRelw version 1.46® (Rohlf 2009c) and tpsRegr version 1.37® (Rohlf 2009d). Differences among populations were assessed by the Mann-Whitney test (centroid size) and Wilk's Lambda (relative warps) (Statistica 9.0®). We used Statistica 9.0® to calculate the Mahalanobis distance matrix and to carry out the multivariate analysis, MANOVA.

Molecular analyses

Total nucleic acid extractions from thorax and legs of individual specimens followed the protocol for alcohol-preserved specimens by Han & McPheron (1997). A fragment of 1,050 bp within the mitochondrial COI gene was amplified by polymerase chain reaction (PCR) using primers tRNA-cys2 (ACTCCTTTAGAATTGCAGTCTAAT) and COld-r (GGGCTCATACAATAAATCCTAAT) (Ruiz-Arce 2009). PCR reactions were carried out in 25 μl according to Gasparich *et al* (1995) using from 3-5μl of genomic DNA. The cycle program consisted of an initial denaturation step of 3 min at 94°C, followed by 39 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, with a final extension step of 10 min at 72°C. The amplified fragments were purified using exonuclease I and shrimp alkaline phosphatase (SAP) (Fermentas®) and incubated in a thermocycler for 1h at 37°C, followed by 15 min at 80°C. DNA sequencing was carried out at the Centro de Estudos do Genoma Humano at the Universidade de São Paulo (CEGH-USP) using the DYEnamic ET Dye Terminator Kit in a MegaBACE 1000 automated DNA sequencer. We sequenced one specimen per location for each population of *A. pickeli* and one specimen for each species used as outgroups. Sequences were aligned using the Bioedit Sequence Alignment Editor 7.0.9® (Hall 1999) which uses the ClustalW Multiple Alignment® tool (Thompson *et al* 1994).

Phylogenetic analyses were conducted using maximum parsimony (MP) and neighbor-joining (NJ) methods using the MEGA 4.1® software (Tamura *et al* 2007). A consensus NJ tree was generated using the Jukes-Cantor distance (chosen based upon criteria in Kumar *et al* 1993). Bootstrapping of the MP and NJ analyses (1,000 replicates) was performed. Two species of the *Anastrepha serpentina* group, *Anastrepha serpentina* (Wiedemann) and *Anastrepha striata* Schiner were used as outgroups.

Results

Traditional morphometric variables L1 (aculeus apex length), L4 (aculeus width at the apex of the cloacal opening), and L5 (serrate part length) showed significant differences among the analyzed populations (Wilk's Lambda test, $P < 0.05$). The principal component analysis showed that these three morphometric characters were significantly different among Amazonas and Espírito Santo, Bolivia and Rio Grande do Norte, Paraguay and Rio Grande do Norte population pairs (Wilk's Lambda test, $P < 0.05$), whereas Amazonas and Bolivia, and Rio Grande do Norte and Espírito Santo populations were not statistically different (Wilk's Lambda test, $P > 0.05$).

In all characters analyzed by traditional morphometry, the population of *A. pickeli* from Bahia was significantly different from all other populations (Wilk's Lambda test, $P < 0.0001$). The variables L1, L4 and L5 explained 62.6% of the data variability. The dendrogram (Fig 3)
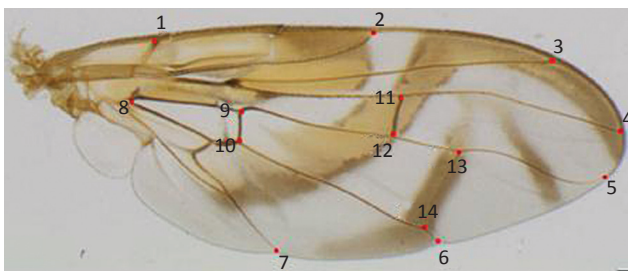

Fig 2 Wing of *Anastrepha pickeli* from Natal, RN, with landmarks 1 = intersection of the veins umeral and costal; 2 = intersection of the veins R_1 and costal; 3 = intersection of the veins R_{2+3} and costal; 4 = intersection of veins R_{4+5} and costal; 5 = intersection of vein M with apical margin; 6 = intersection of vein CuA_1 with apical margin; 7 = intersection of vein CuA_2 with posterior margin; 8 = intersection of vein CuA_1 and M; 9 = intersection of the veins M and bm-cu; 10 = intersection of veins CuA_1 and bm-cu; 11 = intersection of the veins r-m and R_{4+5}; 12 = intersection of the veins r-m and M; 13 = intersection of the dm-cu and M; 14 = intersection of veins dm-cu and CuA_1.
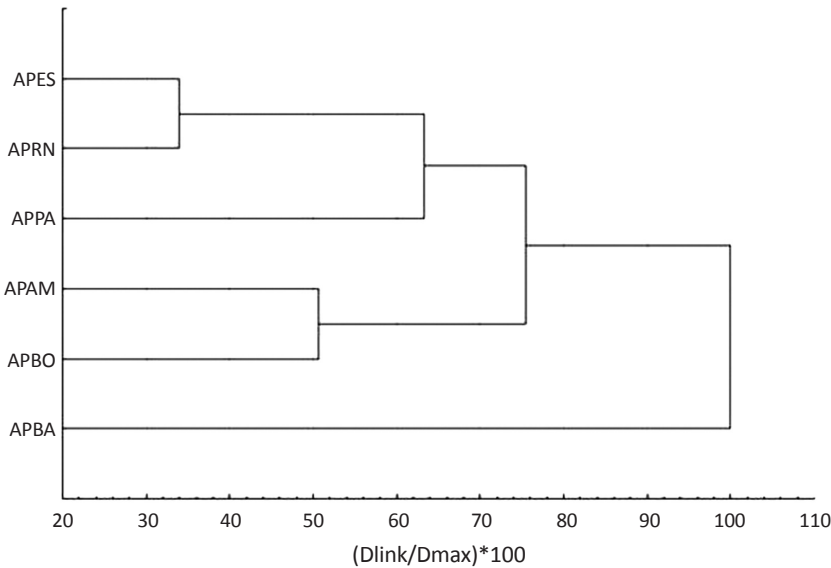
Fig 3 Dendrogram of populations of *Anastrepha pickeli* from Amazonas (APAM), Bahia (APBA), Espírito Santo (APES), Rio Grande do Norte (APRN), Bolivia (APBO), and Paraguay, generated from distances of aculeus by clustering analysis (UPGMA).

shows that the population from Bahia did not cluster with the remaining populations analyzed, which formed two clusters, one with populations from Paraguay, Rio Grande do Norte and Espirito Santo, and a second one with populations from Bolivia and Amazonas. However, the MANOVA of the traditional morphometric data indicated that populations were not statistically different (Wilk's Lambda test = 0.33, P > 0.05).

Centroid size ranged between 7.0 and 8.0. Average and standard deviation values are graphically represented in Fig 4. Centroid size analysis indicated that most populations were not statistically different among themselves at the 95% confidence level (Mann-Whitney test, P > 0.05), except for the population from Paraguay
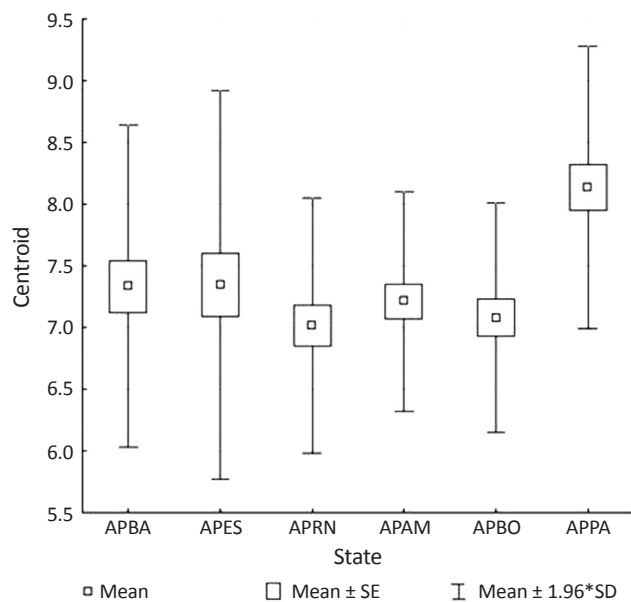
that was significantly different from all remaining populations (Mann-Whitney test, P < 0.05), and the population from Bolivia that was significantly different from that of Rio Grande do Norte (Mann-Whitney test, P < 0.05). The significant differences between populations from Paraguay and Bolivia indicate that they harbor individuals with larger wings when compared to the remaining populations.

A total of 24 relative warps was generated (k = 2n - 4), where k represents the number of relative warps and n the number of anatomic landmarks. The MANOVA of the canonical variables of relative warps showed significant differences among populations (Wilk's Lambda = 0.006; P < 0.05). Significant differences were observed between populations from Paraguay and Espírito Santo (Wilk's Lambda test, P < 0.05), but they were not significantly different from populations from Bahia and Rio Grande do Norte (Wilk's Lambda test, P > 0.05), which, in turn, were significantly different from each other (Wilk's Lambda test, P < 0.05). Populations of *A. pickeli* from Bolivia and Amazonas can be readily distinguished from each other and also from the other populations (Wilk's Lambda test, P < 0.05). The two first canonical variables explained, respectively, 42.7% and 24.5% of the data variability (Fig 5).

The Mahalanobis distance matrix indicated the largest distances between population pairs from Bolivia and Bahia (35.9%), Amazonas and Rio Grande do Norte (30.6%), and Amazonas and Bahia (29.3%) (Table 2).

Aligned sequences, including the outgroups, resulted in a data matrix containing 685 characters, of which 129 were variable and 59 sites were informative for parsimony analysis. Sequences were deposited in GenBank under accession numbers JN002428 - JN002435.

Three most parsimonious trees were recovered from

Fig 4 Graph comparing average values and standard-error of the centroid size of the wing of *Anastrepha pickeli* females.
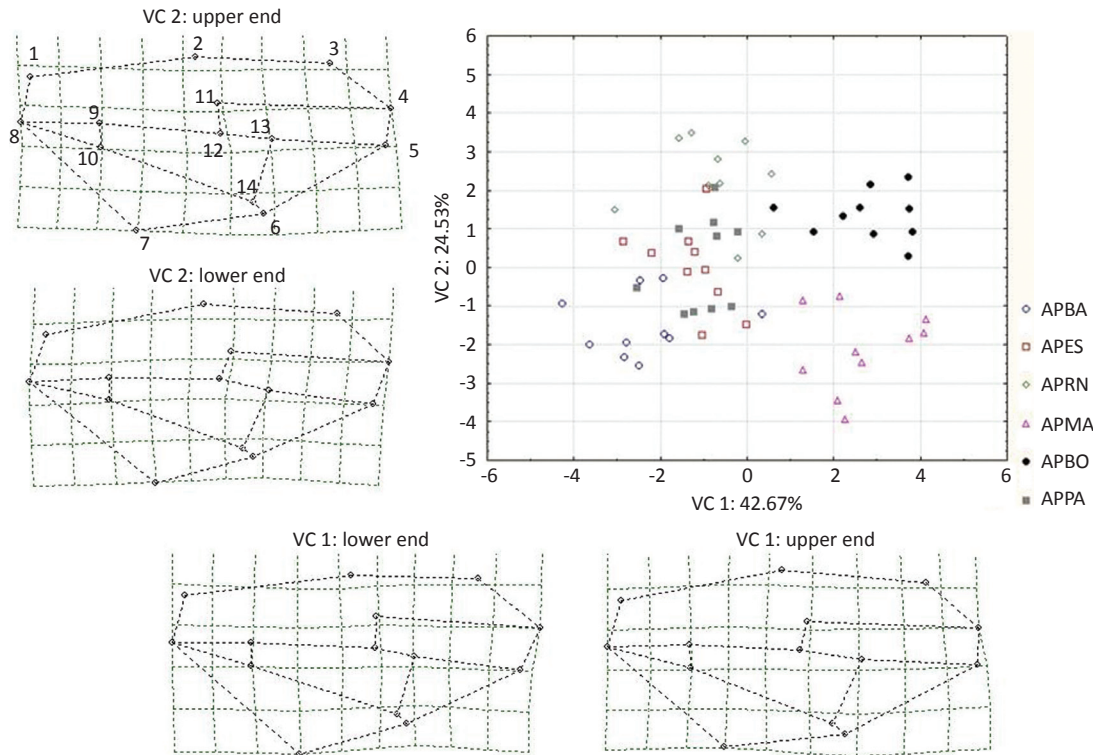
Fig 5 Dispersion graph of *Anastrepha pickeli* females from Bahia (APBA), Espírito Santo (APES), Rio Grande do Norte (APRN), Amazonas (APMA), Bolivia (APBO), and Paraguay (APPA) in the bidimensional space of canonical variables VC1 and VC2. The deformation diagrams indicate the presumable wing conformations for individuals in the superior and inferior ends of the canonical variables. Deformation magnitudes were amplified 3x for visualization.

MP analysis of the COI data and the strict consensus tree is shown in Fig 6. The neighbor-joining tree is very similar in its placement of the various populations to the MP tree (Fig 7). The average distance among *A. pickeli* populations was 0.040 ± 0.031. The level of sequence divergence ranged from a minimum of 0.003 to a maximum distance of 0.085 between populations. The lowest distance observed within *A. pickeli* (0.003) was between the populations from Amazonas and Rio Grande do Norte and the highest level of divergence (0.085) was verified between those from Paraguay and Bahia.

Table 2 Mahalanobis distances of *Anastrepha pickeli* populations from Amazonas (APMA), Bahia (APBA), Espírito Santo (APES), Rio Grande do Norte (APRN), Bolivia (APBO), and Paraguay (APPA) based on wing shape components.

	APBA	APES	APRN	APMA	APBO	APPA
APBA	0.00					
APES	9.49	0.00				
APRN	21.40	13.77	0.00			
APMA	**29.35**	26.05	**30.64**	0.00		
APBO	**35.93**	23.13	22.17	18.63	0.00	
APPA	14.37	17.75	15.93	26.48	25.17	0.00

Discussion

The MANOVA indicated that the differences among the populations were not statistically significant based on traditional morphometry, as opposed to the significant differences observed with the geometric morphometry. *Anastrepha pickeli* is a specialist species and therefore it would be expected to have a more uniform morphology among its populations. However, these morphological differences may be attributed to variation in both biotic (host plants) and abiotic factors (temperature, pluviosity and humidity among others). For example, aculeus measurements for five species in the *fraterculus* group have been shown to vary along the geographic distribution range and also among specimens from the same host (Araujo & Zucchi 2006). Alencar-Souza (1998) analyzed the aculeus of two generalist species, *Anastrepha fraterculus* (Wiedemann) and *Anastrepha zenildae* Zucchi, and one specialist, *A. pickeli*, from Rio Grande do Norte and found remarkable intraspecific differences among *A. pickeli* populations and concluded that there were two new species in the sample. It is likely that in the sample studied by Alencar-Souza (1998), besides specimens of *A. pickeli*, there were specimens of a species that is not yet formally described and has been denominated *Anastrepha* sp. aff. *pickeli* (Araujo *et al* 2005).
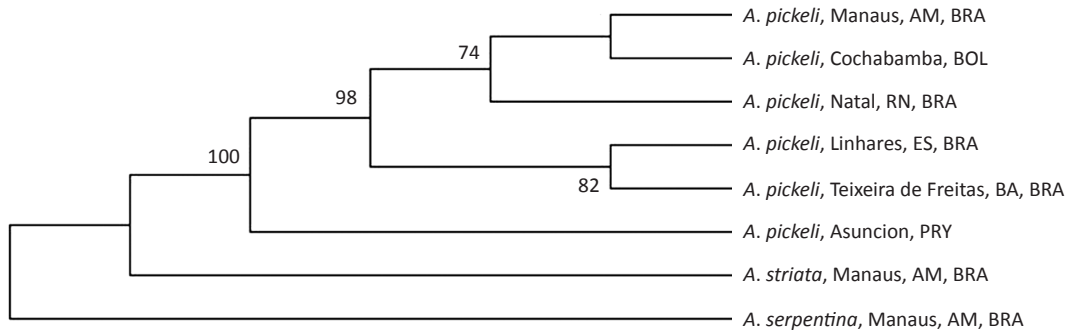
Fig 6 Phylogenetic relationships inferred from the strict consensus of three most parsimonius trees. Numbers in the branches indicate bootstrap confidence limits higher than 50% (1,000 replications). Statistics of each most parsimonious tree: length = 151; consistency index = 0.90; retention index = 0.84.

In our study, samples were collected in various geographic regions (Table 1), which can explain the differentiation among *A. pickeli* populations. According to some researchers, the morphometric differences related to the size of the structures among the populations studied are doubtful (Rohlf & Marcus 1993, Adams *et al* 2004). However, the significant difference in wing size observed in populations from Bolivia and Paraguay relative to the remaining populations generated by the centroid size analysis, minimizes the influence of some external factors as this measurement represents the geometric center of the wing as well as the mass center of a configuration, which is paramount for size definition (Monteiro & Reis 1999). Our results agree with the centroid size reported by Nascimento (2005), who analyzed a laboratory population of *A. pickeli* and other populations of generalist *Anastrepha* species. However, further comparisons were prevented as that author did not specify the origin of the laboratory colony of *A. pickeli* studied.

The canonical variables analysis revealed that populations of *A. pickeli* tended to form clusters; however, most of them showed partial or total overlap. Populations from Amazonas and Bolivia showed a tendency to be located in the superior end of VC 1, as opposed to those

from Rio Grande do Norte and Espírito Santo (Fig 5). The specimens of *A. pickeli* analyzed by Nascimento (2005) had wings widened at the base and narrowed apically and were distributed along the axis between the two ends when compared to generalist species in different infrageneric groups. The deformation diagram shows that populations from Amazonas and Bolivia diverged from the remaining populations, showing differences mainly in the landmark 4 (apex of vein R_{4+5}), which indicated a tendency to narrow the wing, and landmark 6 (apex of vein CuA_1), which widened the wing.

This is the most extensive molecular study of *A. pickeli* populations to date, both in number of included samples and localities, even though we could not obtain specimens from the entire range of distribution of this species in Brazil. Populations of *A. pickeli* were recovered as a monophyletic group with strong support in the NJ tree. The COI data obtained indicated the existence of three groups within the populations analyzed with reasonable support. The first group included populations from Amazonas, Bolivia and Rio Grande do Norte, the second from Bahia and Espirito Santo, and the third was represented by a single population from Paraguay, which is a strongly supported and highly divergent clade at the
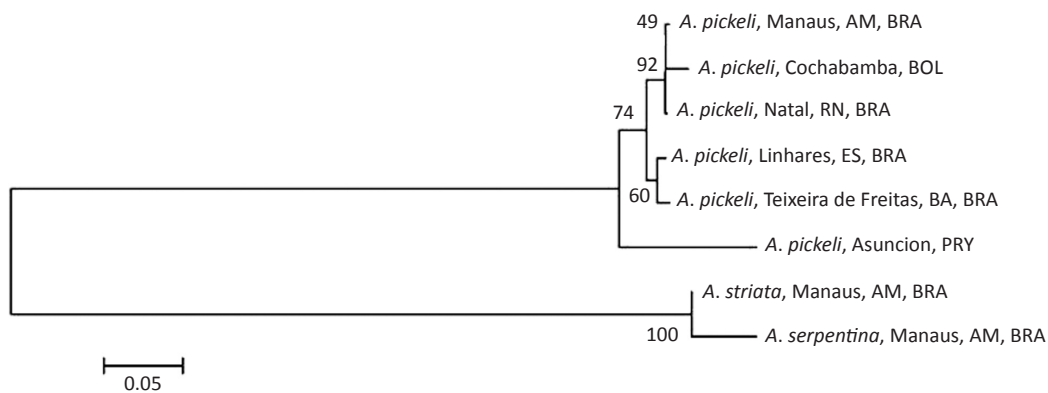

Fig 7 Phylogenetic relationships inferred from neighbor-joining tree based on Jukes-Cantor distances. Numbers on the branches indicate bootstrap values higher than 50% (1,000 replications).

base of the remaining *A. pickeli* populations in the trees. Interestingly, the topology of both MP and NJ trees reflects the geographic proximity among samples from north southwards. This is consistent with the fact that northern Amazonia is considered the place of domestication of cassava (Nassar 1978), which is the main host of *A. pickeli* throughout its geographic range.

Our data revealed a high level of genetic variation within *A. pickeli* populations from various geographic regions. There is a strong molecular divergence (JC distance = 0.074-0.085) between the population from Paraguay, situated at the extreme south of the distribution range, and the remaining populations. Additional studies, including sampling across the species distribution, are warranted to explore the boundaries of this species, since recent studies have revealed that what were believed to be widespread species are actually cryptic species complexes, such as *A. fraterculus* (see Norrbom *et al* 1999).

Further morphometric and genetic studies of populations along the entire distribution range would help to better evaluate variations in *A. pickeli*. In order to advance further with molecular approaches, a gene or genes with an even higher level of divergence may be required to actually track patterns of the evolutionary history of the *spatulata* group.

Acknowledgments

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