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Integrative approach reveals the identity of Brazilian specimens previously recognized as *Anastrepha dissimilis* Stone, 1942 (Diptera: Tephritidae)

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Abstract

Anastrepha dissimilis is currently considered to be widely distributed in Brazil, occurring in 20 of 27 states. However, morphological differences between the holotype (from Plaisance, Haiti) and a paratype (from Pernambuco, Brazil) suggest that the Brazilian specimens are not *A. dissimilis*, because their aculeus tip is similar to the paratype not to the holotype. Therefore, considering the importance of integrative taxonomy for species delimitation, we used geometric and linear morphometrics and *cytochrome c oxidase subunit I* sequences integrated with the morphology of the aculeus tip to clarify the identity of populations previously identified as *A. dissimilis* from multiple Brazilian localities. Morphological data show a uniform pattern among the Brazilian populations, with some variation among specimens from the south and northeast. In addition, the geometric and linear morphometrics suggest considerable geographic variation among these populations, suggesting the existence of at least two morphs. The molecular analysis revealed that specimens from Brazil previously identified as *A. dissimilis* belong to *Anastrepha chiclayae* Greene, with a genetic distance ranging from 0.00 to 0.015%. According to our integrative analyses, specimens from Brazil formerly identified as *A. dissimilis* actually are *A. chiclayae*. Therefore, this is the first record of *A. chiclayae* in Brazil, and we also report that *A. dissimilis* does not occur in Brazil.

Key words: Taxonomy, Trypetinae, Fruit flies, *Anastrepha pseudoparallela* group, Morphometrics, COI

Introduction

The genus *Anastrepha* Schiner, 1868 (Tephritidae) is widely distributed from the extreme southern USA to Argentina (Malavasi & Zucchi 2000; Hernández-Ortiz 2007; Norrbom 2010). It includes multiple important fruit pests, whose larvae feed on internal tissues of many cash crops (Aluja 1994; Schutze *et al*. 2017). Currently, more than 300 species of *Anastrepha* are known (Norrbom *et al*. 2021), of which 128 occur in Brazil (Zucchi & Moraes 2022).

Anastrepha is divided into various species groups, one of which is the *pseudoparallela* group with 31 species which are specialized to develop in fruits of the family Passifloraceae (Norrbom & Kim 1988; Norrbom *et al*. 1999a; Norrbom *et al*. 2012; Tigrero & Norrbom 2020; Norrbom *et al*. 2021; Rodriguez & Norrbom 2021). Eleven species of this group have been reported in Brazil, and eight of them are known to attack fruits of Passifloraceae (Lima

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1934; Stone 1942; Zucchi 1978; Malavasi & Zucchi 1980; Norrbom 1997; Aguiar-Menezes *et al*. 2004; Uramoto *et al*. 2004; Leal 2008; Sá *et al*. 2008; Garcia & Norrbom 2011; Figueiredo *et al*. 2013; Marsaro Junior 2014; Dutra *et al*. 2013; Almeida *et al*. 2019; Marinho *et al*. 2021; Zucchi & Moraes 2022).

Anastrepha dissimilis Stone, a species of the *pseudoparallela* group, has been reported from Argentina, Brazil, Colombia, Guyana, Haiti, Peru, and Trinidad (Norrbom *et al*. 1999b). In Brazil, it has been considered widely distributed, with records from all Brazilian geographic regions (Zucchi & Moraes 2022). It has been associated with several host plants, such as *Passiflora edulis* Sims (Garcia & Norrbom 2011), *Passiflora caerulea* L., *Passiflora elegans* L. (Marsaro Junior 2014), *Sarcomphalus joazeiro* (Mart.) (Sá *et al.* 2008) and *Psidium guajava* L. (Zucchi & Moraes 2022).

The original description of *A*. *dissimilis* was based on specimens from Haiti (holotype), Tumatumari, British Guiana (Guyana), and Pernambuco, Brazil (paratypes) (Stone 1942). In his description, Stone (1942) depicted the aculeus tip of the paratype from Brazil, which differs slightly but significantly from that of the holotype (see Norrbom *et al*. 2012). Further investigation of these specimens suggested that they are not conspecific, thus the identifications of all specimens from Brazil reported as *A*. *dissimilis* needed to be reassessed.

Considering the importance of accurate taxonomic identification of pest insects, such as species of *Anastrepha*, for pest management, it is essential to provide clear species delimitation hypotheses for these groups (Schutze *et al*. 2017). Thus, based on morphological, morphometric, and DNA barcoding analyses, this study aimed to clarify the identity of Brazilian populations originally determined as *A*. *dissimilis*.

Material and methods

Identification and sampling. Only female specimens were analyzed as the characters of the ovipositor are important in *Anastrepha* taxonomy and could be used in morphometric studies. These specimens were preliminarily identified based mainly on morphological characters of the aculeus tip, following taxonomic keys (Stone 1942; Steyskal 1977; Zucchi 2000; Norrbom *et al*. 2012). Morphological terminology follows Norrbom *et al*. (2012) and Cumming & Wood (2017).

In our study an integrative approach (considering data from morphology, morphometrics and DNA barcoding) was conducted only with samples from Assú and Mossoró (Rio Grande do Norte—RN); Cruz das Almas, Jaguaripe and Nova Soure (Bahia—BA); Monte Alegre do Sul and Presidente Prudente (São Paulo–SP); Janaúba (Minas Gerais–MG); Lages and Nova Veneza (Santa Catarina–SC); Morada Nova (Ceará—CE); Pelotas and Vacaria (Rio Grande do Sul—RS). Specimens from Jacupiranga and Piracicaba (SP) were only used in morphological study. Further information about the examined specimens, including localities, geographic coordinates, method of collection, and voucher specimens are in Table 1.

Morphological study. A comparison of the morphology of the aculeus tip was conducted with 95 specimens, fitting the previous concept of *A*. *dissimilis*, from Assú and Mossoró (RN); Cruz das Almas, Jaguaripe and Nova Soure (BA); Jacupiranga, Monte Alegre do Sul, Presidente Prudente and Piracicaba (SP); Janaúba (MG); Lages and Nova Veneza (SC); Morada Nova (CE); Pelotas and Vacaria (RS) (Fig. 1).

For morphological study, the abdomen of each female was removed using microforceps and cleared with heated KOH 10% solution for 3–4 min. It was transferred to a Petri dish and washed with distilled water, then put into a microvial filled with glycerin and attached to the pin of the specimen.

The aculeus was everted using microforceps and mounted on a temporary microscope slide with a drop of glycerin, then examined (ventral view) under an optical microscope. A photograph of the aculeus tip of one specimen from each population was taken using an SCMOS Digital Camera coupled with a Nikon Eclipse E200 microscope.

In addition, a Scanning Electron Microscope (SEM) was used for a more detailed observation of the morphology of one or two specimens of each population. The abdomen with the aculeus everted was dried and ventrally positioned and attached to metal stub using double-sided carbon tape and sputter-coated using Balzers SCD050. The photographs of the aculeus tip were taken in a JSM-IT300 In TouchScopeTM Scanning Electron Microscope.

Voucher specimens were pinned and deposited in the Museum of Entomology Luiz de Queiroz (MELQ), Department of Entomology and Acarology, Luiz de Queiroz College of Agriculture (ESALQ/USP).

TABLE 1. Localities, geographic coordinates, collection methods, voucher numbers, and number of specimens used in morphological, morphometric and molecular analyses.

States	**Localities**	**Geographic coordinates**		Specimens (n)				Collection Methods	**Voucher Numbers (MELQ)**
		Latitude	Longitude	Morphological Study	Linear Morphometrics	Geometric Morphometrics	Molecular Analysis		
Bahia	Nova Soure	11°14′20″S	38°28′48″W	13	9	13	-	McPhail	ESALQENT000145-159
	Cruz das Almas	12°40′10″S	39°6′23″W	7	5	7	-	McPhail	ESALQENT000129-135
	Jaguaripe	13°6′45″S	38°53′34″W	1	-	-	1	McPhail	ESALQENT000580
Ceará	Morada Nova	5°6′20.2″S	38°22′1.5″W	2	-	-	2	McPhail	ESALQENT0001568-1569
Minas Gerais	Janaúba	15°48′23″S	43°18′29″W	18	11	18	4	McPhail	ESALQENT0001570-1587
Rio Grande do Norte	Assú	5°35′48″S	36°54′41″W	3	2	2	-	McPhail	ESALQENT000121-123
	Mossoró	5°11′15″S	37°20′39″W	9	7	7	-	McPhail	ESALQENT000160-168
Rio Grande do Sul	Vacaria	28°30′7″S	50°56′14″W	39	14	21	-	McPhail	ESALQENT000180-237
	Pelotas	31º37′16.7″S	52º31′39.2″W	1	-	-	1	Fruits of *Passiflora caerulea*	ESALQENT0001588
São Paulo	Jacupiranga	24°42′13″S	48°0′29″W	4	-	-	-	McPhail	ESALQENT000238-241
	Monte Alegre do Sul	22°40′56.1″S	46°40′50.5″W	3	-	-	2	McPhail	ESALQENT0001589-1591
	Piracicaba	22°42′31″S	47°37′58″W	5	-	-	-	McPhail	ESALQENT000124-128
	Presidente Prudente	22°7′21.2″S	51°23′17.1″W	1	-	-	1	McPhail	ESALQENT0001592
Santa Catarina	Lages	27°48′55.7″S	50°19′35″W	4	-	-	-	McPhail	ESALQENT0001593-1596
	Nova Veneza	28°38′13″S	49°29′54″W	1	-	-	1	McPhail	ESALQENT000579

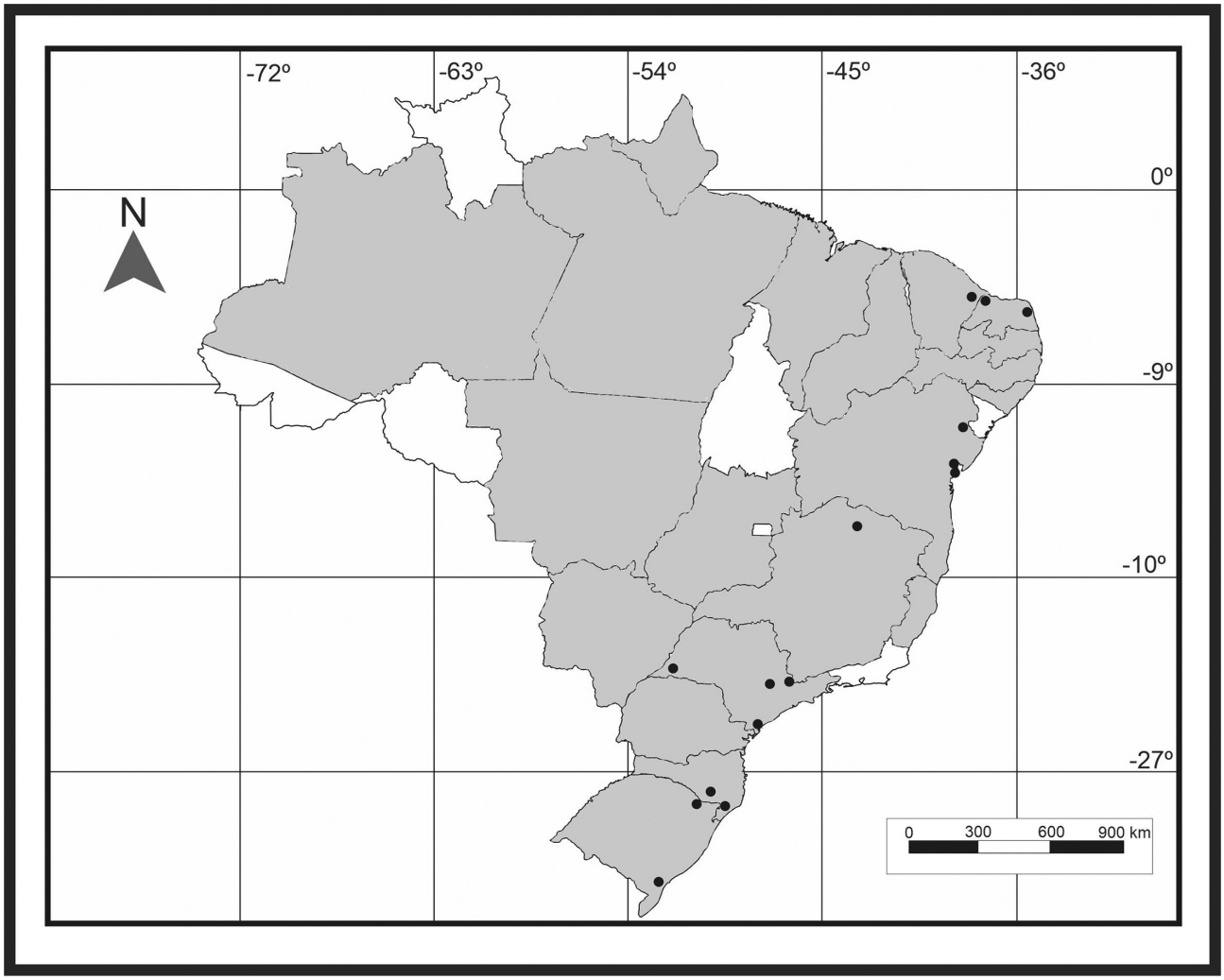

FIGURE 1. Localities of the populations studied (black circles). Gray areas in the map represents the reported distribution of specimens previously identified as *A. dissimilis* by state in Brazil.

Linear morphometrics. We measured the aculeus and mesonotum of 48 specimens from Mossoró-Assú (RN), Cruz das Almas and Nova Soure (BA), Janaúba (MG), and Vacaria (RS). Considering their geographical proximity and morphological similarity, we considered the specimens from Mossoró and Assú as a single population (Mossoró-Assú).

For measurements of the mesonotum, the specimens were mounted on a pin and photographed using a Moticam 2000 camera coupled to a Nikon SMZ 168 stereomicroscope. The aculeus was mounted on a microscopic slide with glycerin and photographed with a Moticam 2000 camera coupled with a Nikon Eclipse E200 microscope (aculeus tip) or a Nikon SMZ 168 stereomicroscope (whole aculeus).

For the linear analysis, a tps file containing the mesonotum and aculeus photographs was created in the software TpsUtil 32 version 2.3.1. (Rholf 2015). Seventeen measurements were taken following Hernández-Ortiz *et al*. (2004; 2012; 2015) (Fig. 2a and b) in TpsDig2 (Rholf 2015): (M1) length of the mesonotum, (M2) width of the mesonotum at the level of the post-sutural supra-alar seta, (M3) length from the apex of the scutellum to the left post-sutural supra-alar seta, (A0) aculeus length, (A1) length of non-serrated part of aculeus tip, (A2) length of serrated part of aculeus tip, (A3) lateral length of serrated part of aculeus tip, (A4) width of aculeus tip at its base, (A5) width of the base of the serrated part, (A6) length of aculeus tip (A1+A2), (P1) ratio of length of non-serrated part and length of serrated part (A1/A2), (P2) ratio of length of the aculeus and length of the aculeus tip (A0/A6), (P3) ratio of length of aculeus tip and length of serrated part (A6/A2), (P4) ratio of length and width of aculeus tip (A6/A4), (P5) ratio of length and width of base of serrated part of tip (A2/A5), (P6) ratio of length of aculeus and length of serrated part of aculeus tip (A0/A2), (P7) ratio of length and width of mesonotum and level of post-sutural supra-alar seta (M1/M2) (Fig. 2a and b; Table 2).

TABLE 2. Linear measurements and ratios used in MANOVA and results from post hoc test.

Structures	Variables	Descriptions	Degrees of freedom (Populations)	F	*p*
Mesonotum	M1	Length	4	9.841	<0.001*
	M2	Width at level of postsutural supra-alar seta	4	27.675	0.039*
	M3	Distance from apex of scutellum to left postsutural supra-alar seta	4	35.587	0.013*
Aculeus	A0	Length	4	3.473	0.015*
	A1	Length of non-serrated part of tip	4	7.243	<0.001*
	A2	Length of serrated part of tip	4	8.6464	<0.001*
	A3	Lateral length of serrated part of tip	4	2.6393	0.046*
	A4	Width of aculeus tip	4	0.4547	0.768
	A5	Width of base of serrated part of tip	4	17.550	<0.001*
	A6	Length of aculeus tip (A1+A2)	4	11.399	<0.001*
Ratios	P1	Length of non-serrated part to length of serrated part of aculeus tip (A1/A2)	4	6.776	<0.001*
	P2	Length of aculeus to length of aculeus tip (A0/A6)	4	13.101	<0.001*
	P3	Length of aculeus tip to length of serrated part (A6/A2)	4	6.776	<0.001*
	P4	Length to width of aculeus tip (A6/A4)	4	0.470	0.756
	P5	Length of serrated part to width of base of serrated part of aculeus tip (A2/A5)	4	8.684	<0.001*
	P6	Length of aculeus to length of serrated part of tip (A0/A2)	4	7.051	<0.001*
	P7	Length of mesonotum to width of mesonotum at level of postsutural supra-alar seta (M1/M2)	4	3.174	0.02*

The linear measurements were used to perform a Principal Component Analysis (PCA) and a Canonical Variate Analysis (CVA). Furthermore, the values of Mahalanobis distance extracted from the CVA were used to plot a dendrogram using the Unweighted Pair Group Method (UPGMA) in the software Past 4.03 (Hammer 2020). Finally, to know which measurements and proportions were statistically significant among the populations, a Multivariate Analyses of Variance (MANOVA) was performed.

For all other analyses, we used the free software R (R Core Team 2020) using the packages ggfortify (Tang *et al*. 2016; Horikoshi & Tang 2018), morpho (Schlager *et al*. 2021), ggplot2 (Wickham 2011), and dplyr (Wickham *et al*. 2022).

Geometric morphometrics. The right wing of 68 females from Mossoró-Assú (RN), Cruz das Almas (BA), Nova Soure (BA), Janaúba (MG), and Vacaria (RS) was detached from the thorax with microforceps and submerged in Cellossolve ($C_4H_{10}O_2$) for 3–5 days. Then, the wing was mounted on a permanent slide using Euparal and dried in a laboratory oven at 35 ºC for 10–15 days. Photographs were taken with the Moticam 2000 camera coupled to the Nikon SMZ 168 stereomicroscope.

A tps file was created in the software TpsUtil version 2.3.1 (Rholf 2015). Nineteen homologous points of intersection between wing veins, apices, and maximum curvature of the veins (Bookstein 1991; Hernández-Ortiz *et al*. 2015) were marked using the software TpsDig2 version 2.26 (Rholf 2015) (Fig. 2c). Then, Procrustes Superimposition was performed using the software MorphoJ (Klingenberg 2011) to remove the effect of size, rotation, and orientation by scaling, shifting, and aligning the set of landmarks (Klingenberg 2013). The data from the Procrustes superimposition were submitted to a PCA and a CVA in the software MorphoJ (Klingenberg 2011). Finally, a clustering plot was done in Past 4.03 (Hammer 2020) using the Procrustes distance values extracted from the CVA.

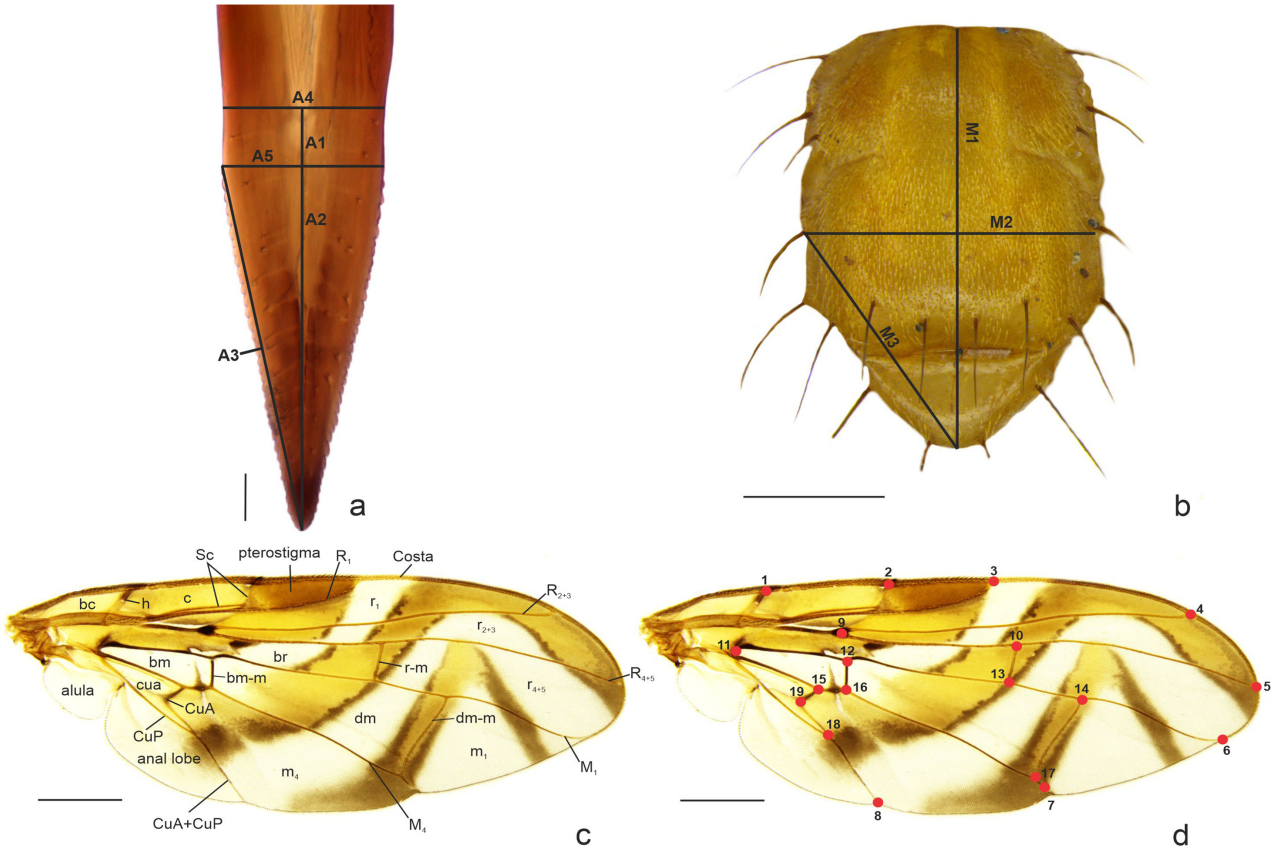

FIGURE 2. Measurements and landmarks for the aculeus tip (a), mesonotum (b), and wing (c–d). A1) Length of non-serrated part of aculeus tip; A2) Length of serrated part; A3) Lateral length of serrated part; A4) Width of aculeus tip at its base; A5) Width of base of serrated part; M1) Length of mesonotum; M2) Width of mesonotum at level of postsutural supra-alar seta; M3) Distance from apex of scutellum to left postsutural supra-alar seta; (1) Intersection of costa and humeral crossvein; (2) intersection of subcosta and costa; (3) apex of R_1; (4) apex of R_{2+3}; (5) apex of R_{4+5}; (6) apex of M_1; (7) apex of M_4; (8) apex of CuA+CuP; (9) basal bifurcation of R_{2+3} and R_{4+5}; (10) intersection of R_{4+5} and r-m; (11) base of cell bm; (12) intersection of M_1 and bm-m; (13) intersection of M_1 and r-m;(14) intersection of M_1 and dm-m; (15) intersection of M_4 and CuA; (16) intersection of CuA and bm-m; (17) intersection of M_4 and dm-m; (18) intersection of CuA and CuP; (19) point of maximum curvature of CuA. Scale bars: a = 0.05 mm; b–d = 1 mm.

DNA Barcoding. DNA Barcoding was performed using specimens from Jaguaripe (BA), Morada Nova (CE), Janaúba (MG), Nova Veneza (SC), and Pelotas (RS). The DNA was obtained by maceration of both midlegs with attached thoracic tissue in a digestion buffer [$CaCl_2$ (1M), SDS (2%, DTT (1M), Tris-HCl (1M; pH = 8,0), NaCl (5M), and H_2O MiliQ] following the protocol from Lima *et al*. (2022). First, 12.5 µl of proteinase K (20 µg/mL) was added, and the solution was incubated for 14 h at 65 ºC. Next, the extraction product was transferred to a microvial, and a solution containing chloroform and ethanol (24:1) was added and mixed for 2 min. Finally, this mixture was centrifuged at 14,000 rpm for 20 min (25 ºC).

The supernatant was transferred to a new microvial, where 0.1 of the total volume of sodium acetate, 2.5 µl of glycogen, and 0.7 of cold 100% isopropanol were added and incubated overnight at -20 ºC. It was then centrifuged at 14,000 rpm for 30 min at 4 ºC. Next, the DNA was washed in different ethanol concentrations (500 µl—70% and 95%), and then dried in an airflow chamber and eluted in 35 µl of H_2O MiliQ.

The Polymerase Chain Reaction (PCR) was conducted using the primers LepF1/Lep R1 (Hebert *et al*. 2004) and LCO1490/HCO2198 (Folmer *et al*. 1994) targeting the so called DNA barcode or Folmer region of COI. The PCR solution was composed of: 9.5 µl of MilliQ-H_2O (9.5 µl), 2.5 µl 10X PCR Buffer Mg^{2+} free (Thermo Fisher Scientific™), 4 µl $MgCl_2$ (50 mM) (Thermo Fisher Scientific™), 0.8 µl dNTP (10 mM) (Sinapse Inc®), 0.5 µ of LCO1490 and HCO2198 (10 µM), 1 µl of LepF1 and LepR1 (5 µM), 0.2 µl Platinum® Taq DNA Polymerase (5 U $µl^{-1}$) (Thermo Fisher Scientific™), and 5µl of DNA (5.0 µl).

The amplification reaction was performed with the following steps: primary denaturation for 3 min at 94 ºC, then 35 cycles of denaturation at 94 ºC (30 s), annealing at 53 ºC (45 s), elongation at 72 ºC (22 s), and a final extension at 72 ºC (10 min). The PCR products' aliquots were put into an electrophorese gel (1.5% agarose) and observed under ultraviolet light.

The purification was conducted using 1 µl of the enzyme Exo+Sap (Cellco Biotec) for each 10 µl of PCR final product. The bidirectional sequencing followed the Sanger method performed in the Agricultural Biotechnology Laboratory (CEBTEC) at ESALQ/USP. After that, the sequences were manually edited and aligned using the software BioEdit 7.2.

The COI sequences of the specimens were compared with the sequences of *Anastrepha* registered in the Barcode of Life Data Systems (Bold Systems). The genetic distance among the *A*. *dissimilis* sensu lato specimens and two *A*. *chiclayae* (extracted from Bold Systems: MT655023 and MT644035) was performed using Kimura 2-parameters model (Tamura *et al*. 2004) with 10,000 bootstraps in Mega 7 software (Kumar *et al*. 2016).

A Bayesian analysis was also performed using COI sequences from nine *A*. *dissimilis* sensu lato specimens and 28 specimens of selected *Anastrepha* spp. of the *pseudoparallela* and *fraterculus* groups deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The Bayesian phylogenetic tree was constructed using the GTR + G + I nucleotide substitution model (Waddell & Steel 1997). The most suitable model and parameters were selected using the MrModeltest v2 software (Nylander 2004). Finally, the phylogenetic analysis was performed using MrBayes (Huelsenbeck & Ronquist 2001) with the support generated by ten million replicates.

Results

Morphological study. The morphology of the aculeus tip of the Brazilian samples identified as *A*. *dissimilis*, following the concepts and keys of Stone (1942) and Zucchi (1978), revealed a uniform pattern among the Brazilian populations. Their aculeus tips are triangular and finely serrated on the distal 0.82–1.00, with a weak or no strong constriction before the serrations (Figs. 3–4). In contrast, in the holotype of *A*. *dissimilis*, there is a stronger constriction before the serrated part and the serrations extend over the distal 0.68 of the aculeus tip (Fig. 3p), and in two other specimens from the Dominican Republic the distal 0.62–0.72 of the tip is serrate.

The specimens from the northeast (Assú, Cruz das Almas, Jaguaripe, Morada Nova, and Mossoró) and some from the southeast of Brazil (Janaúba and Monte Alegre) have the lateral margin of the non-serrate part of the aculeus tip straighter and with a slight angle at the base of the serrate part (Figs. 3a–g; 4a–e). In these specimens, the serrations also tend to be smaller (Figs. 4i–n) than in specimens from other localities herein studied. In another group, which includes specimens from southern (Lages, Nova Veneza, and Vacaria) and southeast (Jacupiranga, Piracicaba, and Presidente Prudente), the junction of the non-serrated and serrated parts of the aculeus tip is smoothly rounded or slightly widened, sometimes with a smooth constriction before the serrations (Figs. 3h–o; 4f–h), tending to have larger teeth (Figs. 4o–q) than in the previous group.

Linear morphometrics. The PCA generated 16 Principal Components to explain 100% of the variance contained in the dataset. The PC1 and PC2 were used for the scatter plot because they comprised more than 50% of the variance (Table 3). The PCA separated Janaúba from the other localities (Fig. 5a). The Cruz das Almas and Mossoró-Assú specimens were separated from specimens of the other localities except Nova Soure (Fig. 5a). Nova Soure does not separate from Vacaria and Mossoró-Assú (Fig. 5a). Finally, Vacaria is also separated from Janaúba, Mossoró-Assú, and Cruz das Almas (Fig. 5a).

The CVA generated four Canonical Variates. The CV1 and CV2 were responsible for 88.02% of the whole variance in our dataset (Table 3). According to CVA scatter plot, Janaúba is separated from Cruz das Almas, Mossoró-Assú, Nova Soure, and Vacaria (Fig. 5b). On the other hand, Vacaria is separated from Cruz das Almas, Mossoró-Assú, and Nova Soure (Fig. 5b). Also, Cruz das Almas and the samples from the northeast of Brazil (Mossoró-Assú and Nova Soure) are from the same group (Fig. 5b). The linear morphometrics showed the existence of three groups. One group comprising samples from Janaúba, another with the specimens from Mossoró-Assú and Nova Soure, and other with flies from Vacaria (Fig. 5c).

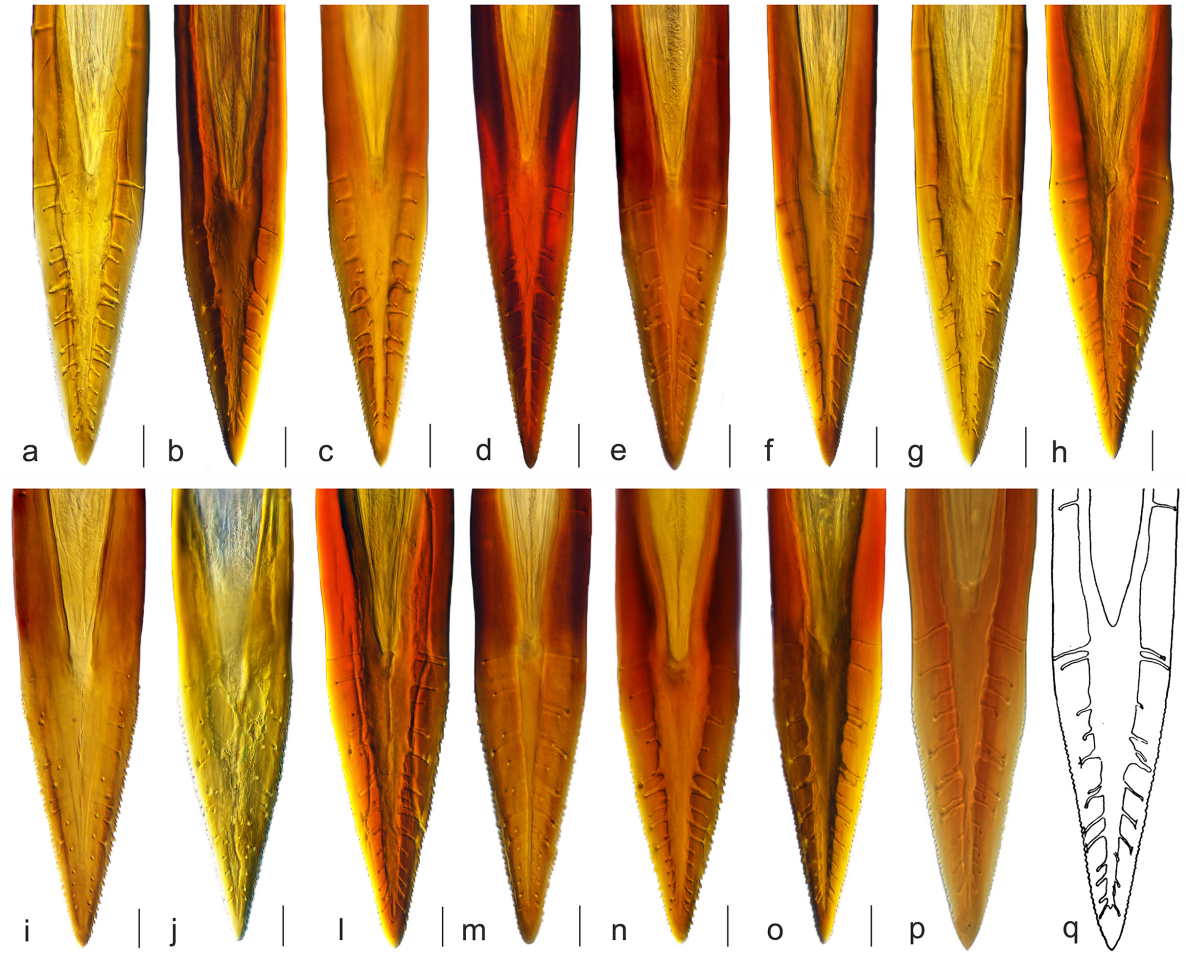

FIGURE 3. Aculeus tip (ventral view). a) Assú (RN); b) Cruz das Almas (BA); c) Janaúba (MG); d) Jaguaripe (BA); e) Monte Alegre do Sul (SP); f) Mossoró (RN); g) Morada Nova (CE); h) Jacupiranga (SP); i) Lages (SC); j) Pelotas (RS); l) Piracicaba (SP); m) Presidente Prudente (SP); n) Nova Veneza (SC); o) Vacaria (RS); p) Plaisance, Haiti (holotype) (Norrbom *et al*. 2012); q) *Anastrepha dissimilis* (paratype) Bonito, Pernambuco (Stone 1942). Scale bars: 0.05 mm.

The Multivariate Analysis of Variances was statistically significant (Pillai's Trace = 28.065, df = 4.43, $p<0.001$). The post hoc test indicated that only the aculeus tip width (A4) and the ratio of aculeus tip length and width are not statistically different among the populations (Table 2).

Geometric morphometrics. The PCA performed with the data from Procrustes Superimposition created 34 PCs, with the variance distributed in the first four Principal Components (Table 3). The landmarks that most contributed to the PC1 were: apex of M_4 (7); apex of CuA+CuP (8), intersection of R_{4+5} and r-m (10), and intersection of M_4 and dm-m (17). For the PC2, the most important landmarks were: apex of R_{4+5} (5), apex of M_1 (6), apex of M_4 (7), apex of CuA+CuP (8), intersection of R_{4+5} and r-m (10), and intersection of M_1 and r-m (13).

The exploratory PCA showed that specimens from Vacaria differed from those from the other localities (Fig. 6), while specimens from Cruz das Almas, Janaúba, Mossoró-Assú, and Nova Soure overlapped (Fig. 6). According to the outline graph, shape changes in the posterior and proximal regions of the wings (Fig. 6) represent the differences among specimens from Vacaria and the other populations.

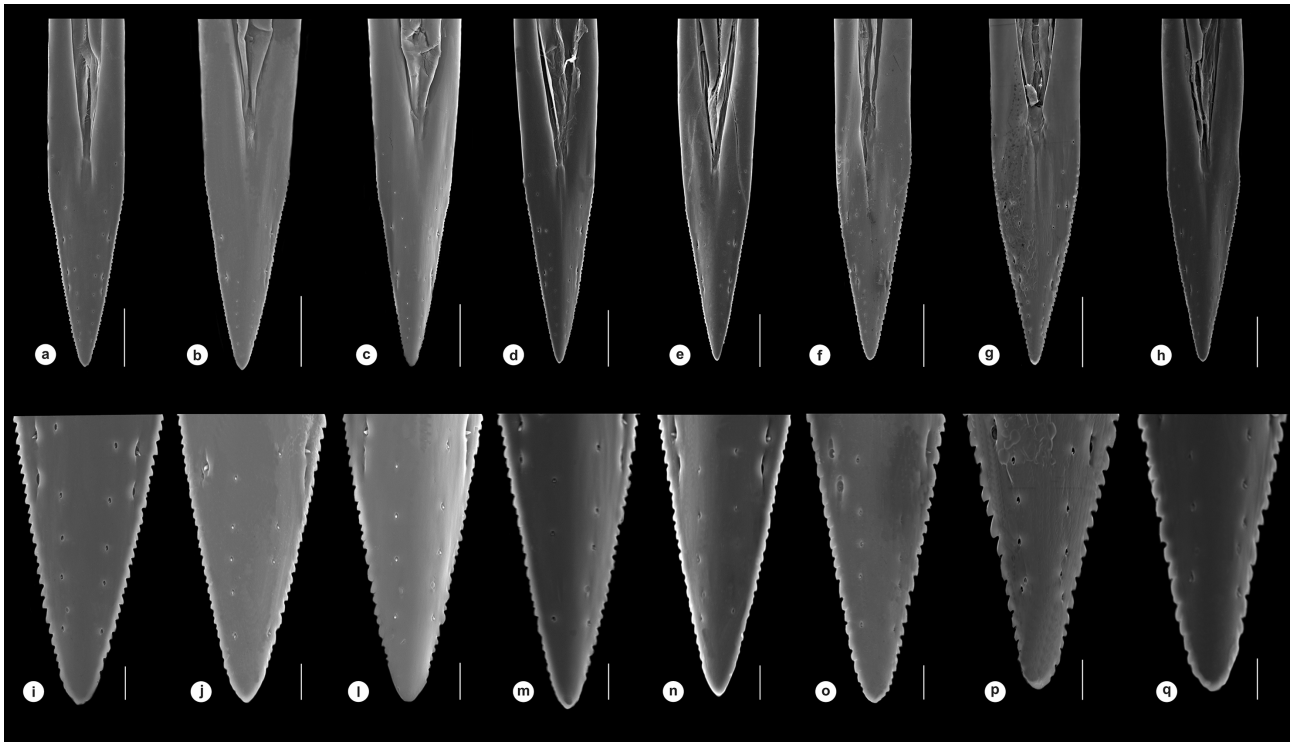

FIGURE 4. Scannig Electron Microscope photographs of aculeus tip (ventral view). a) Assú (RN); b) Cruz das Almas (BA); c) Janaúba (MG); d) Mossoró (RN); e) Nova Soure (BA); f) Lages (SC); g) Piracicaba (SP); h) Vacaria (RS). Detail of tip. i) Assú; j) Cruz das Almas; l) Janaúba; m) Mossoró; n) Nova Soure; o) Lages; p) Piracicaba; q) Vacaria. Scale bars, a–h: 0.1 mm; i–q: 0.02 mm.

TABLE 3. Proportion of variance and cumulative variance of the Principal Components (PCs) and Canonical Variates (CVs) extracted from the Principal Component Analysis (PCA) and Canonical Variate Analysis (CVA) of the morphometric study.

Linear Morphometrics			***Geometric Morphometrics***		
Principal Component (PCs)	Proportion of Variance (%)	Cumulative Variance (%)	Principal Component (PCs)	Proportion of Variance (%)	Cumulative Variance (%)
PC1	30.02	30.02	PC1	14.44	14.44
PC2	26.91	56.92	PC2	10.88	25.33
PC3	12.77	69.69	PC3	10.56	35.89
PC4	8.92	78.61	PC4	9.63	45.52
...	...	...	...	...	...
PC16	0.00	100	PC34	0.05	100
Canonical Variate (CVs)	Proportion of Variance (%)	Cumulative Variance (%)	Canonical Variate (CVs)	Proportion of Variance (%)	Cumulative Variance (%)
CV1	55.35	55.35	CV1	73.67	73.67
CV2	32.67	88.02	CV2	13.88	87.56
CV3	7.71	95.74	CV3	8.18	95.74
CV4	4.25	100	CV4	4.25	100

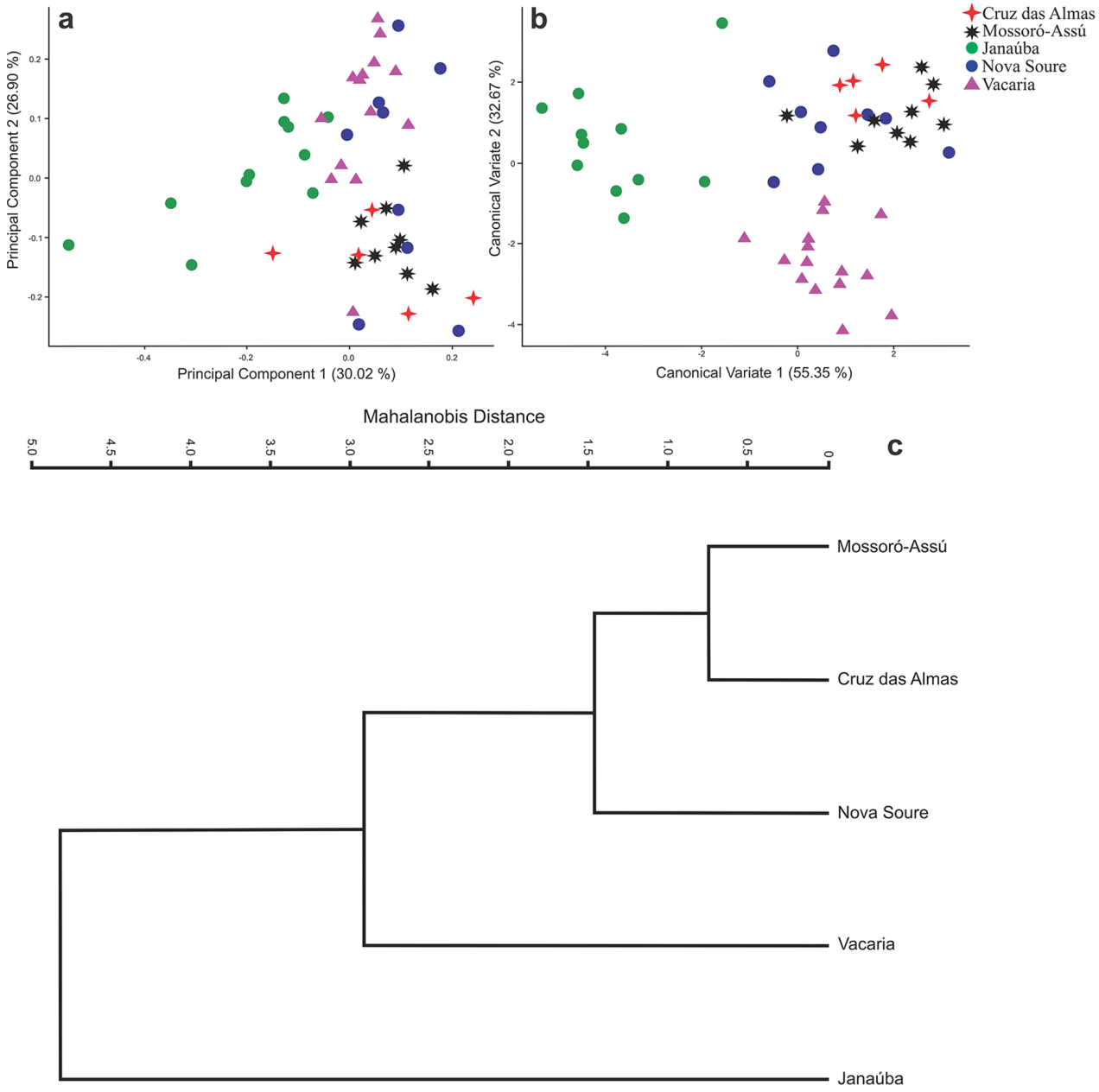

FIGURE 5. Graphs of linear morphometric analyses. a) Principal Components Analysis scatter plot; b) Canonical Variate Analysis scatter plot; c) Dendrogram of Mahalanobis distance from canonical variate analysis.

The Canonical Variate Analysis generated only four Canonical Variates, where most variation was concentrated in CV1 and CV2 (Table 3). The CVA showed that specimens from Vacaria were separated from Cruz das Almas, Janaúba, Mossoró-Assú, and Nova Soure, suggesting the existence of two morphotypes (Figs. 7 and 8). Based on Procrustes distance, the shape patterns of specimens from Vacaria were significantly different from the other localities (Permutation test, $p<0.001$). The Procrustes distance observed among the specimens from the Northeast (Cruz das Almas, Mossoró-Assú, and Nova Soure) and from the Southeast (Janaúba) was not statistically significant (Permutation test, $p>0.001$).

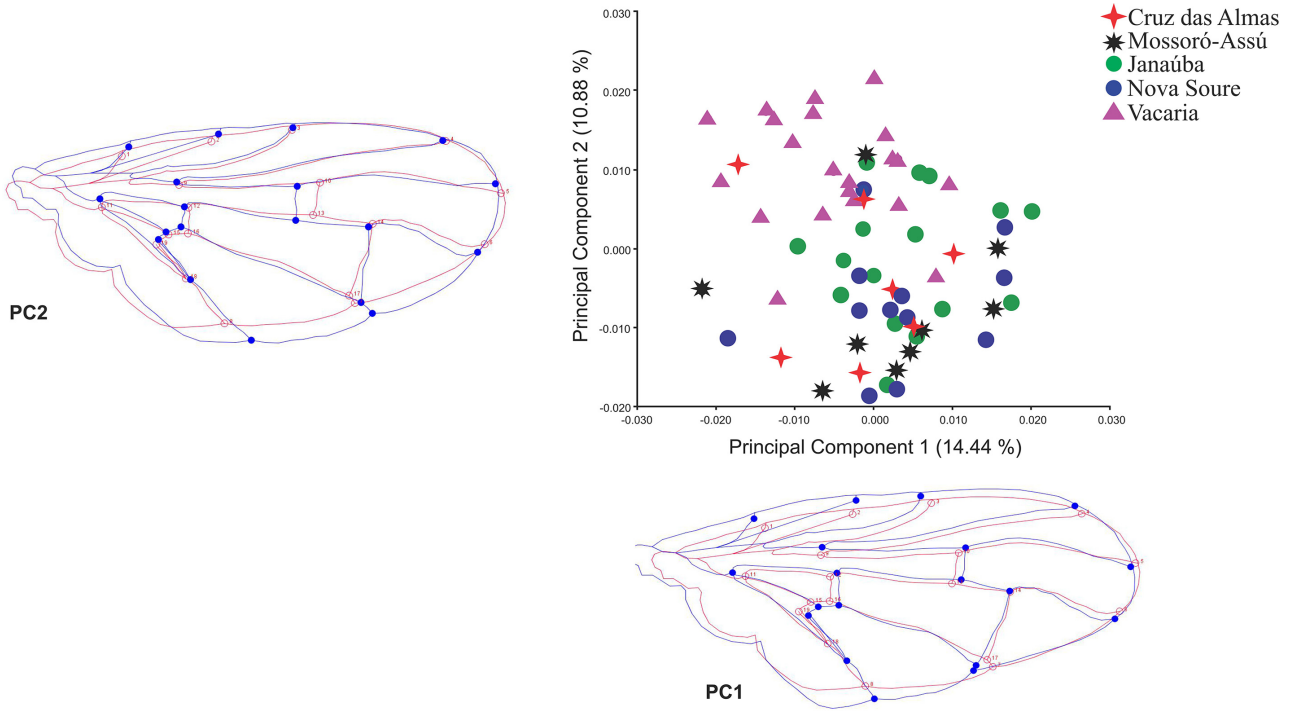

FIGURE 6. Scatter plot and outline drawing from Principal Component Analysis performed with the procrustes superimposition data from wing.

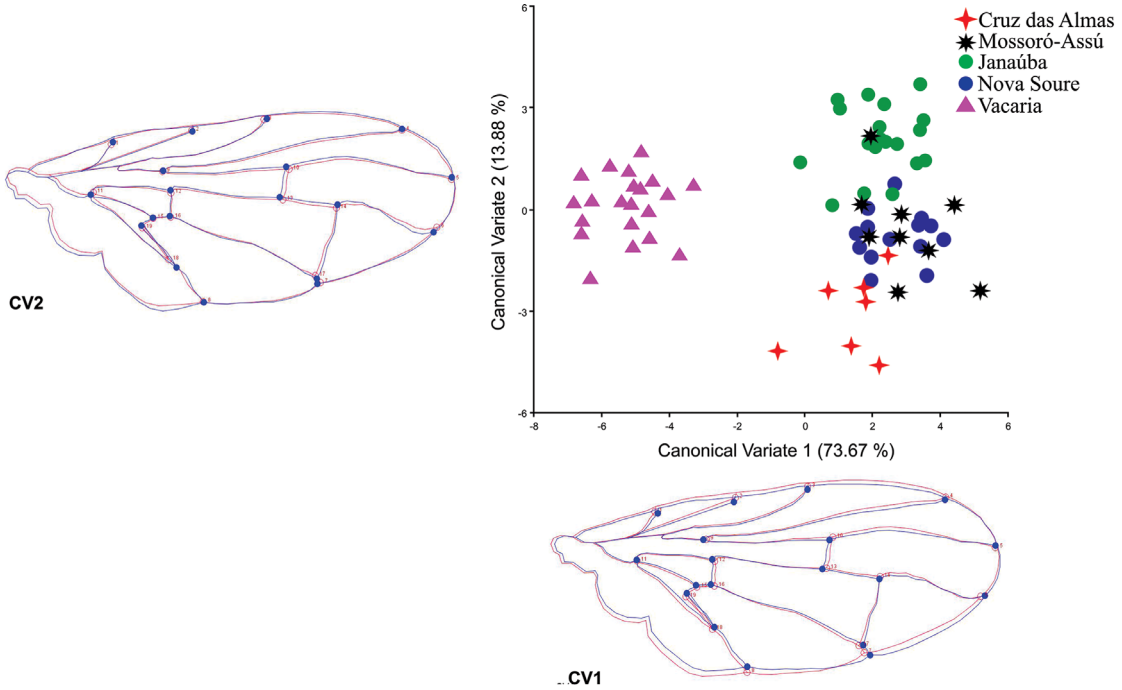

FIGURE 7. Scatter plot and outline drawing from Canonical Variate Analysis performed with the procrustes superimposition data from wing.

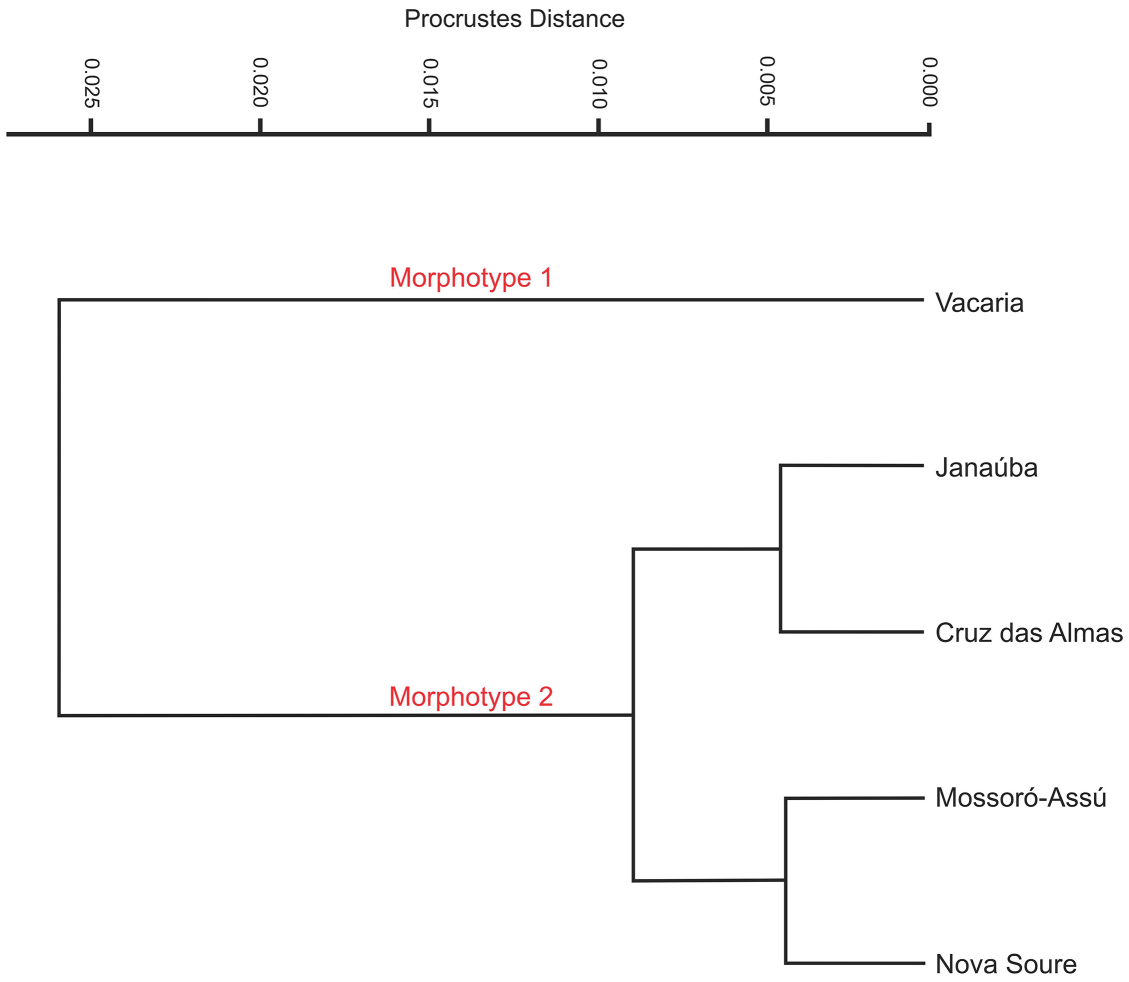

FIGURE 8. Dendrogram made with procrustes distance values from the Canonical Variate Analysis of Geometric Morphometrics of wing.

TABLE 4. Molecular analysis of specimens previously identified as *A. dissimilis* from different populations in Brazil, based on Barcode sequences deposited on Bold System. See Table 1 for detailed information of each specimen.

Samples	Bold Identifications	% ID	Best ID
Morada Nova (ON500545)	*Anastrepha chiclayae*	99.56	*Anastrepha chiclayae*
Morada Nova (ON500546)	*Anastrepha chiclayae*	99.81	*Anastrepha chiclayae*
Janaúba (ON500547)	*Anastrepha chiclayae*	99.39	*Anastrepha chiclayae*
Janaúba (ON500548)	*Anastrepha chiclayae*	100	*Anastrepha chiclayae*
Janaúba (ON500549)	*Anastrepha chiclayae*	99.69	*Anastrepha chiclayae*
Janaúba (ON500550)	*Anastrepha chiclayae*	99.69	*Anastrepha chiclayae*
Jaguaripe (ON500544)	*Anastrepha chiclayae*	99.63	*Anastrepha chiclayae*
Nova Veneza (ON500551)	*Anastrepha chiclayae*	99.83	*Anastrepha chiclayae*
Pelotas (ON500552)	*Anastrepha chiclayae*	99.81	*Anastrepha chiclayae*

DNA barcoding. Sequences of the Folmer fragment of *cytochrome c oxidase subunit I* (COI) were obtained from samples collected in Janaúba, Jaguaripe, Morada Nova, Nova Veneza, and Pelotas (Table 1). According to BOLD Systems, all the specimens studied herein were identified as *Anastrepha chiclayae* Greene, with 99.39 to 100% similarity (Table 4). Among the sequences from Brazilian specimens, the shortest genetic distance was 0.00 and the longest was 0.006% (Table 5). Considering the DNA barcodes of *A. chiclayae* from Ecuador and Peru, the genetic distance was 0.000% to 0.015% (Table 5). Finally, the Bayesian tree formed a high supported (1.0) monophyletic branch with *A. dissimilis* sequences from our study and *A. chiclayae* sequences from Peru (MT644035 and KY428263) and Ecuador (MT655023) (Fig. 9).

TABLE 5. Pairwise genetic distance (Kimura 2-parameters) among specimens previously identified as *A. dissimilis* from Brazil and of *A. chiclayae* from Peru and Ecuador based on Folmer fragment of cytochrome c oxidase subunit I.

Samples	Jaguaripe | Brazil | ON500544	Morada Nova | Brazil | ON500545	Morada Nova | Brazil | ON500546	Janaúba | Brazil | ON500547	Janaúba | Brazil | ON500548	Janaúba | Brazil | ON500549	Janaúba | Brazil | ON500550	Nova Veneza | Brazil | ON500551	Pelotas | Brazil | ON500552	*A. chiclayae* | Ecuador | MT655023
Jaguaripe | Brazil | ON500544	-	-	-	-	-	-	-	-	-	-
Morada Nova | Brazil | ON500545	0.006	-	-	-	-	-	-	-	-	-
Morada Nova | Brazil | ON500546	0.003	0.003	-	-	-	-	-	-	-	-
Janaúba | Brazil | ON500547	0.006	0.006	0.003	-	-	-	-	-	-	-
Janaúba | Brazil | ON500548	0.003	0.003	0.000	0.003	-	-	-	-	-	-
Janaúba | Brazil | ON500549	0.006	0.000	0.003	0.006	0.003	-	-	-	-	-
Janaúba | Brazil | ON500550	0.003	0.003	0.000	0.003	0.000	0.003	-	-	-	-
Nova Veneza | Brazil | ON500551	0.006	0.006	0.003	0.006	0.003	0.006	0.003	-	-	-
Pelotas | Brazil | ON500552	0.003	0.003	0.000	0.003	0.000	0.003	0.000	0.003	-	-
A. chiclayae | Ecuador | MT655023	0.015	0.015	0.012	0.015	0.012	0.015	0.012	0.009	0.012	-
A. chiclayae | Peru | MT644035	0.006	0.012	0.009	0.012	0.009	0.012	0.009	0.006	0.009	0.009

*All nucleotide positions containing gaps and missing data were eliminated. There were 342 nucleotide positions in the final dataset. Analyses were conducted in MEGA7 (Kumar *et al*. 2016).

Discussion

In his description, Stone (1942) illustrated the aculeus tip of the paratype of *A. dissimilis* from Brazil. For this reason, all identifications of specimens in Brazil as *A. dissimilis* have been based mostly on that illustration. Seven decades later, Norrbom *et al.* (2012) published a copy of Stone´s drawing of the aculeus tip of the paratype from Brazil alongside a photograph of the aculeus tip of the holotype from Haiti. These illustrations reveal that aculeus tip of the holotype differs from that of the paratype.

The aculeus tip of the paratype (Fig. 3q) from Bonito, Pernambuco, is similar to other Brazilian specimens studied here, especially those from the northeast and part of the southeast (Janaúba and Monte Alegre). These specimens share similarities including the aculeus tip without a strong constriction before the serrations and the serrations extending from the distal 0.82–1.00 of the aculeus tip. The aculeus tip shape of the specimen of *A. dissimilis* from Brazil illustrated by Zucchi (1978) is also similar to that of the paratype depicted by Stone (1942). Consequently, the identification of *A. dissimilis*, based on the paratype, resulted in misidentification of this species in Brazil.

The morphological findings suggest some apparent similarities among the southeastern and south Brazilian specimens with *Anastrepha correntina* Blanchard, considered a junior synonym of *A. dissimilis* by Steyskal (1977). Therefore, the illustration of this species (Blanchard 1961; Korytkowski & Ojeda 1968) shows that the aculeus tip has the same morphological pattern found in southern and southeastern Brazilian specimens. Probably, *A. correntina* was wrongly synonymized with *A. dissimilis*, since it is more similar to Brazilian specimens than the holotype from Haiti, but its status needs further study.

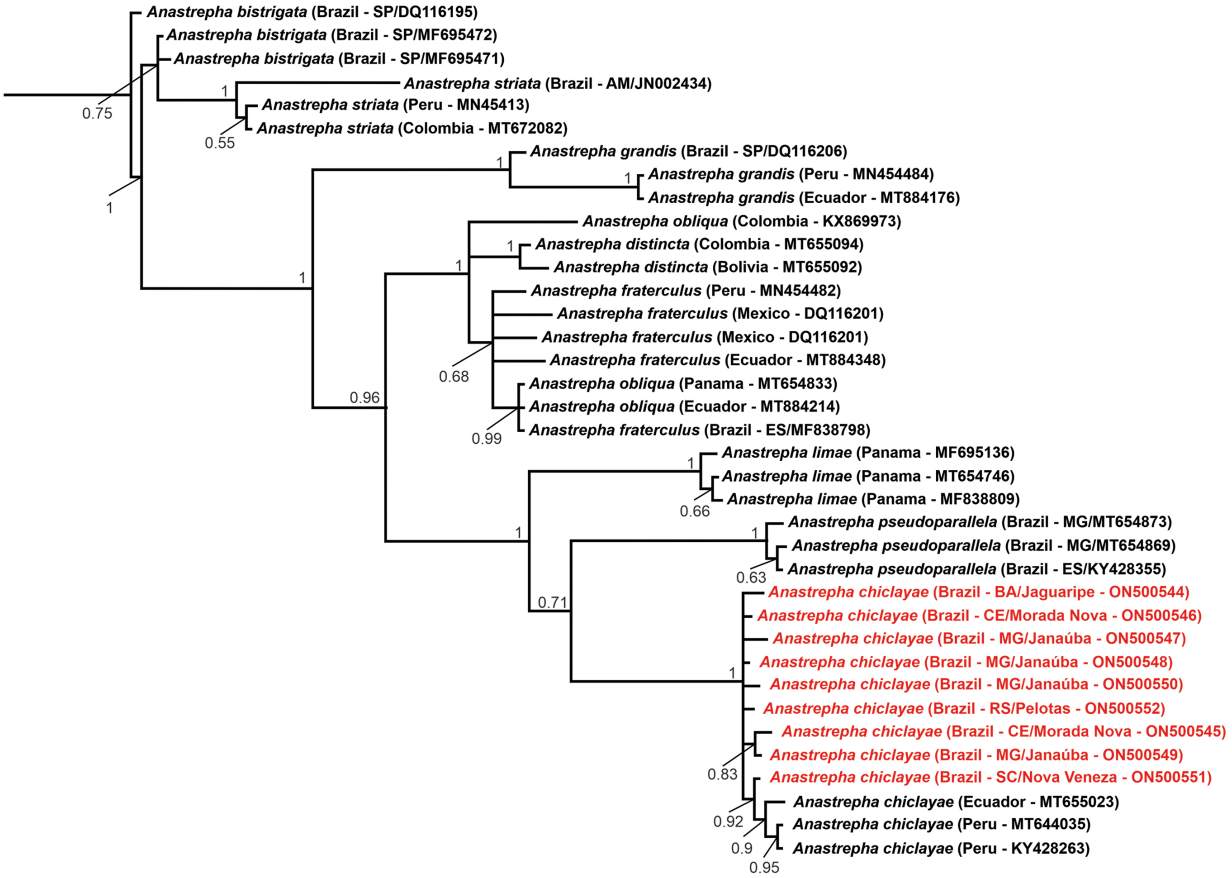

FIGURE 9. Phylogenetic Bayesian inference based on COI mtDNA sequences. The numbers on the branches represent the supporting values for the nodes. The sequences in red are from the specimens previously identified as *A. dissimilis*.

The aculeus of *A. chiclayae* depicted by Stone (1942) and photographed by Norrbom *et al.* (2012) has a tip without constriction before the serrations and the base of the serrated part straight, which is strongly similar to the populations studied herein. In addition, the serrations begin very close to the base of the tip (see Norrbom *et al.* 2012). This morphological pattern corresponds to the aculeus tip shape found in the populations studied here and to the paratype of *A. dissimilis* illustrated by Stone (1942) (Fig. 3q).

According to Norrbom *et al*. (2012), *A*. *dissimilis* has microtrichia on all or almost all of the scutum. However, in Brazilian specimens, the scutum is mostly nonmicrotrichose (Fig. 10), like in *A*. *chiclayae* (Norrbom *et al*. 2012). Therefore, the morphological findings suggest that the Brazilian specimens that have been identified as *A*. *dissimilis* correspond to *A*. *chiclayae*.

Additionally, we noted considerable geographic variation among these samples. Specimens from southern (Lages, Nova Veneza, and Vacaria) and southeast Brazil (Jacupiranga, Piracicaba, and Presidente Prudente) tend to have the base of the serrate part of the tip slightly wider than the non-serrate part, and the teeth more conspicuous. In contrast, the specimens from the northeast (Assú and Mossoró, Cruz das Almas and Jaguaripe, Morada Nova) and two localities from the southeast (Janaúba and Monte Alegre do Sul) tend to have the base of the serrate part of the aculeus tip no wider than the straight non-serrate part, and moderately smaller teeth.

Geographic variation among populations of fruit flies has been reported previously. For example, geographic variation was found in different populations of *Anastrepha pickeli* Lima (Bomfim *et al*. 2011) and *Anastrepha obliqua* (Macquart) (Ruiz-Arce *et al*. 2012; Castañeda *et al*. 2015; Scally *et al*. 2016; Passos *et al*. 2018). Multidisciplinary approaches indicated that different morphotypes of the *Bactrocera dorsalis* complex were the same species and that these morphological variations were caused by geographic factors (Schutze *et al*. 2012; 2015), although Drew & Hancock (2022) still dispute this interpretation. Also, insects reared under different abiotic conditions often show phenotypic variation, however, this variation does not necessarily represent the rise of new lineages and is probably caused by phenotypic plasticity (Birdsall *et al*. 2000; Bubliy *et al*. 2008; Parreño *et al*. 2017).

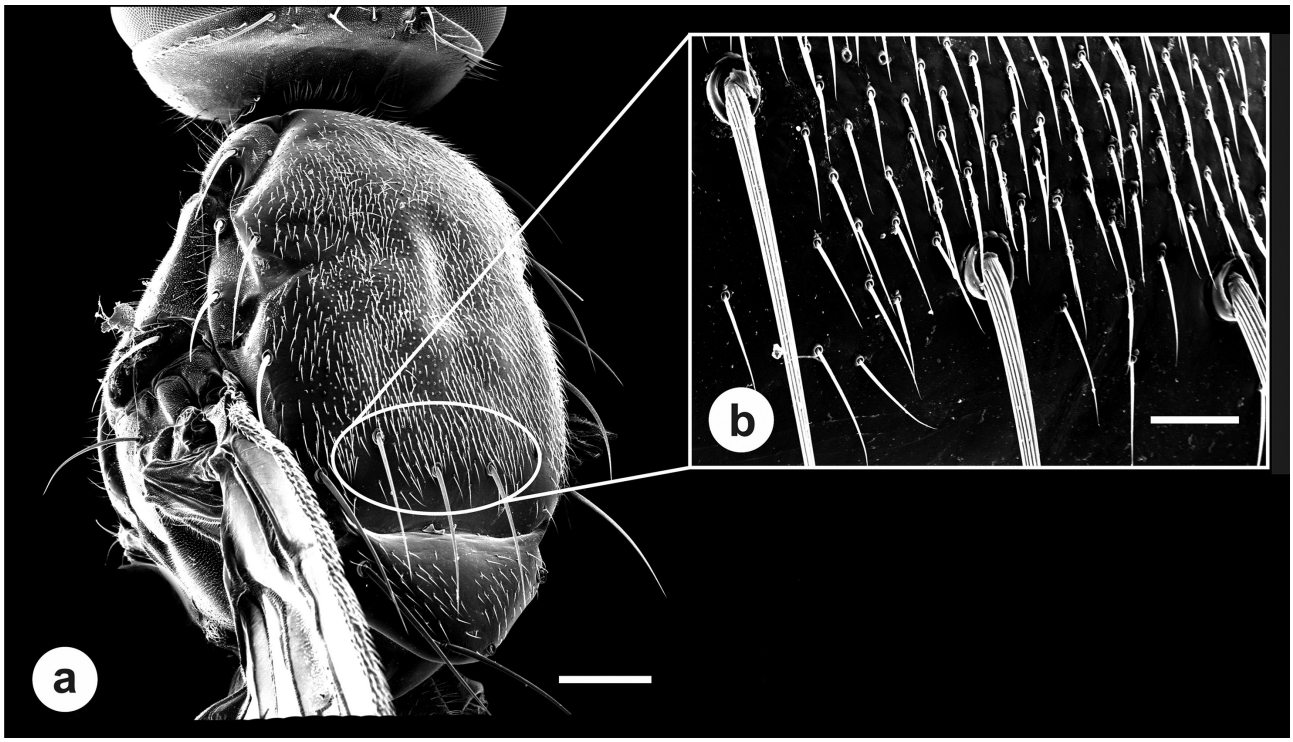

FIGURE 10. SEM of the scutum of a specimen previously identified as *A*. *dissimilis* from Janaúba (MG) (ESALQENT0001587). (B) Detail of the scutum. Scale bars: a = 0.5 mm; b = 0.1 mm.

The data from the wing shape analysis (geometric morphometry) revealed that specimens from Vacaria are statistically different from those of Mossoró-Assú, Cruz das Almas, Nova Soure, and Janaúba, corroborating the morphological findings. Conversely, the data from the mesonotum and aculeus analysis (linear morphometrics), despite showing that specimens from Vacaria are different from the other samples, indicate the existence of another group comprising only Janaúba. Morphometric analysis of the aculeus, wing, and mesonotum have been helpful in the taxonomic separation of species of the *Anastrepha fraterculus* complex (Hernández-Ortiz *et al*. 2015; Perre *et al*. 2014).

The morphometric results suggest the existence of at least two morphs in our samples, which may more likely represent geographic variation in Brazil rather than species-level differentiation. One morphotype is reported for the Caatinga municipalities (Assú, Cruz das Almas, Jaguaripe, Janaúba, Morada Nova, Mossoró, and Nova Soure)

(Barton 1988; Morrone 2001), another for the Brazilian Atlantic Forest (Ombrophilous Mixed Forest, Ombrophilous Dense Forest, and Seasonal Semideciduous Forest) localities (Jacupiranga, Lages, Nova Veneza, Pelotas, Piracicaba, Presidente Prudente, and Vacaria) (Morrone 2001; Ribeiro *et al*. 2011). These ecoregions probably were separated by vicariant events characterized by a savanna corridor and mountains and valleys (Morrone 2004; Pinto-da-Rocha *et al*. 2005), suggesting the existence of geographic barriers between specimens from the south and southeast and those from northern Minas Gerais and northeast Brazil.

The DNA barcoding approach also showed that individuals previously classified as *A*. *dissimilis* in Brazil are *A*. *chiclayae*, supported by high sequence homology (> 99.39%) between Brazilian individuals and DNA barcode sequences of *A*. *chiclayae* in the Barcode of Life Data Systems—Bold Systems database. Additionally, in the Bayesian phylogeny (Fig. 9) the Brazilian specimens and *A*. *chiclayae* form a monophyletic branch. DNA barcoding based on the Folmer fragment of the COI gene has been helpful for the molecular identification of many insect groups (Yang *et al*. 2013; Guerra *et al*. 2014; Hosseininaveh *et al*. 2016; Hickmann *et al*. 2019; Lima *et al*. 2022). Although in some Tephritidae, including some *Anastrepha*, DNA Barcoding failed to differentiate all species (Barr *et al*. 2012; 2018), it was a helpful tool to confirm the presence of *A*. *chiclayae* in Brazil previously misidentified as *A*. *dissimilis*.

According to Schlick-Steiner *et al*. (2010), disagreement among disciplines used in integrative approaches is a typical outcome. In our study, agreement occurred between morphological and molecular data, which suggests that *A*. *chiclayae* occurs in Brazil, and previous identifications of *A*. *dissimilis*, based on Stone (1942), are misidentifications. However, these results were not in full concordance with the morphometric data, probably because there is a geographical morphological variation among the samples.

Our morphometric results showed considerable geographic variation among Brazilian specimens of *A*. *chiclayae*. However, DNA Barcoding of *A*. *chiclayae* showed a low genetic distance among the southern and northeastern Brazilian individuals, which confirms the occurrence of *A*. *chiclayae* in Brazil, but does not explain the morphological diversity among specimens from different regions. Thus, future studies based on genomic molecular data could investigate the evolutionary events that influence in the morphological variation among *A*. *chiclayae* populations from Brazil.

Our work used an integrative approach to confirm that the specimens identified in Brazil as *A*. *dissimilis* correspond to *A*. *chiclayae*, which is, for the first time, reported in Brazil. No specimens that we examined match the holotype of *A*. *dissimilis* which thus appears not to occur in Brazil and may be restricted to Hispaniola. Further study is needed to resolve the status of populations from other countries in South America reported as *A*. *dissimilis* (Stone 1942; Blanchard 1961; Carrejo & González 1994; Korytkowski 2001; Arias *et al*. 2014; Bartolini *et al*. 2020; Ramos *et al*. 2021).

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Authors' contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Alexandre S. Araujo, Marcoandre Savaris, Roberto A. Zucchi, Alberto S. Corrêa, and Frederico Nanini. The first draft of the manuscript was written by Alexandre S. Araújo and all authors commented on subsequent versions of the manuscript. All authors read and approved the final manuscript.

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