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Two new species of *Mycodrosophila* (Diptera, Drosophilidae) proposed by molecular and morphological approaches, with a key to American species

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ABSTRACT

There are approximately 130 species of *Mycodrosophila* Oldenberg, 1914 worldwide, although only nine species were recorded in American countries so far, three of which are exclusively Nearctic, five exclusively Neotropical and one found in both biogeographic regions (*Mycodrosophila projectans*). Such a small number of American species is likely a consequence of collecting bias, which favors the capture of frugivorous drosophilids, and to the general absence of Neotropical *Mycodrosophila* studies in the last 50 years. Here, we describe two commonly sampled species of *Mycodrosophila* from the Amazonian and Pampa Brazilian biomes, which share morphological similarities with *Mycodrosophila neoprojectans* and *M. projectans*, respectively. We compared sequences of the mitochondrial gene cytochrome oxidase subunit I (COI), external morphology characteristics and male terminalia among these species. Based on a DNA barcoding approach coupled to morphological differences, we proposed the delimitation of two new species, *Mycodrosophila hofmanni* sp. nov. and *Mycodrosophila valentae* sp. nov. An updated key to identifying Neotropical and Nearctic *Mycodrosophila* species is also provided.

Introduction

Mycodrosophila Oldenberg, 1914 is usually known for its association with fungal fructification bodies of the genus *Polyporus* and is embedded within the *Zygothrica* genus group (Wheeler and Takada, 1963; Bock, 1980; Grimaldi, 1990). There are almost 130 described *Mycodrosophila* species (Bächli, 2015) and the genus is distributed worldwide, although most of the described *Mycodrosophila* species occur in Africa, Asia and Australia (Burla, 1954; Okada, 1956, 1965, 1968, 1986; Bock, 1980). Six species (*Mycodrosophila brunnescens*, *Mycodrosophila elegans*, *Mycodrosophila neoprojectans*, *Mycodrosophila nigropleura*, *Mycodrosophila projectans* and *Mycodrosophila pseudoprojectans*) have been recorded for the Neotropics, mostly in Central America and Brazil, and all of them were described or redescribed in Wheeler and Takada (1963). In fact, according to Grimaldi (2010), Wheeler and Takada's study can be considered the most important work for identifying American species of *Mycodrosophila* because it characterizes all species that have been reported from this continent, and there has been no other study encompassing the systematics or taxonomy of *Mycodrosophila* in this region since then. Such a delay, associated with the straightforward fruit bait bias in drosophilid sampling, can be responsible for the scarce knowledge regarding the Neotropical *Mycodrosophila* fauna.

Here, we report the collection of two *Mycodrosophila* morphotypes, putatively common to the Brazilian Amazonia and Pampa biomes, which could not be correctly matched to any species in the key provided by Wheeler and Takada (1963). After comparing molecular sequences of the mitochondrial cytochrome oxidase subunit I (*COI*) gene and re-evaluating the morphological patterns among these and other previously described species, we were able to validate these as two new species. Thus, in this manuscript, we provide the validation of two new *Mycodrosophila* in a DNA barcoding like analysis and achieve their description using external morphology and male terminalia illustrations. Moreover, we also provide an update to Wheeler and Takada (1963) key to American *Mycodrosophila* species.

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Fig. 1. Flies resting over fungal fructification bodies of *Mycena* sp. (Pers.) Roussel 1806 (Agaricales, Mycenaceae).

Material and methods

DNA manipulation

Mycodrosophila specimens were collected flying over or resting on fungi fructification bodies (Fig. 1) using a manual entomological aspirator (Machado et al., 2014) in forested areas of five municipalities distributed along Amazonia and Pampa biomes (Table 1). The identification was performed by analysis of external morphology and male terminalia patterns, following Wheeler and Takada (1963).

Total DNA was extracted from each of 14 individuals (Table 1) using NucleoSpin Tissue XS kit (Macherey-Nagel, Düren, Germany) and polymerase chain reactions (PCR) were performed using the TYJ1460 and C1N2329 primers described by Simon et al. (1994), for the amplification of approximately 900 bp of the COI mitochondrial gene. PCR products were purified using a solution of polyethylene glycol (PEG) 13% and NaCl 1.6 M and then directly sequenced in a MegaBace 500 automatic sequencer, using the DYEnamic ET® Sequencing Kit (Amersham, GE Healthcare, Chalfont St. Giles, UK) according to the manufacturer's protocol. Both, forward and reverse sequences were determined, so that each base was read at least twice.

Molecular data matrix assembly

The sequences so obtained were assembled, visualized and corrected in the GAP 4 program from the Staden Package (Staden, 1996), and aligned using the Clustal W algorithm as implemented in Mega 5.0 (Tamura et al., 2011). Table 1 presents GenBank accession numbers for the 14 new *COI Mycodrosophila* sequences, which were added to the orthologous sequences from the Nearctic *Mycodrosophila dimidiata* and *Mycodrosophila claytonae*. Additionally, *COI* sequences were also downloaded from GenBank for different species of *Acletoxenus*, *Amiota*, *Leucophenga* and *Ceratitis*, which were used as outgroups because they belong to different Drosophilidae subfamilies or even to different Dipteran families.

Molecular phylogenetic analyses

The phylogenetic analyses were performed using different methods/algorithms and softwares: Bayesian analysis (BA), as performed in MrBayes 3.2.1 (Ronquist et al., 2012); maximum likelihood (ML) analysis, as implemented in PhyML 3.0 (Guindon and Gascuel, 2003); maximum parsimony (MP) analysis, as executed in Paup 4.0b10 (Swofford, 2003); and Neighbor-Joining (NJ) analysis, as achieved in Mega 5.0. BA analysis was performed using 1,000,000 generations of Markov Chain Monte Carlo (MCMC) search, adopting the GTR+G+I model selected by the Akaike information (AIC) evaluation criterion implemented in MrModelTest 2.3 (Nylander, 2004), with tree sampling every 100 generations and discarding 25% of the initial results as burn-in. The ML analysis was carried out with the GTR+G+I model selected by the AIC criteria in ModelTest 3.7 (Posada and Crandall, 1998), using an optimized NJ starting tree. The MP trees were inferred by heuristic search with Tree Bisection and Reconnection branch swapping optimization applied to a group of 100 random stepwise addition starting trees. Finally, the NJ algorithm was implemented using the Tamura–Nei substitution model (Tamura and Nei, 1993) (the most similar to General Time Reversible (GTR) among Mega 5.0 options available for NJ

Table 1
Taxonomic position, geographical source and *COI* sequences GenBank accession numbers for *Mycodrosophila* species and outgroups used for the molecular phylogenetic reconstruction. *Mycodrosophila* sp. 1, *M. hofmanni* sp. nov.; *Mycodrosophila* sp. 2, *M. valentae* sp. nov.

Family	Subfamily	Species	Origin	Accession number[a]
Drosophilidae	Drosophilinae	*Mycodrosophila hofmanni* sp. nov. 1	Colorado do Oeste, RO, Brazil	KC987477
		Mycodrosophila hofmanni sp. nov. 2	Colorado do Oeste, RO, Brazil	KC987478
		Mycodrosophila valentae sp. nov. 1	Santa Maria, RS, Brazil	KC987479
		Mycodrosophila valentae sp. nov. 2	Rio Grande, RS, Brazil	KC987481
		Mycodrosophila valentae sp. nov. 3	Santa Maria, RS, Brazil	KC987480
		Mycodrosophila valentae sp. nov. 4	Rio Grande, RS, Brazil	KC987482
		Mycodrosophila valentae sp. nov. 5	Rio Grande, RS, Brazil	KC987483
		M. elegans 1 Wheeler and Takada, 1963	Diamante do Norte, PR, Brazil	JX497750
		M. elegans 2	Teodoro Sampaio, SP, Brazil	KC987476
		M. projectans 1 Wheeler and Takada, 1963	Santa Maria, RS, Brazil	JX497751
		M. projectans 2	Santa Maria, RS, Brazil	KC987484
		M. projectans 3	Santa Maria, RS, Brazil	KC987485
		M. projectans 4	Santa Maria, RS, Brazil	KC987486
		M. projectans 5	Santa Maria, RS, Brazil	KC987487
		M. claytonae	Leon Co., Florida, USA	GU597470
		M. dimidiata	–	EU493682
	Steganinae	*Acletoxenus indicus* Loew, 1864	Hainan, Qionghai, China	HQ701131
		Amiota setigera Malloch, 1924	–	EU493568
		Leucophenga albfasciata Macquart, 1851	–	EU493569
		L. angusta Okada, 1956	Hachioji, Tokyo, Japan	HQ842780
		L. quadripunctata de Meijere, 1908	Hachioji, Tokyo, Japan	HQ842781
		L. varia Walker, 1849	Florida, Leon Co., USA	GU597446
Tephritidae	Dacinae	*Ceratitis capitata* Wiedmann, 1824	–	NC_000857

[a] New sequences obtained in this study are underlined.

Table 2
Morphological characters used for the morphological phylogenetic reconstruction and their respective class and state in the analysis.

Morphological character	Class/states
C index	Continuous
4v index	Continuous
5x index	Continuous
Ac index	Continuous
Length Or1/Length Or2	Continuous
Setulae on paraphysis	Continuous
Outer branches on surstyles	Continuous
Setae on gonopod	Continuous
Rows of acrostichal setae	Categorical – 6/8/10
Tergite 2 – pale area	Categorical – presence/absence
Tergite 3 – pale area	Categorical – presence/absence
Tergite 4 – pale area	Categorical – presence/absence
Tergite 5 – pale area	Categorical – presence/absence
Tergite 6 – pale area	Categorical – presence/absence
Cloud band on apical CII	Categorical – presence/absence
Cloud band on 1st costal break	Categorical – presence/absence
Cloud band on 2nd costal break	Categorical – presence/absence
Short pile on paraphysis	Categorical – presence/absence

searches) with a Gamma correction, whose alpha value was set to 0.17, following ModelTest results. Clade support was inferred with a bootstrap test with 1000 replications for ML, MP and NJ analyses, while for BA, the posterior probability (PP) of each clade was estimated. Moreover, intra- and interspecific COI distances were calculated with the Kimura 2-Parameters (K2P) evolutionary model (Kimura, 1980), with standard error estimated through 1000 bootstrap replicates.

Morphological phylogenetic analysis

A morphological phylogenetic analysis was performed with 18 categorical and continuous characters (14 based on external morphology and four based on male terminalia patterns) (Table 2) obtained from the descriptions of *Mycodrosophila* species of Sturtevant (1916), Wheeler and Takada (1963) and of the new species presented below. We used the average of each measure for continuous characters, because this was the only data available in the literature. Additionally, continuous characters were first categorized in an ascending order and latter weighted in inverse proportion to their minimum number of steps, using a scaled MP search, as performed in Paup 4.0b10. Other heuristic search properties were set according to the features described above for the molecular analysis. Given the difficulties in establishing homologies for these characters to other species, the Nearctic species were used as outgroup, which seemed justifiable given the results of our molecular phylogenetic analysis (see below).

New species descriptions

After morphological and molecular validation regarding the sampling of two new species, descriptions were performed from individuals preserved in ethanol 70° according to the measurements and nomenclature of structures suggested by Bächli et al. (2004). The measurements were performed on a stereoscopic microscope coupled with micrometric reticulum. The average values for each characteristic are presented accompanied by the maximum and minimum observed values (in brackets). The postabdomen of all the specimens was disarticulated and the male terminalia were mounted in permanent slides with Canada balsam. Illustrations of male terminalia of the holotypes were made using a *camara lucida* attached to an optical microscope (Olympus BX40) with a 40× objective lens and a 10× ocular lens. We obtained photomicrographs of the external morphology structures and male terminalia of flies with a Zeiss Discovery V.20 stereomicroscope.

Whenever possible, we photographed the host fungi and sent the photographs to Dr. Eduardo Bernardi (Universidade Federal de Pelotas) for identification. The types series of the new species will be deposited in the Museu de Ciências Naturais of the Fundação Zoobotânica do Rio Grande do Sul (MCNZ, Porto Alegre, Brazil). One paratype of each new species will be deposited in the Museu Nacional do Rio de Janeiro of Universidade Federal do Rio de Janeiro (MNRJ, Rio de Janeiro, Brazil).

Results

Species validation and phylogenetic analyses

The sampled *Mycodrosophila* specimens were determined to four morphotypes: *M. projectans*, *M. elegans* and two initially putative new species. The multiple alignments of the 16 *Mycodrosophila* sequences (14 new + two available in GenBank) spanned 748 base pairs from the 5′ region of *COI* and contained 170 variable sites, of which 156 were parsimoniously informative. The general frequencies for each nucleotide were A = 29.9, C = 16.0, G = 17.0 and T = 37.1, and the average transition/transversion ratio was 0.98. Minimum, maximum and average of intraspecific and interspecific K2P *COI* distances with standard errors are presented in Table 3. In this case, whereas the two sequences obtained for the first putative new species (hereafter *M. hofmanni* sp. nov., see below) exhibited 0.4% divergence, the intraspecific distances for the second putative new species (hereafter *M. valentae* sp. nov., see below) sequences ranged from 0.0 to 0.3%. The interspecific distances between the new species and each of the other two sampled congeneric Neotropical species were much higher, and always greater than 12%. The K2P distances between the two putative new species were also high, presenting values of the order of 13%.

The *COI* supported phylogram is depicted in Fig. 2, where it can be seen that all the sampled morphotypes are reciprocally monophyletic in regard to each other, a result presenting high support for all methods. Concerning the interspecific relationships, the phylogeny displays *Mycodrosophila* as monophyletic in regard to the included Steganinae species (PP = 0.97), clustering the two Nearctic species (PP = 1.00), and the pair *M. hofmanni* sp. nov. and *M. valentae*

Table 3
Intra- and interspecific K2P *COI* distances and their respective standard errors in regard to the comparisons involving *Mycodrosophila hofmanni* sp. nov. and *M. valentae* sp. nov. plus the other evaluated Neotropical *Mycodrosophila* species.

	Species	Distances values		
		Minimal	Average	Maximal
Intraspecific distances	*M. hofmanni* sp. nov.	–	0.004 ± 0.002	–
	M. valentae sp. nov.	0.000 ± 0.000	0.001 ± 0.001	0.003 ± 0.002
Interspecific distances	*M. hofmanni* sp. nov. × *M. projectans*	0.143 ± 0.017	0.145 ± 0.017	0.149 ± 0.017
	M. hofmanni sp. nov. × *M. elegans*	0.163 ± 0.018	0.164 ± 0.018	0.165 ± 0.018
	M. valentae sp. nov. × *M. projectans*	0.127 ± 0.014	0.134 ± 0.015	0.144 ± 0.016
	M. valentae sp. nov. × *M. elegans*	0.146 ± 0.014	0.148 ± 0.015	0.150 ± 0.016
	M. hofmanni sp. nov. × *M. valentae* sp. nov.	0.133 ± 0.016	0.135 ± 0.016	0.137 ± 0.016

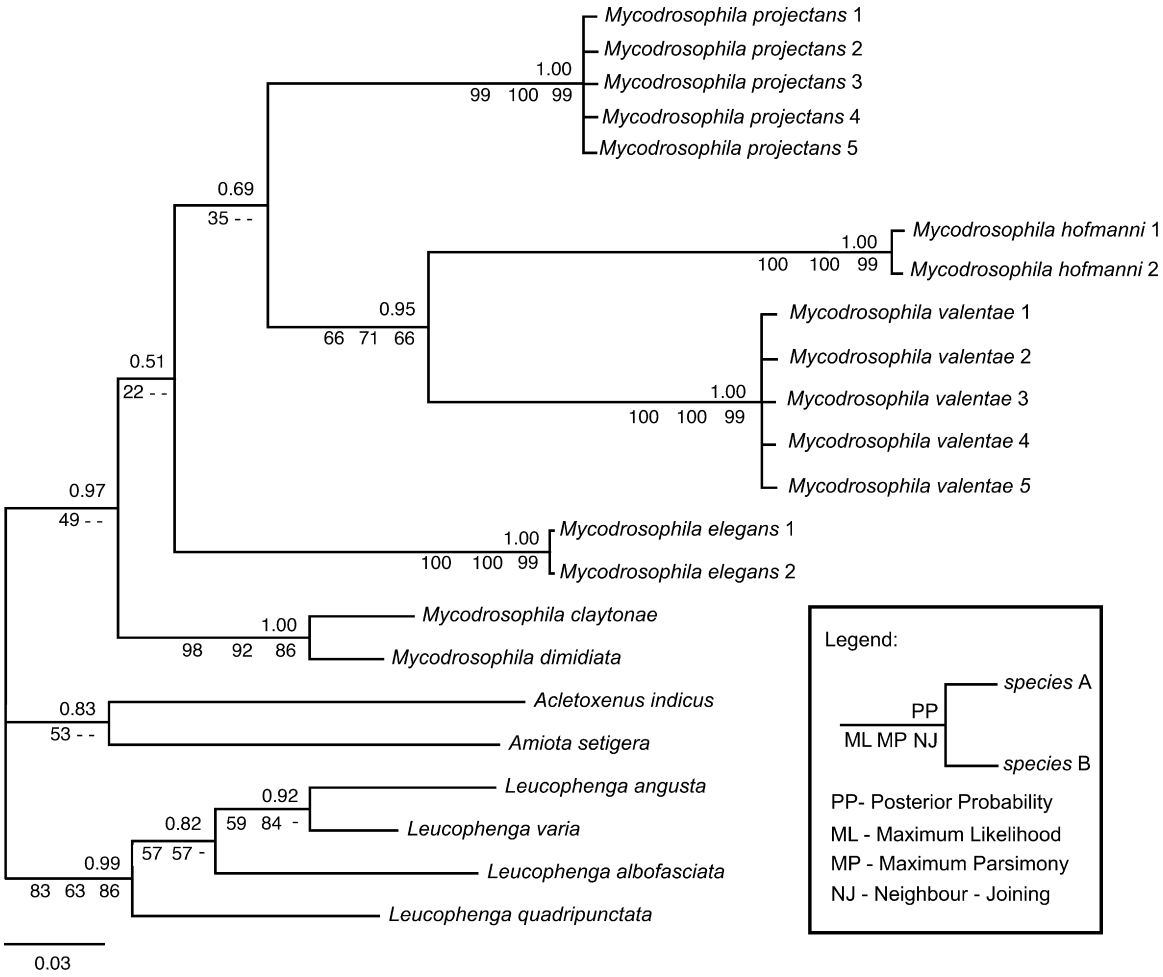

Fig. 2. COI majority-rule consensus phylogeny inferred by Bayesian analysis using the GTR+G+I model for the 23 nucleotide sequences (Table 1). The values above the nodes are the posterior probabilities in Bayesian Analysis and the values below are the bootstrap values for the maximum likelihood, maximum parsimony and Neighbor-Joining trees, respectively. The "-" signal indicates absence of support for the considered relationship. This tree was rooted with *Ceratitis capitata* sequences. Branch lengths are proportional to the scale, given in substitutions per nucleotide.

sp. nov. (PP = 0.95) as sister species. Although with low support, the tree also recovered the early separation between Neotropical and Nearctic species (PP = 0.51), and presented *M. elegans* as an early Neotropical offshoot (PP = 0.69).

The morphological weighted parsimony analysis recovered six trees with the same number of steps (280), all of which presented a *ci* of 0.85 and a *ri* of 0.68. Without considering relationships among the Nearctic species, this set of trees was further reduced to two, one of which is presented in Fig. 3. Instead of clustering *M. hofmanni* sp. nov. and *M. valentae* sp. nov., the two most parsimonious trees recovered *M. hofmanni* sp. nov. as morphologically similar to *M. neoprojectans*. Moreover, although the positioning of *M. valentae* sp. nov. differed between the two trees with the same number of steps, one of the topologies presented this species as sister to *M. projectans* (Fig. 3), whose morphological resemblance was already recognized by some of the authors (MSG and JJ) at a first sight.

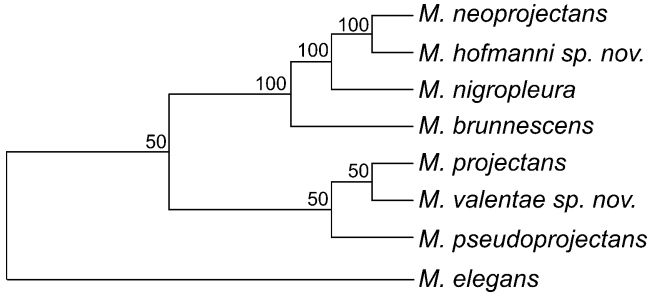

Fig. 3. Majority-rule consensus tree recovered through maximum parsimony analysis of the morphological characters listed in Table 2 showing the external similarities among the eight known Neotropical *Mycodrosophila* species. This tree was rooted with the three Nearctic's species (*M. claytonae*, *M. dimidiata* and *M. stalkeri*). The values above the nodes represent the percentage of maximum parsimonious trees recovering the clade, so that conflicting relationships are highlighted by intermediate support values (i.e. 50%).

Descriptions

Based on the molecular analyses presented above and on the analysis of body morphology and male terminalia patterns, we propose two new species of *Mycodrosophila*, as described below:

Mycodrosophila hofmanni *sp. nov.*

(Figs. 4A–C, 5A, 6A, 7A–E)

Type material. HOLOTYPE: ♂, labeled "Brasil, RO. Colorado do Oeste 13°00′37.7″ S; 60°35′24.9″ W, Junges, J. col. 5.I.2012/*M. hofmanni* sp. nov. Junges, Gottschalk, Loreto and Robe ♂ Holótipo", post-abdomen disarticulated and the terminalia mounted on slide with Canada balsam. PARATYPES: ♂, labeled "Brasil, RO. Colorado do Oeste. Zona rural, 13°00′37.7″ S; 60°35′24.9″ W, Junges, J. col. 5.I.2012/*M. hofmanni* sp. nov. Junges, Gottschalk, Loreto and Robe ♂ Parátipo", post-abdomen disarticulated and the terminalia

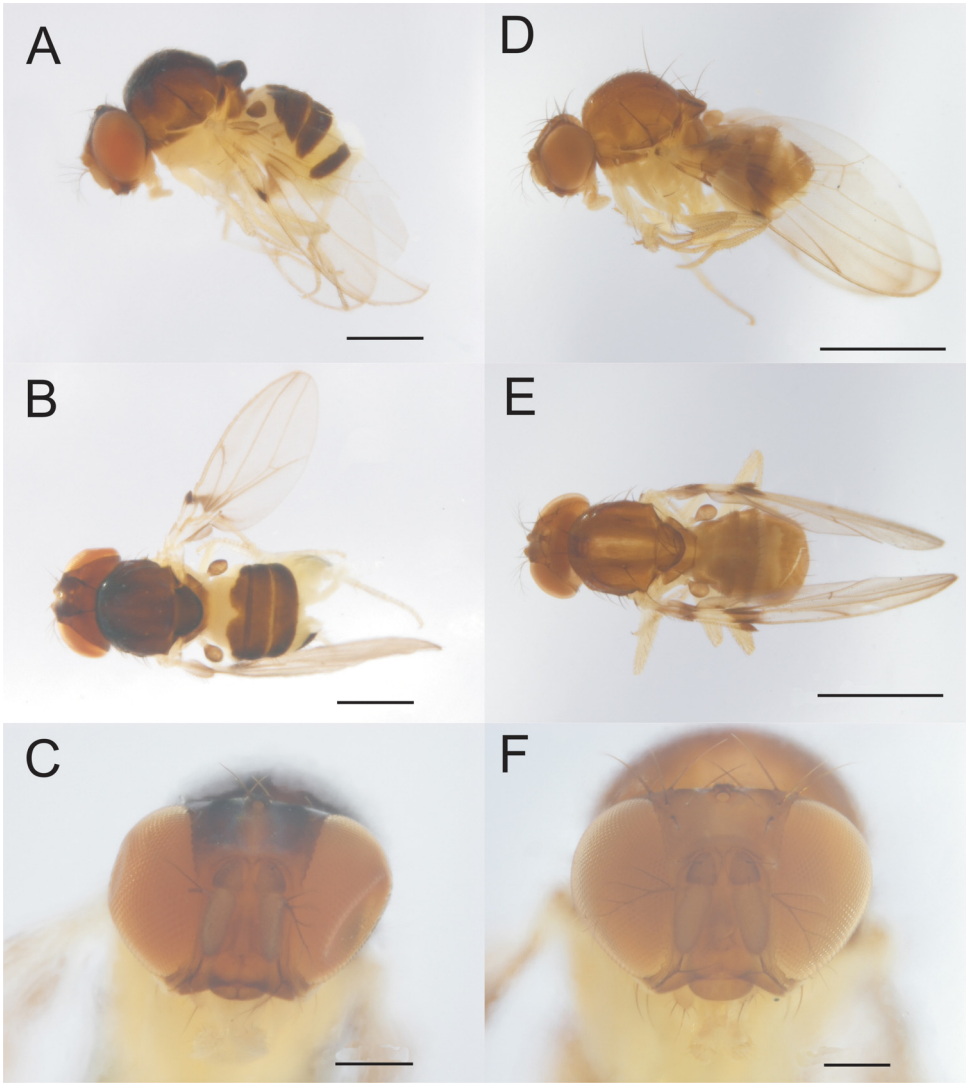

Fig. 4. *Mycodrosophila hofmanni* sp. nov., paratype male, terminalia removed. (A) Lateral view; (B) dorsal view; (C) head, frontal view. *Mycodrosophila valentae* sp. nov., paratype male, terminalia removed. (D) Lateral view; (E) dorsal view; (F) head, frontal view. Bars: A and B = 0.5 mm; D and E = 1.0 mm; C and F = 0.2 mm.

mounted on slide with Canada balsam; male, slide with terminalia labeled "Brasil, RO. Colorado do Oeste. Zona rural, 13°00′37.7″ S; 60°35′24.9″ W, Junges, J. col. 5.I.2012/*M. hofmanni* sp. nov. Junges, Gottschalk, Loreto and Robe ♂ Parátipo" (GenBank accession number KC987477).

Type locality: Amazonian forest fragment, Colorado do Oeste city, Rondonia State, Brazil (13°00′37.7″ S; 60°35′24.9″ W).

Diagnosis: Main color dark brown. Thorax with dorsal region dark brown and pleura pale yellowish (Fig. 4A and B). Abdomen

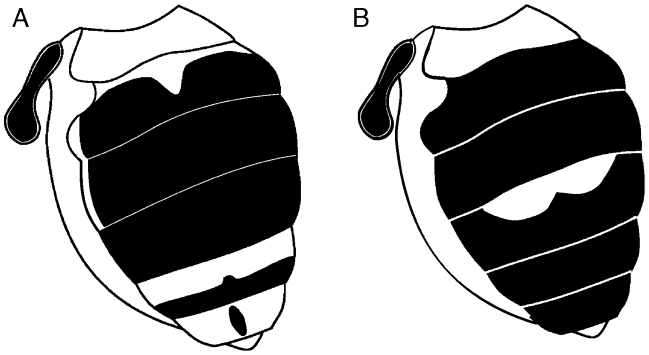

Fig. 5. Illustration of the abdominal patterns for *Mycodrosophila* species. (A) *M. hofmanni* sp. nov.; (B) *M. valentae* sp. nov. Drawing outlines based on Wheeler and Takada (1963).

Fig. 6. Wings of *M. hofmanni* sp. nov. (A) and *M. valentae* sp. nov. (B). Bar = 0.5 mm.

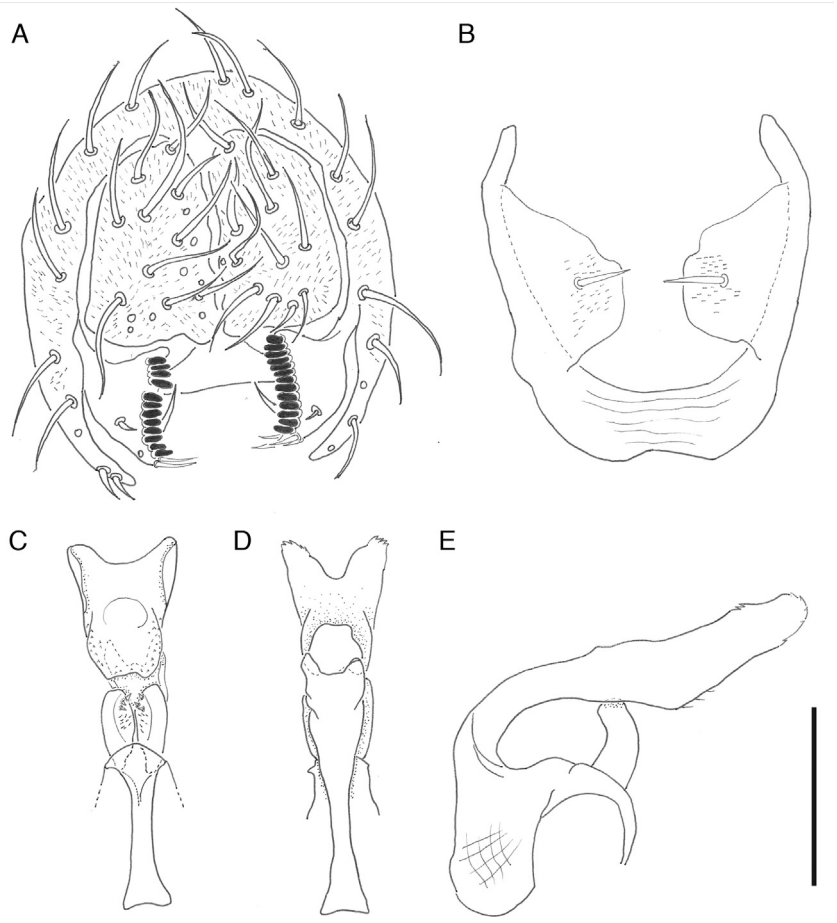

Fig. 7. Male terminalia of *M. hofmanni* sp. nov. holotype. (A) Epandrium, cerci, surstyli and decasternum, posterior view; (B) hypandrium and gonopods, posterior view; (C–E) aedeagus, aedeagal apodeme and paraphyses, respectively in ventral, dorsal and lateral views. In A, arrow indicates decasternum position. Bar = 0.1 mm.

with dark brown bands covering tergites 2–5, tergite 2 with a pale central area backward projected centrally, tergite 5 with the dark band covering only the posterior margin, with a slight central and anterior projection, and tergite 6 with a central longitudinal stripe (Fig. 5A). Wing bearing two clouded bands extending dorso-ventrally below to each costal vein breaks (Fig. 6A), similar to *M. projectans*. Aedeagus projected anteriorly with apical region bifurcated and jagged (Fig. 7C–E).

Male description

Head (Fig. 4C): Frons dark brown. Frontal length = 0.24 mm (0.21–0.25 mm); frontal index = 0.81 (0.86–0.91); frontal tapering ratio = 1.18 (1.13–1.22), length ratio of orbital setae: or2/or1 = 0.16, or2/or3 = 0.33, or1/or3 = 1.00; vertical setae broken; ocellar triangle yellowish about 32% (30–33%) of frontal length. Facial carina proeminent and nose like. Antennae brown, aristae with 4 dorsal and 1 ventral branches plus terminal fork, flagellomere I width to length ratio = 0.90 (0.87–0.93). Palpi dark brown. Red eyes without pile. Genae brown, vibrissal index = 0.60 (0.45–0.75); cheek index = 5.47 (4.43–7.00); eye index = 1.11 (1.07–1.15).

Thorax (Fig. 4A–B): Main color dark brown. Length: 0.67 mm (0.62–0.70 mm), width: 0.54 mm (0.50–0.57 mm). Eight irregular rows of acrostichals, anterior pair of dorsocentral setae absent, distance between posterior dorsocentral = 0.26 mm (0.22–0.30 mm); no prescutellar setae. Scutellum dark brown. Basal scutellar setae convergent. Scut index = 0.54 (0.45–0.64); scut position index = 0.61 (0.50–0.70). Two prominent katepisternal setae. Sterno index = 0.54 (0.45–0.63). Pleurae brownish yellow. Halter dark brown. Legs yellowish.

Wing (Fig. 6A): Yellowish with dark yellow veins, bearing two clouded bands extending dorso-ventrally below each costal vein breaks. Length = 1.53 mm (1.50–1.57 mm). Indices: wing index = 2.27 (2.17–2.33); C = 0.90 (0.88–0.92); ac = 5.95 (5.40–6.25); 4v = 2.04 (1.52–2.38); 5x = 1.85 (1.55–2.00); 4C = 1.79 (1.31–2.15); M = 0.51 (0.42–0.70); hb = 0.63 (0.60–0.66); prox. x = 0.56 (0.42–0.69).

Abdomen (Fig. 5A): Tergites 2–5 with dark brown bands. Tergite 2 with the pale area occupying almost half of the anterior margin, with a backward central projection. Tergite 5 with the band covering only the posterior margin and containing a slight central projection. Tergite 6 bearing one central longitudinal strip.

Body length: 1.51 mm (1.50–1.55 mm).

Terminalia (Fig. 7): Epandrium microtrichose with 5 upper bristles, ventral lobe not microtrichose with 7 large bristles. Cerci microtrichose bearing large bristles, not fused to epandrium. Decasternum as in Fig. 7A. Surstyli with 12–13 prensisetae, 2–3 inner and 3–4 outer setae. Hypandrium (Fig. 7B) in an arc. Gonopods large, fused to hypandrium and containing short piles and one seta. Aedeagus (Fig. 7C–E) projected anteriorly, with the apical region bifurcated and jagged, internal margin of the aedeagus with rows of tiny setae. Aedeagal apodeme flattened laterally and shorter than aedeagus. Ventral rod projected anteriorly and fused with the posteromedian margin of the hypandrium. Paraphyses microtrichose, fused to the gonopods and containing three inner setulae.

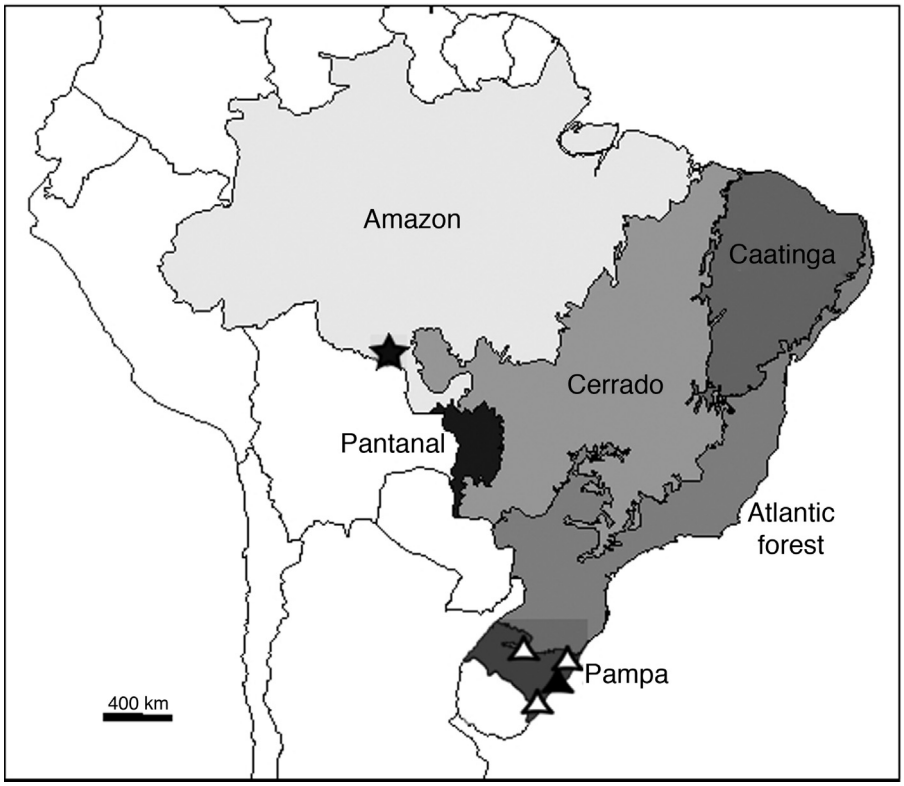

Fig. 8. Brazilian biomes, according to IBGE (2013), and the occurrence records from the two new *Mycodrosophila* species. Stars indicate *M. hofmanni* sp. nov., triangles indicate *M. valentae* sp. nov. The black symbols indicate the type locality of each species.

Female unknown.

Etymology: The epithet is a genitive patronym to honor the Brazilian Drosophilist Paulo Roberto Petersen Hofmann, from Universidade Federal de Santa Catarina.

Geographic distribution: *M. hofmanni* sp. nov. was sampled in two fragmented areas from Brazilian Amazon Forest in Colorado do Oeste city, Rondônia (RO) (13°00′37.7″ S; 60°35′24.9″ W) (Fig. 8).

Ecological notes: The records are restricted to the type locality, and the type series specimens were collected flying over mushrooms of *Mycena* sp. (Agaricales, Mycenaceae) (Fig. 1).

Mycodrosophila valentae *sp. nov.*

(Figs. 4D–F, 5B, 6B, 9A–E)

Type material. HOLOTYPE: ♂, labeled: "Brasil, RS. Capão do Leão. Horto Botânico Irmão Teodoro Luis – UFPel. 31°46′02.05″ S, 52°26′55.34″ W, Blauth, M.L. and Valer, F. col. 29.04.2011/*M. valentae* sp. nov. Junges, Gottschalk, Loreto and Robe, ♂ Holótipo". PARATYPES: 3 ♂, labeled: "Brasil, RS. Capão do Leão. Horto Botânico Irmão Teodoro Luis – UFPel. 31°46′02.05″ S, 52°26′55.34″ W, Blauth, M.L. and Valer, F. col. 29.04.2011/*M. valentae* sp. nov. Junges, Gottschalk, Loreto and Robe ♂ Parátipo". All the specimens (holotype and paratypes) had their post-abdomen disarticulated and the terminalia mounted on slides with Canada balsam.

Type locality: Horto Botânico Irmão Teodoro Luis, Campus of Universidade Federal de Pelotas, Capão do Leão city, Rio Grande do Sul State, Brazil (31°46′02.05″ S, 52°26′55.34″ W).

Diagnosis: Main color dark brown, notum dark brown and pleurae brownish yellow (Fig. 4D and E). Abdomen with dark brown bands covering tergites 2–6, tergite 4 with a pale central area as in *M. projectans* (Fig. 5B). Wing bearing two clouded bands below each costal vein breaks (Fig. 6B). The aedeagus has a tubular form with tiny scales around it, being apically bifurcated and slightly serrated (Fig. 9C–E).

Male description

Head (Fig. 4F): Frons dark brown with a median area light brown. Frontal length = 0.29 mm (0.29–0.30 mm); frontal index = 0.97 (0.96–1.00), frontal tapering ratio = 1.35 (1.30–1.48), length ratios of orbital setae: or2/or1 = 0.20 (0.12–0.29), or2/or3 = 0.21 (0.12–0.38), or1/or3 = 1.05 (0.82–1.3), vt index = 0.99 (0.77–1.15), ocellar triangle dark brown about 35% (29–40%) of frontal length. Face dark brown, facial carina proeminent and nose like. Antennae dark brown, aristae with 4 or 5 dorsal, 1 ventral and 4 internal branches, plus terminal fork; flagellomere I width to length ratio = 0.44 (0.41–0.47). Palpi dark brown. Red eyes without pile. Genae brown, vibrissal index = 0.27 (0.16–0.33); cheek index = 9.00 (5.00–13.00); eye index = 1.12 (1.05–1.26).

Thorax (Fig. 4D and E): Main color dark brown. Length = 0.95 mm (0.92–1.00 mm), width = 0.72 mm (0.69–0.77 mm). Ten irregular rows of acrostichals, anterior pair of dorsocentral setae absent, distance between posterior dorsocentral = 0.34 mm (0.28–0.36 mm), no prescutellar setae. Scutellum dark brown. Basal scutellar setae convergent. Scut index = 0.41 (0.36–0.51), scut position index = 0.63 (0.41–0.82). Two prominent katepisternal setae, sterno index = 0.54 (0.47–0.59). Pleurae brownish yellow. Halter dark brown. Legs brownish yellow.

Wing (Fig. 6B): Yellow with veins dark yellowish, bearing clouded bands below the two costal vein breaks, extending dorso-ventrally. Length = 2.26 mm (2.17–2.33 mm). Indices: wing index = 2.39 (2.17–2.81); C = 0.84 (0.80–0.87); ac = 5.40 (5.06–5.86); 4v = 1.90 (1.81–1.97); 5x = 1.42 (1.25–1.61); 4C = 1.78 (1.64–1.86); M = 0.48 (0.45–0.50); hb = 0.76 (0.72–0.79); prox. x = 0.52 (0.47–0.54).

Abdomen (Fig. 5B): Tergites 2, 6 dark brown, only tergite 4 with an anterior and medial pale area.

Body length: 2.36 mm (2.12–2.61 mm).

Terminalia (Fig. 9): Epandrium (Fig. 9A) highly microtrichose, with 11 upper bristles. Ventral lobe not microtrichose, with 10 large bristles. Cerci microtrichose, bearing large bristles, not fused

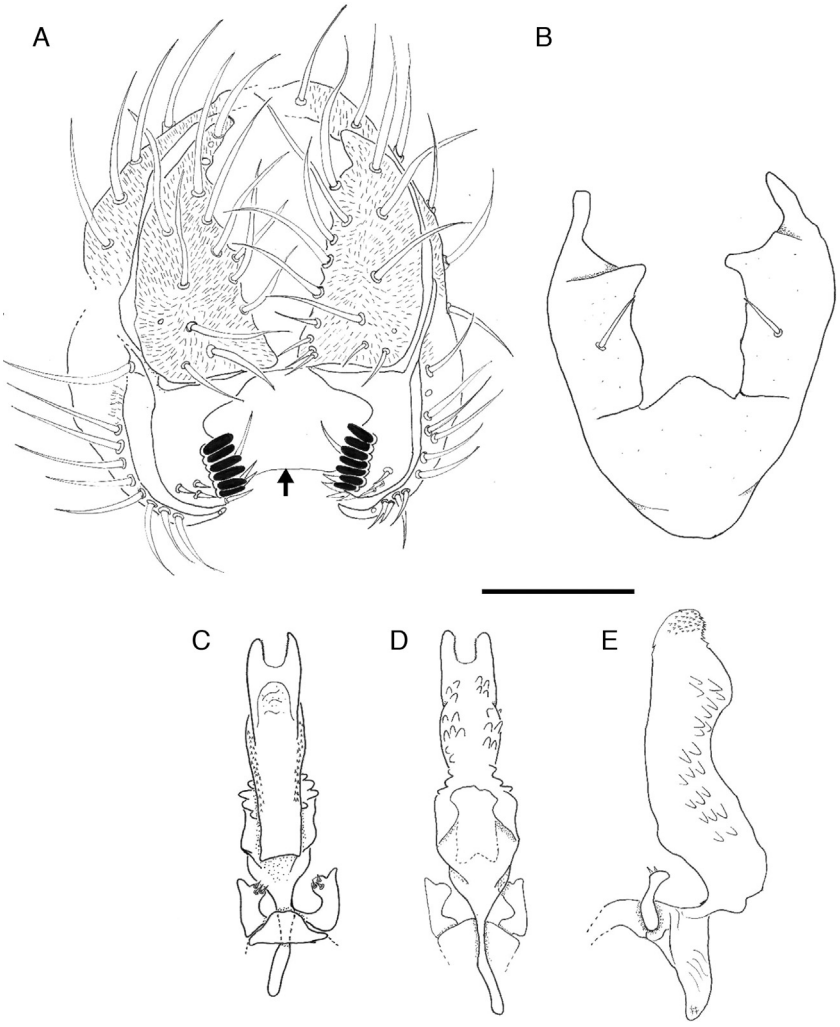

Fig. 9. Male terminalia of *M. valentae* sp. nov. holotype. (A) Epandrium, cerci, surstyli and decasternum, posterior view; (B) hypandrium and gonopods, posterior view; (C–E) aedeagus, aedeagal apodeme and paraphyses, respectively in ventral, dorsal and lateral views. In A, arrow indicates decasternum position. Bar = 0.1 mm.

to epandrium. Decasternum as in Fig. 9A. Surstyli with 6 peg-like prensisetae, 4 inner and 3 outer setae. Hypandrium (Fig. 9B) longer than wide, v-shaped. Gonopods fused to hypandrium, longer than wider, with one long seta. Aedeagus tubular with tiny scales in the medial half, projected anteriorly, with the tip turned to the dorsal region. Apex of aedeagus bifurcated, with marginal small spines and a jagged aspect. Aedeagal apodeme shorter than aedeagus. Ventral rod projected and fused with the posteromedian margin of the hypandrium. Paraphyses linked to the aedeagal apodeme by membranous tissue and containing three tiny setulae internally.

Female unknown.

Etymology: The epithet is a genitive patronym to honor the Brazilian drosophilist Vera Lúcia da Silva Valente, from Universidade Federal do Rio Grande do Sul.

Geographic distribution: *M. valentae* sp. nov. seems to be widely distributed in the Brazilian state of Rio Grande do Sul (Fig. 8). Until now, this species was collected in the municipalities of Capão do Leão (31°46′02.05″ S; 52°26′55.34″ W), Santa Maria (29°43′02″ S; 53°43′34″ W), Rio Grande (32°32′18.28″ S; 52°32′6.74″ W) and Viamão, RS, Brazil (30°05′17″ S; 51°06′07″ W).

Ecological notes: This species is common in Southern Brazil, where specimens of the type series were sampled flying over mushrooms of an unidentified Polyporaceae.

Discussion

Phylogenetic analysis

Our molecular results support the presence of two new Neotropical *Mycodrosophila* species in the Brazilian Amazonian and Pampa biomes. In fact, *M. hofmanni* sp. nov. and *M. valentae* sp. nov. are reciprocally monophyletic species, which showed a broad barcoding gap between intra- and interspecific distances. Since these are two premises for successful application of DNA barcoding approaches (Meyer and Paulay, 2005), our results support the notion that this methodology may aid in species identification/discovery issues within this group, as previously shown for other taxons (Hebert et al., 2003; van Nieukerken et al., 2012). Nevertheless, we need to be aware that these results may suffer from sampling shortages, related to the underestimation of intraspecific variation and/or to the overestimation of interspecific divergence (Moritz and Cicero, 2004; Linares et al., 2009). In fact, whereas the complete distribution range of each of the two new species is not yet understood, we were not able to sample four of the currently known Neotropical *Mycodrosophila* species (*M. brunnescens*, *M. neoprojectans*, *M. nigropleura* and *M. pseudoprojectans*). Even so, morphological distinction from these (see below) attests to the validity of *M. hofmanni* sp. nov. and *M. valentae* sp. nov.

Concerning the recovered phylogenetic patterns, interspecific relationships are difficult to infer because of the low support values presented by our phylogenies. Moreover, comparisons involving a single gene may not accurately reflect species phylogeny (Nixon and Carpenter, 1993; van Nieukerken et al., 2012). Nevertheless, as some similarities were recovered in both molecular and morphological analyses, we may use them to make a first approximation to the group's phylogeny. This is the case, for example, for the clustering of *M. projectans*, *M. hofmanni* sp. nov. and *M. valentae* sp. nov., which formed a monophyletic clade sister to *M. elegans* in both, molecular and morphological phylogenies. The formation of a Neotropical *Mycodrosophila* species group independent of the Nearctic species was inferred through *COI* phylogenetic analysis and—despite its low support values—is concordant with the proposition of Wheeler and Takada (1963). In this sense, it is important to emphasize that no molecular study, to our knowledge, has explored the phylogenetic relationships among American *Mycodrosophila* species.

Two new species of Neotropical Mycodrosophila

The two new species described here are included in the *Mycodrosophila* genus by the presence of the arista with a single ventral branch, only one pair of dorsocentral setae, a costal lappet and the entirely colored and shining scutum, without spots at the bases of the setae (Wheeler and Takada, 1963; Grimaldi, 1990). *M. hofmanni* sp. nov. and *M. valentae* sp. nov. are also embedded within the subgenus *Mycodrosophila*, with a developed costal lappet and a cloud band below the second costal vein.

The patterns of the tergites are remarkable in distinguishing the two new species between each other and in relation to other *Mycodrosophila* species. This distinction is commonly related to the presence of pale areas between black bands in the tergites, which seems to be widespread in *Mycodrosophila* (Bock, 1980; Chassagnard and Lachaise, 2000; Okada, 1956; Tsacas and Chassagnard, 1991). In this case, whereas the Nearctic species present pale areas in all the last six tergites (Wheeler and Takada, 1963), in the species described here these are restricted to some of the tergites. The presence of these pale areas also allows the distinction of *M. hofmanni* sp. nov. and *M. valentae* sp. nov from *M. brunnescens*, *M. nigropleura* and *M. elegans*, which have their abdomen completely dark (Wheeler and Takada, 1963).

Although the differentiation of the two new species in regard to the remaining Neotropical *Mycodrosophila* species is not so remarkable, the color pattern of the tergites is also very informative in this task. In this sense, *M. hofmanni* sp. nov. differs from *M. projectans* by the absence of pale areas in tergites 3 and 4, and from *M. pseudoprojectans* by having a dark spot on tergite 6. In fact, as suggested by our morphological phenogram, *M. hofmanni* sp. nov. is most similar to *M. neoprojectans*, although both species differ by the color pattern of tergite 2, where a pale area is encountered only in the new species. In the case of *M. valentae* sp. nov., it differs from *M. neoprojectans*, *M. projectans* and *M. pseudoprojectans* by having a pale area only in tergite 4, whereas the other three species have additional pale areas in their abdomens.

The male terminalia of the two new species also presents the regular form found in *Mycodrosophila* flies, with a tubular, elongated and apically bifurcated aedeagus (Bock, 1980; Chassagnard and Lachaise, 2000; McEvey and Polak, 2005; Wheeler and Takada, 1963). In fact, the aedeagus with a bifurcation or bifurcated and jagged in the apical region is a characteristic shared by a large number of *Mycodrosophila* species, being found in eight of the nine previously known American *Mycodrosophila* species (the male genitalia of *M. brunnescens* is unknown), in addition to the two new species described here. The ventrally curved aedeagus found in *M. hofmanni* sp. nov. is also encountered in *Mycodrosophila diversa* Bock, 1980 and *Mycodrosophila erecta* Okada, 1968, two species inhabiting other Zoogeographical Regions, although *M. diversa* has a pale area on tergite 6 and a thinner apical aedeagus region, whereas *M. erecta* has the aedeagus more straight and with some notched bifurcations. Otherwise, the aedeagus of *M. valentae* sp. nov. is similar to that found in *M. claytonae*, with a straight format and tiny spines in the apical region, although the abdominal pattern is largely different among these two species.

Key to American species of *Mycodrosophila* (updated from Wheeler and Takada, 1963)

1. Dark bands of tergites 2 and 3 broadly interrupted medially and all tergites with pale areas; Nearctic species . . . 2
1′ Dark bands of tergites 2 and 3 without broad median interruptions; Neotropical species . . . 4
2. Dark band of tergite 4 broadly interrupted medially, thus appearing like the preceding ones; terminalia as in Wheeler and Takada, 1963: 396, figs. 3–6 . . . *M. dimidiata* Loew, 1862
2′. Dark band of tergite 4 not interrupted, medially shaped like an inverted V; terminalia not as above . . . 3
3. Costal index about 2.0, tergite 6 dark or with small pale areas in the lateral region. Abdominal pattern as shown in Wheeler and Takada, 1963: 394, fig. 2; terminalia as in Wheeler and Takada, 1963: 396, figs. 7–10 . . . *Mycodrosophila stalkeri* Wheeler and Takada, 1963
3′. Costal index about 1.7, tergite 6 with large pale areas in the lateral. Abdominal pattern as shown in Wheeler & Takada, 1963: 394, fig. 2; terminalia as in Wheeler and Takada, 1963: 396, figs. 11–14) . . . *M. claytonae* Wheeler and Takada, 1963
4. Wing with five prominent black clouds, including one over dm-Cu crossvein; R_{2+3} bent abruptly to Costa; terminalia as in Wheeler and Takada, 1963: 397, figs. 31–34) . . . *M. elegans* Wheeler and Takada, 1963
4′. Wing less clouded, posterior crossvein (dm-Cu) never in a cloud; R_{2+3} vein approaching Costa gradually; terminalia not as above . . . 5
5. Pleura largely brown, with a whitish area extending obliquely from the axillar area (wing base) to the pronotum (prosternum); legs brown. Male: unknown . . . *M. brunnescens* Wheeler and Takada, 1963
5′. Pleura pale yellowish, at most the anepimeron (pteropleura) dark; legs mostly pale . . . 6
6. Anepimeron (pteropleura) dark, forming a short oblique band from the base of the haltere to the katepisternum (sternopleural) corner; abdomen black; terminalia as in Wheeler and Takada, 1963: 396, figs. 27–30 . . . *M. nigropleura* Wheeler and Takada, 1963
6′. Anepimeron (pteropleura) yellowish pale or brownish pale; some tergites with pale areas . . . 7
7. Tergite 6 whole shining black . . . 8
7′. Tergite 6 yellow with and elongate, narrow, median black stripe; tergite 5 with an apical dark band expanded basally in midline . . . 9
8. Tergites 3, 4 and 6 shining black, tergite 5 with paramedian basal pale areas; humeral break (costal incision) weak, lappet scarcely developed; terminalia as in Wheeler and Takada, 1963: 396, figs. 23–26 . . . *M. pseudoprojectans* Wheeler and Takada, 1963
8′. Tergites 3 and 4 or only tergite 4 with some pale areas; tergites 5 and 6 shining black; costal lappet large and black; terminalia not as above . . . 10
9. Tergite 2 without pale areas; dark band in tergite 5 not touching the lateral margin; terminalia as in Wheeler and Takada, 1963: 397, figs. 19–20) . . . *M. neoprojectans* Wheeler and Takada, 1963
9′. Tergite 2 with a pale area in the anterior margin and a dark band with bilobed aspect (Fig. 5A; terminalia as in fig. 7A–E. . . *M. hofmanni* sp. nov.
10. Both tergites 3 and 4 with pale areas in the anterior margin; tergite 3 with a pale and rounded central area and tergite 4 with an expanded pale area; terminalia as in Wheeler and Takada, 1963: 396, figs. 15–18 . . . *M. projectans* Sturtevant, 1916
10′. Only tergite 4 with a pale area expanded in the anterior margin, tergite 3 dark brown (Fig. 5B); terminalia as fig. 9A–E . . . *M. valentae* sp. nov.

Conflicts of interest

The authors declare no conflicts of interest.

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