Revista Brasileira de Entomologia 62 (2018) 283–287

SOCIEDADE BRASILEIRA
DE ENTOMOLOGIA
FUNDADA EM 1937

REVISTA BRASILEIRA DE
Entomologia
A Journal on Insect Diversity and Evolution

www.rbentomologia.com

Biology, Ecology and Diversity

Male and female association in *Trichomyia* Haliday in Curtis, 1839 using a molecular approach (Diptera, Psychodidae, Trichomyiinae), and description of new species from Brazil

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ARTICLE INFO

Article history:
Received 28 May 2018
Accepted 23 August 2018
Available online 5 September 2018
Associate Editor: Andrzej Grzywacz

Keywords:
DNA-barcoding
COI
Neotropical Region
Psychodid
Trichomyiinae

ABSTRACT

A new species of *Trichomyia* from the state of Bahia, Brazil, is described and illustrated, and male and female are associated using DNA barcoding. Additionally, fragments of the COI of two other species, *Trichomyia cerdosa* Araújo & Bravo, 2016 and *Trichomyia ituberensis* Araújo & Bravo, 2016, and the females of two unidentified species, are sequenced.

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Introduction

Taxonomy of *Trichomyia* Haliday in Curtis is mainly based on males. Duckhouse (1978), for example, described 30 species, only two of which included females, and Araújo and Bravo (2016) described all of the forty-four *Trichomyia* species based only on males. It is possible that the absence of females in taxonomic treatments is due to the fact that they are not attracted to the same baits that attract males (Duckhouse, 1978: 202); alternatively, the morphological association between males and females in this genus is difficult when several species are collected from the same locality.

Males of nine species of *Trichomyia* are unknown: *T. barretoi* Barreto, *T. coutinhoi* (Barretto), *T. squamosa* (Enderlein), *T. eatoni* Satchell, *T. travassosi* (Barretto), *T. vazi* (Barretto) and *T. wasmanni* (Holmgren) from the Neotropical Region and *T. batu* Quate from the Oriental Region.

The DNA barcoding technique (Hebert et al., 2003), i.e., the analysis of a short region of the mitochondrial cytochrome c oxidase gene subunit I (COI) (< 650 pb), has been used with relative success in animals to differentiate species, including in Diptera (Ekrem et al., 2010; Kurina et al., 2011). The technique has also been used extensively to associate life stages such as males and females (Willassen, 2005; Zhang et al., 2013) or immatures and adults (Contreras-Gutierrez et al., 2013; García-Robledo et al., 2013; Vivero et al., 2017). In Psychodidae, DNA barcoding has been used extensively for species differentiation in Phlebotominae (e.g. Kumar et al., 2012; Gutiérrez et al., 2014; Nzelu et al., 2015; Pinto et al., 2015), but also for other subfamilies including Psychodinae (Kvifte and Andersen, 2012; Kvifte and Boumans, 2014; Kvifte and Menzel, 2016), Sycoracinae (Ježek et al., 2015) and Bruchomyiinae (Polseela et al., 2018). In this paper, sex association by DNA barcoding is used for the first time in *Trichomyia*. Additionally, male and female of a new species from Brazil are described.

Material and methods

The specimens studied are deposited at Coleção Entomológica Professor Johann Becker do Museu de Zoologia da Universidade Estadual de Feira de Santana, Feira de Santana, Brazil (MZFS). The specimens for DNA extraction were collected with Malaise and light traps between 2012/2013 from Reserva Ecológica da Michelin, state of Bahia, Brazil (13°50′16.0″S/39°14′28.9″W; 139 m).

The specimens were collected in 70% ethanol, transferred and stored in 100% ethanol, then packed in a freezer at −20 °C. The head,

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https://doi.org/10.1016/j.rbe.2018.08.004

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wing and genitalia of each specimen studied were separated and mounted on permanent slides with Canada balsam after a diaphanization process with potassium hydroxide (10% KOH). The thorax and abdomen of the studied specimens were used in the DNA extraction. All vouchers are deposited at the MZFS.

Terminology

The morphological terminology is based on Cumming and Wood (2009), except for the antenna (Ibáñez-Bernal, 2004). The posterior projection of gonocoxite is named here as 'arm of gonocoxite'.

Molecular techniques

The sequences were obtained in the Laboratory of Molecular Systematics of Plants (Lamol) of the Universidade Estadual de Feira de Santana. The extraction was performed with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, USA) following the protocol provided by the manufacturer with the following modifications: for more concentrated DNA, elution was performed in two successive steps of 50 μl each with Buffer AE.

A partial sequence of the mitochondrial cytochrome oxidase gene subunit I (COI) was amplified and sequenced with the primer pairs listed in Table 1. The DNA primers LCO/HCO were used to amplify some samples. When the primer pair mentioned above failed, a smaller fragment, obtained with the primers MtD6 and MtD9, was amplified instead (see Table 1). All primers at 10 nmol/μl.

A solution for polymerase chain reaction (PCR) was prepared with the following concentrations: 0.7 μl MilliQ water; 2 μl of additive; 0.15 μl of each primer and 5 μl of Top *Taq* Master Mix kit (Qiagen) for each 2 μl of concentrated DNA. Subsequently, the PCR was performed with 37 cycles of the following steps: an initiation of 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 48 °C for 40 s, extension at 72 °C for 40 s and a final extension of 72 °C for 5 min.

Table 1
Primer pairs used for the amplification of COI gene fragments.

Primer	Sequence (5′ → 3′)	References
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
MtD6	GGAGGATTTGGAAATTGATTAGTTCC	Simon et al. (1994)
MtD9	CCCGGTAAAATTAAAATATAAACTTC	Simon et al. (1994)

The result of this amplification was analyzed on a 1.0% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator. The strong bands were measured as an indirect measure of the amount of DNA, which was confirmed by measurement in Nanodrop® using 1 μl of the PCR reaction. According to these results the samples were considered good for the sequencing reaction and subjected to a PEG (polyethylene glycol) cleaning.

The mix for the pre-reactions of sequencing were made with 10 μl (in both directions, forward and reverse) using 0.75 μl of BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), 1.75 μl of buffer for sequencing (*Save Money* 5×) and 1.0 μl of the 5 pmol/μl primer. The amount of DNA and ultra pure water used in this solution depended on the values obtained in the count in *Nanodrop®*.

The sequencing reaction followed this schedule in the Biocycler® MJ96G thermal cycler: 30 cycles of initial temperature of 96 °C for 3 min, denaturation of 96 °C for 15 s, annealing of 50 °C for 10 s, extension of 60 °C for 4 min and finally final extension of 60 °C for 7 min. The samples were then purified for sequencing and inserted into an automatic sequencer (ABI 3130XL Genetic Analyzer).

All sequences are deposited in GenBank (Table 2).

Alignment

Each nucleotide sequence was compared to the sequences deposited in the NCBI (National Center of Biotechnology Information) database through the BLAST (Standard Nucleotide Basic Local Alignment Search Tool) algorithm. The sequences obtained were edited and aligned using the program BioEdit 7.1.9 (www.mbio.ncsu.edu/BioEdit/BioEdit.html). For each sequence, the agreement between the chromatogram and the nucleotides, and between the two complementary strands, was maximized.

Intra and interspecific genetic divergences were calculated using the p-distances in the MEGA X 10.0.4 program (Kumar et al., 2018).

Taxonomy

Trichomyia pseudoannae Araújo & Bravo sp. nov.
(Figs. 1.1–9 and 2.1–3)

Diagnosis. Head with one row of supraocular alveoli and one row of occipital alveoli. Palpus with three segments. Male terminalia with hypandrium fused to gonocoxites and expanded posteriorly as a apically slightly bifucate plate covering the aedeagus, gonocoxite

Table 2
Specimens analyzed in this work, including the species names; BR, Brazil; gender (M, male; F, female); code, primer, pair base sequence, GenBank accession numbers and locality.

Species	Gender	DNA extraction code	Primer	Sequence (pb)	GenBank accession numbers	Locality
T. cerdosa Araújo & Bravo, 2016	M	T15	lco/hco	657	MH042537	BR, Bahia, Igrapiuna, Reserva Ecológica Michelin, Pancada Grande, 15.VI.2013 (light trap), M. Aragão & E. Menezes leg.
T. cerdosa Araújo & Bravo, 2016	M	P4	mtd6/mtd9	480	MH042535	BR, Bahia, Igrapiuna, Reserva Ecológica Michelin, Pancada Grande, 15.VI.2013 (light trap), M. Aragão & E. Menezes leg.
T. pseudoannae sp. nov.	M	P28	lco/hco	468	MH042538	BR, Bahia, Igrapiuna, Reserva Ecológica Michelin, Pancada Grande, 18.V.2013 (light trap), M. Aragão & E. Menezes leg.
T. ituberensis Araújo & Bravo, 2016	M	P21	mtd6/mtd9	480	MH042540	BR, Bahia, Igrapiuna, Reserva Ecológica Michelin, Vila 5, 28.IV–19.V.2013 (Malaise), M. Aragão & E. Menezes leg.
T. pseudoannae sp. nov.	F	P46	lco/hco	657	MH042536	BR, Bahia, Igrapiuna, Reserva Ecológica Michelin, Pancada Grande, 22.IX.2012 (light trap), M. Aragão & E. Menezes leg.
Trichomyia sp1	F	P29	mtd6/mtd9	467	MH042539	BR, Bahia, Igrapiuna, Reserva Ecológica Michelin, Pancada Grande, 18.V.2013 (light trap), M. Aragão & E. Menezes leg.
Trichomyia sp2	F	P44	lco/hco	633	MH042541	BR, Bahia, Igrapiuna, Reserva Ecológica Michelin, Pancada Grande, 24.III.2013 (light trap), E. Mota, M. Aragão & E. Menezes leg.

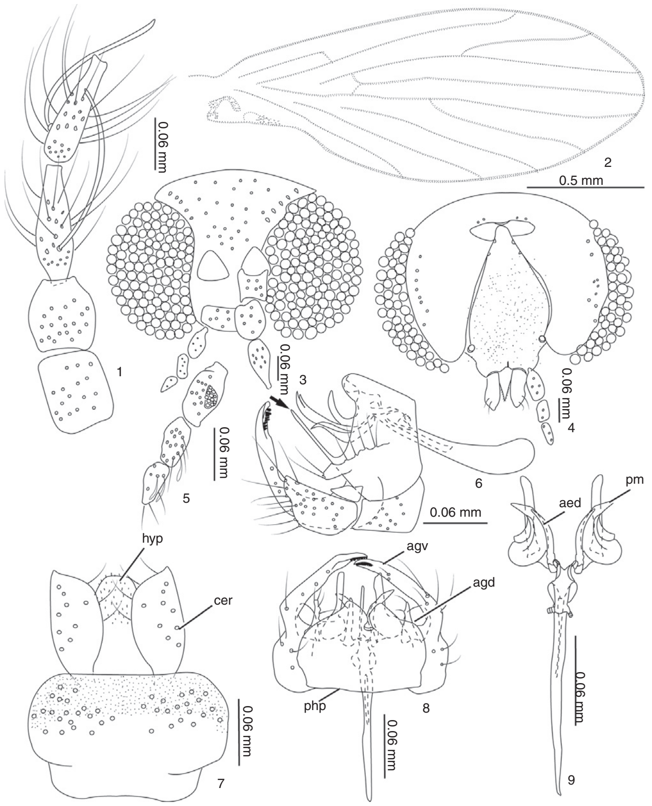

Fig. 1. 1–9 *Trichomyia pseudoannae* sp. nov. 1. Scape, pedicel and flagellomeres 1 and 2; 2. Right wing; 3. Head, dorsal view; 4. Head, ventral view; 5. Palpus; 6. Male terminalia, lateral, arrow in projection in the gonocoxal apodeme; 7. Cerci, epandrium, hypoproct; 8. Male terminalia, ventral; 9. Aedeagus and parameres (agv, ventral arm of gonocoxite; agd, dorsal arm of gonocoxite; cer, cercus; aed, aedeagus; hyp, hypoproct; pm, paramere; php, post-hypandrial plate).

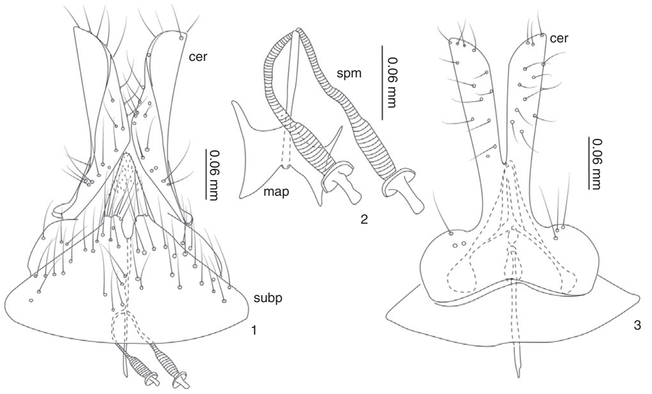

Fig. 2. 1–3 *Trichomyia pseudoannae* sp. nov. 1. Female terminalia, ventral; 2. Median apodeme and spermathecae; 3. Female terminalia, dorsal (map, median apodeme; cer, cercus; spm, spermathecae; subp, subgenital plate).

with two pairs of arms. Female with the subgenital plate trapezoidal and bifurcated apically, cerci elongated.

Description.

Male. Head subcircular, eyes rounded. Supraocular setae in single row (Fig. 1.3). Occipital setae arranged in single row (Fig. 1.4). Antennal pit subtriangular, short distance between antennae (less than 1/3 of the width of the pits and with sclerotic fold). Scape subcylindrical and pedicel subespherical, basal flagellomeres pyriform and eccentric; with a pair of mediobasal digitiform and S-shaped ascoids, first and second flagellomere equal in length, ascoids 1.4 times length of flagellomere (Fig. 1.1). Palpus three-segmented; first segment with sensilla in depressed pit on inner side; palpus formula: 1.0:0.6:0.9 (Fig. 1.5). Wing (Fig. 1.2). Sc-r sclerotized but not microsetose, r-m present, radial fork distal of apices of CuA$_2$ and medial fork, base of M$_2$ sclerotized but without microsetae. Male terminalia. Hypandrium fused with gonocoxites and expanded posteriorly as an apically slightly bifucate plate covering the aedeagus. Two pairs of arms of gonocoxite (Fig. 1.8: agd, agv), one dorsal, directed to apical region and with fine bristles distributed irregularly and a ventral pair, longer than dorsal one, directed to internal region of genitalia at an angle of 60°. Pair of dorsal arms digitiform, with row of rod-like setae at apex and simple bristles distributed irregularly. Gonostylus sub-circular, slightly sclerotized and with fine bristles, articulated with ventral region of gonocoxite (Fig. 1.8). Gonocoxal apodeme with medium, narrow and sclerotized projection directed to dorsal region of genitalia (Fig. 1.6 and 1.8). Aedeagus bifid; two pairs of parameres, dorsal lanciform and ventral digitiform, ejaculatory apodeme long, 1.75 times length of parameres (Fig. 1.9). Cercus cuneiform with bristles distributed irregularly. Hypoproct with micropilosity and apex rounded. Epandrium trapezoidal and pilose, with alveoli distributed in two lateral patches (Fig. 1.7).

Female. Head, antennae, mouthparts, palpi and wings as in male. Female terminalia. subgenital plate trapezoidal bifurcated apically. Cerci elongate, about 5.2 times longer than wide; sclerotized arch between cerci acuminate and with microseate, 0.4 as long as cerci (Fig. 2.1 and 2.3). Spermathecae with ducts annulated, inflated apically, apex slightly truncated. Median apodeme with two sclerotized projections anteriorly and three posteriorly; median posterior projection three times longer than other projections (Fig. 2.2).

Material examined: Voucher #m and holotype #m (MZFS) Brazil, Bahia, Igrapiuna, Reserva Ecológica da Michelin, Pancada Grande, 18.V.2013, M. Aragão & E. Menezes cols.; 1 paratype #m (MZFS) the same locality and collector as holotype, 15.VI.2013; 22 paratypes #m (MZFS) Brazil, Bahia, Igrapiuna, Reserva Ecológica da Michelin, Pacangê, M. Aragão & E. Menezes cols. 27–28.X.2012 (1 paratype); 22.IX–28.X.2012 (5 paratypes); 16.XII–20.I.2013 (11 paratypes); 24.II–31.III.2013 (1 paratype); 21–22.VII.2012 (1 paratype); 27–28.IV.2013 (2 paratypes); 30–31.III.2013 (1 paratype); 1 paratype #m (MZFS) Brazil, Bahia, Igrapiuna, Reserva Ecológica da Michelin, Vila 5, 24.II–31.III.2013, M. Aragão & E. Menezes cols.; Voucher #f (MZFS) Brazil, Bahia, Igrapiuna, Reserva Ecológica da Michelin, Pancada Grande, 22.IX.2012, M. Aragão & E. Menezes cols.; 1 paratype #f (MZFS) the same locality and collector as allotype.

Etymology. The epithet refers to morphological similarity with *Trichomyia annae* Bravo, 2001.

Distribution. Known only from the type locality.

Comments. The new species is morphologically similar to *Trichomyia annae*. The differences are in the male terminalia, the plate expanded posteriorly of hypandrium and gonocoxites has a small bifurcation apically with projections on rounded apex and not lanciform as in *T. annae*. Both species, to date, have not been included in any subgenus.

Trichomyia ituberensis Araújo & Bravo

Trichomyia ituberensis Araújo & Bravo, 2016: 30–31, figs. 13A–I.

Comments. Males of *T. ituberensis* are recognized by the genitalia, with a hypandrium fused with gonocoxites and expanded posteriorly as an apically strongly bifucate plate covering the aedeagus. There are two pairs of parameres and rod-like setae in the arm of gonocoxite.

Material examined. Brazil, Bahia, Igrapiuna, Reserva Ecológica Michelin, Vila 5, 28.IV–19.V.2013 (Malaise), M. Aragão & E. Menezes cols., 1 #m (MZFS).

Distribution. Brazil (Bahia).

Table 3
Matrix of p-distances among males and females of specimens of *Trichomyia*. Bold denotes shortest distances; F, female.

	#P4 *T. cerdosa*	#P46 *T. pseudoannae* (F)	#T15 *T. cerdosa*	#P28 *T. pseudoannae*	#P29 *Trichomyia* sp1 (F)	#P21 *T. ituberensis*	#P44 *Trichomyia* sp2 (F)
#P4 *T. cerdosa*		0.156	**0.010**	0.162	0.197	0.216	0.276
#P46 *T. pseudoannae* (F)	0.156		0.166	**0.002**	0.188	0.139	0.287
#T15 *T. cerdosa*	**0.010**	0.166		0.168	0.201	0.220	0.294
#P28 *T. pseudoannae*	0.162	**0.002**	0.168		0.202	0.154	0.304
#P29 *Trichomyia* sp.1 (F)	0.197	0.188	0.201	0.202		0.218	0.388
#P21 *T. ituberensis*	0.216	0.139	0.220	0.154	0.218		0.314
#P44 *Trichomyia* sp.2 (F)	0.276	0.287	0.294	0.304	0.388	0.314	

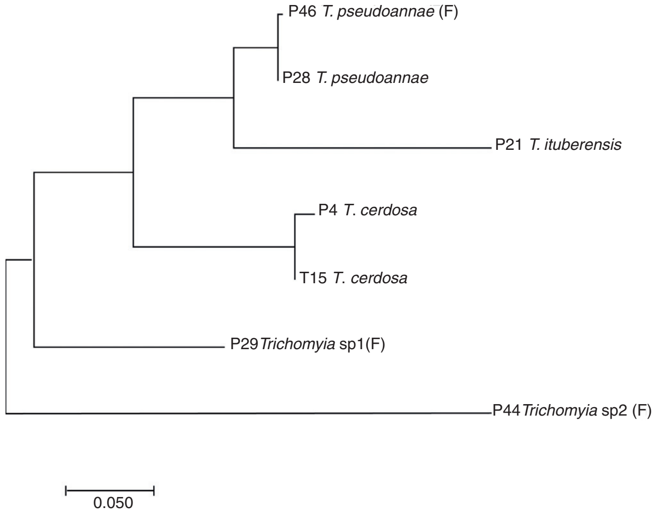

Fig. 3. Dendrogram of genetic similarity among *Trichomyia* species analyzed, F, female.

Trichomyia cerdosa Araújo & Bravo

Trichomyia cerdosa Araújo & Bravo, 2016: 39–40, figs. 20A–H.

Comments. Males of *T. cerdosa* are recognized by the genitalia, with an elongate arm of gonocoxite with elongate apical bristles. The cercus has four apical bristles rod-like.

Material examined. Brazil, Bahia, Igrapiuna, Reserva Ecológica Michelin, Pancada Grande, 15.VI.2013 (light trap), M. Aragão & E. Menezes cols., 2 #m (MZFS).

Distribution. Brazil (Bahia).

Sexual association by DNA barcoding

Fragments of COI of seven specimens of at least three species were sequenced (Table 2).

The male–female association was possible only in *Trichomyia pseudoannae* sp nov. The COI sequences from male and female diverged in only in nucleotide position 361 (G in the sequenced female and A in the male). Data from morphology, close DNA-barcoding (0.002) and the fact that both specimens were collected from the same locality corroborate our hypothesis that of that they belong to the same species (Fig. 3).

Concerning the other specimens analyzed here, the distance between the sequences of two male specimens of *T. cerdosa* was 0.011. In general, the intra/inter specific genetic distances range from 0.002–0.010/0.390–0.314 respectively (Table 3).

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors would like to thank Dr. Gunnar Mikalsen Kvife and Dr. Rüdiger Wagner for your valuable input that have helped to improve this manuscript. CJBC and FB thanks the CNPq for support (process # 309873/2016–9 and 306441/2015–2, respectively).

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