Article

ZOOTAXA

Description of two new species closely related to *Doryctobracon areolatus* (Szépligeti, 1911) (Hymenoptera, Braconidae), based on morphometric and molecular analyses

CLÁUDIA F. MARINHO[1], FERNANDO L. CÔNSOLI[1], ANGÉLICA MARIA PENTEADO-DIAS[2] & ROBERTO A. ZUCCHI[1,3]

[1]*Departamento de Entomologia e Acarologia, ESALQ, Universidade de São Paulo (USP), Caixa Postal 9, 13418-900, Piracicaba, SP.*
E-mail: cfmarinhoo@gmail.com; fconsoli@usp.br; razucchi@usp.br

[2]*Universidade Federal de São Carlos UFSCAR, Caixa Postal 676, CEP 13565-905, São Carlos, SP*
E-mail: angélica@power.ufscar.br

[3]*Corresponding author*

Abstract

This study clarifies the identity of two morphs that appear similar but not identical to the braconid wasp *Doryctobracon areolatus* (Szépligeti), by analyses of fore wing morphometry and the ITS2 and D2 region of 28S ribosomal DNA. Wing measurements of *D. areolatus* and the two morphs, one with a yellow stigma and one with a brown stigma, from several Brazilian localities, were taken and subjected to geometric morphometry. Evaluation of 20 anatomical points on the fore wing by means of multivariate analysis revealed that these morphs consistently differ from *D. areolatus*. Intraspecific size variation in both molecular markers (ITS2 and 28S-D2 rDNA) was also detected among populations of *D. areolatus* from the states of Amapá, São Paulo, Goiás, and Tocantins, but no such difference was observed among samples of the two morphs. However, high sequence variability was observed for both markers among *D. areolatus* and these morphs. Morphometric analysis yielded similar results and produced dendrogram congruent with those based on the molecular markers, also indicating that *D. areolatus* corresponds to a complex of cryptic species. Therefore, our morphometric and molecular data, in addition to revealing that *D. areolatus* includes cryptic species, showed that the unidentified morphs actually represent two distinct, previously undescribed species of *Doryctobracon*. These new taxa are formally described herein, as *D. whartoni* **sp. nov.** and *D. adaimei* **sp. nov.**

Key words: geometric morphometry, canonical variate analysis, ITS2, 28S-D2 rDNA, molecular taxonomy

Introduction

Species of koinobiont parasitoids in the genus *Doryctobracon* Enderlein, 1920 are exclusive to the Americas (Ovruski 2003). This genus comprises 15 species of which ten are associated with larva/pupa of tephritid flies in fruits (Fischer 1977; Wharton & Marsh 1978; Wharton 1997b; Yu *et al.* 2012), one in flowers (Wharton & Norrbom 2013), and the remaining four have unknown hosts (Yu *et al.* 2012). Four of the species that parasitize tephritids in fruits are known to Brazil: *Doryctobracon areolatus* (Szépligeti, 1911), *D. brasiliensis* (Szépligeti, 1911), *D. fluminensis* (Costa Lima, 1937) and *D. crawfordi* (Viereck, 1911) (Zucchi *et al.* 2011). *Doryctobracon areolatus* is widely distributed in Brazil, and it is the most common and abundant of species parasitizing tephritid fruit flies (Canal Daza & Zucchi 2000).

Field surveys identified two morphospecies of *Doryctobracon* that are morphologically similar to *D. areolatus*, but clearly distinct from the other species known to *Doryctobracon*. These two morphs fit the *D. areolatus* group by the positioning of veins of their front wings, but mainly by the areolate sculpture of their propodeum. This study focused on the use of geometric morphometrics and molecular analyses of the ITS2 and 28SD2 regions of the ribosomal DNA to shed light on taxonomical identity of the two morphs due to the high level of morphological similarities they share between them and with *D. areolatus*.

Material and methods

Samples. Braconid wasps were collected by several Brazilian researchers using the traditional procedure for obtaining parasitoids associated with fruit flies (e.g. Leonel Júnior *et al*. 1996). Samples were obtained at irregular intervals, and host associations were not established. After identification, some of the wasps were transferred to absolute ethanol and stored at -80ºC for later extraction of genomic material, while the rest were kept in 70% ethanol and used for morphometrics. Specimens close to *D. areolatus* (DA) were separated according to the color of their stigma—one morph with yellow stigma (YS), and the other with brown stigma (BS) (Fig 1).
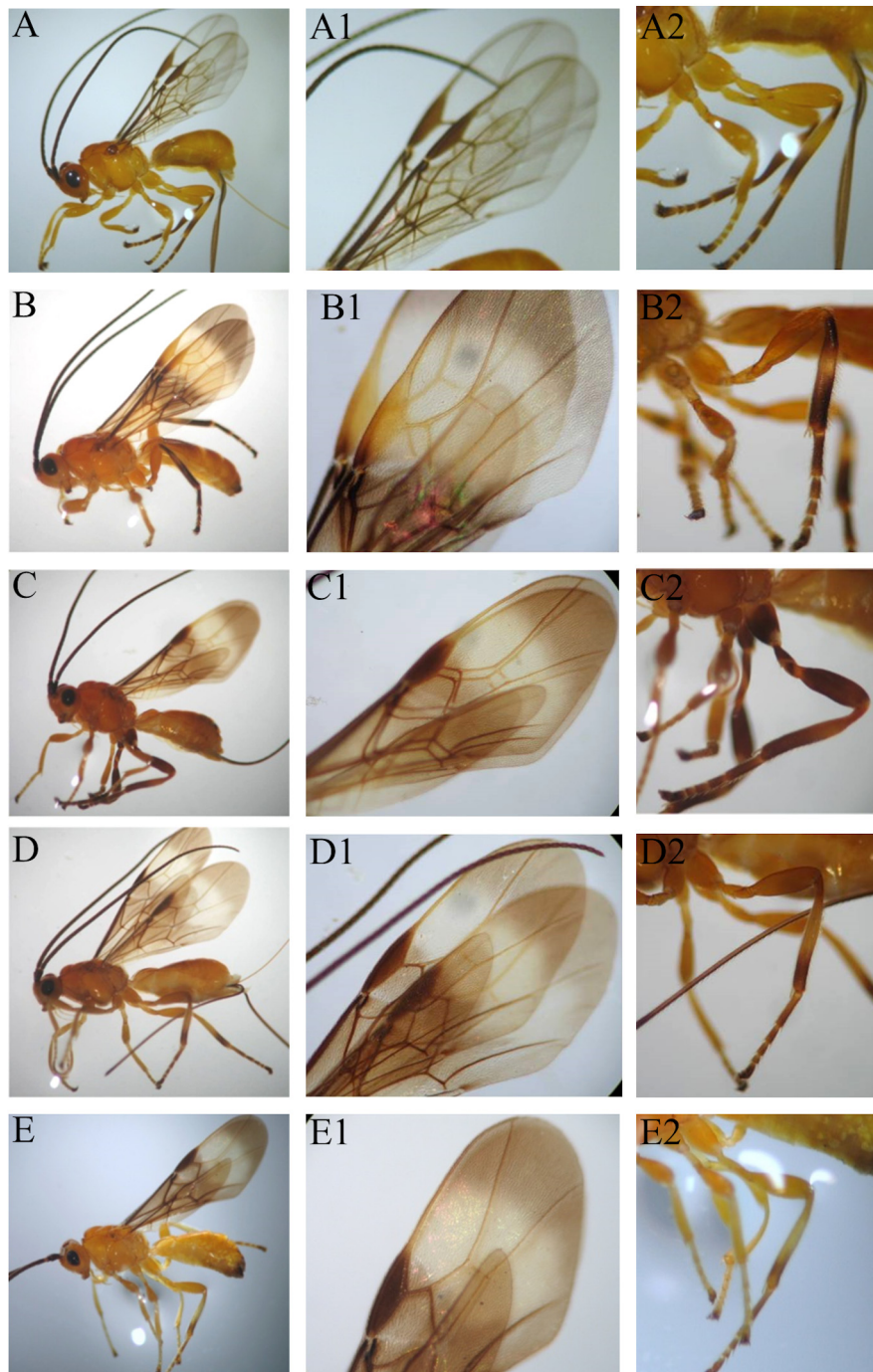

FIGURE 1. Chromatic variation on the wings and legs. *Doryctobracon areolatus* (A-A2), *Doryctobracon whartoni* **sp. nov.** (yellow stigma – YS) (B-B2), *Doryctobracon adaimei* **sp. nov.** (Goiás) (C-C2), *D. adaimei* **sp. nov.** (Tocantins) (D-D2), *D. adaimei* **sp. nov.** (Amapá) (brown stigma – BS) (E-E2). (the figures are not on the same scale).

Terminology. Descriptive terminology used for body morphology and wing nervures broadly follows Sharkey & Wharton (1997) and Wharton (1997a). Identification of anatomical structures was based on the Hymenoptera Ontology Anatomy (HAO) (Yoder *et al*. 2010). Wharton (1997b) was used for the description of the ovipositor. Measurements were performed using the software Motic Image Plus 2.0, coupled to a light microscope. For clarity, the first metasomal ridge, referred to as the petiole, is termed T1, and the following median ridges are termed T2 and T3 (Walker & Wharton 2011). The measurements broadly follow Ovruski (2003) and Walker & Wharton (2011).

Morphometric analysis. Brazilian populations of *D. areolatus* from the states of Amapá (North), Tocantins and Goiás (Midwest), and São Paulo (Southeast) were included in this study. The YS morph from Amapá; and the BS morph from Amapá and Goiás were studied by means of analysis of the left anterior fore wing from 224 specimens (Table 1). The left anterior fore wing of each specimen was removed and laid on a millimeter slide for image scanning and capturing. Digital images of fore wings were used for marking 20 fixed homologous anatomical points defined by intersections of wing nervures. These points were recorded using the software TpsDig version 1.40 (Rohlf 2004) (Fig 2), and consensus configurations were computed based on the original coordinates of each specimen by the least-squares orthogonal overlay method, using the program TpsRewl (Monteiro & Reis 1999; Rohlf 2007). Mahalanobis distance means were used in cluster analysis to construct the UPGMA dendrogram. Multivariate analyses were performed, and charts and dendrograms were constructed using STATISTICA version 7.0.

TABLE 1. Parasitoid samples (*Doryctobracon*) used for the morphometric analysis.

Collection sites	*Doryctobracon areolatus*			*D. whartoni* **sp. nov.**			*D. adaimei* **sp. nov.**		
	F	M	Codes	F	M	Codes	F	M	Codes
Amapá (AP)	20	20	DAAP	2	3	YSAP	20	20	BSAP
Goiás (GO)	20	20	DAGO	-	-	-	20	14	BSGO
Tocantins (TO)	13	12	DATO	-	-	-	-	-	-
São Paulo (SP)	20	20	DASP	-	-	-	-	-	-
Total	73	72		2	3		40	34	

Doryctobracon areolatus (DA), population from Amapá (DAAP), Goiás (DAGO), Tocantins (DATO) and São Paulo (DASP). *D. whartoni* **sp. nov.** (yellow stigma) population from Amapá (YSAP); *D. adaimei* **sp. nov.** (brown stigma) population from Amapá (BSAP) and Goiás (BSGO).
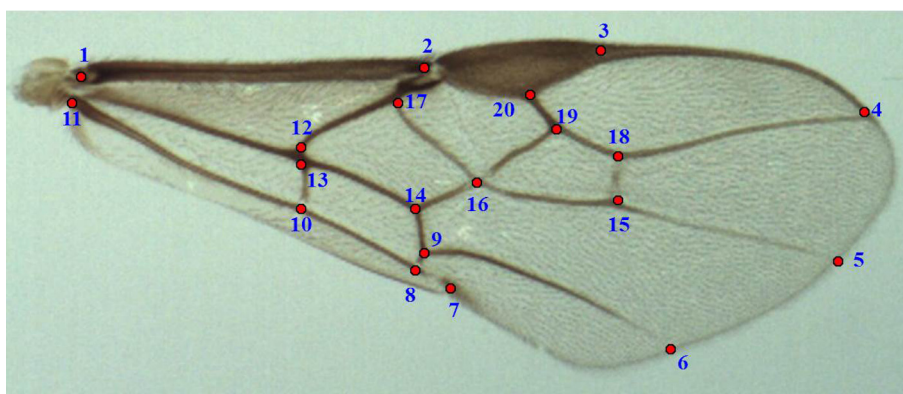

FIGURE 2. Landmarks on fore wing of *Doryctobracon areolatus*. 1. base of vein costal; 2. Intersection of the parastigma, 1RS and wing margin; 3. Intersection of the end of stigma and R1a; 4. Intersection of the radial sector 3RSb and wing margin; 5. Intersection of vein 3M and wing margin; 6. Intersection of vein 3CU and wing margin; 7. Intersection of vein 3-1A and wing margin; 8. Intersection of the veins 3-1A and 2cu-a; 9. Intersection of the veins 2CUa, 3CU and 2cu-a; 10. Intersection of the veins 1-1A, 1cu-a and 2-1A; 11. Intersection of the veins 1-1A and base of wing; 12. Intersection of the veins M+CU, 1M and 1CU; 13. Intersection of the veins M+CU, 1cu-a and 1CU; 14 Intersection of the veins 2CUa, 1CU and 1m-cu; 15. Intersection of the veins 2M, r-m and 3M; 16. Intersection of the veins 1m-cu, (RS+M)a, 2RS and 2M; 17. Intersection of the veins 1M, (RS+M)a and 1RS; 18. Intersection of the veins 3RSa, 3RSb and r-m; 19. Intersection of the veins 3RSa, r and 2RS; 20. Intersection of the vein r and base of stigma (venational terminology according to Sharkey & Wharton 1997).

Molecular analysis. Specimens of *D. areolatus* (states of Amapá, Tocantins, and Goiás), collected in the same geographical regions of morph YS (state of Amapá) and morph BS (states of Amapá, Tocantins, and Goiás), were selected and used for the assessment of intra and interspecific variations. Only one population of *D. areolatus,* from the state of São Paulo, was included for comparison. A sequence from *D. crawfordi* Viereck (access number NCBI Z93646.1) was also added for analysis.

DNA extraction and PCR. Genomic DNA was extracted from the abdomens of individual specimens, following Aljanabi & Martinez (1997). Briefly, isolated abdomens were homogenized in 400 µL Tris-EDTA-NaCl buffer (TEN) (10 mM Tris-HCl pH 8.0; 2 mM EDTA pH 8.0; 400 mM NaCl), 40 µL 20% SDS and 8 µL 20 mg/mL proteinase-K. Samples were incubated at 55°C for 1 h, and centrifuged (14,000g × 25 min at 25C) after addition of 300 µL 6M NaCl and vortexing (30 s). The supernatant was collected and the DNA precipitated by adding one volume of cold absolute ethanol, incubating at -20°C × 1 h, and centrifuging 14,000g × 20 min × 4°C. The DNA pellet obtained was washed with absolute ethanol (×2), dried at room temperature, resuspended in autoclaved Milli-Q water, and stored at -20ºC until further use.

The DNA samples were subjected to amplification of two nuclear ribosomal molecular markers, the Internal Transcribed Spacer (ITS2) and the D2 domain of the 28S rDNA (28S-D2). ITS2 was amplified using the primer set 5.8S (5'-GTG AAT TCT GTG AAC TGC AGG ACA CAT GAA C-3') and 28S (5'-ATG CTT AAA TTT AGG GGG TA-3') (Porter & Collins 1991), while the primer set F3665 (5'-AGA GAG AGT TCA AGA GTA CGT G-3') and R4047 (5'-TTG GTC CGT GTT TCA AGA CGG G-3') (Belshaw & Quick 1997) was used for 28S-D2 amplification. Amplification reactions contained 1.0 µL gDNA, 1× PCR buffer, 1.5 mM MgCl$_2$, 200 µM dNTP/ each, 0.32 µM of each primer and 0.25U of Taq polymerase (PROMEGA) in a final volume of 25 µL. The thermocycler was set at 94ºC for 2 min (1×); 94ºC for 30 s, 49ºC for 30 s and 72ºC for 1 min (35×); and 72ºC for 20 min (1×) for ITS2 amplification. Thermocycling conditions for 28S-D2 amplification were: 93ºC for 3 min (1×); 98ºC for 15s, 48ºC for 30 s and 72ºC for 40 s (35×); and 72ºC for 3 min (1×). The amplicons obtained were resolved by agarose gel electrophoresis using a 1.5% gel slab, with added 0.5 µg/mL ethidium bromide in Tris-acetate-EDTA buffer (TAE) at 5V/cm. Amplicon visualization, image acquisition and digitalization were done under a transilluminator (UV) coupled with an image analysis system.

All PCR products were purified by using the QIAquick® PCR Purification Kit (Quiagen®), following the manufacturer's guidelines, and either subjected to direct bidirectional sequencing or inserted into a plasmid vector system (pGEM®-T Easy Vector System I - Promega Corporation) for chemical transformation of *Escherichia coli* NEB5-Alpha-F' (BioLabs) competent cells for plasmid multiplication, according to the manufacturer's guidelines. Positive clones were selected, and plasmids were purified and subjected to bidirectional sequencing. Sequencing was conducted at the Centro de Estudos do Genoma Humano (CEGH-USP) (http://genoma.ib.usp.br/servicos/ sequenciamento.php), using the DYEnamic ET Dye Terminator Kit. All sequences obtained were viewed and edited using the BioEdit Software (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html).

Sequence analysis. 5.8S-ITS2-28S (from here on referred to as ITS2) and 28S-D2 rDNA sequences obtained for the three species of *Doryctobracon* were subjected to heuristic blast search against the nucleotide database of the National Center for Biotechnology Information (NCBI) using the Blastn tool. Due to the lack of sequences available for this genus in the database, only the 28S-D2 available for *Doryctobracon crawfordi* (access number NCBI: Z93646.1) was subjected to comparative analysis with the sequences we obtained for *D. areolatus*, and morphs YS and BS. Alignments of the ITS2 and 28S-D2 sequences among the different samples studied were done using the Clustal W software, with a gap open penalty = 13 and a gap extension penalty = 7.6. The best-fit nucleotide substitution model was selected based on the Bayesian information criterion, corrected Akaike information criterion and Maximum-likelihood value as implemented in MEGA 7.0 (Kumar *et al*. 2016). Maximum-likelihood trees using ITS2, 28S-D2 and ITS2-28SD2 concatenated sequences were constructed using the best-fit substitution model selected with 1,000 bootstrap iterations using MEGA7.0 (Kumar *et al*. 2016). All sequences obtained for ITS2 and 28S-D2 for the specimens studied are available in GenBank under access numbers FJ560534 to FJ560549.

Figures. Electron microscopy images were obtained in Zeiss DSM 940A and LEO 435VP scanning electron microscopes at the Research Support Center, Electron Microscopy Applied to Agricultural Research (NAP/MEPA) – ESALQ/USP. Digital images were produced using a Motic® 2000 2.0 MP digital camera coupled to a Nikon® E200 stereoscopic light microscope.

Type material is deposited in the collection of the Department of Entomology (ESALQ/USP), in Piracicaba,

São Paulo, Brazil, and in the collection of the Department of Ecology and Evolutionary Biology at the Federal University of São Carlos (UFSCar) in São Carlos, São Paulo, Brazil (DCBU).

Results

Descriptions

The morphospecies YS (yellow stigma) is herein described as *Doryctobracon whartoni* **sp. nov.**, and the morph BS (brown stigma) as *Doryctobracon adaimei* **sp. nov.**

Taxonomy

Doryctobracon whartoni Marinho and Penteado-Dias sp. nov.
(Figs 1 B–B2, 3 A, 4 A–F)
http://www.zoobank.org/NomenclaturalActs/4D24EABE-41BE-44E5-8AE6-AC27CD421D19
Doryctobracon sp. 1: Deus *et al*. 2010 (Pedra Branca do Amapari, AP, ex *A. atrigona* in *Geissospermum argenteum*).

Diagnosis. This species differs from *Doryctobracon areolatus* (Szépligeti, 1911), by having the fore wing infuscate with a hyaline band near the base (restricted to the subbasal and anal cell) and a second transverse hyaline band on the apical half (Figs 1 B1, 3A). Stigma yellow, veins and bristles in the hyaline band yellow, veins and setae in the infuscate band dark brown (Fig 3A). *Doryctobracon adaimei* **sp. nov.** also has a hyaline area, but it is rounded and is situated after the stigma (dark brown) and before the wing apex (in the middle of R1a). This spot does not reach the posterior margin of the wing and ends in the middle of the second subdiscal cell (2nd disc) (Fig 3B). In general, *D. whartoni* **sp. nov.** is more setose than *D. areolatus* and *D. adaimei* **sp. nov.** (Figs 1B, 4A–D). On the propodeum, laterally the anterior region of the areola, one short basal transverse keel projects to reach the lateral longitudinal keel, a characteristic also found in *D. adaimei* **sp. nov.** (Fig 4D); in *D. areolatus* two transverse keels reach the lateral longitudinal keel. Tegula yellowish orange (dark brown in *D. areolatus*). Ovipositor apex with a dorsal node as in *D. areolatus*, but ventral serrations are much more developed (Fig 4E).
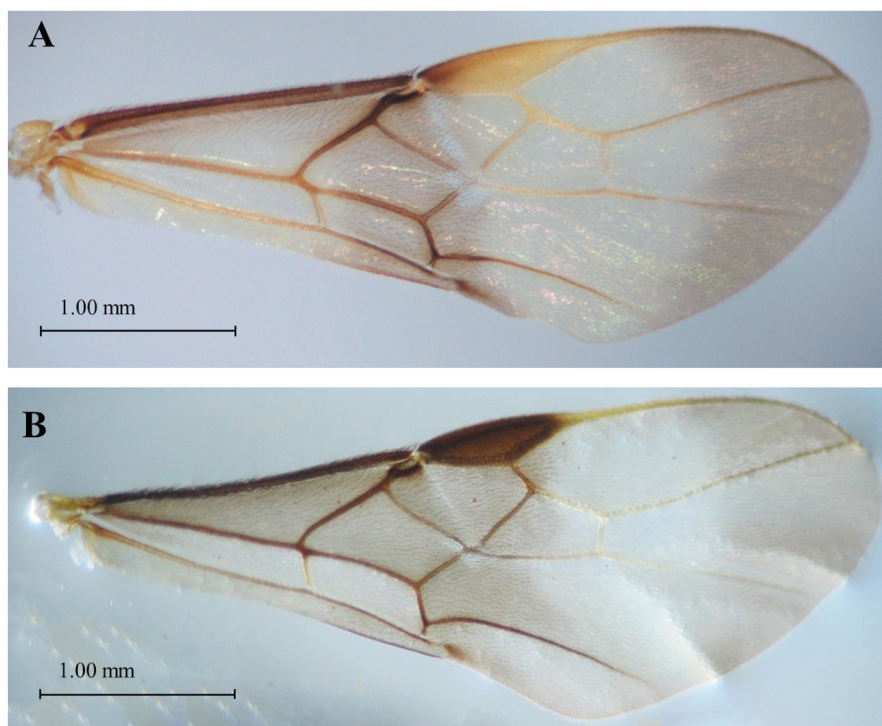

FIGURE 3. Fore wing. (A) *Doryctobracon whartoni* **sp. nov.** (yellow stigma); (B) *Doryctobracon adaimei* **sp. nov.** (brown stigma).
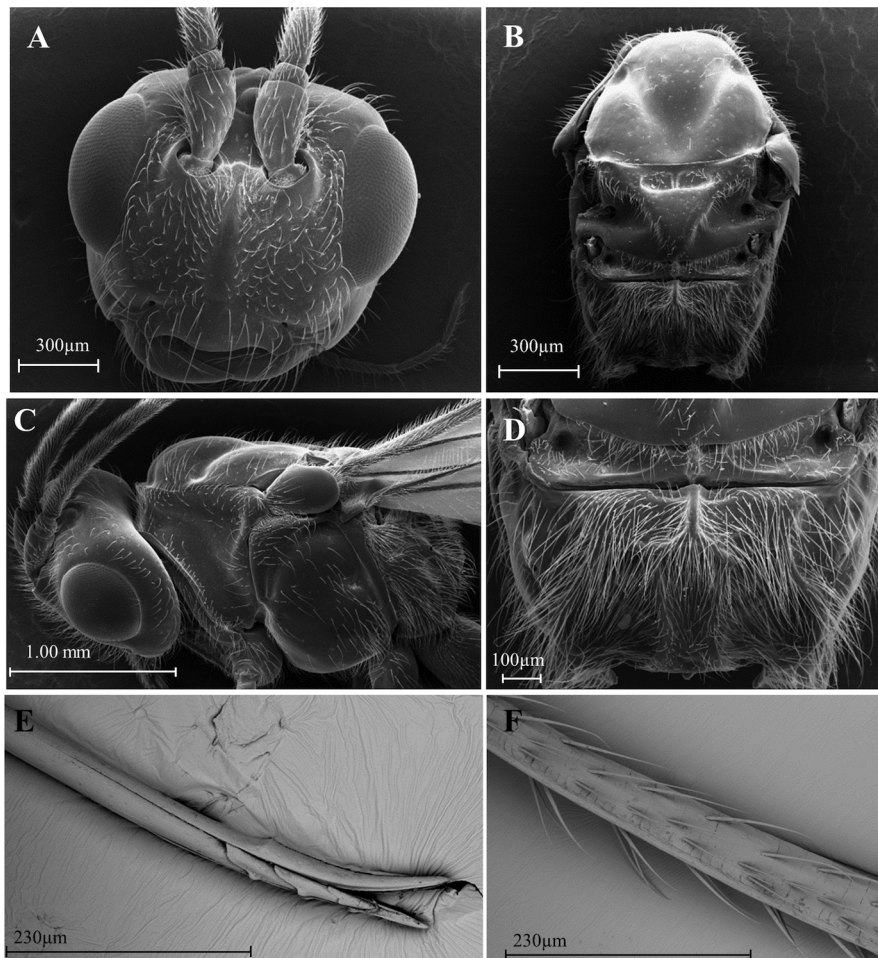
FIGURE 4. *Doryctobracon whartoni* **sp. nov.** head, front view (A), mesosoma, smooth notaulices, dorsal view (B), head and mesosoma, smooth mesopleura (C), setaceous propodeum, dorsal view (D), ovipositor apex (E), pattern of ovipositor sheath bristles (F).

Description Female. Length of body, excluding ovipositor 6.0–6.9 mm.

Head. 1.5–1.3× wider than long; 1.4–1.3× wider than width of mesoscutum; midridge polished, slightly elevated, narrow between toruli and wider toward clypeus; distance between toruli equal to distance from toruli to eye (Fig 4A). Head smooth and polished, with more hairiness concentrated in the clypeus, inner margins of eyes and gena. Antenna slightly longer than body, 7.7–9.5 mm in length, with 58 to 63 flagellomeres; first flagellomere about 0.9–1.2× longer than second; 1.7–1.9× longer than wide. Eyes large, 1.3–1.4× wider than high (Fig 4C); in dorsal view, eye width 1.7–1.9× greater than width of temples; malar space 0.3–0.4× height of eyes. Clypeus 2.9–3.8× wider than high, sinuate, distinctly protruding with median lobe on ventral margin, polished, with sparse setae two to three times longer than those on face (Fig 4A).

Mesosoma. 1.4–1.5× longer than high; 1.9–2.1× longer than wide; 1.3-1.5× higher than wide. Pronotum not visible dorsally; median lobe of mesoscutum and lateral lobes smooth and bright, with few sparse bristles, setae concentrated mainly on margins of lateral lobes (Fig 4B); notaulus smooth, complete, deeper anteriorly, shallower posteriorly, reunited in a large and polished impression without midpit (Fig 4B); scutellar sulcus divided into two large pits by median longitudinal septum; scutellum smooth with small punctures and many setae around margins, posterior portion narrower or sharper than in *D. adaimei* **sp. nov.** and in *D. areolatus* (Fig 4B); propodeum densely setose with median anterior basal keel (0.11–0.15) and complete posterior areola (Fig 4D); laterally, in the anterior region of the areola, one short basal transverse keel extends to reach the lateral longitudinal keel, which is prominent and distinctly curved, from the posterior half of the propodeum (Fig 4D).

Wings. Fore wing 5.8–6.4 mm length; wide stigma 3.8–4.2× longer than wide, with vein r slightly projecting from its midpoint; (RS+M)a straight, 1.4–1.7× longer than 3RSa; 2RS 1.2–1.7× longer than 3RSa, 1.3–1.5× longer

than 1m-cu and 1.7–2.0× longer than r-m; 3RSa 2.3–3.3× longer than vein r; 2M 1.8–2.3× longer than 3RSa; (RS+M)b absent; vein 1cu-a slightly sloping away from 1M for 0.23–0.27 its length. Fore wing infumate with hyaline stripes at base and in median portion. Stigma, veins and setae in hyaline stripe yellow, and veins and setae on infumate stripe dark brown. Hind wing 4.0–4.4 mm length, infumate.

Metasoma. 2.1–2.7× longer than wide and 1.0–1.4× wider than high; T1 0.8–1.0× width at apex; T1 width at apex about 1.2–1.4× width at base; non-sculptured, smooth and bright; ovipositor about 6.0 mm long (twice metasoma length); ovipositor with one subapical dorsal node and developed ventral serrations (Fig 4E); ovipositor sheath with 4–5 rows of bristles (Fig 4F).

General coloration. Yellowish orange; first two pairs of legs bright yellow, third pair with femur, trochanter, and coxa yellow, tibia and tarsi dark brown; ovipositor yellow; antenna and ovipositor sheath dark brown; apex of mandibles black; fore wing infuscate with hyaline stripe, stigma yellow; veins and setae yellow in hyaline stripe, and dark brown in infuscate stripe (Figs 1B1, 3A). Hind wings infumate, sometimes with hyaline stripes at the apex (Fig 1B1).

Male. Similar to female, but dark brown on last tergite. Head, in dorsal view, 1.23–1.30× wider than mesoscutum, 1.49–1.58× wider than face. In dorsal view, eye 1.71–1.74× wider than temple; face 1.6–2.0× wider than high; malar space 0.40–0.48× height of eyes; clypeus 2.7–3.1× wider than high; antenna with 52 to 59 flagellomeres; first flagellomere 1.0–1.2× longer than second, 1.5–1.9× longer than wide. Mesosoma 1.9–2.1× longer than wide, 1.3–1.5× higher than wide; metasoma 2.0–3.0× longer than wide, 1.0–1.5× wider than high.

Type material. Holotype. Female (DCBU 270194), BRAZIL: Amapá, Pedra Branca do Amapari, 00º46'54.9"N and 051º57'01.2"W, 01.ii.2006, reared from fruit fly larva *Anastrepha atrigona* in "Quina" (*Geissospermum argenteum*), coll R. A. Silva. *Paratypes*, with same data as holotype, 1 female, (DCBU 270195), 2 males (DCBU 270196, DCBU 270197) and 1 female, 1 male (ESALQ).

Etymology. This species is named after Robert A. Wharton, in recognition of his outstanding contributions to the taxonomy of Braconidae.

GenBank accession numbers. *Doryctobracon whartoni* **sp. nov.** Amapá: FJ560534 (ITS2) and FJ560542 (28SD2).

Doryctobracon adaimei Marinho and Penteado-Dias sp. nov.
(Figs 1 C–C2, D–D2, E–E2, 3 B, 5 A–H)
http://www.zoobank.org/NomenclaturalActs/7E53462B-1C9E-4FEC-A205-4D9877675D4A

Doryctobracon sp. 2: Veloso *et al.* 1996 (Cerrados de Goiás, GO ex *Anastrepha* spp. in *Pouteria gardneriana* (DC.) Radlk and *P. ramiflora* Radlk). Bomfim *et al.* 2007 (Tocantins, TO ex *Anastrepha coronilli* in *Bellucia grassularioides*); Braga Filho *et al.* 2001, Leopoldo Bulhões, GO in *Salacia crassifolia* (Mart.) Peyr; Deus *et al.* 2010 (Pedra Branca do Amapari, AP, ex *Anastrepha atrigona* in *Geissospermum argenteum*); Silva *et al.* 2011 (Laranjal do Jari, ex *Anastrepha atrigona* in *Geissospermum argenteum*; ex *A. fraterculus* in *Psidium guajava*; Vitória do Jari, AP, ex *A. atrigona* in *Geissospermum argenteum*; ex *A. striata* in *Psidium guajava*).

Diagnosis. Differs from *D. areolatus* in having the fore wing infumate, with a broad rounded hyaline spot from immediately after the stigma to the middle of R1a and not reaching the posterior margin of the fore wing, ending at the middle of the second subdiscal cell (2nd disc) (Fig 3B). Stigma dark brown (Fig 3B). In *D. whartoni* **sp. nov.** the fore wing has a hyaline spot in the anal area and a hyaline stripe in the central portion, and the stigma is yellow (Fig 3A). Hind legs with chromatic variations (orange, orange-brown, dark brown to black) on the entire leg or on part of it (Fig 1C2, D2, E2). Tegula yellowish orange as in *D. whartoni* **sp. nov.** (dark brown in *D. areolatus*). Propodeum with one short basal transverse keel projecting laterally in the anterior portion of the areola reaching the longitudinal lateral keel, as in *D. whartoni* **sp. nov.** (Fig 5D). Ovipositor apex similar to *D. areolatus,* with a dorsal node and ventral serrations (Fig 5G). Ventral serrations morphologically similar to those of *D. areolatus.* In *D. whartoni* **sp. nov.**, the serrations are more conspicuous and distinct (Fig 4E).

Description Female. Length of body, excluding ovipositor 5.5–6.3 mm.

Head. 1.4–1.2× wider than long; 1.5–1.3× wider than width of mesoscutum. Face polished, bright, and distinctly setose; midridge smoother, restricted between toruli (Fig 5A); distance between toruli equal to distance from toruli to eye (Fig 5A). Antenna longer than body, 8.4–8.7 mm in length, with 53 to 55 flagellomeres, first

flagellomeres about 1.1–1.2× longer than second; 1.5–2.1× longer than wide. Eye large, 1.2–1.3× wider than high (Fig 5C); in dorsal view, eye 1.6–2.0× wider than temples; malar space 0.4–0.5× longer than height of eyes. Clypeus 2.6–3.9× wider than high, polished, slightly convex and lateral margin slightly curved with sparse setae two to three times longer than setae on face (Fig 5A); clypeus sinuate, similar to *D. whartoni* **sp. nov.**, protruding as lobe medially on ventral margin; labrum partially covered by clypeus; distinct opening between clypeus and mandibles (Fig 5A).
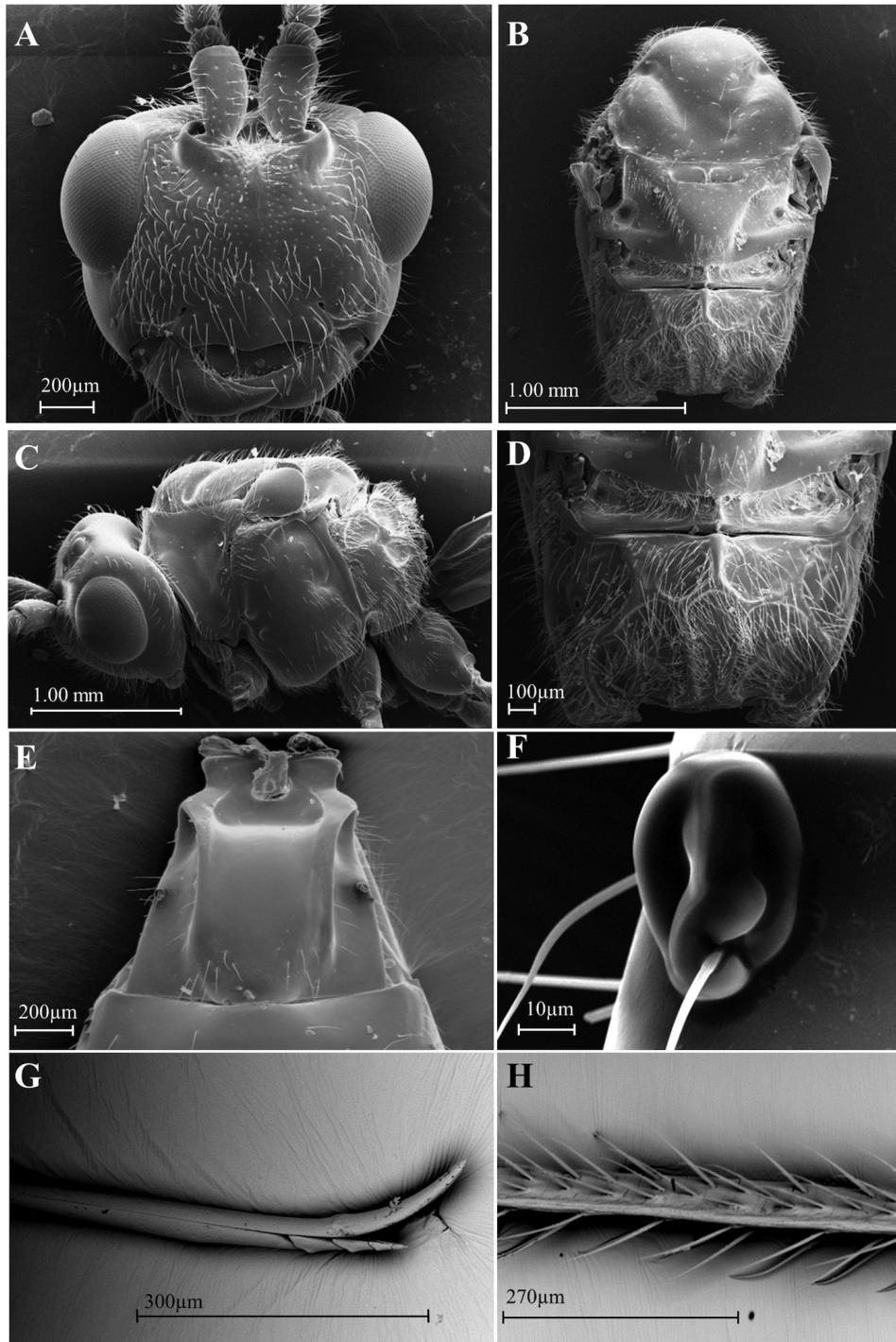

FIGURE 5. *Doryctobracon adaimei* **sp. nov.** head, front view (A), mesosoma smooth notaulices, dorsal view (B), head and mesosoma smooth mesopleura (C), propodeum, dorsal view (D), dorsal view of the petiole (E), modified spiracle (F), ovipositor apex (G), pattern of ovipositor sheath bristles (H).

Mesosoma. 1.2–1.5× longer than high; 1.7–2.0× longer than wide; 1.3–1.5× higher than wide; pronotum not visible dorsally; median lobe of mesoscutum and lateral lobes as in *D. whartoni* **sp. nov.**, polished, shiny with few sparse setae; margins of lateral lobes with few setae and shorter than *D. whartoni* **sp. nov.** (Fig 5B); notaulus smooth, complete, deeper anterior to margin of mesoscutum and later shallower, reunited in broad and polished impression without midpit (Fig 5B); scutellar groove divided into two large pits by median longitudinal septum; scutellum smooth with few or weak punctures, few setae on margins, robust or wide in apical portion, similar to *D. areolatus* (Fig 5B); mesopleuron smooth; propodeum setose with median anterior basal keel (0.11–0.15) and complete posterior areola. As in *D. whartoni* **sp. nov.**, laterally, in the anterior region of the areola, one short basal transverse keel extends to reach the lateral longitudinal keel, which is prominent and distinctly curved, from the posterior half of the propodeum (Fig 5D).

Wings. Fore wing 5.3–6.3 mm length; stigma wide, 3.0–4.2× longer than wide, with vein r slightly projecting from midpoint; (RS+M)a slightly sinuate posteriorly, 1.0–1.6× longer than 3RSa; 2RS 0.7–1.3× longer than 3RSa, 1.2–1.5× longer than 1m-cu and 1.6–2.6× longer than r-m; 3RSa 1.9–3.0× longer than r; 3RSb ending almost at wing tip; 2M 1.6–1.9× longer than 3RSa; (RS+M)b absent; 1cu-a straight line separated from 1M by 0.17–0.30 in length. Fore wing with a rounded hyaline spot that begins from immediately after stigma and ends in the middle of R1a, and before the wing posterior margin at second subdiscal cell (2nd disc). Length of hind wing 3.4–5.0 mm with m-cu, curved, distinctly pigmented just beyond the half toward to the wing margin.

Metasoma. 1.2–2.3× longer than wide and 1.0–2.3× wider than high; T1 length 0.9–1.0 mm greater than apex width; metasoma not sculptured, smooth and bright; T1 apex about 1.2–1.4× width of base; T1 with two dorsal keels developed at the base that gradually decrease from the posterior half, becoming weak or indistinct (Fig 5E); spiracles in half of T1 modified in two oval structures that protrude from the smooth surface, with or without setae (Fig 5E, F); ovipositor about 5.5–6.3 mm long; ovipositor tip with a dorsal node and ventral serrations that are less developed than in *D. whartoni* **sp. nov.** (Fig 5G); ovipositor sheath twice metasoma length, with 4–5 rows of setae (Fig 5H).

General coloration. Yellowish orange; first and second pair of legs bright yellow, third pair quite variable, may have all segments with blackish or dark-brown spots (Fig 1C2); tibiae and tarsi brown (Fig 1D2) or coxa, trochanters, and femur yellow (Fig 1E2); tegula yellowish orange; ovipositor sheath and antenna dark brown; apices of mandibles black; T2, T3, and T4 may have black stripes; wings infumate; fore wing with broad rounded hyaline spot that begins from immediately after stigma to middle of R1a and in middle of second subdiscal cell (2nd disc). Veins and setae yellow in the hyaline spot; stigma, veins, and bristles dark brown in infumate area (Fig 3B).

Male. Similar to females, but usually last tergite with dark-brown or black spots. Head in dorsal view 1.7× wider than mesoscutum width, 2.0× wider than face; in dorsal view, eye 1.85× wider than temple; face 1.5–1.7× wider than high; malar space 0.42–0.44× height of eyes; clypeus 2.9–3.2× wider than high; antennae with 50 to 51 flagellomeres; first flagellomere 1.1-1.2× longer than second, 1.8–1.9× longer than wide; mesosoma 1.9× longer than wide, 1.3–1.4× higher than wide; metasoma 2.4–2.7× longer than wide, 1.1–1.4 wider than high.

Type material. Holotype. Female (DCBU 270198), BRAZIL: Amapá, Serra do Navio, 00º55'57.0"N, and 051º55'14.8"W 01.ii.2006, reared from fruit fly larva in fruits of guava (*Psidium guajava*), collr. R. A. Silva. Paratypes, with same data as holotype, 2 females (DCBU 270199, DCBU 270200), 1 male (DCBU 270201), collr. R. A. Silva and 1 female (ESALQ); 1 male, Tocantins, 28.ii.2005, reared from fruit fly larva *Anastrepha coronilli* in "Goiaba-de-anta" (*Bellucia grossularioides* L.), collr. M. A. Uchôa-Fernandes; 2 females, 1 male, Goiás, Leopoldo Bulhões, 14. ix. 1999, reared from fruit fly larva *Anastrepha* spp. In "bacupari" (*Salacia crassifolia*) collr. V.R.S Veloso, (ESALQ).

Etymology. This species is named after Ricardo Adaime, who has provided valuable information of many *Anastrepha* species, their host fruits, and parasitoids in Amapá, Brazil.

GenBank accession numbers. *Doryctobracon adaimei* **sp. nov.** Amapá, FJ560535 (ITS2); Tocantins, FJ560536 (ITS2); Goiás, FJ560537 (ITS2); Amapá, FJ560543 (28SD2); Tocantins, FJ560544 (28SD2); Goiás, FJ560545 (28SD2).
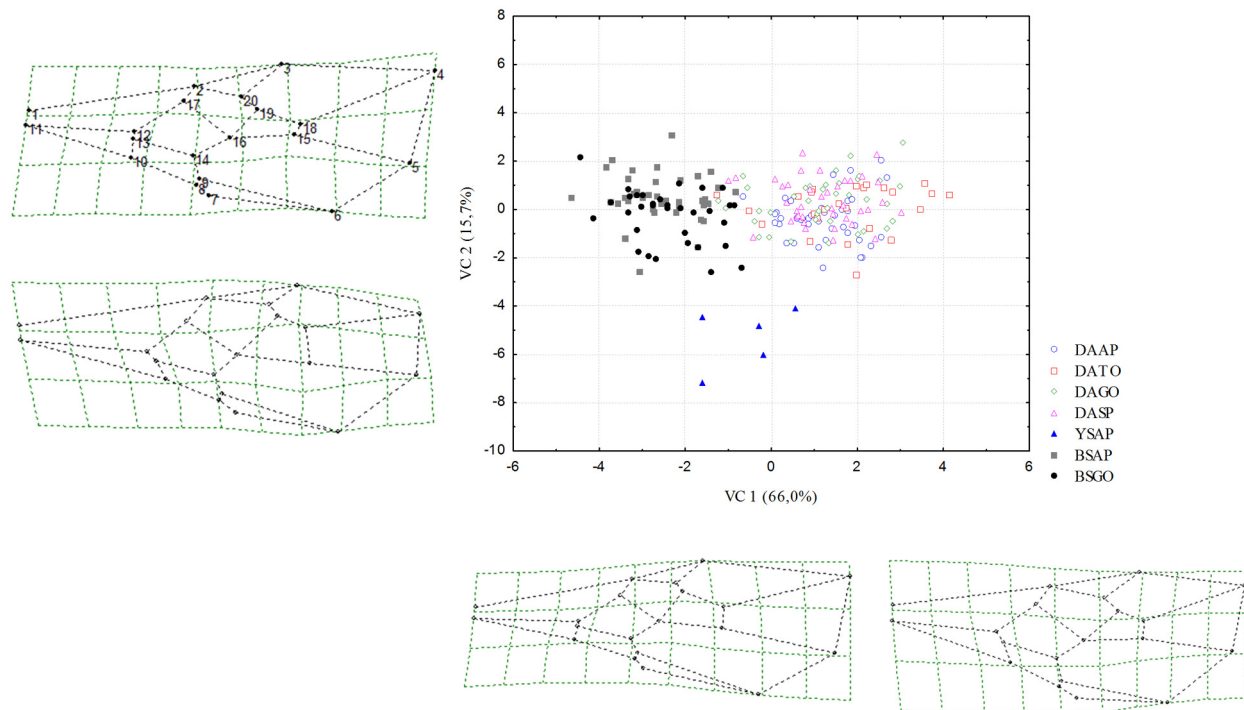
FIGURE 6. Dispersion graphic of *Doryctobracon areolatus* males and females from Amapá (DAAP), Tocantins (DATO), Goiás (DAGO) and São Paulo (DASP); *Doryctobracon whartoni* **sp. nov.** yellow stigma, Amapá (YSAP); *Doryctobracon adaimei* **sp. nov**. brown stigma, Amapá (BSAP) and Goiás (BSGO) in the bidimensional space of canonical variables VC1 and VC2. The deformations diagrams indicate the presumable wing conformations for individuals in the superior and inferior ends of the canonical variables. Deformation magnitudes were amplified 3x for visualization.
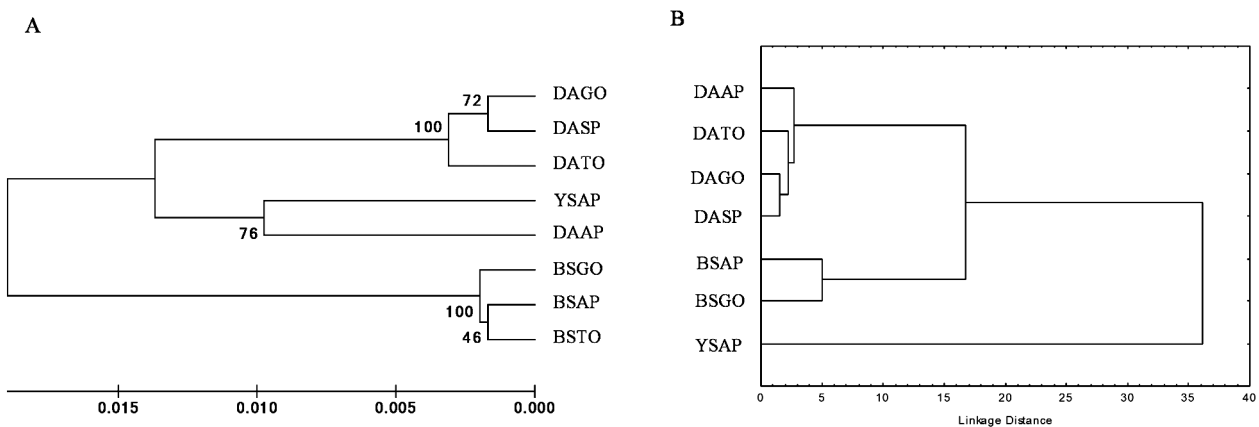

FIGURE 7. Comparison concatenated tree ITS2 – 28S rDNA D2 (UPGMA) produced from the nucleotide sequences (A), cluster analysis of the Mahalanobis distance (UPGMA) calculated from the shape of the wings components (B). (DAAP = *Doryctobracon areolatus* from Amapá, DAGO = *D. areolatus* from Goiás, DATO = *D. areolatus* from Tocantins, DASP = *D. areolatus* from São Paulo, YSAP = *Doryctobracon whartoni* **sp. nov.** (yellow stigma) from Amapá; BSAP = *Doryctobracon adaimei* **sp. nov.** (brown stigma) from Amapá, BSGO = *D. adaimei* **sp. nov.** from Goiás, BSTO = *D. adaimei* **sp. nov.** from Tocantins.

Morphometry. Geometric morphometry generated 36 relative deformation measurements (k=2n–4), where *k* represents the total number of relative deformations, and *n* the number of anatomical markers (20). The results of the multivariate analysis (CVA) of populations of male and female *D. areolatus*, *D. whartoni* **sp. nov.** and *D. adaimei* **sp. nov.** were statistically significant, Wilks' Lambda ($p<0.0001$), Pillai's Trace ($p<0.0001$), Hotelling-Lawley Trace ($p<0.0001$), and Roy's Greatest Root ($p<0.0001$). The first two canonical axes explained 66.0% and 15.7% of the data variability, respectively. In the scatter plot, we observed the complete separation of the

population groups in the space of canonical variables CV1 and CV2 (Fig 6). Specimens termed *D. whartoni* **sp. nov.**, collected only in the state of Amapá, formed an isolated group of *D. adaimei* **sp. nov.** and of *D. areolatus*. *Doryctobracon adaimei* **sp. nov.** separated completely from the groups formed by the other species and revealed total overlap of their populations collected in the states of Goiás and Amapá; among the populations of *D. areolatus*, there was also the formation of distinct grouping with *D. whartoni* **sp. nov.** and *D. adaimei* **sp. nov.**, and total overlap of their populations in the states of Amapá, Tocantins, Goiás and São Paulo (Fig 6). The Mahalanobis distance matrix revealed a lower degree of morphological similarity for *D. whartoni* **sp. nov.** in relation to the distinct populations of *D. areolatus* and *D. adaimei* **sp. nov.** We also observed the same result for population of *D. adaimei* **sp. nov.** in relation to the populations of *D. areolatus*; however, the Mahalanobis distances between samples of *D. whartoni* **sp. nov.** and *D. adaimei* **sp. nov.** were fairly wide, i.e. *D. whartoni* **sp. nov.** (AP) - *D. adaimei* **sp. nov.** (AP) = 38.39%, and *D. whartoni* **sp. nov.** (AP) - *D. adaimei* **sp. nov. (GO)** = 34.96% (Fig 7, Table 2).

TABLE 2. Mahalanobis distances of *Doryctobracon areolatus* populations from Amapá (DAAP), Tocantins (DATO), Goiás (DAGO) and São Paulo (DASP); *Doryctobracon whartoni* **sp. nov.** (yellow stigma), Amapá (YSAP); and *Doryctobracon adaimei* **sp. nov.** (brown stigma), Amapá (BSAP), Goiás (BSGO) based on wing shape components.

Parasitoids	DAAP	DATO	DAGO	DASP	YSAP	BSAP	BSGO
DAAP	0.00						
DATO	3.35	0.00					
DAGO	1.60	1.76	0.00				
DASP	3.29	2.70	1.52	0.00			
YSAP	31.89	37.16	37.64	36.68	0.00		
BSAP	16.89	20.95	15.76	15.07	38.39	0.00	
BSGO	15.66	19.11	14.31	16.08	34.96	5.05	0.00

Molecular markers. We detected intraspecific size variation for both molecular markers tested when comparing populations of *D. areolatus* from different geographical regions. The size variation was larger for the ITS2, which ranged from 564 bp for *D. areolatus* from the state of Amapá to 590 bp for specimens from the state of São Paulo. ITS2 was 587 bp long in samples from the states of Goiás and Tocantins. The size of 28S-D2 from samples of *D. areolatus* differed by no more than 5 bp in length, ranging from 381 bp for specimens from Amapá to 386 bp for those from Goiás. Specimens from Tocantins and São Paulo had intermediate sizes (384 and 385 bp, respectively). Despite the variation in size, these markers shared very high sequence similarities (Table 3). However, no size variation was observed for both markers for the species identified as *D. whartoni* **sp. nov.** and *D. adaimei* **sp. nov.** ITS2 for *D. whartoni* **sp. nov.** from Amapá was 585 bp long, while ITS2 for *D. adaimei* **sp. nov.** from Amapá, Tocantins, and Goiás was 567 bp in length. The 28S-D2 of *D. whartoni* **sp. nov.** from Amapá was 385 bp long, and 386 (Amapá and Tocantins) and 387 bp long (Goiás) for specimens of *D. adaimei* **sp. nov.** Pairwise sequence similarities of ITS2 and 28S-D2 among *D. whartoni* **sp. nov.**, *D. adaimei* **sp. nov.**, and *D. areolatus* were very low (Table 3).

Phylogenetic reconstruction of specimens of *Doryctobracon* species based on the isolated and concatenated sequences of the molecular markers ITS2 and 28S-D2, using maximum likelihood (ML) and neighbor-joining (NJ) methods, produced trees sharing similar topologies and well-defined clades (Fig 8). In all cases, molecular analysis consistently indicated variation in *D. areolatus* from different collection sites (Amapá, Goiás, Tocantins, and São Paulo), with specimens from Amapá being the most divergent. The most variation in clade definition between the ML and NJ methods was found for the positioning of *D. areolatus* from Amapá and *D. whartoni* **sp. nov.** (Fig 8). Nevertheless, the topologies produced with the concatenated sequences left no doubts regarding the external grouping of specimens of *D. areolatus* from Amapá from a more internal clade of *D. areolatus* from the other states. In the concatenated analysis, *D. whartoni* **sp. nov.** resolved in a subclade with the divergent *D. areolatus* from Amapá, but was positioned on a long branch away from *D. areolatus* (Fig 8C). The remaining samples of *D. areolatus* grouped together in a more internal subclade (Fig 8). Samples of *D. adaimei* **sp. nov.** always resolved in a defined clade, in the analysis with either the isolated or concatenated molecular markers. In the analysis with the concatenated sequences, the clade of *D. adaimei* **sp. nov.** was the first to branch out from all of the remaining samples. The NJ analysis resolved specimens belonging to *D. adaimei* **sp. nov.** from Amapá and Tocantins in a more internal subclade from specimens from Goiás, while ML placed specimens from all three populations in a single clade separated from each other by short branches (Fig 8).
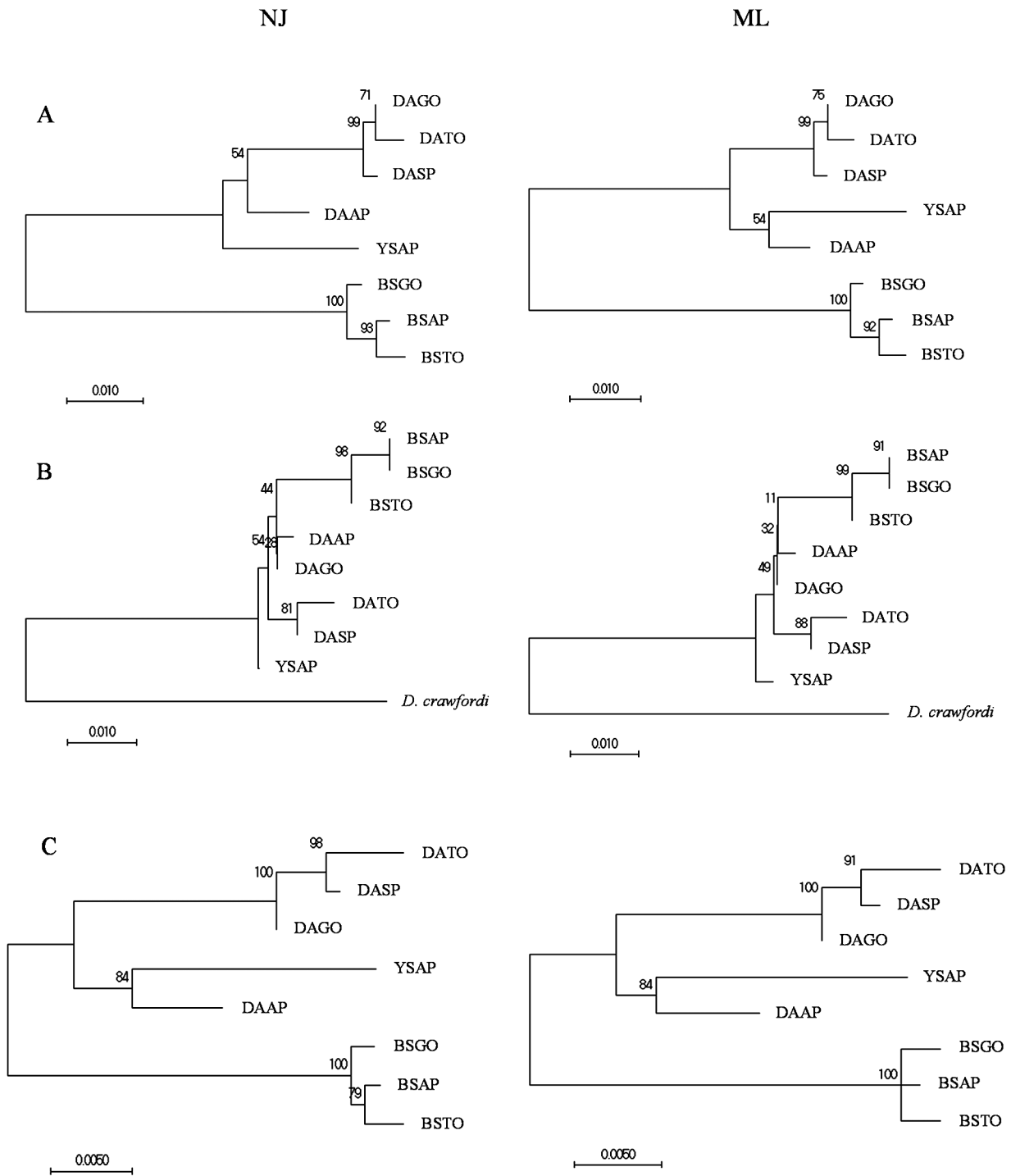
FIGURE 8. Phylogeny reconstruction for *Doryctobracon* species inferred by using the maximum likelihood and Neighbor-joining methods using sequences from the molecular markers ITS2 (A), 28S-D2 (B) and their concatenated sequences (C). ML tree with the highest log likelihood (-1065.5986) and the optimal NJ tree with the sum of branch length (=0.13197667) shown for the ITS2 sequences were based on the Tamura 3-parameter model (Tamura, 1992); ML tree with the highest log likelihood (-719.5043) and the optimal NJ tree with the sum of branch length (=0.11870754) shown for the 28S-D2 sequences were based on the Tamura 3-parameter model; the ML tree with the highest log likelihood (-1598.9138) and the optimal NJ tree with the sum of branch length (=0.076130) for the concatenated ITS2 and 28S-D2 sequences was based, respectively, on the Tamura 3-parameter model+G (=0.1096)+I (=0.001%) and Tamura 3-parameter model+G (=0.1).

TABLE 3. Sample identification, molecular marker used, fragment size, and GenBank access number for each parasitoid specimen analyzed.

*Codes	Parasitoids	Stigma	Markers	Size	GenBank (access)
YSAP	*D.whartoni* **sp. nov.**	yellow	ITS2/28SD2	585pb/385pb	FJ560534 - FJ560542
BSAP	*D. adaimei* **sp. nov.**	brown	ITS2/28SD2	567pb/386pb	FJ560535 - FJ560543
BSTO	*D. adaimei* **sp. nov.**	brown	ITS2/28SD2	567pb/386pb	FJ560536 - FJ560544
BSGO	*D. adaimei* **sp. nov.**	brown	ITS2/28SD2	567pb/387pb	FJ560537 - FJ560545
DAAP	*D. areolatus*	brown	ITS2/28SD2	564pb/381pb	FJ560538 - FJ560546
DAGO	*D. areolatus*	brown	ITS2/28SD2	587pb/ 386pb	FJ560539- FJ560547
DATO	*D. areolatus*	brown	ITS2/28SD2	587pb/384pb	FJ560540 - FJ560548
DASP	*D. areolatus*	brown	ITS2/28SD2	590pb/385pb	FJ560541 – FJ560549

*The first and second letters represents the color of the stigma (YS—yellow stigma, BS—brown stigma and the species DA—*Doryctobracon areolatus*), the second and third letters, the Brazilian states from sample were collected (AP—Amapá, TO—Tocantins, GO—Goiás, SP—São Paulo).

Discussion

Braconid wasp parasitoids (Opiinae) of tephritid fruit flies are often surveyed in Brazil; however, the parasitoid fauna in some areas is still being explored, and recent surveys conducted in the state of Amapá (Adaime *et al.* 2007; Silva & Adaime 2007; Jesus *et al.* 2008; Deus *et al.* 2010; Silva *et al.* 2011; Jesus *et al.* 2012; Deus & Adaime 2013) yielded new species records (Zucchi *et al.* 2011). Color differences among *D. whartoni* **sp. nov.**, *D. adaimei* **sp. nov.**, and *D. areolatus*, and their occurrence in different states of Brazil, have drawn our attention for several years. It is highly likely that these specimens have been misidentified in various surveys, limiting the correct assessment of their distribution ranges in Brazil. Despite their similarities with *D. areolatus*, *D. whartoni* **sp. nov.** and *D. adaimei* **sp. nov.** are characterized by having smoky wings, with a hyaline spot that varies in its base-apex extension, which may or may not reach the stigma (yellow stigma in *D. whartoni* **sp. nov.**; brown stigma in *D. adaimei* **sp. nov.**). This variation in the color of the stigma (Fig 1) led others to wonder if this change might correspond to a process of cline formation in *D. areolatus* (Slatkin 1973; Lenormand 2002).

The color of insects is often an important characteristic in species separation and identification; however, it must be employed with caution. Color differences are often reported as a plastic phenotypic trait. Phenotypic plasticity is the capacity of a genotype to express different phenotypes in accordance with biochemical changes or extreme alterations in environmental conditions (Pigliuci 2001; Whitman & Agrawal 2009). There are a number of examples of genotypes with phenotypic plasticity that resulted in misclassification (for example, see Uvarov 1966; Greene 1989; Schlichting & Pigliucci 1998; Mound 2005). Thus, identity confirmation should not rely exclusively on color differences among specimens. Methods such as geometric morphometry and molecular taxonomy provide reliable additional information for morphology-based species identification, and help avoid misidentification of plastic genotypes. Geometric morphometry uses a series of techniques to describe and represent the geometry of the shapes studied (Moraes 2003). This tool reveals minute morphological variations that are often undetectable by studies based only on traditional morphometry (Villemant *et al.* 2007). Therefore, geometric morphometry is extremely useful in studies involving species complexes that are characterized by high levels of specificity and minimal morphological differences among members (Baylac *et al.* 2003; Villemant *et al.* 2007). In this study, geometric morphometry revealed marked differences in the shape of the fore wings of *D. areolatus*, *D. whartoni* **sp. nov.** and *D. adaimei* **sp. nov.** These differences resulted from the wide variation in the arrangement of nervures that involve the formation of the first subdiscal cell, and especially, the second submarginal cell and nervures connected to it. In *D. whartoni* **sp. nov.**, the second submarginal cell, the segments 2RS (16–19) and r-m (18–15) were quite prolonged, while 3RSa (18–19) was smaller in relation to 2M (15–16), located at the lower end of VC2 (negative score). *Doryctobracon adaimei* **sp. nov.** has 2RS (16–19) and r-m (15–18) of similar sizes, and 3RSa (18–19) slightly longer than 2M (15–16) at the lower end of VC1 (negative score). However, in *D. areolatus*, 3RSa (18–19) was shorter than 2M (15–16), and 2RS (16–19) longer and more inclined than the r-m segment (15–18) located at the upper extreme of VC1 (positive score) (Fig 6). The second submarginal cell is considered one of the

most reliable taxonomic characters in identifying species of Opiinae, and was decisive for separation of populations and species of *Psyttalia* Walker (Billah *et al.* 2008). Here we observed the great divergence among the specimens analyzed by means of morphological variation in the shape of their wings within the space created by the two canonical variables (81.7%), and also highlighted by the Mahalanobis distance (Fig 6, Table 2). The dendrogram based on this distance also corresponded to the genetic proximity trees produced with the molecular markers analyzed (ITS2, 28S-D2 and concatenated ITS2-28S-D2 sequences), which separated specimens of the population groups formed by *D. areolatus*, *D. whartoni* **sp. nov.** and *D. adaimei* **sp. nov.** into well-defined clades and branches (Figs 7, 8).

Our molecular analyses corroborated the morphological divergence observed in the morphometric analysis, and support our proposal of the existence of a cryptic species complex for *D. areolatus* and two other closely related new species. ITS2 and 28S-D2 sequence similarities and size differences for samples of *D. areolatus* were observed, and led to the positioning of *D. areolatus* in highly supported subclades and branches, which were consistent in all topologies obtained with the different methods of phylogenetic reconstruction used (NJ and ML) for the markers either individually or concatenated (Table 3, Fig 8).

The concatenated construction shows a well-defined topology. The same topology was observed, with high bootstrap values, when ITS2 was used alone. The topology of the trees when using the 28S-D2 individually as a marker lost some support in branch definition, but clearly positioned the two new species in separate clades (Fig 8). The analyses support the existence of cryptic species for *D. areolatus*, especially by observing the concatenated tree (ITS2 – 28S-D2) (Fig 8C). Moreover, the sample of *D. areolatus* from Amapá (DAAP) is more closely related to *D. whartoni* **sp. nov.** (YSAP) than are the other samples of *D. areolatus* from Tocantins (DATO), São Paulo (DASP) and Goiás (DAGO) (Fig 8C). However, the nucleotide composition of DAAP is distinct from *D. whartoni* **sp. nov.** (YSAP) (Fig 8C). The 28S-D2 marker allowed the separation of the populations of *D. areolatus* from *D. whartoni* **sp. nov.** (YSAP) and revealed variation among samples of *D. adaimei* **sp. nov.** (Fig 8B). The placement of *D. adaimei* **sp. nov.** from Tocantins (BSTO) on a distinct branch from the populations from Amapá (BSAP) and Goiás (BSGO), with high bootstrap support values (NJ, 98%; ML, 99% ML), may also indicate that *D. adaimei* **sp. nov.** constitutes a species complex. Subtle differences in color of the hind legs of specimens of *D. adaimei* **sp. nov.** from Goiás (BSGO), Tocantins (BSTO) and Amapá (BSAP) (Fig 1) could support the molecular analysis. However, the well-defined clade formed in the ML tree and the complete morphological similarity of these individuals from different localities reported in our morphometric analyses, led us to propose that these specimens belong to the same species. The congruence found in the morphological analysis with the results obtained with the use of molecular markers strengthens the systematic positioning of these individuals, with the separation of the three species analyzed and the discovery of a complex of species for *D. areolatus* (Fig 8).

Doryctobracon areolatus is widely investigated for use in applied biological control of fruit flies (Eitam *et al.* 2003; Nunes *et al.* 2011). This species is considered an efficient parasitoid of different larval instars of several species of Tephritidae (Canal Daza & Zucchi 2000; Murillo *et al.* 2015). In Brazil, successful parasitization of fruit flies is influenced by differences among local environmental conditions, due to these flies' wide geographic distribution and association with a range of host fruits (Canal Daza & Zucchi 2000). We demonstrated here that the taxon *D. areolatus* is composed of cryptic species that are difficult to separate by traditional morphological analysis, but easily differentiated when using geometric morphometry and molecular analysis.

The ribosomal DNA-based markers allow for systematic studies at different taxonomic levels, and are often used in species identification (Fouly *et al.* 1997; Weekers *et al.* 2001). ITS regions accumulate variability that allows the successful differentiation of species or strains of the same species (Silva *et al.* 1999; Ciociola Jr. *et al.* 2001; Gallego & Gálian 2001; Müller *et al.* 2007). The D2-D3 regions of the 28S ribosomal gene provide an alternative method for phylogenetic analysis, which can equally be used to test and supplement hypotheses derived from morphological analyses (Taylor *et al.* 2006; Stouthamer *et al.* 2017).

Our comparative analyses using the molecular markers ITS2 and 28S-D2 showed high genetic diversity among the three species compared. We also detected high genetic diversity among populations of *D. areolatus*. The discovery of new species of *Doryctobracon* and the existence of a species complex in *D. areolatus* have direct implications for the development of biological-control programs against fruit flies in Brazil, because accurate species identification provides the basis for any investigation of a particular species (Zucchi 2002). Moreover, species misidentification is one of the major issues affecting the successful implementation of biocontrol programs (Parra *et al.* 2002). Besides, we have very little, if any, information on the reproductive compatibility, host

specificity, host plant preference, parasitization efficiency, and/or ecological requirements for the parasitoid species identified, all of which are important matters for the successful use of parasitoids as biocontrol agents (Stouthamer *et al*. 2000; Leon *et al*. 2006).

We demonstrated the successful use of combined morphometric and molecular analyses in the identification of two species closely related to *D. areolatus,* and the existence of cryptic species within the taxon *D. areolatus*. These findings may have serious implications for the use of *D. areolatus* as a biocontrol agent of fruit flies, but also for the limitations that cryptic species imposes for the adoption of any other strategies based on conservation (Bickford *et al*. 2007). Our data obtained by using geometric morphometry combined with molecular tools provided information to allow the appropriate treatment of important issues for conservation and biological control.

Acknowledgements

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