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Back from the past: Molecular and morphological support for *Simulium mutucuna* Nunes de Mello & Vieira da Silva, 1974 (Diptera: Simuliidae) as a valid species

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ABSTRACT

Simulium mutucuna, a species described based on a single female from Roraima state, was previously synonymized with *Simulium paynei* and currently is considered a synonym of *Simulium rubrithorax*. In the present paper we present morphological and molecular evidence supporting the validity of *S. mutucuna* based on analysis of specimens from Brazil, Venezuela and Mexico. We redescribe the female and describe, for the first time, the male, pupa and larva of *S. mutucuna* and discuss the morphological differences between this species and the others that are already considered as its senior synonyms. Currently, the distribution of *S. mutucuna* is restricted to Roraima state. The distribution record for *S. rubrithorax* in Brazil's North region needs to be removed, since the previous records were based on occurrence of *S. mutucuna*. Finally, we present new evidence of cryptic diversity in the *S. paynei* complex based on molecular information.

1. Introduction

Poor understanding of cryptic diversity is one of the greatest challenges for biodiversity conservation (Bickford et al., 2007). Uncovering cryptic species to understand diversity patterns more accurately is essential, for example, to assigning special conservation status to regions with high levels of species richness and to propose precise policies to control diseases in medically important taxa (Adler, 2010; Myers et al., 2000; Orme et al., 2005).

The application of molecular methods to systematics has revolutionized the discovery and description of biological diversity. Utilization of short and standardized DNA fragments for species identification (e.g., DNA barcodes - a variable 5′ half of mitochondial gene COl) has proven useful for species recognition in some animal groups (Hebert et al., 2003; Tavares and Baker, 2008). In many cases, DNA barcodes have also aided the detection of cryptic species, whose distinction could later be confirmed by morphology, thus highlighting the importance of integrated analyzes (e.g., Kanturski et al., 2018; Nascimento et al., 2018, 2020).

Simulium mutucuna Nunes de Mello & Vieira da Silva, 1974 was described based on one female collected in the northern portion of Roraima state, Brazil. Due to the lack of accurate taxonomic information for all stages of development (male, pupa, and larva remained unknown), the specific status of this enigmatic species was later contested. Coscarón (1987), in a study on the *Simulium* of South America, was the first to hypothesized it as a synonym of *Simulium paynei* Vargas, 1942, a species described from Mexico and currently considered to be a species complex (Adler et al., 2004; Hernández et al., 2015). Subsequently, various authors began to consider it a synonym of *Simulium rubrithorax* Lutz, 1909 (e.g., Adler and Crosskey, 2008; Coscarón et al., 2008; Hernández, 2011; Hernández et al., 2007; Shelley et al., 2010), a valid species described from Brazil (Adler, 2020). Another species currently in synonymy with *S. rubrithorax* is *Simulium magnum* Lane & Porto, 1940 (Py-Daniel, 1989) which was described based on females collected on

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Table 1
Specimens of *Simulium* (Diptera: Simuliidae) used in morphological and molecular analysis and collection data. Numbers from 1 to 8 on the species column represents the locations of the populations indicated on the map of the Figure 1; N represents the number of sequences. Note: * = near type locality of *S. mutucuna*; ** = near region of the neotype of *S. rubrithorax*; *** = same geographic region of the type locality of *S. magnum*.

Species	Location	N	GenBank accession number reference
Simulium rubrithorax			
1. (RR01)*	• Brazil, Roraima state (RR), Pacaraima municipality, Bananal river, N4°25'46.8" W61°12'45.9"; 13/XII/2000; N.H. & J.O.S., cols.	9	MW346187–MW346195, This study
2. (RR02)	• Brazil, Roraima state (RR), Caracaraí municipality, Pacu river, N01°34'48.0" W61°55'48"; 22/I/2016; K.D.S. col.	9	MW346196–MW346204, This study
3. (MG)**	• Brazil, Minas Gerais state (MG), Juiz de Fora municipality, Fazenda da Cachoeira; S21°48'21.7" W43°27'44.3"; 22/VI/2010; N.H & V.C.O., cols.	5	MW346230–MW346234, This study
4. (MT)***	• Brazil, Mato Grosso state (MT), Cáceres, Jacobina stream, S16°15'40.3" W57°37'36.6"; 18/VII/2004; N.H col.	5	MW346225–MW346229, This study
Simulium paynei complex			
5. (VE)	• Venezuela (VE), Aragua state, Magdaleno municipality, stream near road, N10°6'31.43" W67°40'46.9"; 10.IV.2011; N.H., H.F., U.G.N., cols.	7	MW346218–MW346224, This study
6. (ME01)	• Mexico (ME), Oaxaca, Tuxtepec, stream near road, N17°06′39.8" W96°38′08.9"; 04/XI/2017; N.H., J.M.C.N. & J.A.G.H. cols.	3	MW346205–MW346207, This study
7. (ME02)	• Mexico (ME), Oaxaca, Cuajimoloyas, Verde river, N17°14′44.4" W96°32′15.8"; 04/XI/2017; N.H., J.M.C.N. & J.A.G.H. cols.	5	MW346208–MW346212, This study
8. (ME06)	• Mexico (ME), Morelos, Yautepec municipality, La Poza Azul, N18°54′08.6" W98°57′02.1", 24/V/2015; N.H., J.M.C.N. cols.	5	MW346213–MW346217, This study
Simulium paynei complex (GenBank)	• xCosta Rica, Puntarenas, Buenos Aires, Colinas, Rio Fresco.	1	KP252368.1, Hernandez-Triana et al. (2005)
Simulium paynei complex (GenBank)	• Costa Rica, San Jose, Rd San Jose to Purisca.	1	KP252432.1, Hernandez-Triana et al. (2005)
Simulium paynei complex (GenBank)	• Costa Rica, Heredia, Rio El Angel, near bridge.	1	KP252467.1, Hernandez-Triana et al. (2005)
Outgroups (Bayesian Analysis)			
Simulium tarsatum	• Costa Rica, Cartago, Tapanti (see GenBank informations)	2	GU713663.1; GU713611.1, iBOL Data Release - Unpublished
Simulium lobatoi	• XBrazil, Goiás state, Alto Paraíso, Riacho da Usina, S14°07′31.3" W47°30′9"; 02/VI/2007; N. H. col.	2	MW346185, MW346186, This study
Simulium cristalinum	• Brazil, Roraima state, Rio Surumu, N4°13′8.2" W60°53′17.8; 21/X/2004; N.H. & J.O.S cols.	2	MW346183, MW346184, This study
Simulium metallicum complex	• Mexico, Oaxaca, Waterfall near road, N18°07′41.5" W 96°57′47.5"; 06/XI/2017; N.H., J.M.C. N. & J.A.G.H. cols.	1	MW346182, This study

horse bait in Mato Grosso state, Brazil. According to the literature, females of *S. rubrithorax* and *S. paynei* are zoophilic (Lutz, 1910; Maia--Herzog et al., 1984; Dalmat, 1955), unlike *S. mutucuna,* which was collected on human bait (Nunes de Mello and Vieira da Silva, 1974).

When we analyzed COI sequences of different populations of *S. rubrithorax* from Brazil, we observed considerable genetic divergence between populations from Roraima state and the populations collected in the other states. Based on an integrative approach, the objectives of this paper are to propose the revalidation of the name *S. mutucuna*, redescribe the female and describe, for the first time, the male, pupa, and larva of this species.

2. Material and methods

Four populations of *S. rubrithorax* from Brazil were analyzed, one collected in the same geographic region as its neotype (about 10 km distant), another about 250 km distant of the type locality of *S. magnum* in the state of Mato Grosso, and two populations from Roraima state, one of which was near the type locality of *S. mutucuna* (about 12 km distant) (Table 1). The holotype of *S. mutucuna* deposited in the Invertebrate Collection of the National Institute for Research in Amazonia (INPA), Manaus, Amazonas, Brazil was examined. Additionally, pupae of *S. paynei* complex collected in Venezuela and pupae of three populations of the *S. paynei* complex collected in Mexico were used for morphological comparisons and molecular analyses (Fig. 1; Table 1). *Simulium paynei* deposited at the Institute of Epidemiological Diagnosis and Reference (InDRE), Mexico, were analyzed morphologically for further comparison. Three DNA sequences identified as *S. paynei* from Costa Rica were downloaded from GenBank and used in the molecular analysis (Table 1). Voucher specimens are deposited in the Invertebrate Collection of the National Institute for Research in Amazonia (INPA), Manaus, Amazonas, Brazil.

Collected black-fly larvae and pupae were fixed in absolute ethanol. Specimens were maintained at -20°C in the laboratory until analysis. Pupae with pharate adults were isolated individually in 2-mL plastic vials with damp filter paper until adult emergence. Pharate adults that did not emerge were dissected and the adults were extracted, dehydrated using the Sabrosky (1966) technique and mounted on card points. For morphological analysis, when needed, structures were cleared in hot 95% lactic acid and mounted between slide and coverslip, using Euparal® as the mounting medium. Procedures for measurement of specimens and the terminology used in the species description are detailed in Adler et al. (2004). All color descriptions are based on pinned or alcohol-preserved specimens.

Multi-layer photographs of the morphology were obtained using a Leica M165C stereomicroscope in conjunction with Leica DFC 420 image-capturing equipment and LED dome lighting for uniform and constant reflection of light on the specimens (Kawada and Buffington, 2016) and an Olympus BX51 compound microscope in conjunction with an Olympus digital image-acquisition system (model DP 72 using the Cell D program). The final images were generated using Digital Leica Application Suite v.3.7 and Helicon Focus® (6.7.1 Pro) software.

For molecular analysis genomic DNA was extracted from larvae and pupae through nondestructive methods using a DNeasy Blood & Tissue (Qiagen) kit, following the manufacturer's recommendations. Amplifications of extracted DNA were performed using forward and reverse primers developed by Folmer et al. (1994) for the cytochrome oxidase I (COI) mitochondrial gene, with the PCR technique adapted from Pramual et al. (2005). The amplified fragments were purified using Exonuclease I and Thermosensitive Alkaline PhosphataseTM (FastAP) (Fermentas®). DNA sequencing was carried out in the Laboratório Temático de Biologia Molecular at the National Institute for Research in Amazonia using the DYEnamic ET Dye Terminator Kit, following Platt et al. (2007), in an ABI PRISM® 3130xl DNA Analyzer (Life Technologies - Applied Biosystems) and at the Centro de Estudos do Genoma Humano at the Universidade de São Paulo (CEGH-USP) in an ABI 3730.

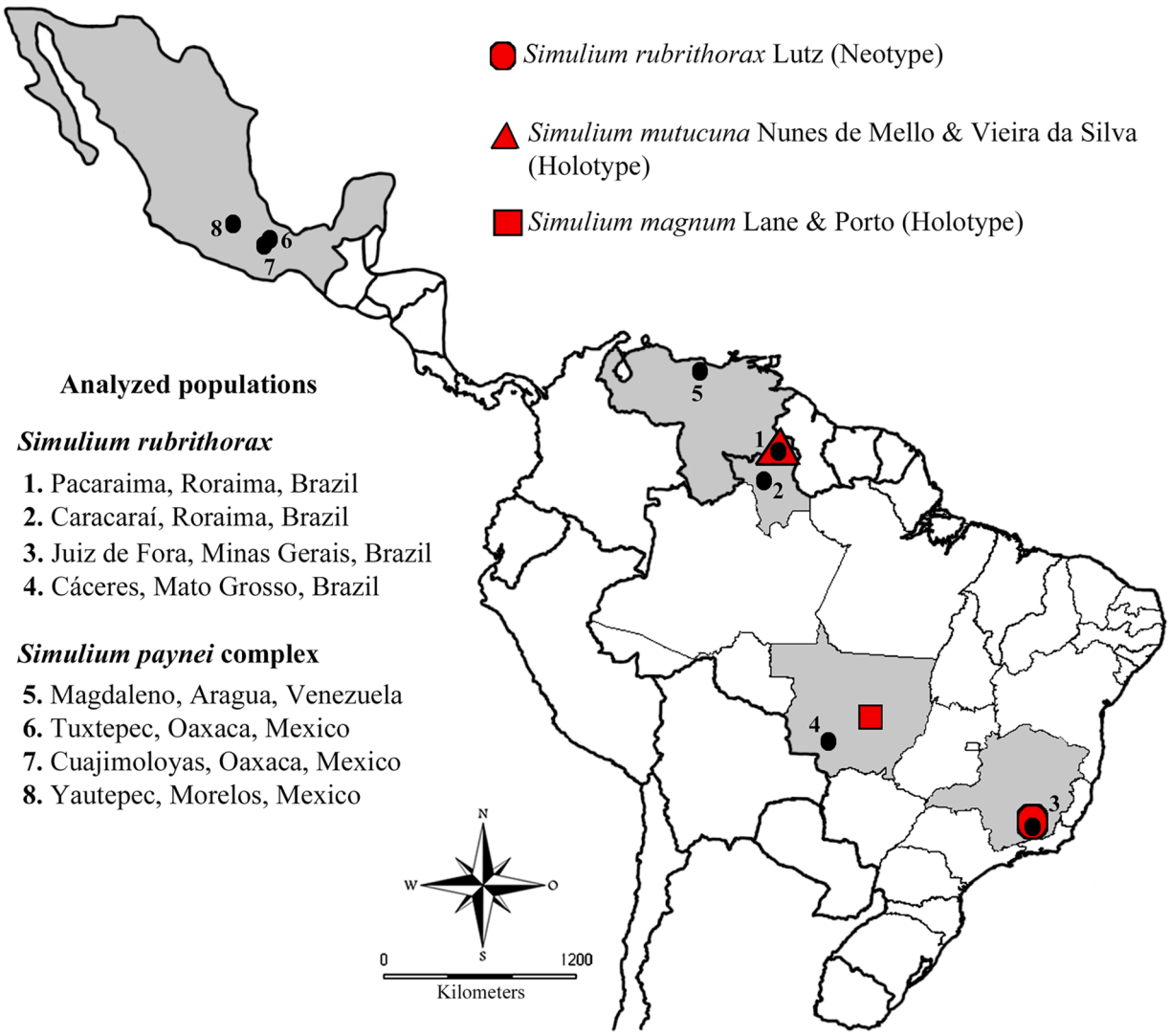

Fig. 1. Partial map of Central and South America, numbers indicate the localities where the analyzed *Simulium* (Diptera: Simuliidae) populations were collected. For details of the sampled sites see Table 1. Red symbols represent the localities of the holotypes of *Simulium mutucuna* and *Simulium magnum* (both synonymous of *Simulium rubrithorax*) and of the neotype of *Simulium rubrithorax*.

Chromatograms were assembled and edited using Geneious v 7.1.3 (Biomatters) software, and multiple sequence alignments were performed with the Clustal W module implemented in Geneious software (Thompson, 1994). DNA polymorphisms were calculated using DnaSP v5.10.1 software (Rozas et al., 2003). The Kimura two-parameter model of base substitution (Kimura, 1980) was used to calculate intra- and interspecific genetic distances with MEGA 6.0 software (Tamura et al., 2013). Sequences were deposited in the GenBank database; the accession numbers are given in Table 1.

Bayesian inference performed in MrBayes v.3.2.2 (Ronquist et al., 2012) was used to examine genetic relationships of the specimens based on COI sequences. For this analysis, the HKY model was chosen as the best-fit model by jModelTest v.2.1.7 (Darriba et al., 2012) under the Bayesian information criterion (BIC). Four independent Markov chain Monte Carlo (MCMC) analyses, each with four chains (three heated and one cold), were run for 30.000.000 generations, sampling trees every 1000 generations. Four species were used as outgroups (Table 1). At the end of the analyses, the initial 25% of sampled trees were discarded as burnins. Convergence among independent analyses was assessed by monitoring values of the standard deviation of the split frequencies (< 0.05) in MrBayes, and parameter sampling was assessed with Tracer v.1.6 (Rambaut et al., 2014) based on the effective sample size (ESS) criterion (> 200). Statistical branch support was obtained through posterior probabilities (PPs), and the reliability of the clades was accepted according to the proposal by Hillis & Bull (1993) as follows: strong (≥0.95) and moderate (0.85–0.94). The final tree was previewed in FigTree v.1.4.2 (Rambaut 2014) and later edited in Adobe Photoshop CC®.

3. Results

3.1. DNA analysis

A total of 48 sequences of approximately 600 base pairs (bp) of the COI gene was obtained from four populations from Brazil previously identified as *S. rubrithorax*, and from one population from Venezuela and three populations from Mexico identified as in the *S. paynei* complex (Table 1). Of the sequences obtained, 115 sites were polymorphic, and 102 sites were parsimony informative. The Bayesian inference analyses recovered the populations collected in Roraima state (RR) as a monophyletic lineage (Fig. 2) with strong support (pp=1). The values for genetic distance between the populations from Roraima state and those from Minas Gerais and Mato Grosso were above 10% (Table 2), corroborating the presence of two independent lineages (cryptic species)

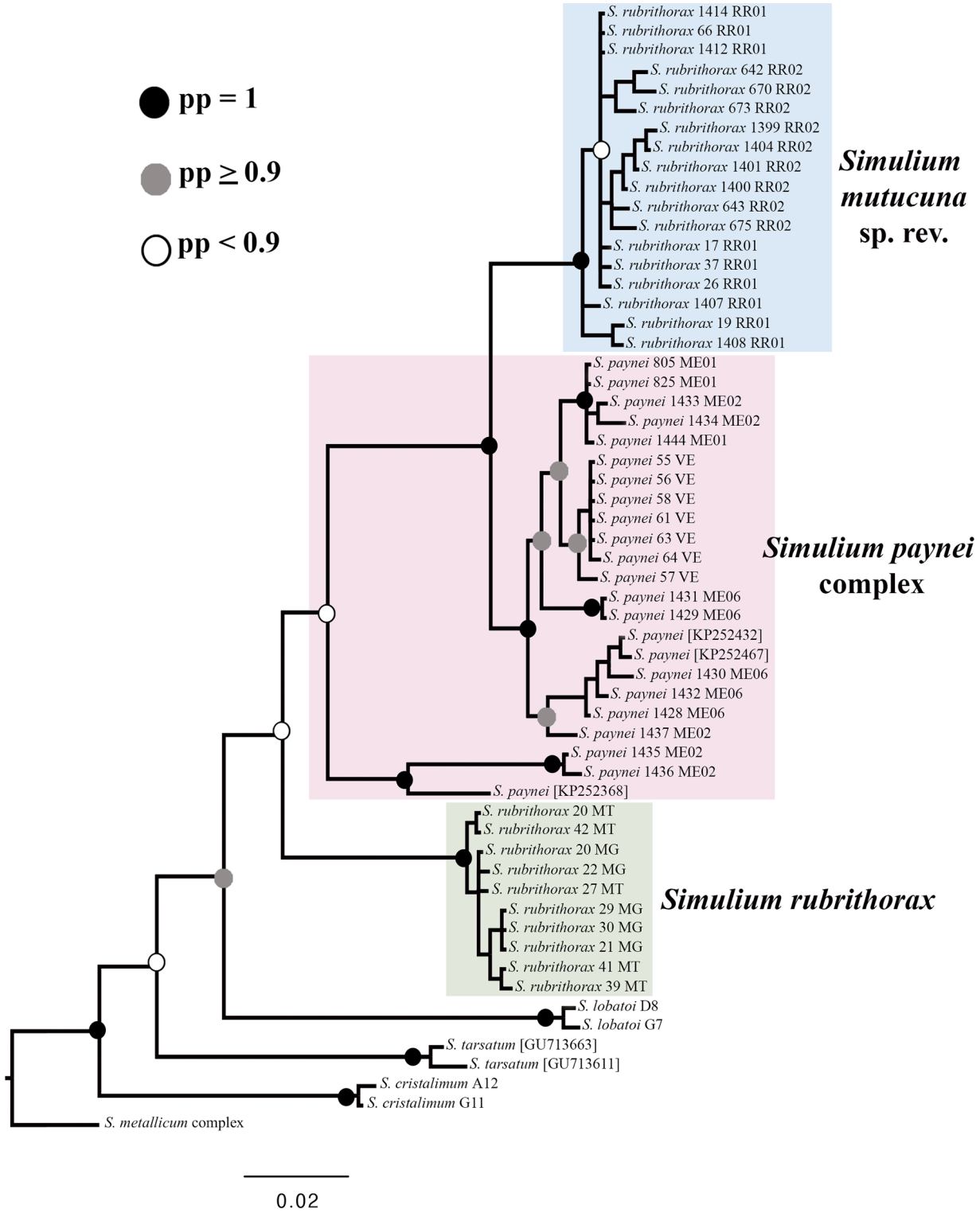

Fig. 2. Bayesian inference consensus of the post-burn-in tree resulting from the analysis of a fragment of the mitochondrial COI gene of species of *Simulium* (Diptera: Simuliidae) analyzed in this study. Black circles in the main branches indicate clades with posterior probability = 1; gray circles indicate clades with posterior probability ≥ 0.9; white circles indicate clades with posterior probability < 0.9.

Table 2
Pairwise genetic distance (%) between different populations of *Simulium* (Diptera: Simuliidae) based on the sequencing of a fragment of the mitochondrial COI gene. Analyses were conducted using the K2P model. Intrapopulational (diagonal and bold) and interpopulational (below the diagonal). Note: for details of the analyzed populations sites see Table 1.

	1	2	3	4	5	6	7
1.*Simulium mutucuna* (Brazil, RR01, Bananal river)	**0.90**						
2.*Simulium mutucuna* (Brazil, RR02, Pacu river)	1.03	**0.69**					
3.*Simulium paynei* complex (Venezuela)	4.15	3.70	**0.19**				
4.*Simulium paynei* complex (México)	5.61	5.17	2.96	**4.34**			
5.*Simulium paynei* complex (Costa Rica – GenBank sequences)	6.40	5.97	4.32	5.23	**6.09**		
6.*Simulium rubrithorax* (Brazil, Minas Gerais)	11.80	10.51	10.32	10.45	9.13	**0.44**	
7.*Simulium rubrithorax* (Brazil, Mato Grosso)	11.16	10.56	10.45	10.65	9.30	0.40	**0.27**

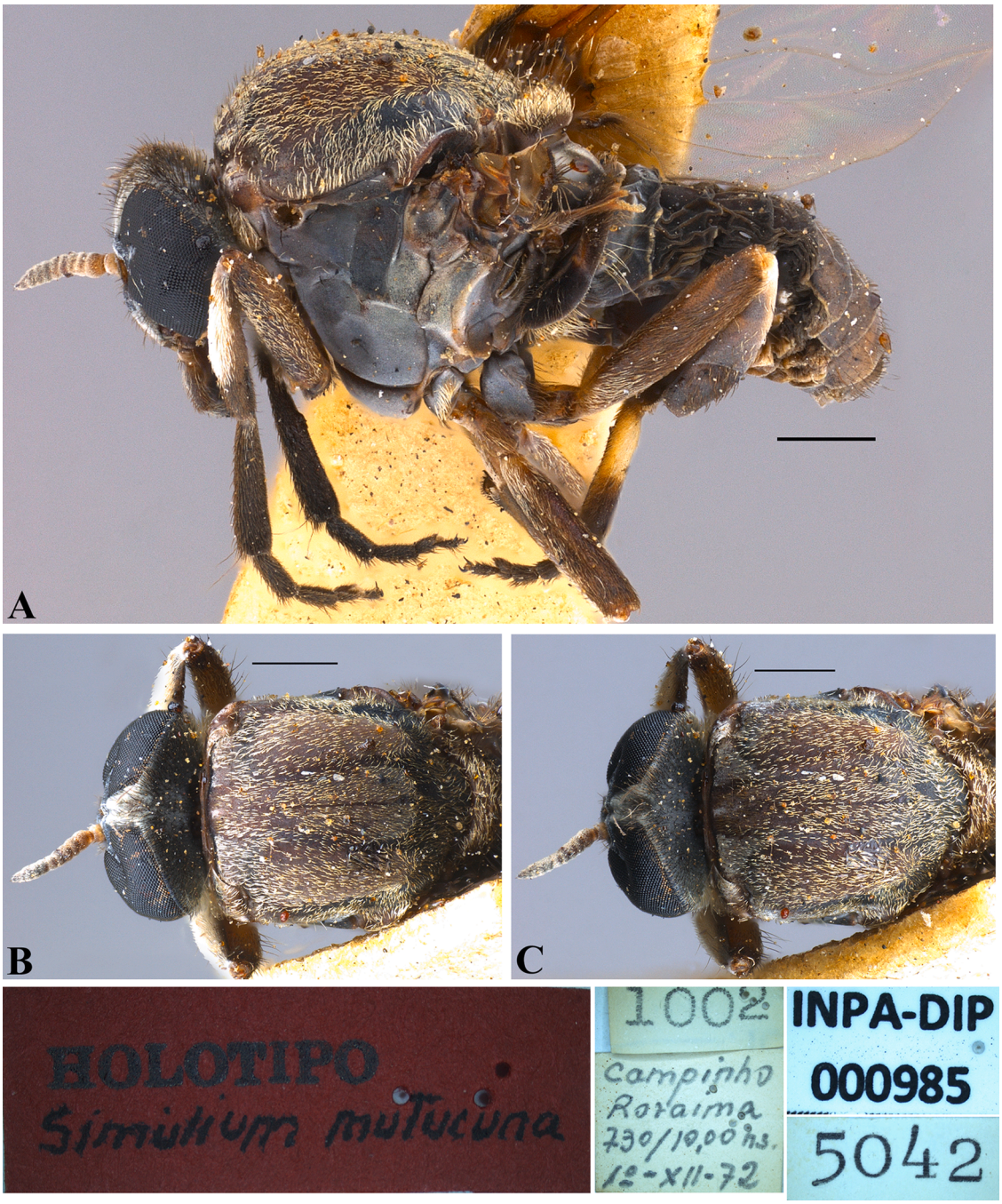

Fig. 3. *Simulium mutucuna* (Diptera: Simuliidae) female holotype collected in Roraima state, near the border with Venezuela. Material deposited in the Invertebrate Collection of the National Institute for Research in Amazonia (INPA). (A) Habitus, lateral view. (B) Scutum, dorsal view, with anterior illumination. (C) Scutum, dorsal view, with posterior illumination. (Bar = 0.5 mm).

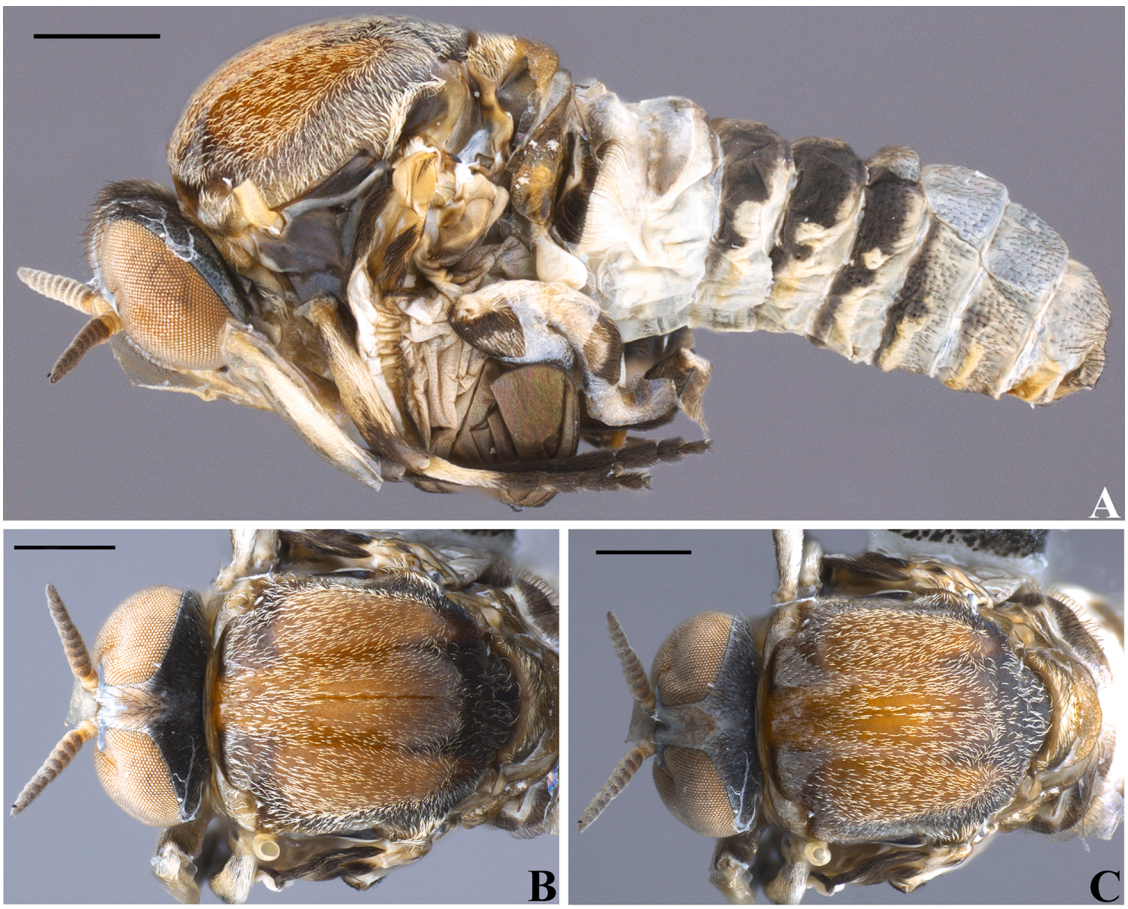

Fig. 4. *Simulium mutucuna* (Diptera: Simuliidae) pharate female extracted from pupa. (A) Habitus, lateral view. (B) Scutum, dorsal view, with anterior illumination. (C) Scutum, dorsal view, with posterior illumination. (Bar = 0.5 mm).

in this nominal species.

Our results indicated that populations of *S. rubrithorax* from Roraima state are more closely related to populations of the *S. paynei* complex than to the populations of *S. rubrithorax* from Minas Gerais and Mato Grosso states (Fig. 2). The genetic distances between the Roraima populations and the *S. paynei* complex were above 3.5% (Table 2). According to Rivera and Currie (2009), genetic divergence between species ranged from 2.83 to 15.33% among Simuliidae from the Nearctic Region. Hernández et al. (2012), in a study with *Simulium* (*Trichodagmia*) and related taxa in the New World, suggested that values of genetic divergence from 3.2 to 3.7% in species complexes are indicative of the presence of cryptic diversity. Based on our results we propose that *S. mutucuna* is a valid species because the analyzed populations in Roraima state were collected approximately 10 km from the type locality of this species.

3.2. Taxonomy

Simulium mutucuna Nunes de Mello & Vieira da Silva, sp. rev.

Simulium mutucuna Nunes de Mello & Vieira da Silva, 1974: 69. Holotype: female. Brazil: Roraima, Campinho, BR 174, near the border with Venezuela, 10.00 hrs; 12.xii.1972, without collector's name, INPA, no. 1002 (Examined). Treated as a synonym of *Simulium rubrithorax* by Crosskey and Howard (1997): 38; Hamada and Grillet (2001): 29–45; Alvan-Aguilar and Hamada (2003): 507–511; McCreadie *et al.* (2004): 183–196; Hernández *et al.* (2007): 12; Coscarón *et al.* (2008): 32; Shelley *et al.* (2010): 460; Hernández (2011): 232; Adler and Crosskey (2008)–2018 [Inventory]; Adler 2019, 2020 [Inventory]. Treated as a synonym of *Simulium paynei* by Coscarón (1987): 36; Coscarón and Coscarón-Arias (2007): 531.

Hemicnetha mutucuna; Py-Daniel and Moreira-Sampaio, 1995: 118.

Diagnosis

Female: scutum dark brown to orangish brown (Figs. 2A–C; 3A–C); hypogynial valves subtriangular, almost entirely membranous, except inner margins slightly sclerotized (Figs. 7A–F); anal lobe, in lateral view, with distal projection subquadrangular, sclerotized distally (Fig. 7C), in posteroventral view (*in situ*), distal projection medially directed (Fig. 7B). Male: scutum orangish yellow, with recumbent, thick golden hairs (Fig. 8A–C); ventral plate with main body rectangular, about three times wider than long, without developed shoulders, (Figs. 10C–F); median projection, in dorsal and ventral views, 2.5 times longer than wide, with a rounded base and apically with a V-shaped incision (Figs. 10C, D). Pupa: cocoon translucent, yellowish brown, boot-shaped, with reticulated area intertwining in a basket shape, with triangular or rhomboid-shaped fenestrations (Figs. 11A, B); head and thorax with gelatinous cover (Figs. 12A–C); with eight grayish-yellow, translucent, slightly wrinkled filaments; filaments distributed in a 2-dimensional pattern: main trunk short, giving rise to two sets of primary branches with four filaments each (Figs. 12A–C; 13D, E).

Holotype redescription

Female (Figs. 3A–C). Body sparsely covered with silver pruinosity; generally black. Body length (cervix to abdominal tip) = 3.7 mm; thorax, lateral length (cervix to anterior region of hind wing articulation) = 1.7 mm. Wing length = 4.5 mm; width = 2 mm.

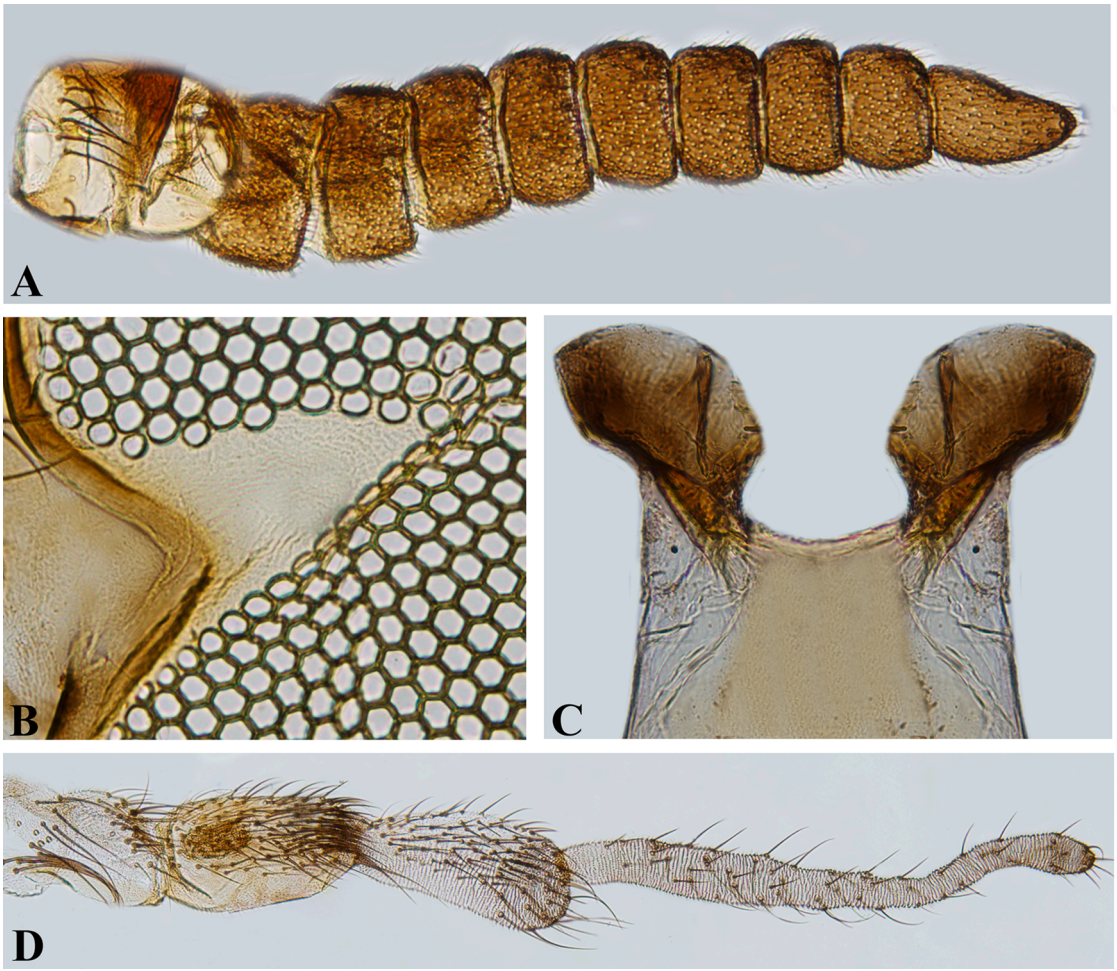

Fig. 5. *Simulium mutucuna* (Diptera: Simuliidae) pharate female extracted from pupa. (A) Antenna. (B) Fronto-ocular triangle. (C). Cibarium. (D). Maxillary palp.

Head. Antenna of 11 articles, with silver pruinosity; scape and pedicel orange, flagellomere dark brown. Eye black. Frons about 1.5 times as long as wide, with silver pruinosity; fronto-ocular suture absent.

Scutum dark brown, with recumbent golden hairs, posterolateral areas blackish. Scutal pattern varying slightly with incident light. With anterior light incidence (Fig. 3B): anterior ¾ dark brown, except for 1+1 sinuous, black marks on submedial region; posterior ¼ black, without evident pruinosity. With posterior light incidence (Fig. 3C): anterior region with 1+1 silver, club-shaped marks; posterior ¼ with silver pruinosity. Anepisternum and katepisternum grayish black, with silver pruinosity (Fig. 3A). Scutellum dark brown, with erect golden and black hairs on posterior margin. Postnotum black, with silver pruinosity. Wing: costa with spine-like setae among hair-like setae; Sc with hair-like, thick setae; R with hair-like and spine-like setae along entire length. Halter with base dark brown, apical region broken. Foreleg: coxa, trochanter, femur and tibia blackish brown, except anteromedial region of tibia whitish yellow; basitarsus and tarsomeres black. Middle and hind legs: coxae, trochanters and femora dark brown; tibiae blackish brown, except anteromedial regions yellowish; basitarsi and tarsomeres black.

Abdomen. Basal fringe with long, thin, golden hairs; segments blackish brown, with silver pruinosity (more evident on first segments).

Description of adult female based on new material

Pharate female extracted from pupa (Figs. 4A–C; 5A–D, 6A–E, 7A–G). Body covered with silver pruinosity. Body length (cervix to abdominal tip) = 3.9–4.5 mm (n = 3); thoracic lateral length (cervix to anterior region of hind wing articulation) = 1.6–1.9 mm (n = 3).

Antenna (Fig. 5A) length = 0.75–0.83 mm (n = 3). Eye orangish brown. Frons orangish gray with silver pruinosity; nudiocular area well developed (Fig. 5B). Maxillary palp grayish brown; palpomere V approximately 2.9–3.3 times as long as palpomere III; 2.5–2.7 times as long as palpomere IV; sensory vesicle small, spherical, approximately 1/3 as long as palpomere III (Fig. 5D). Mandible with 0–4 weak external serrations 35–38 internal teeth (n = 4). Lacinia with 22–30 retrorse teeth (n = 4). Cibarium with sclerotized cornuae, medial area with small teeth (Fig. 5C).

Scutum orangish brown, with blackish brown margins (Figs. 4A–C). Scutal pattern varying with incident light similar to holotype, except on anterior lighting with 1 + 1 anterolateral, subquadrangular, brownish mark (Figs. 4B). Legs (Figs. 6B–E): color pattern similar to the holotype, except coxae, trochanters, femora and tibiae lighter; hind leg with calcipala as broad as long, reaching pedisulcus; tarsal claws with short, conically-shaped, subbasal tooth, about one-quarter length of the main talon (Fig. 6E). Wing setation as in holotype (Fig. 6A); halter yellowish gray, except base blackish brown.

Abdomen. Cercus wider than long in lateral view (Fig. 7C), with long, brown setae. Anal lobe, in lateral view (Fig. 7C), with distal projection subquadrangular, sclerotized distally, with thin, short, golden hairs; proximal region lightly sclerotized, with thick, long, dark-brown hairs; in ventrolateral view (Fig. 7D) with distal projection oval, longer than wide; in posteroventral view (*in situ*, after clearing and partially dissected as in Fig. 7B), distal projection medially directed; inner margin with a membranous projection (Fig. 7B). Hypogynial valves (Fig. 7E) subtriangular, almost entirely membranous, except

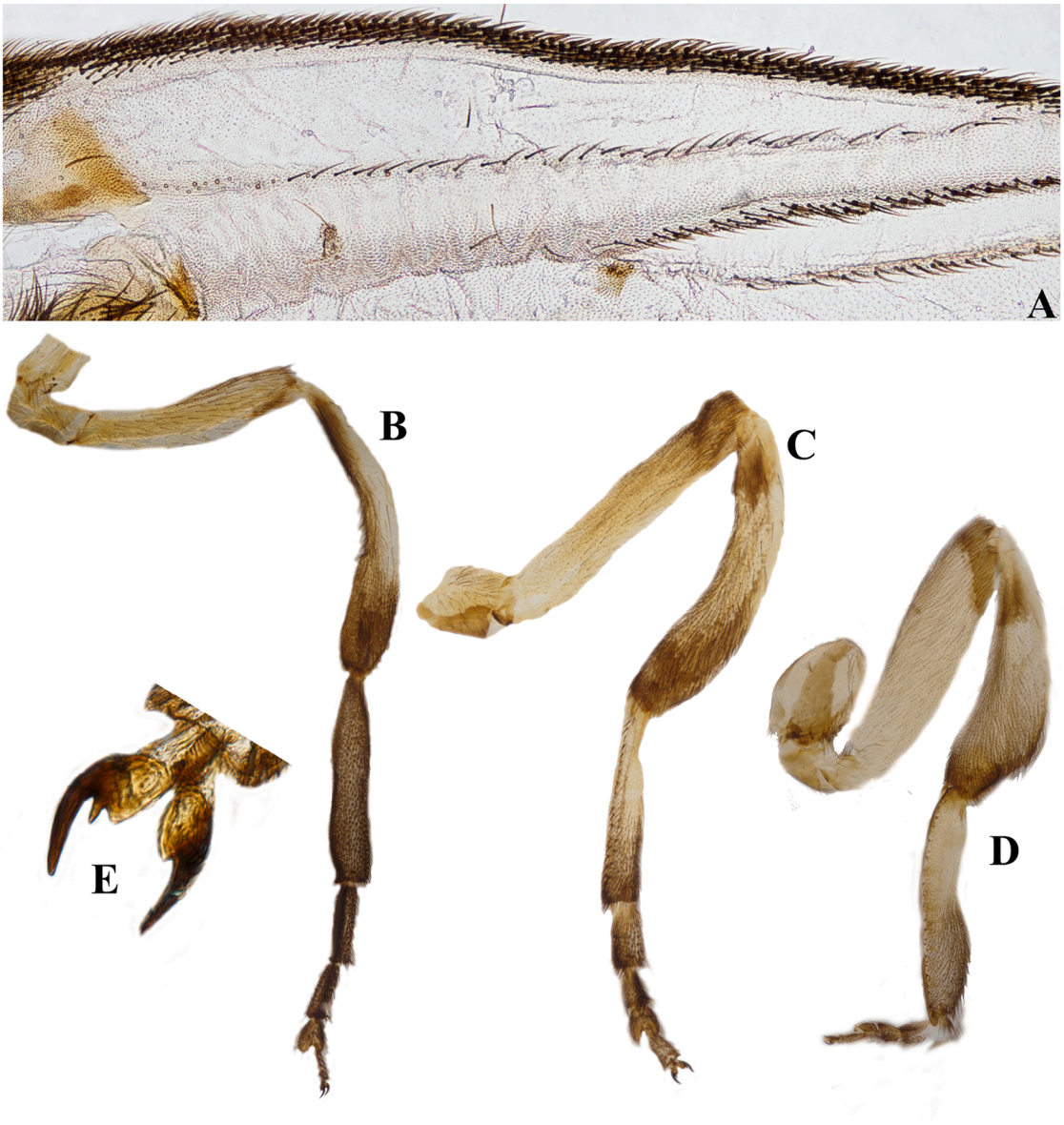

Fig. 6. *Simulium mutucuna* (Diptera: Simuliidae) pharate female extracted from pupa. (A) Base of wing. (B) Foreleg. (C) Middle leg. (D) Hind leg. (E) Tarsal claw with a small subbasal tooth.

inner margins slightly sclerotized; with microtrichia; valves *in situ* closely approximated (Fig. 7A). Genital fork (Fig. 7F) with stem longer than lateral arms, slightly expanded apically; lateral arms forming a broad U at junction with the stem; anteriorly directed apodeme well developed. Spermatheca subspherical, with cuticular microspines (Fig. 7G); spermathecal duct and area of attachment unpigmented.

Descriptions of previously unknown life stages of Simulium mutucuna

Pharate male extracted from pupa (Figs. 8A–C, 9A–F, 10A–I). Body covered with silver pruinosity. Body length (cervix to abdominal tip) = 3.0–4.0 mm (n = 3); thoracic lateral length (cervix to anterior region of hind wing articulation) = 1.16–1.20 mm (n = 3).

Antenna (Figs. 8A–C; 9B) with 11 articles, with silver pruinosity; length = 0.7–0.8 mm (n = 3); scape, pedicel and basal region of first flagellomeres yellowish brown, remainder dark brown. Eye orange. Clypeus with silver pruinosity. Palpus (Fig. 9C): palpomere III brown, palpomeres IV and V grayish brown; palpomere V approximately 3.1–3.5 times as long as palpomere III and 2.4–2.8 times as long as palpomere IV; sensory vesicle small, oval, approximately 1/3 length of palpomere III.

Scutum orangish yellow; with recumbent, thick golden hairs. Scutal pattern varying with direction of incident light. With anterior light incidence (Fig. 8B): anterior region with 1+1 oval, pearly, marks weakly evident; posterior region blackish brown. With posterior light incidence (Fig. 8C): anterior region with 1+1 oval, silver, evident marks; submedial region with 1+1 pearly bands weakly evident, distally incomplete; posterior region grayish brown with silver pruinosity. Anepisternum and katepisternum grayish, with silver pruinosity. Scutellum brown, with erect golden and black hairs on posterior margin. Postnotum brown, with silver pruinosity. Wing: costa with hair-like and spine-like setae; Sc almost entirely bare, except basal 1/3 with 5-6 hair-like setae; R with hair-like and spine-like setae along entire length (Fig. 9A). Halter yellowish white, except base blackish brown. Foreleg: (Fig. 9D): coxa, trochanter and femur yellowish, except distal region of femur brown; tibia dark brown, except medial region yellowish; basitarsus and tarsomeres blackish brown. Middle and hind legs: (Figs. 9E, F): coxae, trochanters and femora brown, tibiae darkish brown, except

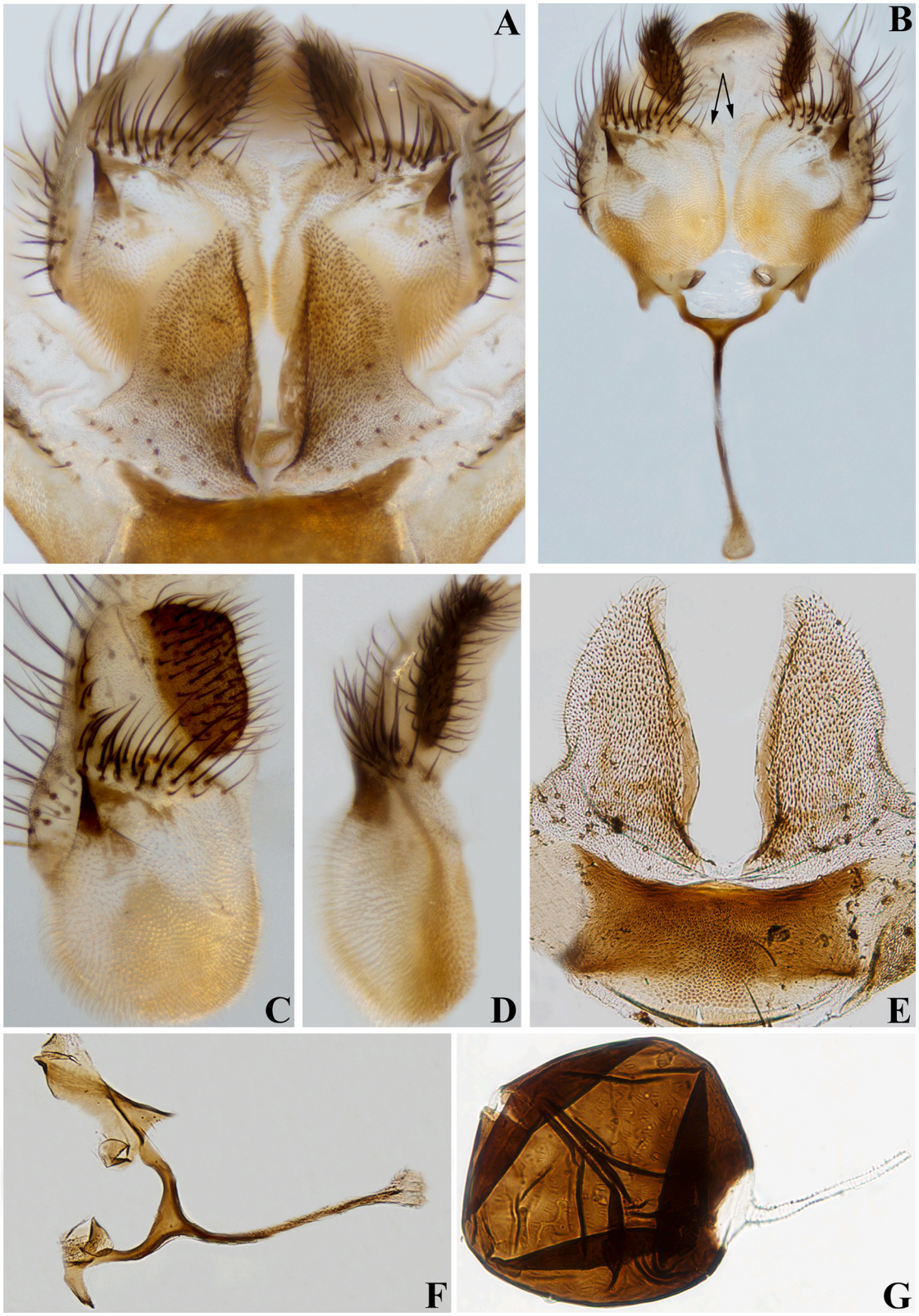

Fig. 7. *Simulium mutucuna* (Diptera: Simuliidae) pharate female extracted from pupa. (A, B) Terminalia after clearing. (A) *In situ*, posteroventral view. (B) Partially dissected; arrows indicate membranous projections on anal lobe. (C, D) Cercus and anal lobe. (C) Lateral view. (D) lateroventral view. (E) Sternite VIII and hypogynial valves, ventral view. (F) Genital fork. (G) Spermatheca.

Fig. 8. *Simulium mutucuna* (Diptera: Simuliidae) pharate male extracted from pupa. (A) Habitus, lateral view. (B) Scutum, dorsal view, with anterior illumination. (C) Scutum, dorsal view, with posterior illumination. (Bar = 0.5 mm).

medial regions yellowish brown; basitarsi and tarsomeres blackish brown, except proximal region of basitarsi yellowish brown.

Abdomen. Basal fringe with long, thin, blackish brown hairs; tergites grayish brown, with silver pruinosity laterally. Gonocoxite and gonostylus (Figs. 10A, B, G) brown, except distal 2/3 of gonocoxite translucent; covered with thick, brown hairs; in ventral view, gonocoxite trapezoidal, wider than long, outer apical angle rounded. Gonostylus approximately two times longer than width, about twice the gonocoxite length; gonostylus (*in situ*, Fig. 10A) with outer margin strongly convex, inner margin slightly concave distally, bearing 1 or 2 stout spinules at apex (Fig. 10G). Ventral plate (Figs. 10C–F): main body rectangular, about three times wider than long, without developed shoulders, with median projection strongly developed, covered with thin setae; in dorsal view, (Fig. 10C), base of median projection occupying the entire central area of the main body, lateral margins of median projection with pleated folds, concave medially; in ventral view (Fig. 10D), median projection longer than wide, base wider than the apex, lateral margins parallel, apically with a V-shaped incision, ventral keel not evident; in lateral view (Fig. 10E), median projection 2.5 times longer than wide, with rounded base and subtriangular apex, margin almost entirely sinuous; in ventral view (with apex tilted dorsally) (Fig. 10F) median projection triangular. Median sclerite club-shaped, with distinct incision medially (Fig. 10H). Paramere with basal process strongly developed and sclerotized; aedeagal membrane with spicules (Fig. 10I). Dorsal plate dome-shaped (Fig. 10J).

Pupa (Figs. 11A, B; 12A–C; 13A–F; 14A–J). Cocoon dorsal length 4.4–5.9 mm (5.2 ± 0,2 mm, $n = 8$); ventral length 2.9–4.2 mm (3.8 ± 0.12 mm, $n = 8$); pupa lateral length 4.3–5.0 mm (4.5 ± 0.13 mm, $n = 8$).

Cocoon boot-shaped, enclosing thorax, constructed of tightly woven yellowish-brown silk; anterior rim thickened, aperture high (anterior region strongly elevated) with reticulated area intertwining in a basket shape, with triangular or rhomboid-shaped fenestrations occupying approximately 50% of the elevated area of the cocoon (Figs. 11A, B; 13F).

Head and thorax covered with gelatinous matrix (Figs. 12A–C). Head projected downward; cephalic plate with 1 + 1 short, thin, unbranched facial trichomes and 2 + 2 short, thin, unbranched frontal trichomes (facial and frontal trichomes similar in length) (Figs. 13A, B), clypeus with short, pointed tubercles (Fig. 13C). Thorax with 5 + 5 thin, unbranched trichomes; with pointed tubercles (similar to the head) located on lateral and posterior region. Gills (Figs. 12A–C; 13D, E) with eight, grayish-yellow, translucent, slightly wrinkled filaments; filaments with rounded apex, covered with microspicules along entire length and distributed in a 2-dimensional pattern: main trunk short, giving rise to two sets of primary branches with four filaments each; filaments branching at similar heights and approximately equal in length, basal fenestra not evident. Abdomen as in Fig. 14. Tergite I weakly sclerotized, with microspines medially near posterior margin (Fig. 14A); tergite II with 3 + 3 unbranched hooks near posteromedial margin (Fig. 14C); tergites III and IV each with 4 + 4 anteriorly directed pairs of stout, unbranched hooks on posterior margin (Fig. 14D); tergites II–IV with comb-like groups of thick microspines on anterior margin (Fig. 14B); tergites III–VIII with comb-like groups of thin microspines near anterior margin (Fig. 14E); tergite IX without terminal spines. Sternites III–VIII with comb-like groups of microspines near anterior margin (Figs 14F, J); sternite IV with 2 + 2 stout, unbranched hooks near posterior margin, members of pair near one another (Fig. 14G); sternites VI and VII with 2 + 2 stout, unbranched hooks medially located, members of pair widely separated (Figs 14H, I).

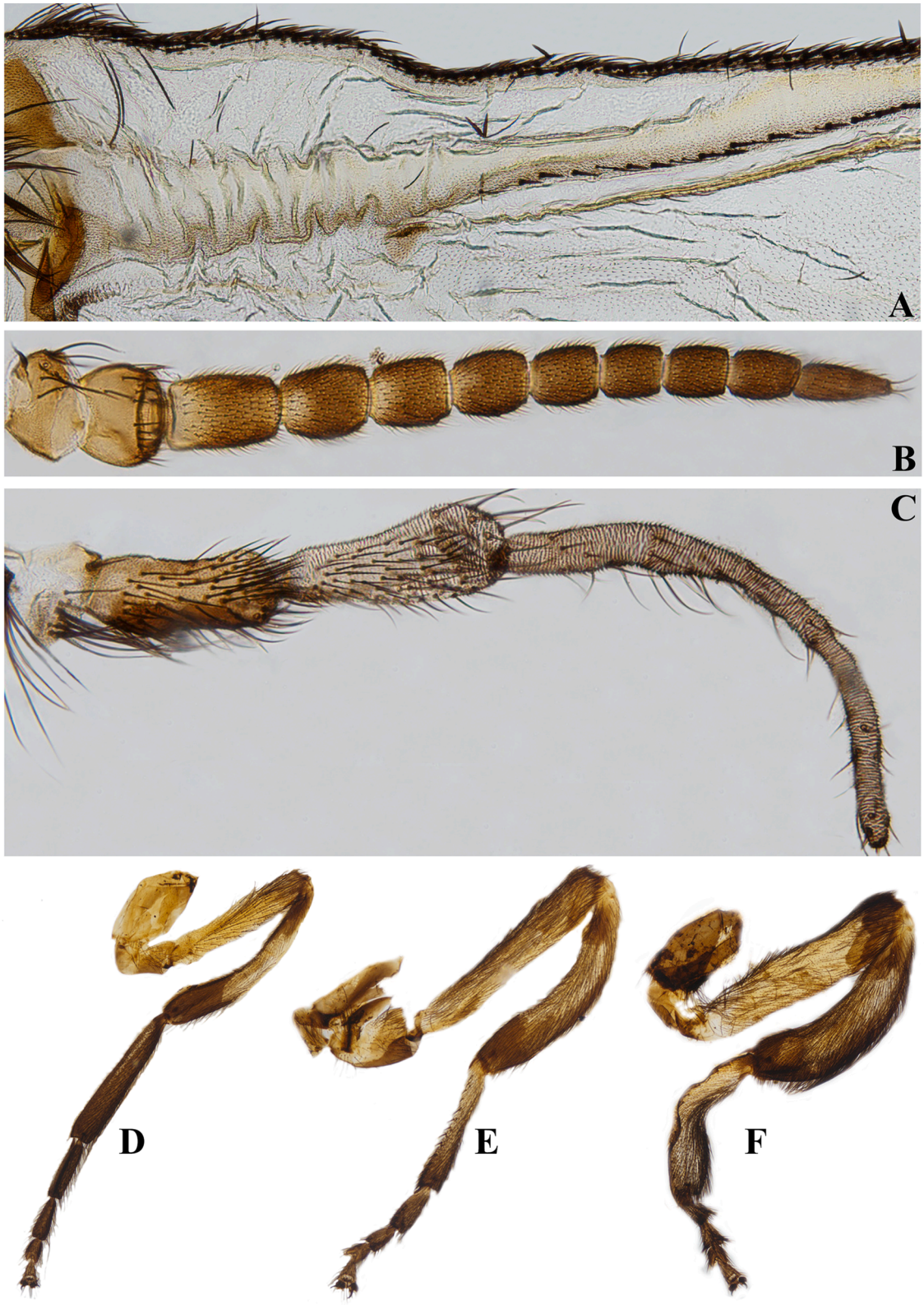

Fig. 9. *Simulium mutucuna* (Diptera: Simuliidae) pharate male extracted from pupa. (A) Base of wing. (B) Antenna. (C). Maxillary palp. (D) Foreleg. (E) Middle leg. (F) Hind leg.

Fig. 10. *Simulium mutucuna* (Diptera: Simuliidae) pharate male extracted from pupa. (A, B) Terminalia *in situ*. (A) Ventral view. (B) Posterior view. (C–F) Ventral plate. (C) Dorsal view. (D) Ventral view. (E) Lateral view. (F) Ventral view with distal margin tilted dorsally. (G) Gonostylus and gonocoxite and detail of the apex of gonostylus showing spinules. (H) Median sclerite. (I) Paramere and aedeagal membrane. (J) Dorsal plate.

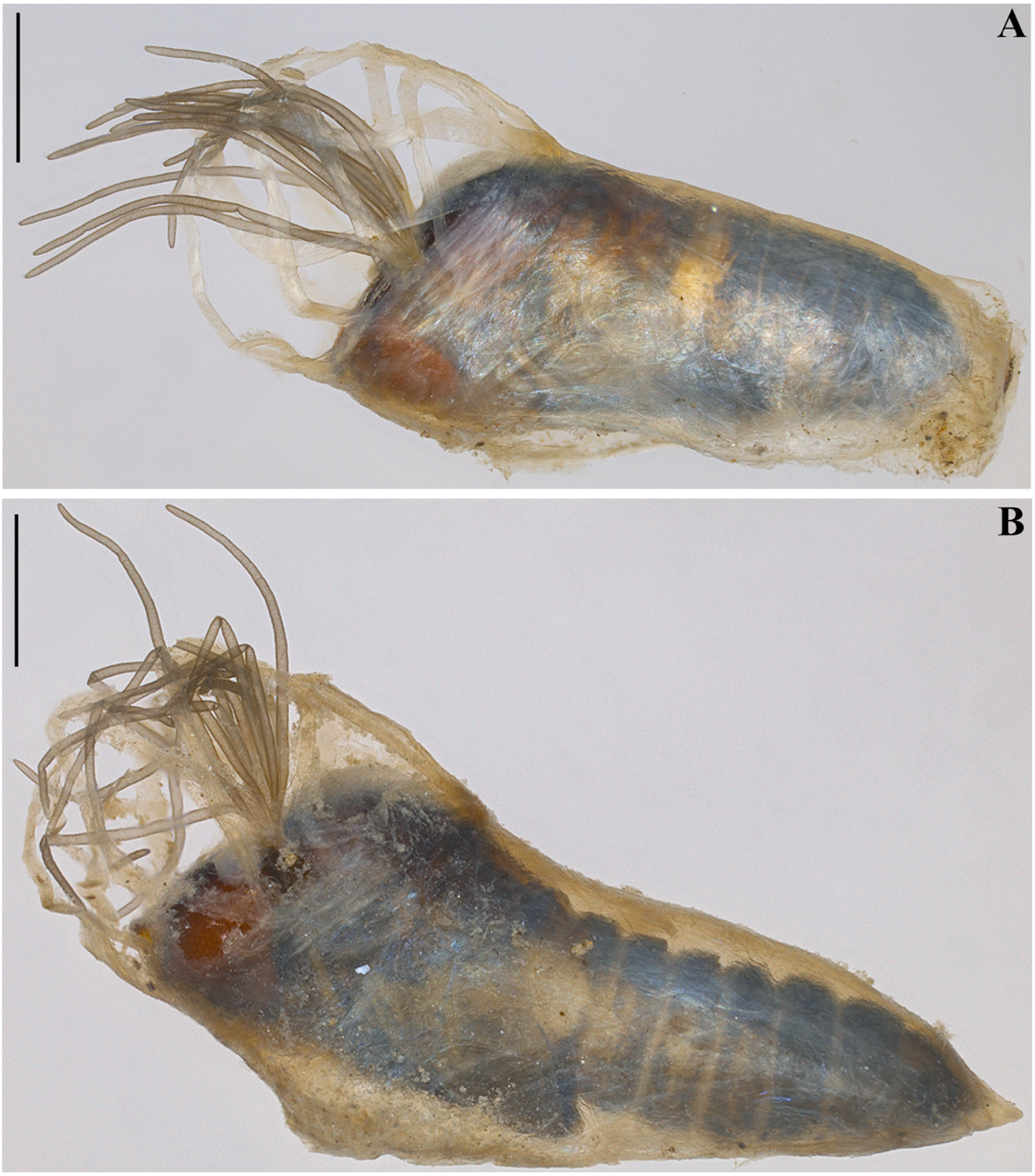

Fig. 11. *Simulium mutucuna* (Diptera: Simuliidae) pupa. (A, B) Lateral views showing variations in the cocoon. (Bar = 1 mm).

Larva (Final Instar) (Figs. 15A, B; 16A, B; 17A–C; 18A–F). Body length 10.8–12.3 mm (11.2 ± 0.60 mm, $n = 8$); head capsule lateral length 0.68–0.79 mm (0.7 ± 0.08 mm, $n = 8$).

Body coloration generally green to greyish green, whitish ventrally; posterior region of abdomen ventrally enlarged (Figs. 15A, B). Head capsule varying from dark brown to yellowish brown, with unbranched, short setae; in dorsal view (Figs. 16A, B; 17C) transversally wrinkled, frontoclypeal apotome with two small, oval marks located submedially on basal 2/3 and one elongated mark located medially near posterior margin. Cervical sclerites small, elliptical, free in membrane (Fig. 18D). Postgenal cleft subtriangular in shape, deep, its apex nearly attaining the hypostomal groove; subesophageal ganglion pigmented (Figs. 17A, B; 18B); postgenal bridge 0.8 times hypostomal length (Fig. 18B). Antenna equal in length to or slightly longer than labral fan stalk, brownish, with some areas translucent (Figs. 16A, B; 17C; 18A), proportions of articles (proximal: medial: distal, excluding apical sensillum) 1.0: 1.11–1.15: 0.65–0.7 (n=4). Labral fan with 49–61 primary rays, brownish. Hypostoma (Figs. 18B, C) with anterior margin slightly convex, strongly pigmented; medial region with a longitudinal light brown band; anterior margin with nine, well-differentiated teeth; median tooth longer than lateral teeth; lateral and sublateral teeth approximately similar in height; 5–7 + 5–7 lateral serrations, 1 + 1 rows of 7 or 8 sublateral setae, and 1 + 1 filiform setae near posterior margin. Lateral mandibular process absent; mandibular teeth: 1 apical, 2 external, 3 subapical, 8 or 9 internal teeth; mandibular serration singular, with small mandibular sensillum (*sensu* Craig and Craig 1986). Gill histoblast *in situ* (Fig. 17C) composed of eight blackish filaments, grayish basally. Body cuticle bare, ventral tubercles absent. Anal sclerite with anterodorsal arms 0.4–0.6 (n = 4) as long as posteroventral arms, associated with thin, short setae (Fig. 18E). Posterior circlet bearing 290–310 rows with 35–45 hooks (n = 4). Rectal papilla (Fig. 18F) with 3 lobes, each with approximately 14–20 lobules (n = 4); total number of lobules 48–53 (n = 4) (Fig. 19).

Fig. 12. *Simulium mutucuna* (Diptera: Simuliidae) pupa without cocoon. (A) Lateral views, seta showing the gelatinous cover on head and thorax. (B) Lateral view, partial view of anterior region. (C) Dorsal view, partial view of anterior region. (Bar: A = 0.2 mm; B, C = 0.5mm).

3.3. Bionomics

Immature stages of *S. mutucuna* were collected in two rivers in two different mountain ranges in Roraima state: Pacaraima and Serra da Mocidade (Table 1). In the Pacaraima mountains the stream width was 3 m, water temperature 23°C, pH 7.2, and electrical conductivity was 20 µS/cm; the stream had a well-preserved riparian forest and riverbed composed of large rocks, gravel and sand. In the Serra da Mocidade mountains, the stream width was 20–25 m (much larger from the one in the Pacaraima mountains) and the collected area was exposed to sunlight, water temperature 22°C, pH 8.0, electrical conductivity 26 µS/cm, and stream riverbed with large rocks and sand. In both streams, larvae and pupae were collected on submerged tree branches and rocks in sections with rapid flow.

4. Discussion

As of now, the known distribution of *S. mutucuna* is restricted to Roraima (see examined material). Hamada and Grillet (2001), in the study on Simuliidae collected in the Pacaraima and Grande Savana regions (northern Roraima and southern Venezuela, respectively) recorded *S. rubrithorax* for the first time in Roraima and northern Brazil, based on pupae and larvae collected in the Bananal stream in Pacaraima municipality. Later, Alvan-Aguilar and Hamada (2003) carried out a biological study with specimens from that same location, reporting, again, *S. rubrithorax* for Roraima state. Based on the information presented here, the record of *S. rubrithorax* for Roraima and the North region of Brazil should be removed and updated as *S. mutucuna*.

With the description of all stages (except egg) of *S. mutucuna* presented here, a series of morphological differences may now be used to differentiate this species from both *S. rubrithorax* and *S. paynei*, species with which *S. mutucuna* has already been hypothesized as synonyms (Coscarón, 1987; Hernández, 2011; Hernández et al., 2007). These synonyms were mainly justified by the general color pattern of the thorax of females, which is similar in these three species (Figs. 3B, C; 4B, C; 19A, B).

In the male adults, the most useful diagnostic characteristics for identification are in the terminalia (Figs. 10A–E; 20A–F; 21A–F). In ventral view, the ventral plate of *S. mutucuna* is most similar to that of *S. rubrithorax* (Fig. 20D) by having the basal area of the median projection wider than the apical area and by the apex having a V-shaped incision. In *S. paynei* the basal area of the median projection is as wide as the apical area, and the apex is almost straight, without an evident V-shaped incision. In *S. mutucuna*, the lateral margins of the median projection are parallel, whereas in *S. rubrithorax*, they are sinuous. In lateral view, *S. mutucuna* has the apical region of the main body of the ventral plate detached from the median projection (Fig. 10E), whereas in *S. paynei* and *S. rubrithorax* the median projection is attached to the apical region of the main body of the ventral plate (Figs. 20E; 21E). In addition, in lateral view, the shape of the median projection in these species is different: in *S. mutucuna*, the outer margin of the projection is sinuous along almost its entire length; in *S. paynei*, the sinuosity is less evident and restricted to the apical region, while in *S. rubrithorax* the outer margin is concave in the median region (peanut-shaped). In ventral view (with the apex tilted dorsally), the median projection in *S. mutucuna* (Fig. 10F) and *S. paynei* (Fig. 21F) is longer and narrower

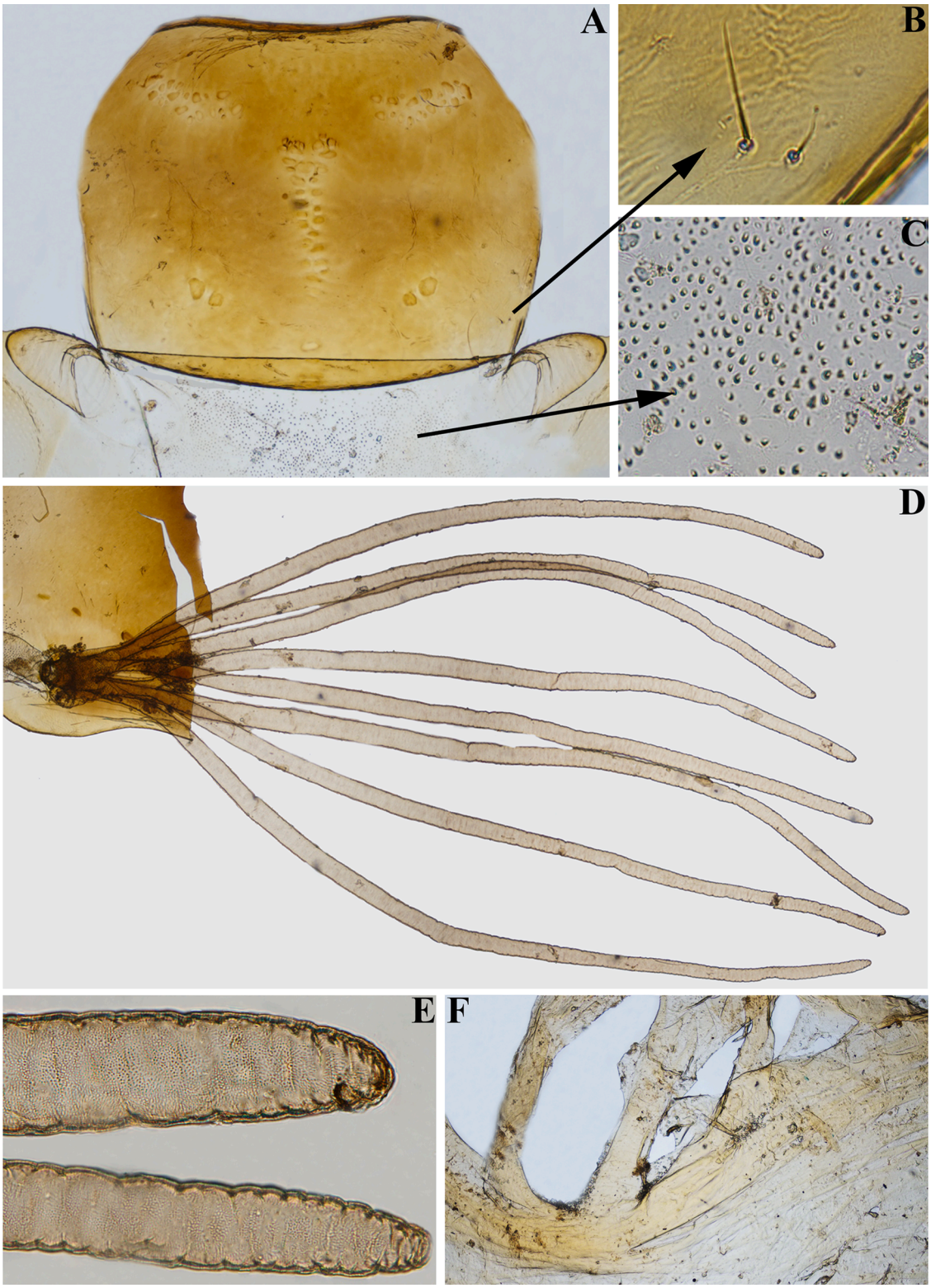

Fig. 13. *Simulium mutucuna* (Diptera: Simuliidae) pupa, slide mounted. (A) Cephalic plate. (B) Cephalic trichomes. (C) Clypeal tubercles. (D) Gill filaments and part of thorax. (E) Gill apices. (F) Middle area of anterior margin of cocoon.

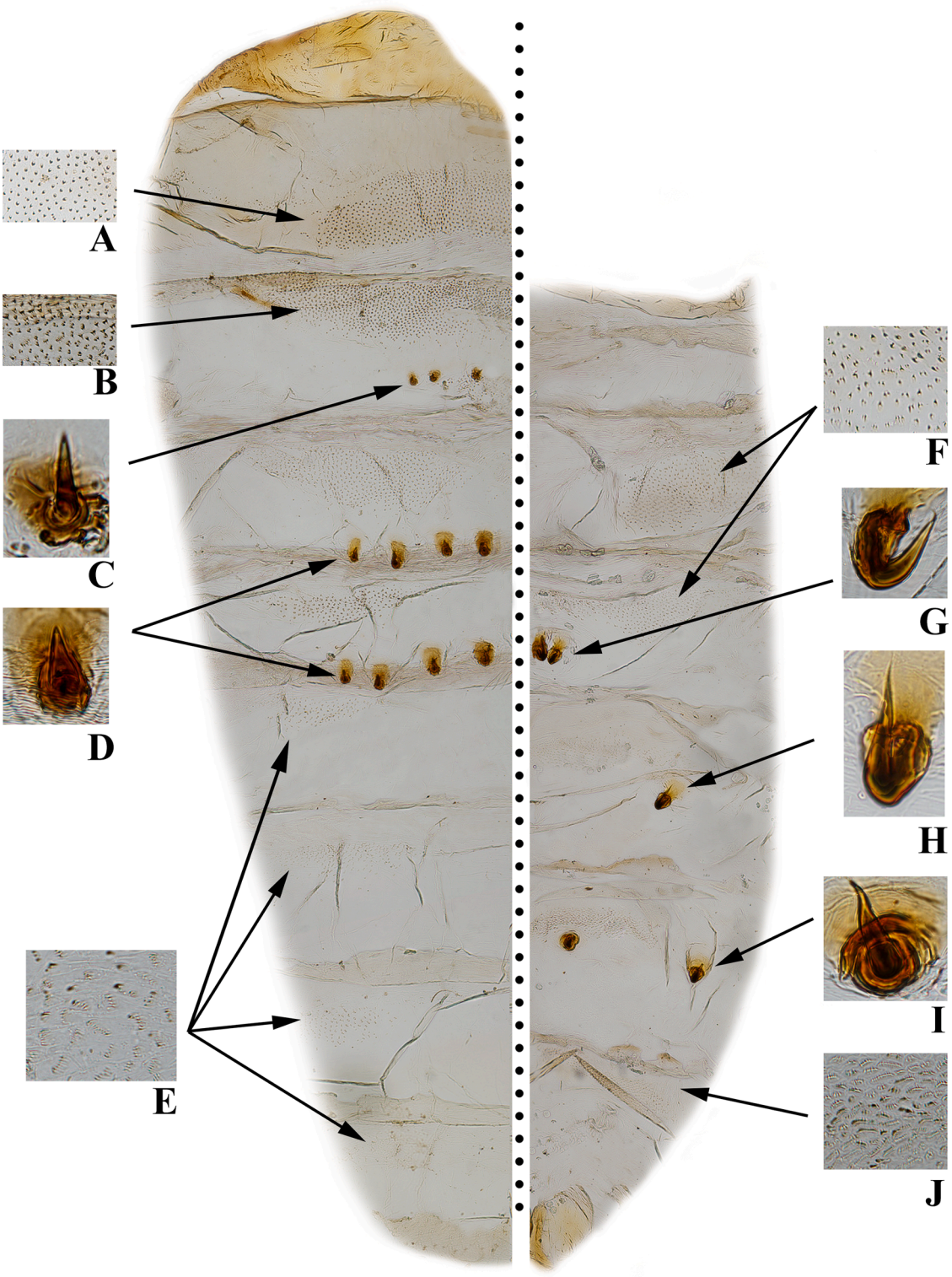

Fig. 14. *Simulium mutucuna* (Diptera: Simuliidae) pupal abdomen; left: tergites, right: sternites. (A) Microspines on tergite I. (B) Comb-like groups of thick microspines on tergites III–VIII. (C, D) Unbranched hooks on tergites III–IV. (E) Comb-like groups of thin microspines on tergites III–VIII. (F, J) Comb-like groups of microspines on sternites III–VIII. (G, H, I) Stout, unbranched hooks on sternites IV–VI.

Fig. 15. *Simulium mutucuna* (Diptera: Simuliidae) larva. (A, B) Habitus, lateral view, showing color variation (Bar: 2 mm).

than in *S. rubrithorax* (Fig. 20F); however, in *S. mutucuna* the projection is triangular, with the basal region wider than the apical region, while in *S. paynei* the projection is rectangular, with the apical and basal regions approximately of the same width. The gonostylus (in ventral view, *in situ*) also can be differentiated as follows: in *S. mutucuna*, the outer margin is strongly convex medially (Fig. 10A), whereas in *S. paynei* and *S. rubrithorax* it is smoothly curved, without abrupt enlargement (Figs. 20A and 21A).

The female terminalia in *S. mutucuna, S. rubrithorax* and *S. paynei* are very similar, as illustrated in the Figs. 7C, E and 22A–D, which illustrate structures that are usually variable in other species groups or subgenera in the family. However, the presence of the sclerotization in the hypogynial valves and in the distal projection of the anal lobe can be useful to differentiate these species. In *S. mutucuna*, the hypogynial valves are almost entirely membranous, except inner margins slightly sclerotized (Fig. 7E); in *S. rubrithorax* and *S. paynei* the central area of the valves is sclerotized (Figs. 22A, C). In the anal lobe, in lateral view, the distal projection in *S. mutucuna* has the apical half almost entirely sclerotized (Fig. 7C), while in *S. rubrithorax* only a small apical area near the margin is sclerotized (Fig. 22B); in *S. paynei* the apical half of the distal projection is totally membranous (Fig. 22D).

Regarding the general coloration of the body, the darker color of the female holotype (Figs. 3A–C), when compared to the additional females analyzed in this study (Figs. 4A–C), is probably due to the fact that it was collected when engaged in hematophagy. However, the general pattern of the thorax, especially with posterior illumination, is similar in the

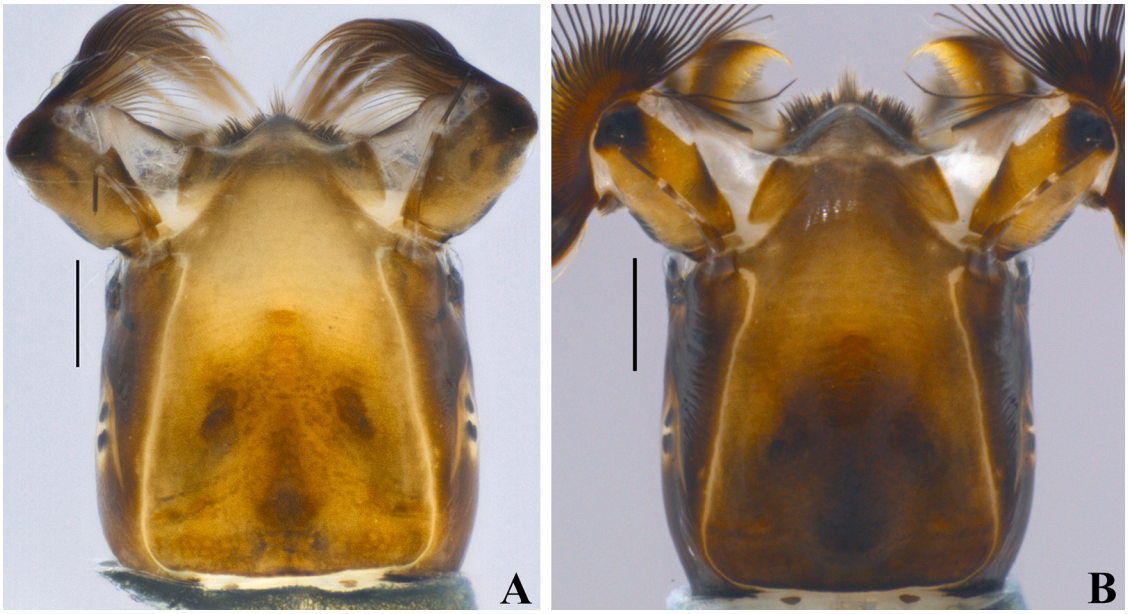

Fig. 16. *Simulium mutucuna* (Diptera: Simuliidae) larval head. (A, B) Dorsal view showing variation in pigmentation. (Bar = 0.2 mm).

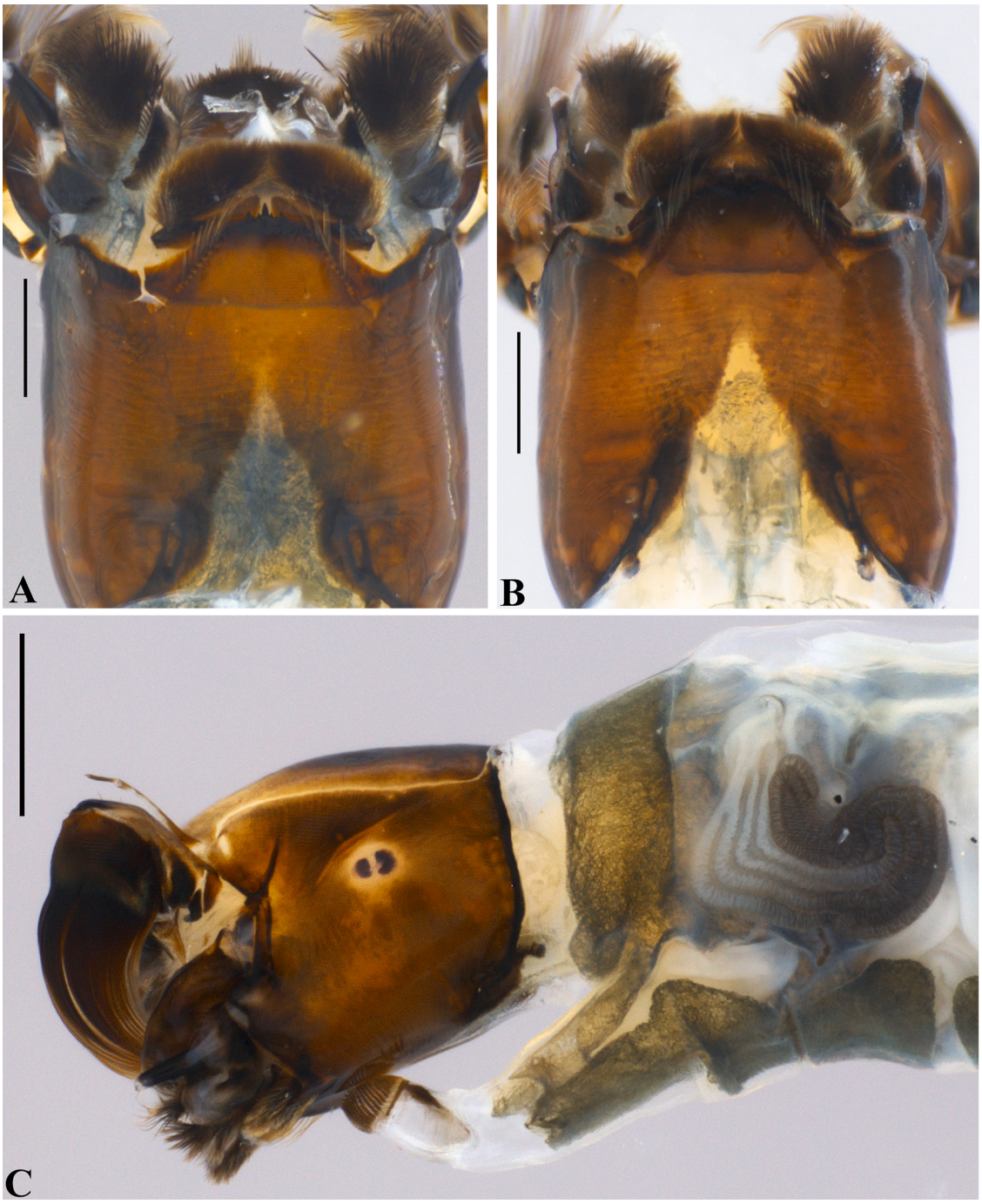

Fig. 17. *Simulium mutucuna* (Diptera: Simuliidae) larva. (A, B) Head, ventral view showing variation in pigmentation. (C) Head and thorax, lateral view, detail. (Bar: A, B = 0.2 mm; C = 0.5mm).

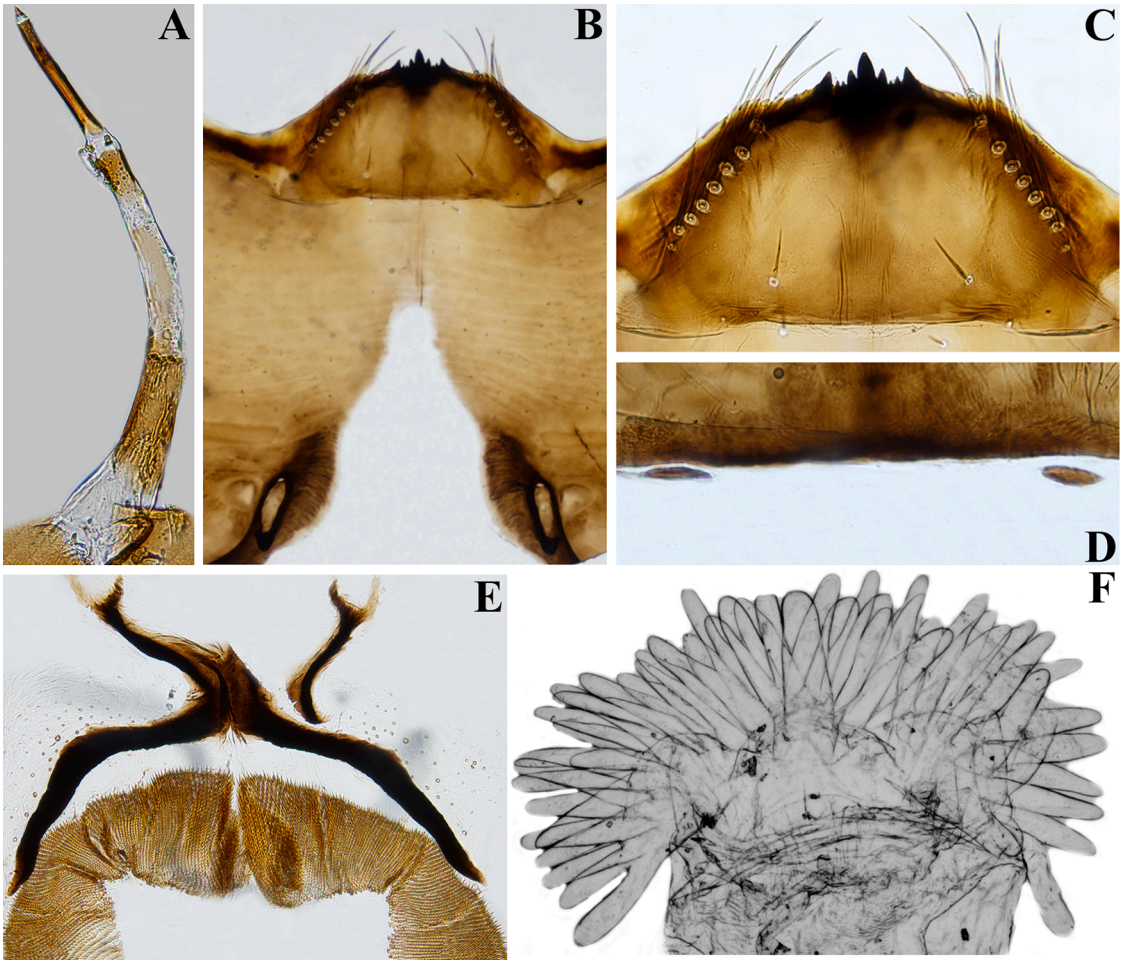

Fig. 18. *Simulium mutucuna* (Diptera: Simuliidae) larva. (A) Antenna. (B) Hypostoma and postgenal cleft, ventral view. (C) Hypostoma, ventral view, detail. (D) Cervical sclerites. (E) Anal sclerite and a partial region of posterior circlet. (F) Rectal papilla.

holotype and in the new females analyzed.

In the pupal stage, *S. mutucuna, S. rubrithorax* and *S. paynei* are very similar, sharing a series of important diagnostic features, such as the number of gill filaments (eight) and branch pattern (main trunk short, giving rise to two sets of primary branches with four filaments each). In addition, the presence of a gelatinous matrix covering the head and thorax and the cocoon with reticulated area intertwining in a basket shape, with triangular or rhomboid-shaped fenestrations, are characteristics shared with other members of the *S. tarsatum* group. Nevertheless, differences in the pattern of the fenestration can be useful in differentiating these three species. In *S. paynei*, the fenestrations have a rectangular appearance (Fig. 23B), whereas in *S. mutucuna* and *S. rubrithorax* they are triangular to rhomboid in shape (Figs. 23A, C). However, the ratio between width and height of the fenestrate area can help in the diagnosis of the latter two species: in *S. mutucuna*, this region is 1.5X wider than long, while in *S. rubrithorax* it is approximately as wide as long.

In the larval stage, morphological differentiation between some species in this group is difficult, mainly due to the similarity in the configuration of the gill histoblast and the lack of consistent diagnostic characters in this life stage. An illustration of this can be found in the study of Alvan-Aguilar and Hamada (2003), who conducted a biometric

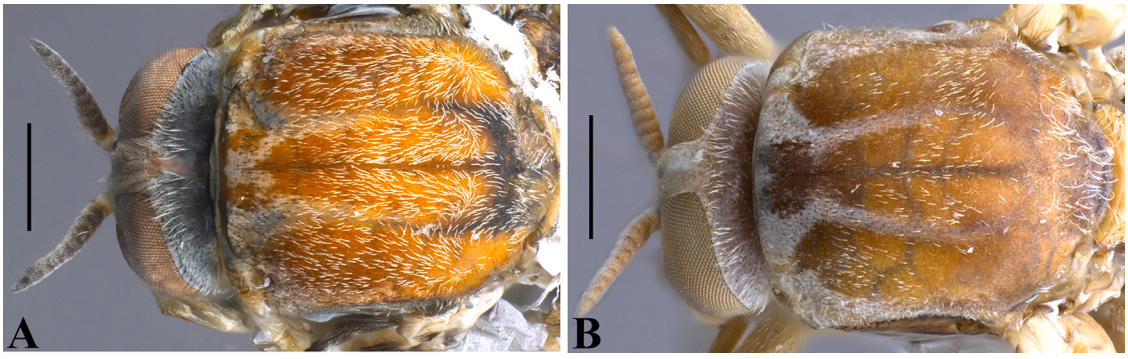

Fig. 19. Pharate females of *Simulium* (Diptera: Simuliidae) extracted from pupa. (A, B) Scutum, dorsal view, with anterior illumination. (A) *Simulium rubrithorax*. (B) *Simulium paynei*. (Bar = 0.5 mm).

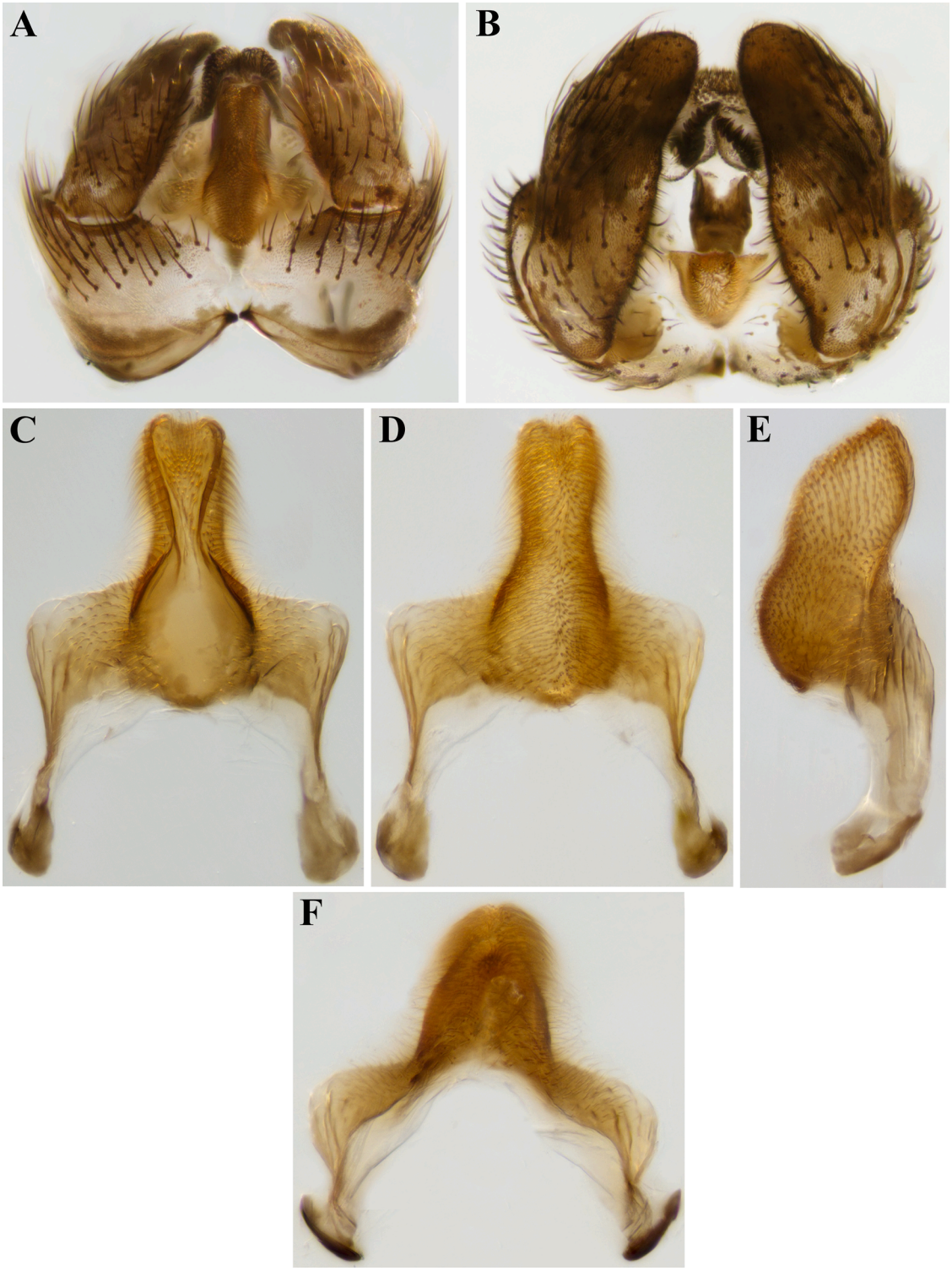

Fig. 20. *Simulium rubrithorax* (Diptera: Simuliidae) pharate male extracted from pupa. (A, B) Terminalia *in situ*. (A) Ventral view. (B) Posterior view. (C–F) Ventral plate. (C) Dorsal view. (D) Ventral view. (E) Lateral view. (F) Ventral view with distal margin tilted dorsally.

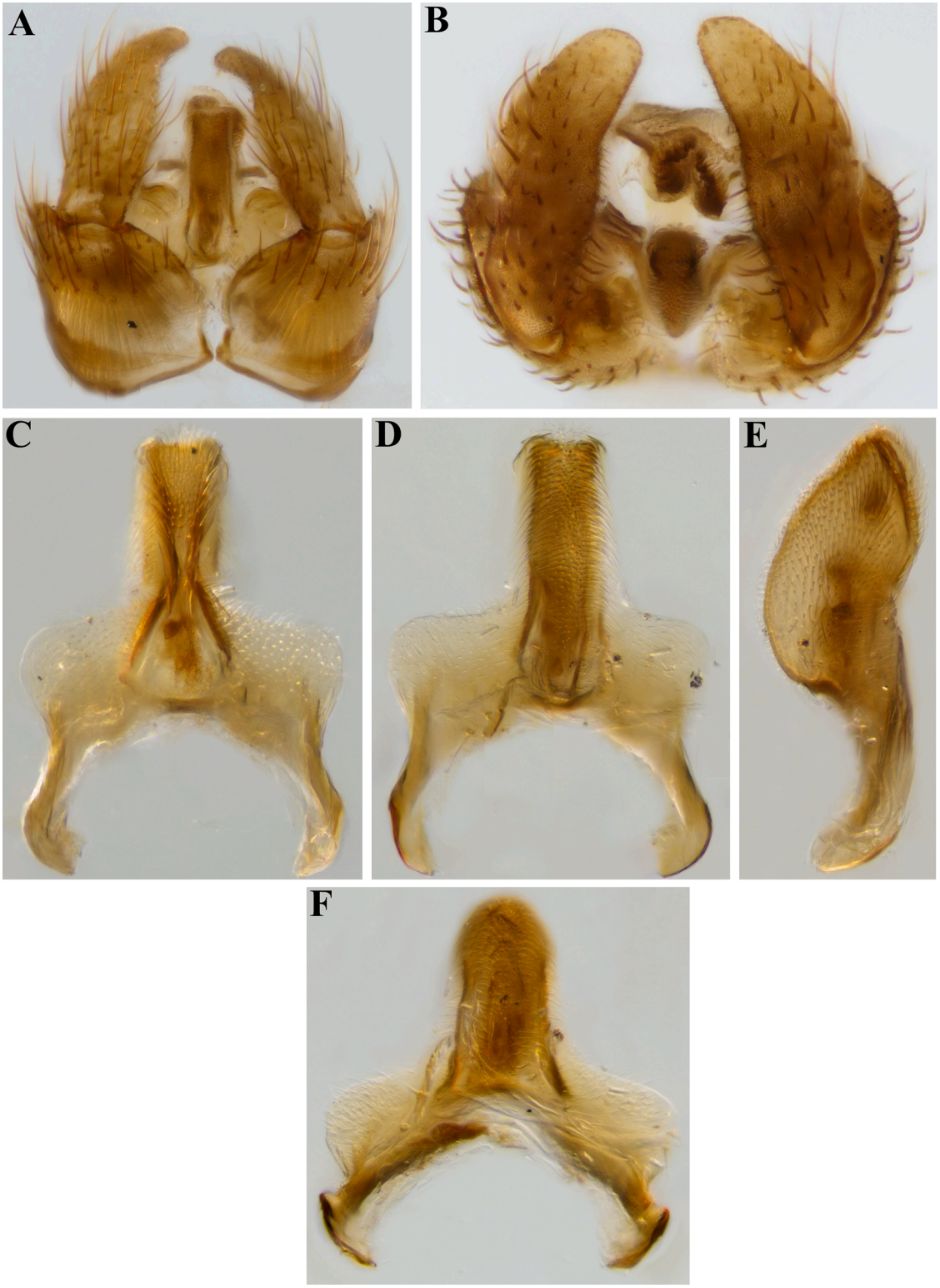

Fig. 21. *Simulium paynei* (Diptera: Simuliidae) male. (A, B) Terminalia *in situ*. (A) Ventral view. (B) Posterior view. (C–F) Ventral plate. (C) Dorsal view. (D) Ventral view. (E) Lateral view. (F) Ventral view with distal margin tilted dorsally.

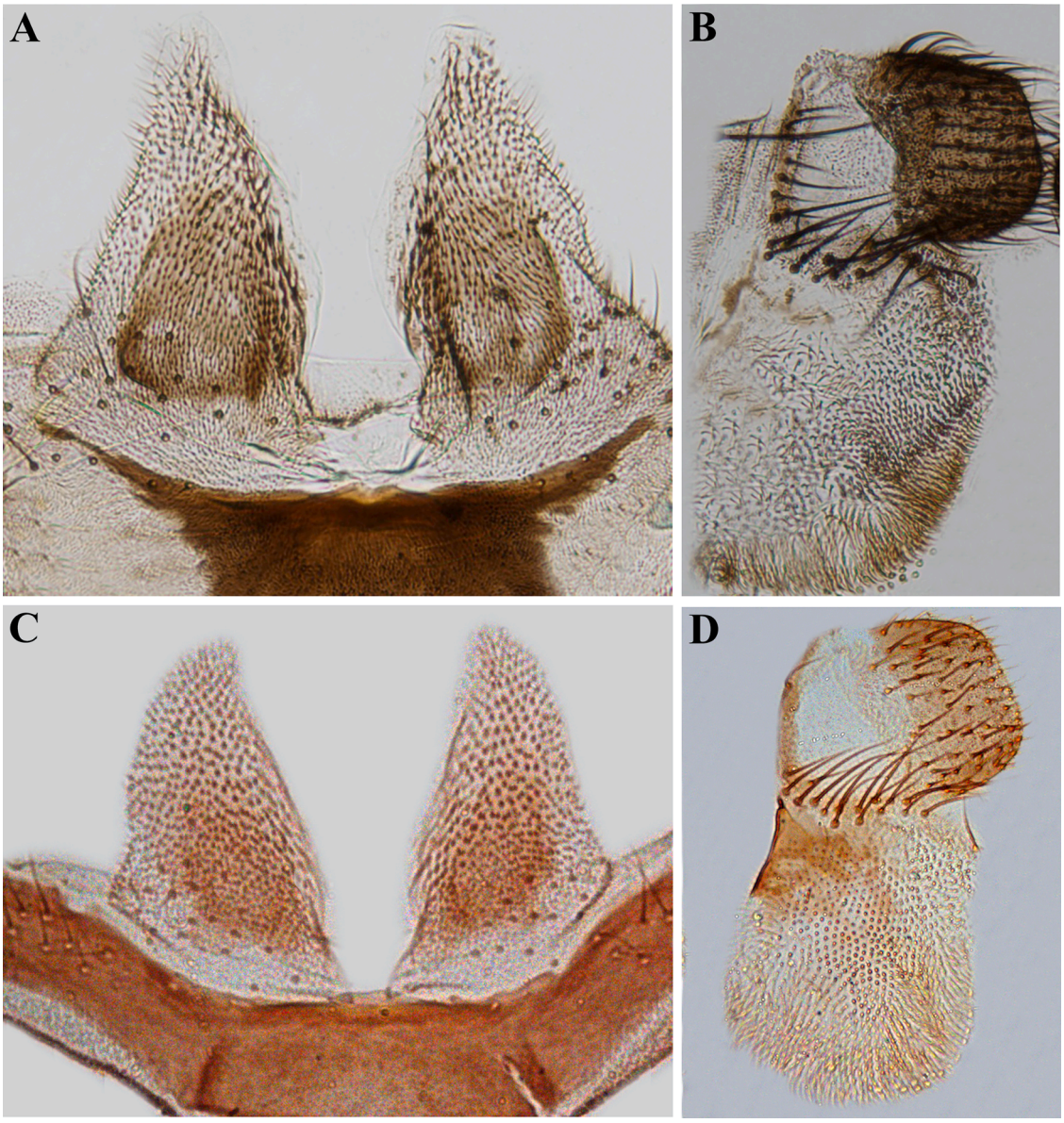

Fig. 22. *Simulium* (Diptera: Simuliidae), female, terminalia partially dissected. (A, C) Sternite VIII and hypogynial valves, ventral view: (A) *Simulium rubrithorax*, (C) *Simulium paynei*. (B, D) Cercus and anal lobe, lateral view: (B) *Simulium rubrithorax*, (D) *Simulium paynei*.

study of larvae from different populations of *S. rubrithorax* (including those from Roraima state, now considered as *S. mutucuna*). No differences were found among the analyzed specimens, even among larvae from distinct sites (e.g., different biomes and rivers with distinct physical and chemical characteristics).

The lack of diagnostic characters in some stages of development is a recurring fact in many groups of Simuliidae. In some cases, informative characteristics are found only in adults, while in other cases they are found in the immature stages, especially in the pupal stage. This fact indicates the importance of the description of all life stages to understand the real diversity is this family, where cryptic diversity is high (Hamada et al., 2010; Nascimento et al., 2018a, b; Nascimento *et al.*, 2020).

DNA-aided species determination has been shown to be useful in solving taxonomic ambiguities, mainly when associated with a detailed morphologic analysis. In this study, we have provided strong support for the view that *S. mutucuna* is a valid taxon. Also, the Bayesian inference analysis provided here indicates the existence of at least four cryptic species in the *S. paynei* complex, corroborating the statements on cryptic diversity by Adler et al. (2004) and Hernández et al. (2015). However, new analysis covering all of the known distribution of this nominal species is necessary to understand the evolutionary history of this taxon. Such findings reinforce the need for molecular and morphological studies of such as this one to investigate the extent of hidden diversity in Simuliidae.

Author statement

Conceptualization: JMCN, NH, VAS
Methodology: JMCN, NH, VAS
Validation: JMCN, NH, VAS
Formal analysis: JMCN, NH, VAS
Investigation: JMCN, NH, VAS, HH, JAG, HF, CF, MEG
Resources: NH, HH, HF, CF
Data curation: NH, JMCN, VAS, HH, JAG, HF, CF, MEG
Writing - original draft: JMCN, NH
Writing - review & editing: JMCN, NH, VAS, HH, JAG, HF, CF, MEG
Funding acquisition: NH

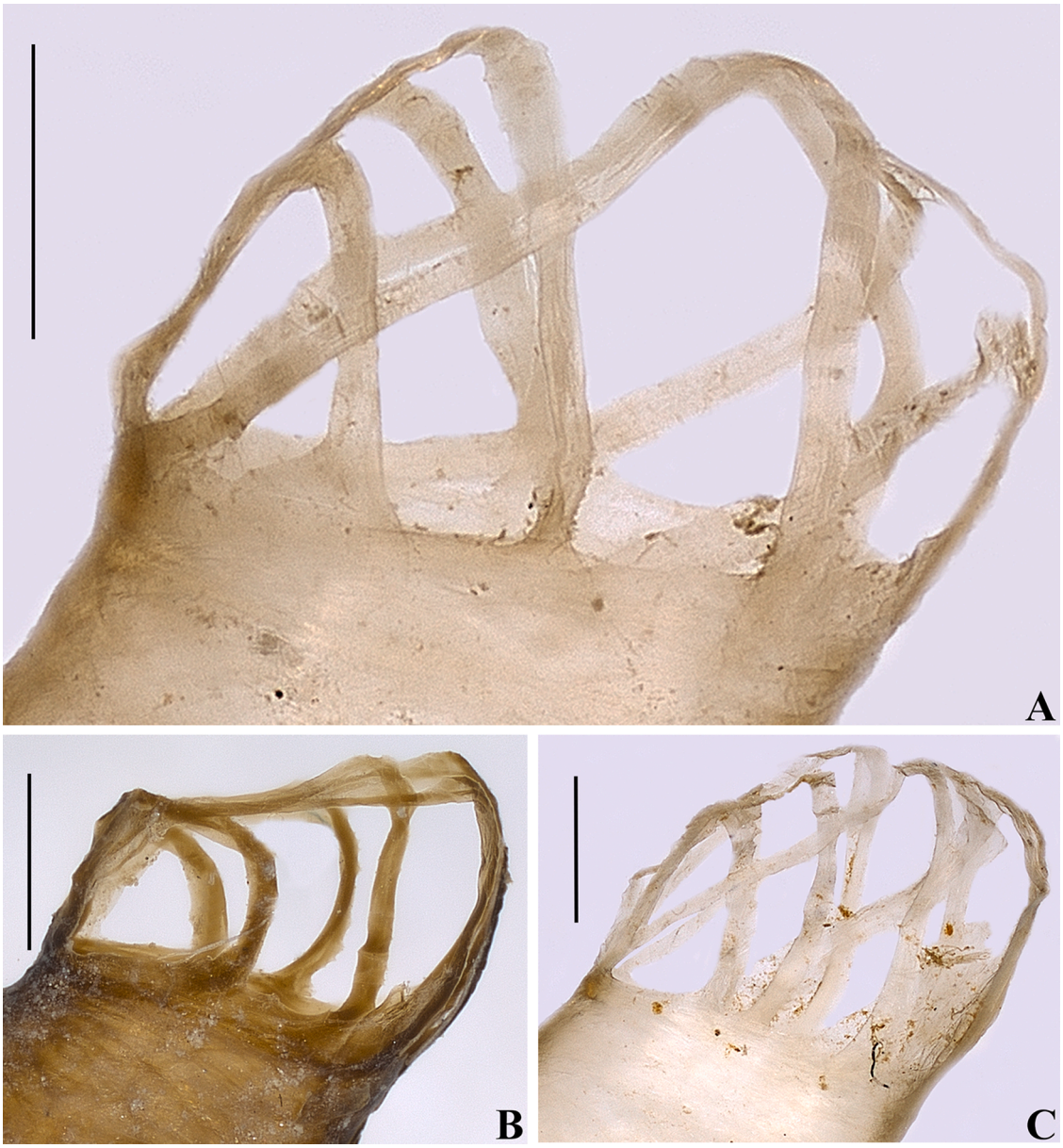

Fig. 23. *Simulium* (Diptera: Simuliidae), pupae, cocoon apices showing the fenestrations. (A) *Simulium mutucuna*. (B) *Simulium paynei*. (C) *Simulium rubrithorax*. (Bar = 1 mm).

Project administration: NH

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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