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Integrative taxonomy and phylogeny of the damselfly genus *Forcepsioneura* Lencioni, 1999 (Odonata: Coenagrionidae: Protoneurinae) with description of two new species from the Brazilian Atlantic Forest

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Abstract. *Forcepsioneura* Lencioni, 1999 is a small genus of eight forest-dependent damselfly species endemic to the Brazilian Atlantic Forest domain. Some of its species are difficult to identify due to their strong morphological similarities. Thus, the use of DNA sequences for taxonomic purposes is warranted. This study examined the diversity among mitochondrial COI and 16S and nuclear PRMT markers in *Forcepsioneura*, identified discrete evolutionary units based on morphological and molecular characters, and described two new species using an integrative approach to propose species-level hypotheses. The first molecular phylogeny of *Forcepsioneura* species, including seven of the 10 valid species, is presented. *Forcepsioneura gabriela* **sp.n.** and *Forcepsioneura janeae* **sp.n.** are described and illustrated based on males. *Forcepsioneura gabriela* **sp.n.** is closely related to *F. garrisoni* Lencioni, 1999 and *F. regua* Pinto & Kompier, 2018 and was included in the light blue group, but was recovered with high K2P COI divergence values relative to *F. garrisoni*. PRMT and ribosomal 16S rDNA sequences were too conservative to distinguish this new species from others of the light blue group. Nevertheless, *F. gabriela* **sp.n.** can be distinguished from other *Forcepsioneura* by its coloration and shape and length of the ventrobasal process of cercus and MP vein. On the other hand, we were unable to get COI sequences for *F. janeae* **sp.n.**, but morphological diagnostic characters, such as, coloration and shape of the posterior lobe of the prothorax and ventrobasal process of cercus supported its proposal as a new species. A concatenated Bayesian analysis of all markers supported the monophyly of both *Forcepsioneura* and the light blue group of species. This study affirmed the value of COI sequence variation for species-level studies but did not support the use of PRMT and 16S for this group of damselflies, as there was very little interspecific variation between some closely related species.

Key words. DNA-barcoding, dragonfly, molecular phylogeny, Protoneuridae, Zygoptera.

1. Introduction

Recent studies on Odonata phylogeny based on morphological and molecular characters have resulted in a comprehensive family-level phylogenetic hypothesis for the suborder Zygoptera (damselflies) (Dijkstra et al. 2014) and suggested Protoneuridae as a polyphyletic group (Bybee et al. 2008; Carle et al. 2008; Pessacq 2008; Dijkstra et al. 2014). These studies, however, recognize all protoneurid species occurring in the New World, including the type-genus *Protoneura* Selys, 1857, as a monophyletic group. This group is currently treated as a subfamily of Coenagrionidae, with Protoneurinae damselflies comprising 123 species distributed in 15 genera (Garrison & von Ellenrieder 2016).

Forcepsioneura Lencioni, 1999 is a small genus of forest-dependent damselflies endemic to the Brazilian Atlantic Forest domain, except for *F. sancta* (Hagen *in* Selys, 1860), also recorded from the Cerrado of the Central Brazilian plateau. Species in the genus occur in

Atlantic Forest remnants from South and Southeastern Brazil associated with specific habitats (PINTO & KOMPIER 2018). Additionally, an unknown species from the State of Rio Grande do Norte, at the northern boundary of the Atlantic Forest domain, was placed in *Forcepsioneura* (IRUSTA & LENCIONI 2015), but it was most likely misidentified (J. Irusta pers. comm.). Eight species are known for the genus, two of which (*F. serrabonita* Pinto & Kompier, 2018 and *F. regua* Pinto & Kompier, 2018) were discovered from material recently collected in the Brazilian states of Bahia and Rio de Janeiro, and more species are yet to be described (see PINTO & KOMPIER 2018; PINTO 2019).

According to PINTO & KOMPIER (2018), two informal groups of species can be recognized in *Forcepsioneura* based on morphological characters and habitat preferences: the light blue group, comprising smaller species with slender cerci and pale areas predominantly bluish-green, which includes *F. garrisoni* Lencioni, 1999 (type-species), *F. haerteli* Machado, 2001, *F. regua* Pinto & Kompier, 2018, and *F. sancta* (Hagen *in* Selys, 1860); and the orange-black group, comprising larger species with robust cerci and pale areas orange-green, which includes *F. grossiorum* Machado, 2005, *F. itatiaiae* (Santos, 1970), *F. lucia* Machado, 2000, and *F. serrabonita*. Species in the first group are associated with lowland habitats and have slender cerci with a comparatively long ventrobasal process, whereas species in the second group are associated with montane habitats and have robust cerci with a short ventrobasal process. In addition, PINTO & KOMPIER (2018) highlighted the morphological similarity of species in the light blue group, especially between *F. garrisoni* and *F. regua* and a putative undescribed species collected in southern Bahia. These species are difficult to identify due to the strong morphological similarities that exist in diagnostic characters. Finally, another interesting case that needs to be carefully addressed is that of populations found in the states of Espírito Santo and Rio de Janeiro identified as *F. lucia*, even though the latter was originally described from the state of Minas Gerais (PINTO & KOMPIER 2018). Any morphological variation, however subtle it may be, that is eventually found between these two populations may indicate that they actually represent distinct species, albeit very closely related to *F. lucia*.

Although morphology has been the basis for taxonomic work, in some cases data from other areas of biology may also provide invaluable information on the species delimitation and phylogeny of a group of species. PADIAL et al. (2010), based on the concept of integrative taxonomy, stressed the importance of using new methods and protocols for species delimitation. With the advance of molecular techniques, molecular data have been increasingly used in taxonomic studies to describe and delimit species and to advance more robust species-level hypotheses. Integrating data from the fields of ecology, geography, population genetics, and behavior into taxonomic studies, as earlier advocated by DAYRAT (2005), has consistently refined species delimitation proposals.

Different types of analytical methods and molecular data have been used to investigate species delimitation in Odonata. For example, genetic distance- and character-based methods supported the description of new species from different regions of Africa (DIJKSTRA et al. 2015), whereas phylogeographic studies helped distinguish levels of polymorphism in specimens of Zygoptera species (FERREIRA et al. 2014a) and identify a large-scale barcoding gap using the ABGD method (KOROIVA & KVIST 2017). In Odonata, the three molecular markers most commonly used in taxonomic and phylogenetic studies are the genes encoding mitochondrial cytochrome c oxidase subunit I (COI), ribosomal 12S rDNA, and 16S rDNA (WARE et al. 2007; BALLARE & WARE 2011; SÁNCHEZ HERRERA et al. 2010; DIJKSTRA et al. 2014, 2015; KOROIVA et al. 2017). COI is widely used in studies that address genetic identity in animals, intra- and interspecific distances, and DNA-barcoding methods. Additionally, the gene encoding arginine methyltransferase (PRMT), a nuclear marker, has been proposed by FERREIRA et al. (2014a) as suitable for species level studies in several Zygoptera but was also used in a study of endangered populations of a new species of Gomphidae (Anisoptera) (FERREIRA et al. 2014b).

The aims of this study were to examine the genetic diversity among *Forcepsioneura* species, to identify discrete evolutionary units based on morphological and molecular characters, and to describe the new species identified. Additionally, the first molecular phylogeny of the genus, including seven of the 10 described species, is presented.

2. Material and methods

2.1. Taxon sampling

Thirty-four specimens of seven species of *Forcepsioneura*, including two new species that are described herein, were used to investigate inter- and intraspecific genetic distances and infer the genus phylogeny based on the molecular markers COI, 16S, and PRMT (Table 1). Specimens examined are deposited in the following collections:

DZRJ – Coleção Entomológica Prof. José Alfredo Pinheiro Dutra, Departamento de Zoologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil; **DZUP** – Coleção Entomológica Pe. Jesus Santiago Moure, Departamento de Zoologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, PR, Brazil; **MNRJ** – Coleção Entomológica do Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil; **MZSP** – Serviço de Entomologia, Museu de Zoologia, Universidade de São Paulo, SP, Brazil.

In addition, outgroup species including representatives of the Protoneurinae, "Teinobasinae", Pseudostigmatinae s.l. (all ridge-faced Coenagrionidae), Ischnurinae (core-Coenagrionidae), and Lestidae (Table 1) were selected to represent different levels of the Zygoptera phylogeny following the most comprehensive molecular hypothesis (i.e., DIJKSTRA et al. 2014).

Table 1. Species included in the phylogenetic analysis of the genus *Forcepsioneura* with voucher specimen code and collection locality in Brazil, and GenBank accession codes for molecular markers sequenced in this study.

Family / Subfamily / Species		Voucher	Locality	COI	16S	PRMT
INGROUP: Protoneurinae						
Epipleoneura venezuelensis	♂	ENT 4355	Una – BA	MN058172		MN080560
Forcepsioneura garrisoni	♂	ENT 4344	Antonina – PR	MN058173		MN080577
Forcepsioneura garrisoni	♂	ENT 4345	Antonina – PR	MN058174		MN080573
Forcepsioneura gabriela **sp.n.**	♂	ENT 2784	Una – BA	MN058175	MN023110	MN080574
Forcepsioneura gabriela **sp.n.**	♂	ENT 3445	Una – BA	MN058176	MN023111	MN080575
Forcepsioneura janeae **sp.n.**	♂	ENT 3403	Santa Teresa – ES		MN023112	
Forcepsioneura aff. *lucia*	♂	ENT 2786	Nova Friburgo – RJ	MN058177	MN023113	MN080572
Forcepsioneura aff. *lucia*	♂	ENT 3404	Itatiaia – RJ	MN058178	MN023114	MN080576
Forcepsioneura aff. *lucia*	♀	ENT 3405	Itatiaia – RJ	MN058179	MN023115	
Forcepsioneura aff. *lucia*	♂	ENT 3606	Nova Friburgo – RJ	MN058180	MN023116	
Forcepsioneura aff. *lucia*	♂	ENT 3615	Itatiaia – RJ	MN058181	MN023117	MN080581
Forcepsioneura aff. *lucia*	♂	ENT 3616	Itatiaia – RJ	MN058182	MN023118	MN080578
Forcepsioneura regua	♂	ENT 2854	Cachoeiras de Macacu – RJ		MN023119	MN080570
Forcepsioneura regua	♂	ENT 3608	Cachoeiras de Macacu – RJ		MN023120	
Forcepsioneura sancta	♂	ENT 2365	Petrópolis – RJ	MN058183	MN023121	MN080563
Forcepsioneura sancta	♂	ENT 2366	Rio de Janeiro – RJ		MN023122	MN094792
Forcepsioneura sancta	♂	ENT 2369	Nova Friburgo – RJ	MN058184	MN023123	MN080564
Forcepsioneura sancta	♂	ENT 2785	Nova Friburgo – RJ	MN058185	MN023124	MN080582
Forcepsioneura sancta	♀	ENT 3506	Itatiaia – RJ	MN058186	MN023125	MN080565
Forcepsioneura sancta	♂	ENT 3507	Itatiaia – RJ	MN058187	MN023126	
Forcepsioneura sancta	♂	ENT 3508	Itatiaia – RJ	MN058188	MN023127	MN080562
Forcepsioneura sancta	♂	ENT 3509	Itatiaia – RJ	MN058189		MN080571
Forcepsioneura sancta	♂	ENT 3510	Itatiaia – RJ	MN058190	MN023128	
Forcepsioneura sancta	♂	ENT 3511	Itatiaia – RJ	MN058191	MN023129	
Forcepsioneura sancta	♂	ENT 3512	Itatiaia – RJ	MN058192	MN023130	MN080569
Forcepsioneura sancta	♂	ENT 3612	Rio de Janeiro – RJ		MN023131	
Forcepsioneura sancta	♀	ENT 4340	Curitiba – PR	MN058193		MN080561
Forcepsioneura sancta	♂	ENT 4341	Antonina – PR	MN058194		
Forcepsioneura sancta	♂	ENT 4343	Itatiaia – RJ	MN058195		
Forcepsioneura sancta	♂	ENT 4346	Antonina – PR	MN058196		MN080566
Forcepsioneura sancta	♂	ENT 4347	Antonina – PR			MN080579
Forcepsioneura sancta	♂	ENT 4348	Antonina – PR			MN080580
Forcepsioneura sancta	♂	ENT 4350	Antonina – PR			MN080567
Forcepsioneura sancta	♂	ENT 4354	Itatiaia – RJ			MN080568
Forcepsioneura serrabonita	♂	ENT 2857	Camacan – BA	MN058197	MN023132	
Idioneura ancilla	♂	ENT 3447	Una – BA	MN058198	MN023133	
Neoneura amelia	—	Na-3	–			KM276629*
Peristicta aeneoviridis	♂	ENT 4342	Maquiné – RS	MN058199		
Roppaneura beckeri	♀	ENT 4337	Curitiba – PR	MN058200		
OUTGROUP						
Lestidae: *Lestes forficula*	♂	ENT 2789	Nova Friburgo – RJ	MN058201	MN023134	
Ischnurinae: *Acanthagrion aepiolum*	♀	ENT 3398	Brasília – DF		MN023135	
Pseudostigmatinae: *Leptagrion andromache*	♂	ENT 2788	Parati – RJ	MN058202	MN023136	
Pseudostigmatinae: *Leptagrion elongatum*	♂	ENT 3408	Cachoeiras de Macacu – RJ		MN023137	
Pseudostigmatinae: *Mecistogaster amalia*	♂	ENT 2860	Rio de Janeiro – RJ		MN023138	
Pseudostigmatinae: *Mecistogaster asticta*	♂	ENT 3406	Itatiaia – RJ		MN023139	
"Teinobasinae": *Metaleptobasis selysii*	♂	ENT 3446	Una – BA	MN058203	MN023140	

2.2. DNA extraction, amplification and sequencing

DNA was extracted from one leg and muscle bundles of adult specimens using the DNeasy Blood & Tissue Kit following the manufacturer's protocol but without macerate the samples (QIAGEN, Hilden, Germany). Fragments of the genes encoding mitochondrial cytochrome c oxidase subunit I (COI, 658 bp), subunit 16S of rDNA (16S, 524 bp), and nuclear arginine methyltransferase (PRMT)

were amplified and sequenced using the following primers: LCO1490 (5' GGTCA ACAAA TCATA AAGAT ATTGG 3') and HCO2198 (5' TAAAC TTCAG GGTGA CCAAA AAATC A 3') (FOLMER et al. 1994); LR-J-12887 (5' CCGGT YTGAA CTCAR ATCA 3') and LR-N-13398 (5' CRMCT GTTTA WCAAA AACAT 3') (TAKIYA et al. 2006); and ARG_F2 (5' TGCCG CCAAG GCTGG AGCAT C 3') and ARG_R3 (5' CCGGA ACTCT ATGTA CCACA AC 3') (FERREIRA et al. 2014a); respectively. The protocol for amplification of COI and 16S consisted of 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C, and a final extension period of 7 min at 72°C. For PRMT, the amplification protocol consisted of 5 min at 94°C followed by 40 cycles of 30 sec at 92°C, 30 sec at 54°C, 45 sec at 72°C, and a final extension period of 10 min at 72°C. Amplified products were separated on a 1% agarose gel and stained with GelRed™ (Biotium, Inc., Fremont, CA, USA). Purification and sequencing of each amplicon (both strands) was performed by Macrogen (Seoul, Korea).

Consensus sequences were generated based on electropherograms with GeneStudio™ Professional Edition v. 2.2.0.0 (Genestudio, Inc., Suwanee, GA, USA). Consensus sequences (and a single PRMT sequence from *Neoneura amelia*; GenBank KM276629) were aligned with MUSCLE 6 (EDGAR 2004) implemented in MEGA 7 (KUMAR et al. 2016) for COI and PRMT, and MAFFT 6 (KATOH et al. 2005) using the online server (http://mafft.cbrc.jp/alignment/server/) with the Q-INS-I algorithm for 16S.

2.3. Genetic distances and phylogenetic analysis

Pairwise genetic distances (*p*-distances) (NEI & KUMAR 2000) of specimens were calculated for both 16S and PRMT. However, for COI, distances were modeled under the Kimura two-parameter (K2P) model (KIMURA 1980) for comparability with other barcoding studies. In addition, cluster analysis was conducted for each gene separately using the neighbor-joining (NJ) distance method (SAITOU & NEI 1987), whereas group support was calculated with 1,000 bootstrap (BS) pseudoreplicates. *P*-distances and NJ analyses were calculated in MEGA 7 (KUMAR et al. 2016).

Bayesian inference analyses were performed based on separate gene alignments and the concatenated dataset under a mixed-model strategy. The most appropriate evolutionary model for COI and 16S markers (GTR+I+G) and PRMT (GTR+I) was selected based on the Akaike information criterion (AKAIKE 1974), implemented in jModeltest2 v.2.1.7 (DARRIBA et al. 2012). Four independent Monte Carlo Markov Chain (MCMC) simulations were run in MrBayes 3.2 (RONQUIST et al. 2012) with four chains for 5,000,000 generations with a sample frequency of 1,000 generations in the concatenated analysis and, for individual-gene analysis, four chains for 1,000,000 generations with a sample frequency of 2,000 generations. Convergence and mixing of sampled parameters was checked in Tracer 1.6 (RAMBAUT et al. 2013) with 10% of trees discarded as "burn-in". Branch support was assessed by posterior probabilities of clades (PP, RONQUIST & HUELSENBECK 2003).

2.4. Morphological analysis and terminology

Morphological terminology and general procedures follow PINTO & KOMPIER (2018). The following morphological abbreviations were used in the text: Ax = antenodal crossvein; Fw = fore wings; GL = genital ligula; Hw = hind wings; MBP = mediobasal process; Px = postnodal crossvein; Pt = pterostigma; S1 – 10 = abdominal segments; and VBP = ventrobasal process. Measurements and photographs were taken with a Leica DFC 500 digital camera mounted on Leica MZ16 and M205C stereomicroscopes. Multiple focal plane images were compiled using LAS MONTAGE v.4.7 and LAS CORE v.4.6 software.

3. Results

3.1. Genetic distances

Pairwise genetic distances between sequences of all three markers studied are shown in Supplementary Tables S1 – S3. Maximum intraspecific variation of COI sequences (Table 2) varied among *Forcepsioneura* species, from 0% in *F. gabriela* **sp.n.** and *F. garrisoni* and 1.1% in *F.* aff. *lucia* to 5.0% in *F. sancta*. Other markers studied showed no intraspecific variation among sequences, except for 16S (1.1%) and PRMT (1.5%) in *F. sancta*.

Interspecific distances among *Forcepsioneura* species ranged from 3.8 to 18.4% for COI, from 0.0 to 5.2% for 16S, and from 0.0 to 3.0% for PRMT (Tables 3 – 5). Interestingly, no genetic variation was found between 16S sequences of *F. regua* and *F. gabriela* **sp.n.** and PRMT sequences of *F. regua* and *F. garrisoni.*

3.2. Proposal of new taxa

Both new species proposed herein have diagnostic morphological characteristics that justify their description as new taxa (see Table 6). In addition, DNA sequence divergences were assessed to support their distinction.

The new species *F. gabriela* **sp.n.**, described herein, was not recovered as closely related to and with high divergence (3.8%) from the most similar species, *F. garrisoni*, in K2P distances and the neighbor joining tree of COI sequences (Fig. 1, Table 3). However, 16S and PRMT failed to distinguish the new species from the closely related *F. garrisoni* and *F. regua* (Figs. 2, 3). Nevertheless, we believe that COI results in addition to morphological evidence are sufficient to propose this new taxon.

Table 2. Range (and mean) of intraspecific genetic variation of the three molecular markers (COI, 16S, and PRMT) sequenced for *Forcepsioneura*, including numbers of individuals (N) analyzed.

Species	N	K2P distances (COI)	N	p-distances (16S)	N	p-distances (PRMT)
F. gabriela **sp.n.**	2	0.0	2	0.0	2	0.0
F. garrisoni	2	0.0	0	—	2	0.0
F. aff. *lucia*	6	0–0.011 (0.004)	6	0.0	4	0.0–0.015 (0.008)
F. regua	0	—	2	0.0	1	—
F. sancta	14	0–0.050 (0.025)	11	0–0.011 (0.004)	14	0–0.015 (0.008)

Table 3. Range (and mean) of interspecific K2P distances between COI sequences of *Forcepsioneura* species.

	F. gabriela **sp.n.**	*F. garrisoni*	*F.* aff. *lucia*	*F. serrabonita*
F. garrisoni	0.038 (0.038)			
F. aff. *lucia*	0.112–0.122 (0.117)	0.126–0.136 (0.132)		
F. serrabonita	0.162 (0.162)	0.158 (0.158)	0.173–0.184 (0.178)	
F. sancta	0.079–0.096 (0.091)	0.089–0.095 (0.092)	0.105–0.133 (0.122)	0.140–0.158 (0.153)

Table 4. Range (and mean) of uncorrected interspecific distances between 16S sequences of *Forcepsioneura* species.

	F. gabriela **sp.n.**	*F.* aff. *lucia*	*F. janeae* **sp.n.**	*F. regua*	*F. sancta*
F. aff. *lucia*	0.036 (0.036)				
F. janeae **sp.n.**	0.030 (0.030)	0.006 (0.006)			
F. regua	0.000 (0.000)	0.036 (0.036)	0.030 (0.030)		
F. sancta	0.025–0.033 (0.029)	0.030–0.039 (0.035)	0.025–0.033 (0.029)	0.025–0.033 (0.029)	
F. serrabonita	0.052 (0.052)	0.041 (0.041)	0.036 (–)	0.052 (0.052)	0.039–0.047 (0.043)

Table 5. Range (and mean) of uncorrected interspecific distances between PRMT sequences of *Forcepsioneura* species.

	F. gabriela **sp.n.**	*F. garrisoni*	*F.* aff. *lucia*	*F. regua*
F. garrisoni	0.008 (0.008)			
F. aff. *lucia*	0.000–0.015 (0.004)	0.008–0.023 (0.012)		
F. regua	0.008 (0.008)	0.000 (0.000)	0.008–0.023 (0.012)	
F. sancta	0.000–0.015 (0.011)	0.008–0.023 (0.019)	0–0.030 (0.012)	0.008–0.023 (0.019)

Unfortunately, only a single individual of the other new species proposed, *F. janeae* **sp.n.**, was available for DNA extraction and only 16S was successfully sequenced. Nonetheless, the *p*-distance between the new species and its most related species (Fig. 4), *F.* aff. *lucia*, was 0.6% (Table 4), which was higher than the variation among some species of the light blue group. Thus, the morphological evidence gathered and the additional variation in 16S sequences was found to be sufficient to propose this new taxon.

3.3. Taxonomy

Coenagrionidae Kirby, 1890

Protoneurinae Yakobson & Bianchi, 190

3.3.1. *Forcepsioneura gabriela* sp.n.
(Figs. 5A,B, 6A–C, 8A,B, 9A–C)

Material examined. ***Type material***: Holotype ♂, BRAZIL. Bahia State, Una municipality, Reserva Biológica de Una, Expedição Gabriela Cravo e Canela II, first order stream [?], after Fazenda Piedade (15°09′36.2″S 39°10′31.1″W, 100 m a.s.l), 14–15.vi.2014, A.P. Pinto leg. (DZUP 498858, DNA voucher ENT2784). Paratype ♂ same data as holotype but Expedição Gabriela Cravo e Canela IV, 07.viii.2016, A.P. Pinto, A.P.M. Santos, D.M. Takiya & P.M. Souto leg. (DZRJ 3555; DNA voucher ENT3445).

Etymology. Specific name in apposition after the strong female character of the famous novel "Gabriela, cravo e canela" by Brazilian writer Jorge Amado. The novel is set in the region of the type locality at the beginning of the 20th century, when the southern coast of Bahia prospered from the exploitation of cacao trees.

Diagnosis. A small, dorsally brown with metallic green reflections, lateroventrally light blue and yellow protoneurid. Frons angulated, rear of head pale; CuA&AA indistinct; and with genital ligula (GL) with long inner fold and distal lateral lobe elongated into a flagellum according to current concept of *Forcepsioneura* (see Pinto & Kompier 2018). The long ventrobasal process of cercus (VBP), with length ≥ 0.65 of total cercus length, distinguishes the new species from *F. grossiorum*, *F. lucia*, *F. itatiaiae* and *F. serrabonita* (as long as 0.55 in *F. itatiaiae* and ≤ 0.4 the

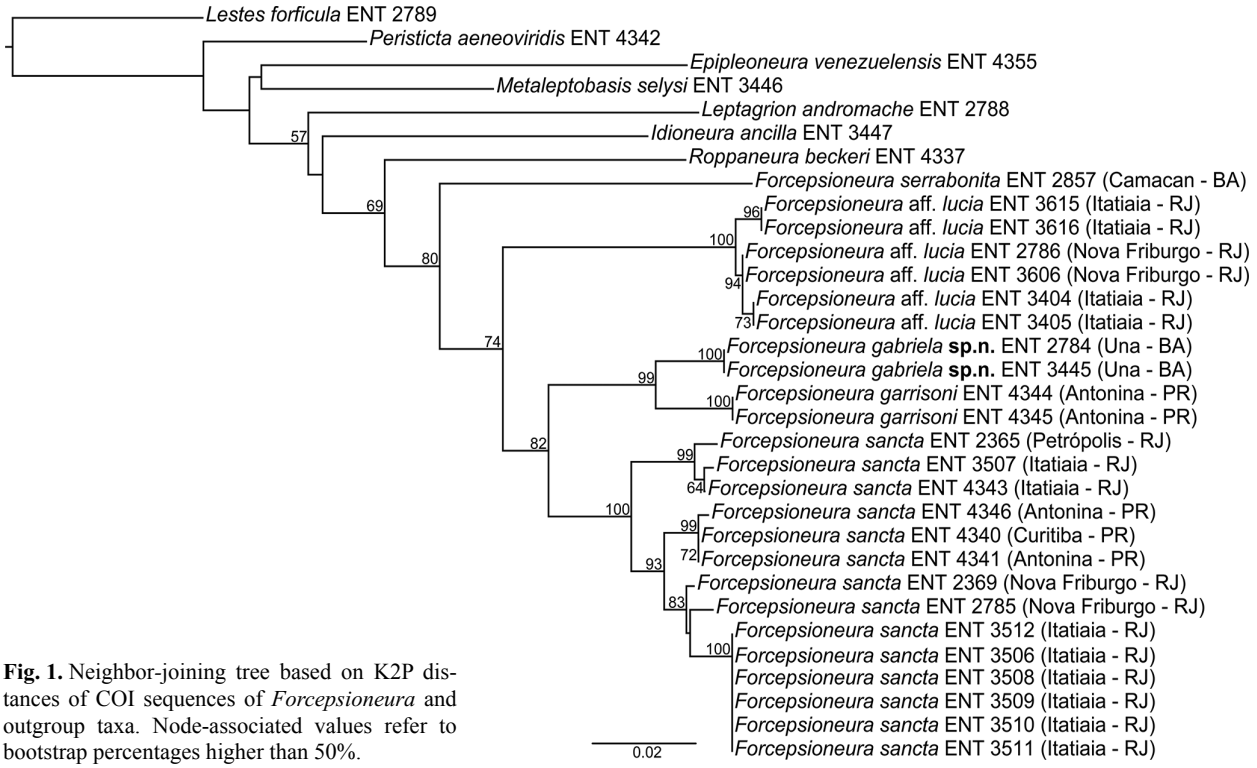

Fig. 1. Neighbor-joining tree based on K2P distances of COI sequences of *Forcepsioneura* and outgroup taxa. Node-associated values refer to bootstrap percentages higher than 50%.

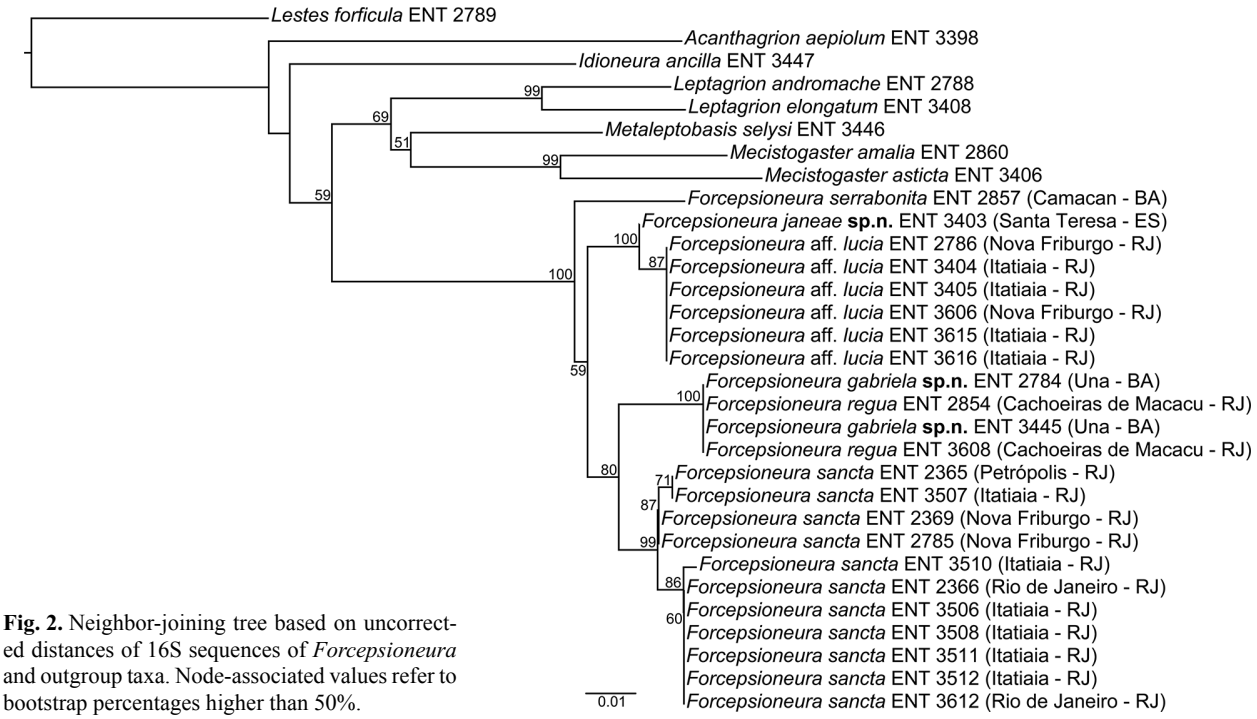

Fig. 2. Neighbor-joining tree based on uncorrected distances of 16S sequences of *Forcepsioneura* and outgroup taxa. Node-associated values refer to bootstrap percentages higher than 50%.

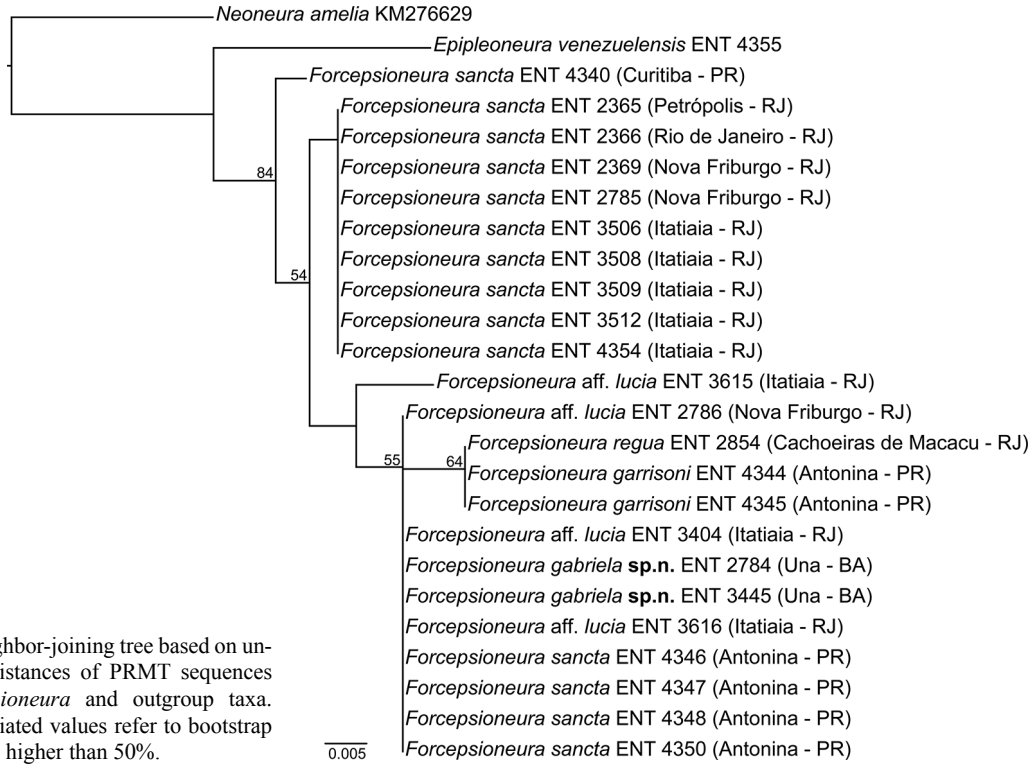

Fig. 3. Neighbor-joining tree based on uncorrected distances of PRMT sequences of *Forcepsioneura* and outgroup taxa. Node-associated values refer to bootstrap percentages higher than 50%.

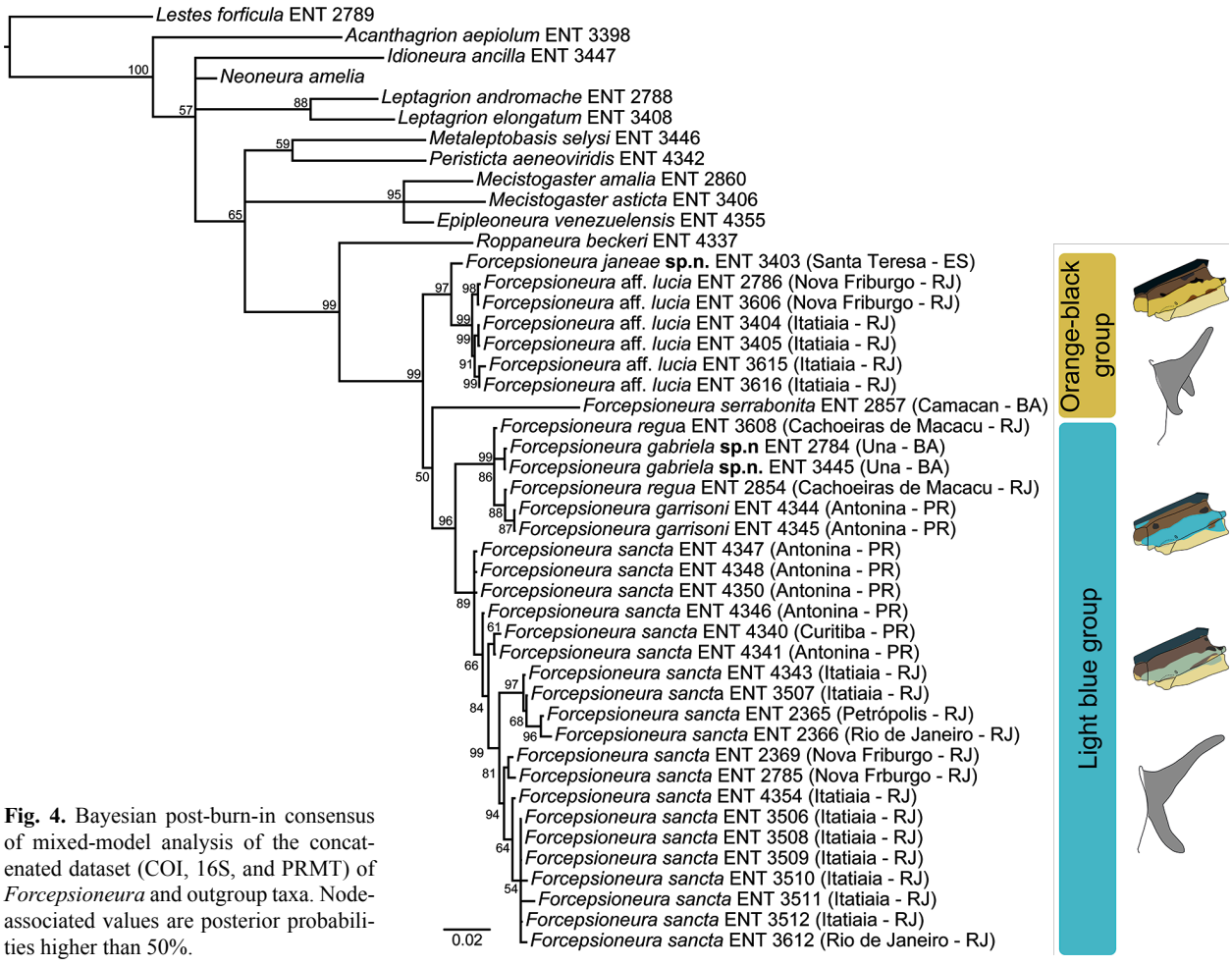

Fig. 4. Bayesian post-burn-in consensus of mixed-model analysis of the concatenated dataset (COI, 16S, and PRMT) of *Forcepsioneura* and outgroup taxa. Node-associated values are posterior probabilities higher than 50%.

Table 6. Diagnostic morphological characteristics for species of *Forcepsioneura* (* sensu MACHADO 2001).

Taxa	1. Process of median lobe of prothorax	2. Posterior lobe of prothorax	3.Stripes on synthorax	4. Length of MP vein in Hw	5. Internal fold of GL	6. VBP/cercus length ratio in lateral view	7. Length of VBP of cercus	8. Apex of VBP in lateral view	9. Shape of MPB	10. MBP in lateral view	11. Anteromesal margin of MBP in dorsal view
F. gabriela **sp.n.**	Well-defined	Concave mesally	Bluish-green	Short, reaches anal margin usually at the vein descending from subnodus (rare distally)	Thin and oblique	≥ 0.5	Short, not reaching S10	Broadly rounded and abruptly curved inwardly	Small fin-shaped plate with an acute and strongly anteriorly margin	Not visible	Acute
F. garrisoni	Well-defined	Sinuous	Bluish-green	Short, reaches anal margin usually at the vein descending from subnodus (rare distally)	Thin and oblique	≥ 0.5	Long, exceeding S10	Strongly curved inwardly	Small fin-shaped plate with an acute and strongly anteriorly margin	Not visible	Acute
F. regua	Ill-defined	Asymmetrically convex	Bluish-green	Short, reaches anal margin usually at the vein descending from subnodus (rare distally)	Thin and oblique	≥ 0.5	Short, not reaching S10	Strongly curved inwardly	Small fin-shaped plate with an acute and strongly anteriorly margin	Not visible	Acute
F. haerteli	Well-defined	Convex, with lateral margins straight	Greenish-red	—	—	≥ 0.5	Long, reaching S10	Rounded, slightly curved inwardly	Small slightly rounded plate with na acute anteriorly margin	Not visible	Acute
F. sancta	Well-defined	Straight or slightly concave	Bluish-green	Long, reaches anal margin distally 0.3–0.5 from the vein descending from subnodus	—	≥ 0.5	Short, not reaching S10	Rounded	Small rounded plate with a rounded anteriorly margin	Not visible	Rounded*
F. itatiaiae	Well-defined	Sinuous, almost straight	Greenish-orange	Long, reaches anal margin distally 0.3–0.5 from the vein descending from subnodus	—	≥ 0.5	Short, not reaching S10	Truncate	Large fin-shaped plate with a truncate anteriorly margin	Visible	Truncate
F. grossiorum	Well-defined	Strongly sinuous; two broad concavities with convex median elevation, lateral margin forming a flap	Greenish-orange	Long, reaches anal margin distally 0.3–0.5 from the vein descending from subnodus	—	≤ 0.4	Short, not reaching S10	Strongly curved inwardly	Large, slightly rounded plate with an acute anteriorly margin	Visible	Acute
F. janeae **sp.n.**	Well-defined	Rectangular	Orange-yellow	Long, reaches anal margin distally 0.3–0.5 from the vein descending from subnodus	Large and upright	≤ 0.3	Short, not reaching S10	Slightly acute	Large, rounded and flat plate with an acute and strongly anteriorly margin	Visible	Acute
F. lucia	Well-defined	Convex, with lateral margin slightly acute	Greenish-orange	Long, reaches anal margin distally 0.3–0.5 from the vein descending from subnodus	—	≤ 0.4	Short, not reaching S10	Strongly curved inwardly	Large, rounded and flat plate with an acute and strongly anteriorly margin	Visible	Acute
F. serrabonita	Well-defined	Laterally as small flat processes	Orange-yellow	Long, reaches anal margin distally 0.3–0.5 from the vein descending from subnodus	Large and upright	< 0.4	Short, not reaching S10	Rounded	Small, rounded plate with a rounded anteriorly margin	Visible	Rounded

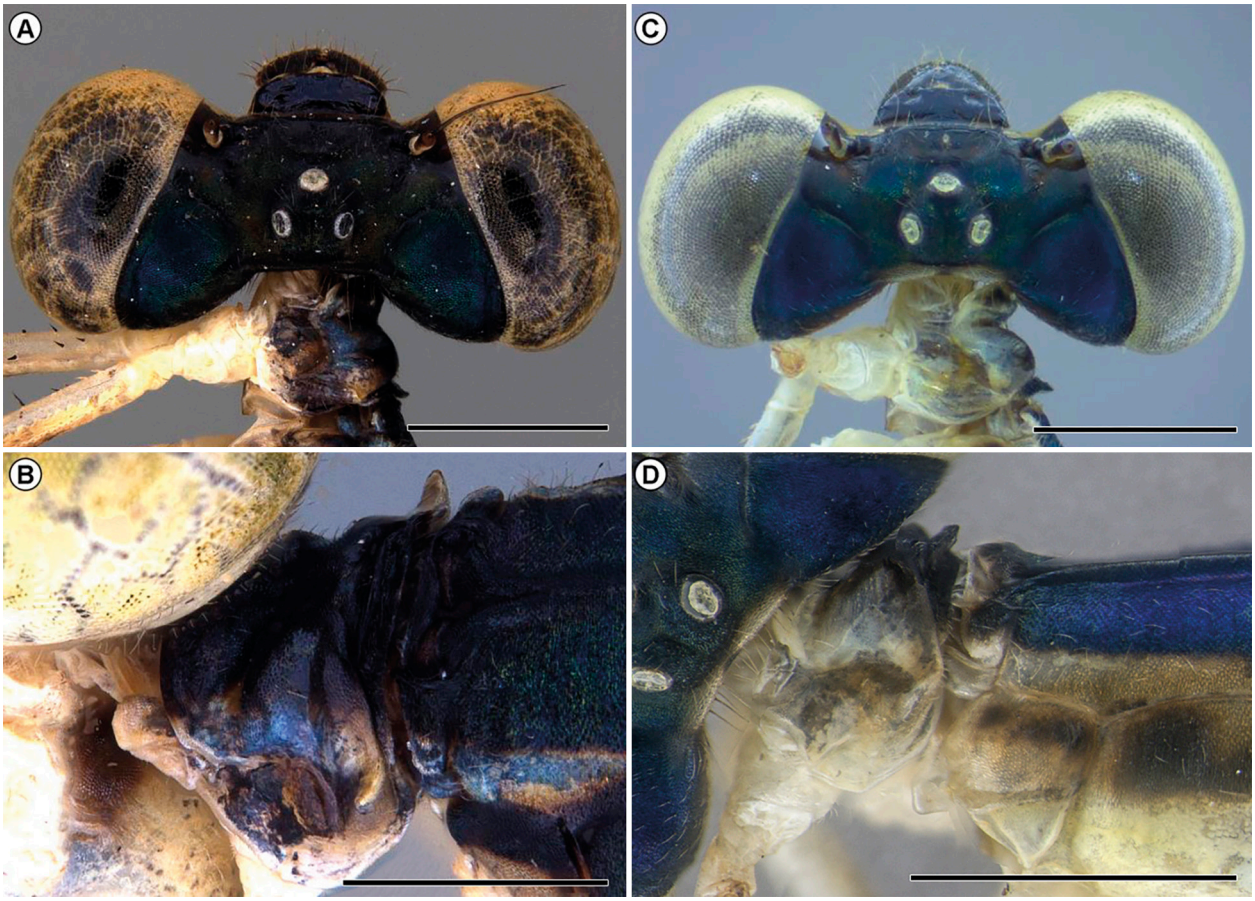

Fig. 5. *Forcepsioneura gabriela* **sp.n.** holotype ♂ (Brazil, Bahia: Reserva Biológica de Una, (DZUP 498858,). **A**: head, dorsal view; **B**: prothorax, lateral view. *Forcepsioneura garrisoni* ♂ (Brazil, Rio de Janeiro: Praia de Tarituba, DZRJ 0325). **C**: head, dorsal view; **D**: prothorax, dorsolateral view. – Scale bars: 1 mm.

length of cercus in the others); MP short, reaching distally at the level of the vein descending from subnodus, distinguishes it from *F. haerteli* and *F. sancta* (MP reaches distally at 0.3–0.5 the vein descending from subnodus). *Forcepsioneura gabriela* **sp.n.** is very similar to *F. garrisoni* and *F. regua* by their blue coloration of the lateral portion of the synthorax and shape of the cercus. However, the well-defined tubercle-like process on the posterolateral margin of the prothorax distinguishes it from *F. regua* (prothorax with an ill-defined process), whereas the robust VBP, with apex not reaching the ventral margin of S10 distally and curved ventrally allows separation from *F. garrisoni* (apex of VBP reaching distally the ventral margin of S10 and curved mesally).

Description of male holotype. ***Head*** (Fig. 5A): Labium, visible parts of maxilla and mandibles ivory-yellow, except apex brown. Genae ivory-yellow. Labrum black encircled by yellow that separates into two large lateral and small mesal spots, yellow occupying ventrally 0.25 of labrum length. Anteclypeus ivory-yellow with mesal wide C-shaped black spot; postclypeus shining black. Antefrons, postfrons, and remainder of epicranium black with shining bluish-green reflections, except by a pair of ill-defined blue spots on antefrons. Antennifer and scape black; posterior surface of pedicel yellow; distal apex of scape ivory-yellow; flagellum dark brown. Posterior region of cranium ivory-yellow, dorsal part 0.40 brownish-black extending ventrally close to occipital foramen.

Thorax (Figs. 5B, 9A): Prothorax black, whitish-blue stripe laterally on notum interrupted at posterior margin of median lobe; lateral of anterior lobe, posterolateral margin of median lobe, and ventral 0.4 of propleuron yellow; posterolateral margin of median lobe strongly projected into a tubercle-like process; posterior lobe convex, rectangular, narrower than median lobe, whitish-blue on lateral apex. Synthorax dorsally dark brown to black with bluish-green metallic reflections; mesepisternum entirely black with bluish-green metallic reflections, anterior 0.25 with whitish-blue wedge-shaped spot; mesepimeron and metepisternum dark brown with wide light blue longitudinal stripe running from posterolateral angle of mesinfraepisternum to antealar process, occupying maximum 0.33 of mesepimeron to 0.9 of metepisternum width; metepimeron ivory-yellow; metapostepimeron ivory-yellow with black spot at lateroventral angle. Legs ivory-yellow with irregular dark brown to black areas on dorsal surface of femora and tibia; articulations of femur-tibia, tarsal segments, apices of pretarsal claws and spurs black, except by scale-like ivory-yellow proximal femoral spurs and tibial comb of prothoracic legs; femora with anteroventral surface armed with four

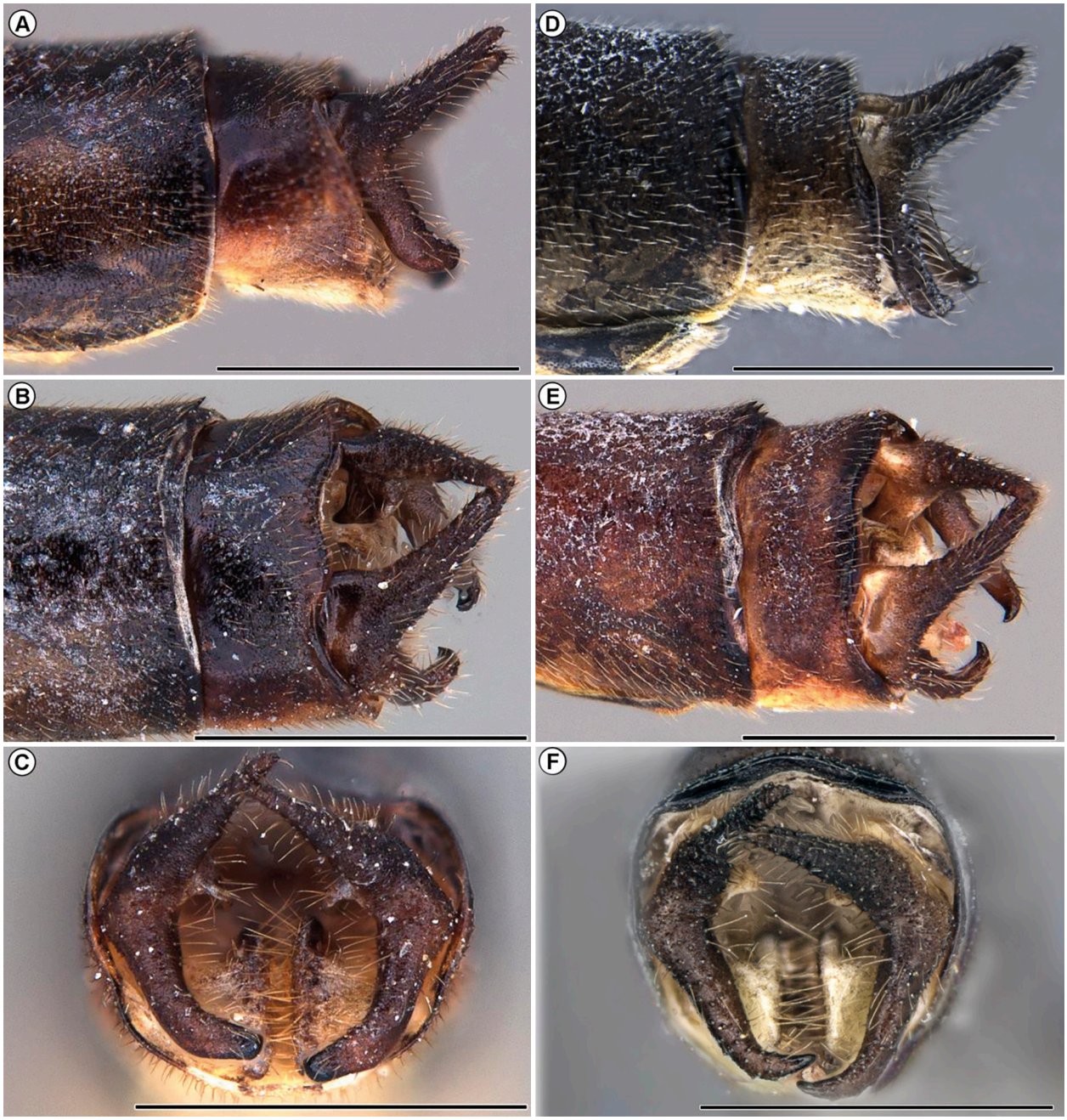

Fig. 6. Caudal appendages of *Forcepsioneura gabriela* **sp.n.** holotype ♂ (Brazil, Bahia: Reserva Biológica de Una, DZUP 498858) (**A**–**C**) and *Forcepsioneura garrisoni* (Brazil, Rio de Janeiro: Praia de Tarituba, DZRJ 0325) (**D**–**F**). A, D: lateral view; B, E: dorsolateral view; C, F: posterior view. – Scale bars: 1 mm.

long and robust spurs; femora with posteroventral surface with short and thinner spurs, 4 on pro-, 6 on meso-, and 7 on metathoracic leg; tibiae with anteroventral surface armed with 10 spurs on pro- (5 of tibial comb), 5 on meso-, and 6 on metathoracic leg; tibiae with posteroventral surface with 8 on pro-, 7 on meso-, and 12 on metathoracic leg.

Wing: Membrane hyaline; venation dark brown to black; Pt black, quadrangular, encircled by thin hyaline line; MP reaches anal margin at level of vein descending from subnodus or very slightly distal, covering 2 cells on all wings; Px on Fw 12; Hw 10; RP2 originating at Px 6 on Fw, at Px 5 on Hw.

Abdomen (Figs. 6A–C, 8A,B, 9A,B): S1–10 tergites dark brown to black dorsally, lateroventrally light brown to ivory-yellow, pale areas of S1–3 with blue shade, darker in S8–10, dorsal carina of S1–8 with a very thin pale line along; sternites similar in color to adjacent areas of tergites; pale longitudinal stripe occupying about 0.5 ventral of S1–7 tergites laterally, gradually narrowing to ca 0.2 in S8, a narrow line in S9 and ill-defined areas in S10; S3–7 with anterior pale ring ≤ 0.1 of total length of segment, separated dorsally in S7; S1–7 cylindrical; S8–10 distinctly wider than others segments (S7 width 0.7 of posterior part of S8); S9–10 dorsally covered by whitish-grey pruinosity, less amount on S10; posterior

margin of S10 with slight concavity. Secondary genitalia (Fig. 8A,B) typical of Coenagrionoidea; anterior lamina with deep and acute incision; anterior hamule dark brown, quadrangular, with anteroventral angle acutely projected; posterior hamule almost entirely internalized with curved thumb-shape; VS longer than wide, maximum width 0.3 of total length in ventral view. Genital ligula (Fig. 8A,B) with L1 smooth, without any kind of special setae; L2 with posterolateral portion of flexure projected distally beyond median region, making its margin slightly concave in ectal view, distal margin (tip of ligula) with mesal concavity; lateral margins prolonged into two curved long flagella, in ectal view basally almost perpendicular, posteriorly twisted; internal fold proximal to flexure, long, ca 0.4 of L2 length in lateral view; no sclerotized tubercle at flexure. Epiproct reduced to membranous-like plate. Cercus (Fig. 6A–C) brown to dark brown, apex of MBP and VBP black; in lateral view slightly directed obliquely dorsad, gradually tapering distally; VBP in lateral view perpendicular to dorsal branch, length ca 0.7 of cercus, apex stoutly rounded, at distal 0.35 distinctly curved posteriorly, distal edge of apex curved ventrally, reaching distally 0.8 from distance of VBP base to margin of S10; MBP not visible in lateral view; tip of cercus blunt; in dorsal view forcipate, proximally wide, slender distally; lateral margin almost straight, internal margin very slightly curved; apexes converging (Fig. 6C); MBP as an acute fin-shaped plate dorsally, apex strongly directed anteriorly, positioned at basal 0.25, in posterior view directed ventrally obliquely; apex of VBP broadly rounded and abruptly curved inwardly with apical edge ventrally in posterior view. Paraproct light brown with dorsal margin black, plate-like.

***Measurements* (*mm*)**: Total length (incl. caudal appendages) 35; abdomen length (excl. caudal appendages) 29.7; head maximum width 2.9; Fw length 19.5; Hw length 18; Fw maximum width 3.4; Hw maximum width 3.2; Pt length on Fw 0.5 and on Hw 0.52; length of metathoracic femur 1.8; metathoracic tibia 1.7; length of S9+10 in lateral view 1.1; length of cercus (dorsal branch) in lateral view 0.45; length of VBP in lateral view 0.37.

Variation of male paratype. The single male paratype is very similar to the holotype, but is generally lighter in coloration, with more extensive pale and darker areas well defined. Minor differences are described below.

Head: Labrum lighter, dark areas brown; labrum with ventral 0.4 yellow. Antefrons yellow, including base of antennifer. Posterior region of the cranium ivory-yellow; 0.2 dorsal brownish-black, not extending ventrally close to occipital foramen.

Thorax: Prothorax black, lighting to brown and yellow ventrally; posterior lobe with small mesal concavity. Mesepisternum, about half of length, with rounded light brown lateral area close to mesopleural suture; whitish-blue wedge-shaped spot at anterior 0.3 larger; mesepimeron and metepisternum with dark spots larger and black. Legs with femora with anteroventral surface armed with 3 on pro- and 4 spurs on meso- and metathoracic legs long and robust; posteroventral surface spurs 3 on pro-, 4 on meso-, and 6–7 on metathoracic leg; anteroventral surface of tibiae armed with 10 spurs (4 of tibial comb) on pro-, 5 on meso-, and 5–6 on metathoracic leg, posteroventral surface with 9 on pro-, 10 on meso- and 11–14 on metathoracic leg.

Wings: MP reaches anal margin at vein descending from subnodus on Fw and very slightly distal on Hw; Px on Fw 12–13; Hw 10; RP2 originating at Px 6 on Fw, at Px 4–5 on Hw.

Abdomen: Pale areas larger than in holotype.

***Measurements* (*mm*)**: Total length (incl. caudal appendages) 35.2; abdomen length (excl. caudal appendages) 31; head maximum width 3.0; Fw length 19.7; Hw length 18.5; Fw maximum width 3.5; Hw maximum width 3.3; Pt length on Fw and on Hw 0.5; length of metathoracic femur 1.9; metathoracic tibia 1.9; length of S9+10 in lateral view 1.1; length of cercus (dorsal branch) in lateral view 0.45; length of VBP in lateral view 0.4.

Female. Unknown.

Larva. Unknown.

Ecology and behavior. Specimens were collected at a slow-running, non-perennial, forested swamp next to a small stream densely covered by aquatic and semiaquatic plants with a soft mud-silt-clay bottom (Fig. 9B). The collection site, at a secondary forest at 100 m a.s.l., was visited twice under very different conditions in the rainy and dry seasons when dramatic changes in the length of the water body occurred. The mesohabitat is very similar to places where *F. regua* and *F. garrisoni* have been collected, i.e., a slow-running stream with shallow water column and fine substrate (silt and clay) under shaded, forested Atlantic Forest formations. Males were seen flying in sunflecks at low height, close to ground level and water surface among dense herbaceous-shrubby vegetation. Other species associated with shaded and forested habitats such as *Perilestes eustaquioi* Machado, 2015, *Metaleptobasis selysii* Santos, 1956, *Idioneura ancilla* Selys, 1860, and a new species of *Heteragrion* were collected at the same site.

Remarks. *Forcepsioneura gabriela* **sp.n.** was recovered forming a group of closely related species together with *F. garrisoni* and *F. regua* (Fig. 4). K2P interspecific distances for COI between *F. gabriela* **sp.n.** and its closest species and *F. garrisoni* was 3.8%, which is generally considered high for intraspecific divergences. This result and the morphological evidence gathered above are considered sufficient for supporting the hypothesis that individuals of *F. gabriela* **sp.n.** represent a species distinct from *F. garrisoni* and *F. regua*.

3.3.2. *Forcepsioneura janeae* sp.n.
(Figs. 7A–D, 8C,D, 9D)

Forcepsioneura lucia nec Machado, 2000; Lencioni (2005: 192, in part, misidentification from specimen from Espírito Santo State, Brazil); Pessacq et al. (2012: 64, record from Espírito

Santo State based on LENCIONI 2005); LENCIONI (2017: 203, in part, replication of the record from Espírito Santo State, Brazil based on LENCIONI 2005).

Material examined. ***Type material***: Holotype ♂, BRAZIL, Espírito Santo State, Santa Teresa municipality, [Biological Station of Santa Lúcia], collecting point 16, limpo 21º [19°56′55″S 40°32′23″W, 796 m a.s.l], 04.iii.2014, Jane Peter Egert Buss & Wander Antônio Martinelli leg. (DZUP 499056). Paratype ♂, same data as holotype, but no additional data further than point 16 (MNRJ 0141; DNA voucher ENT 3403). Paratype on indefinite loan to DZRJ.

Etymology. Specific name of feminine gender, in the genitive form, dedicated to the biologist and fellow colleague Jane Peter Egert Buss, who kindly sent type specimens for study.

Diagnosis. A medium-sized, dorsally brown with bluish-green metallic reflections, laterally to ventrally orange-yellow protoneurid. Frons angulated; rear of the head pale; CuA&AA indistinct; GL with long inner fold and distal lateral lobe elongated into a flagellum according to current concept of *Forcepsioneura* (see PINTO & KOMPIER 2018). Ventrobasal process (VBP) short, with length < 0.3 the length of cercus, distinguishes the new species from *F. gabriela* **sp.n.**, *F. garrisoni*, *F. haerteli*, *F. itatiaiae*, *F. regua* and *F. sancta* (VBP length ≥ 0.55). *Forcepsioneura janeae* **sp.n.** is similar to *F. lucia*, *F. grossiorum*, and *F. serrabonita* by the general orange-black coloration and robust cercus. The new species differs from *F. grossiorum* by the apex of VBP slightly acute (strongly curved ventrally in *F. grossiorum*) and from *F. lucia* by the ratio of VBP and cercus length ≤ 0.3 (as long as ≤ 0.4 in *F. lucia*) and rectangular posterior lobe of prothorax (rounded convex in *F. lucia* and *F. serrabonita*).

Description of male holotype. ***Head*** (Fig. 7A): Labium, visible parts of maxilla and mandibles orangish-yellow, except apex brown. Genae ivory-yellow. Labrum black, expect by transversal orangish-brown stripe occupying 0.25 ventral of labrum length. Anteclypeus black, irregularly spotted with ivory-yellow ventrally; postclypeus black. Antefrons shining black with a pair of elongated pale spots; postfrons and remainder of epicranium opaque black with weak greenish-copper luster. Antenna black; distal apex of scape and posterior surface of pedicel ivory-yellow. Posterior region of cranium ("postgena" plus "occiput") dark yellow to pale-brown, probably ivory-yellow in life; dorsal part 0.40 brownish-black.

Thorax (Fig. 7B): Prothorax black, lightening to yellow laterally; anterior margin of anterior lobe yellow; propleura almost yellow with irregular dark areas; median lobe with lateral margin black, posterolateral margin strongly projected into tubercle-like process; posterior lobe convex, rectangular, width similar to median lobe, posterior margin almost straight, ca 0.13 lateral folded ventrally. Synthorax dorsally dark brown to black with bluish-green metallic reflections; lateral to ventrally orangish-yellow; mesepisternum entirely black with metallic reflections; mesepimeron dark-brown to black with orangish-yellow longitudinal stripe running from of mesinfraepisternum to ca 0.8 posterior, narrowing posteriorly, occupying maximum 0.33 of mesepimeron; metepisternum ivory-yellow with brown longitudinal stripe anterior to metapleural suture, running from to metinfraespisternum to antealar carina; metepimeron ivory-yellow; metapostepimeron ivory-yellow with black spot at lateroventral angle. Legs ivory-yellow with irregular dark-brown to black areas on dorsal surface of femora and tibia; articulations of femur-tibia, tarsal segments, apex of pretarsal claws and spurs black, except scale-like ivory-yellow proximal femoral spurs and tibial comb of prothoracic leg; femora with anteroventral surface armed with long and robust spurs, 3 on pro- and 4 on meso- and metathoracic legs; posteroventral with short and thinner spurs, 4 on all legs; tibiae with anteroventral surface armed with 8 spurs on pro- (3 of tibial comb), 5–6 on meso-, and 6–7 on metathoracic leg; tibiae with posteroventral surface with 8 on pro-, 5–6 on meso- and 11–13 on metathoracic leg.

Wing: Membrane hyaline; venation black, light brown at base; Pt black, quadrangular; MP reaches anal margin at distal 0.25 on Fw, 0.33–0.40 on Hw to vein descending from subnodus, covering 2 cells on all wings; Px on Fw 14; Hw 11–12; RP2 originating at 0.5 distal to Px 6 on Fw, slightly distal at Px 4 on Hw.

Abdomen (Figs. 7C,D, 8C,D): S1–10 tergites dark brown to black dorsally, lateroventrally brown to orange, darker in S8–10, pale areas of S1–3, sternites similar in color to adjacent areas of tergites; pale longitudinal stripe occupying about 0.5 ventral of S1–7 tergites laterally, gradually narrowing to ca 0.2 in S8, narrow line in S9 and ill-defined areas in S10; S3–7 with anterior pale ring ≤ 0.1 of total length of segment, separated dorsally in S3 and S5–7; S1–7 cylindrical, S8–10 distinctly wider than others segments; posterior margin of S10 with slight concavity in dorsal view. Secondary genitalia (based on paratype, Fig. 8C,D) typical of Coenagrionoidea; anterior lamina with deep and acute incision; anterior hamule dark brown, quadrangular, with anteroventral angle acutely projected; posterior hamule almost entirely internalized with a curved thumb-shape; VS longer than wide, maximum width 0.3 of total length in ventral view. Genital ligula (based on paratype, Fig. 8C,D) rectangular in ectal view with L1 smooth, without any kind of special setae; L2 with posteromedial portion of flexure projected distally beyond median region, making its margin slightly convex in ectal view, distal margin (tip of ligula) with mesal concavity; lateral margins prolonged into two curved long flagella, in ectal view basally almost perpendicular; internal fold proximal to flexure, long, ca 0.4 of L2 length in lateral view; no sclerotized tubercle at flexure. Epiproct reduced to membranous-like plate. Cercus (Fig. 7C,D) orange, dark brown on VBP and ventral margin of MBP; in lateral view slightly directed obliquely dorsad, gradually tapering distally; VBP process in lateral view perpendicular to dorsal branch, short, length ca 0.32 of cercus, apex slightly acute, distal edge less than half distance from

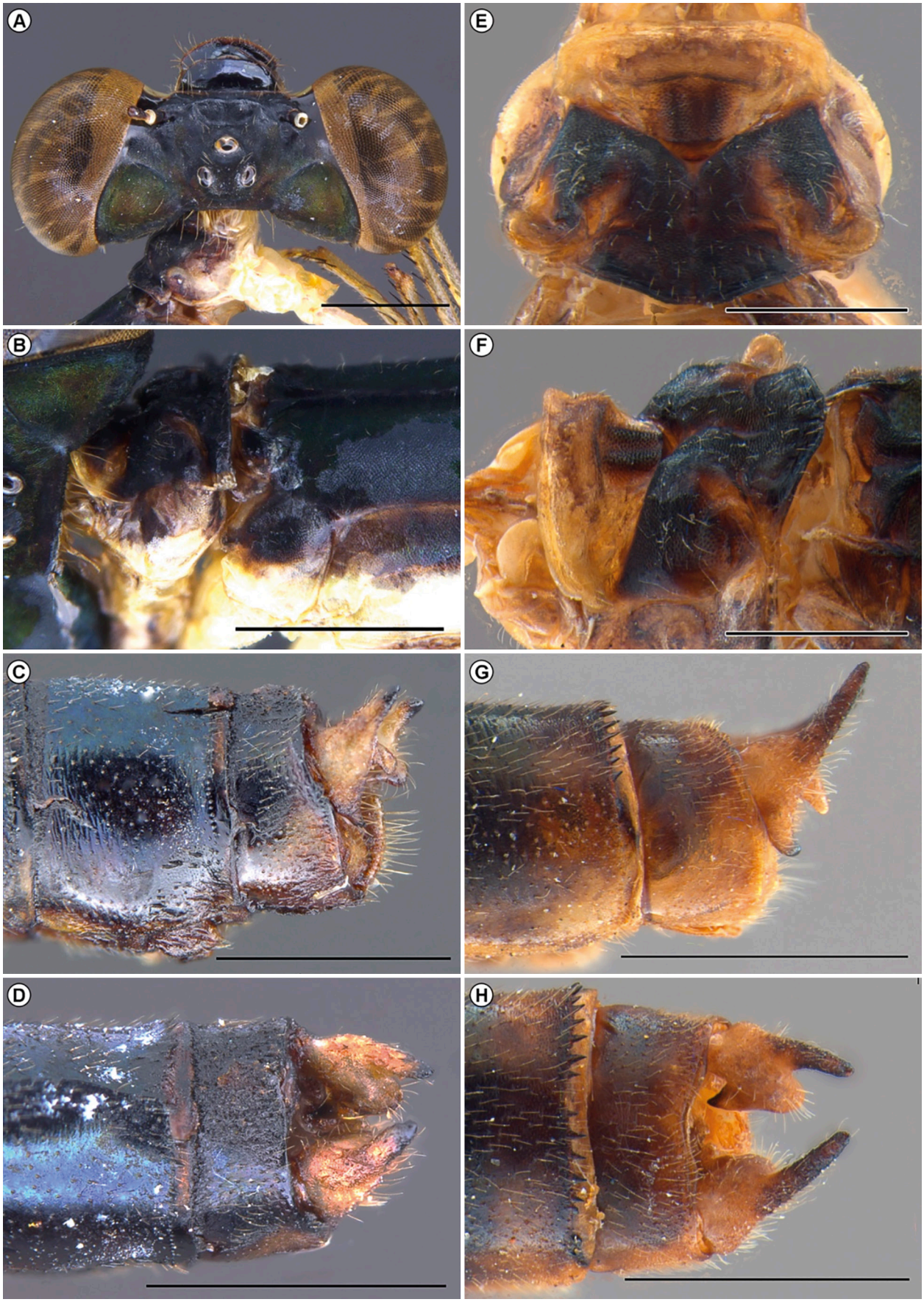

Fig. 7. A–**D**: *Forcepsioneura janeae* **sp.n.** holotype ♂ (Brazil, Espírito Santo: Estação Biológica de Santa Lúcia, DZUP 499056). **A**: head, dorsal view; **B**: prothorax, lateral view; **C**: caudal appendages, lateral view; **D**: caudal appendages, dorsolateral view. **E**–**H**: *Forcepsioneura lucia* paratype ♂ (Brazil, Minas Gerais, Parque Estadual do Rola Moça, DZUP 499902); **E**: prothorax, dorsal view; **F**: prothorax, lateral view; **G**: caudal appendages, lateral view; **H**: caudal appendages, dorsolateral view. – Scale bars: 1 mm.

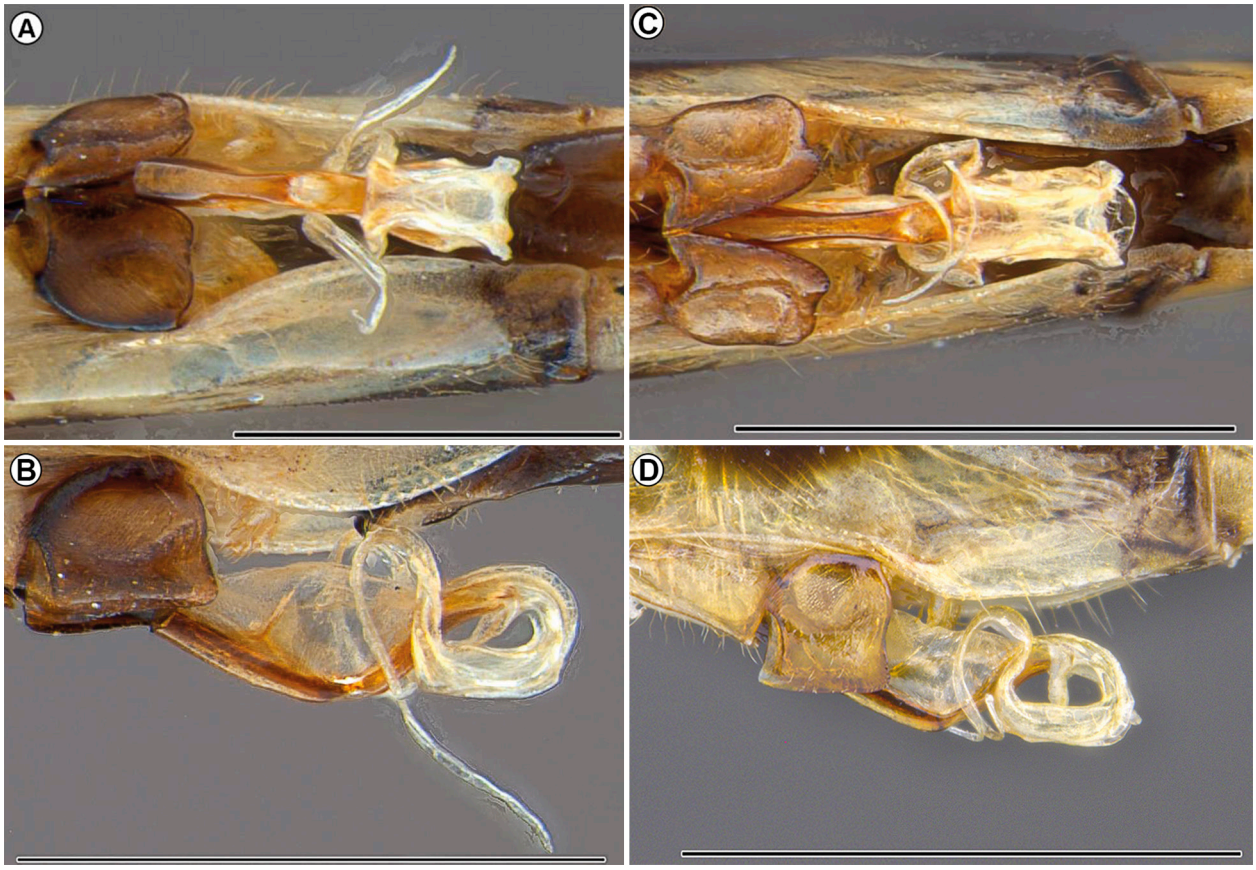

Fig. 8. Genital ligula of *Forcepsioneura* species. *Forcepsioneura gabriela* **sp.n.** holotype ♂ (Brazil, Bahia: Reserva Biológica de Una, DZUP 498858) in ventral (**A**) and lateral (**B**) views. *Forcepsioneura janeae* **sp.n.** paratype ♂ (Brazil, Espírito Santo: Estação Biológica de Santa Lúcia, MNRJ 0141) in ventral (**C**) and lateral (**D**) views. – Scale bars: 1 mm.

ventral margin of S10; MBP largely visible in lateral view such as rounded flat tubercle; tip of cercus blunt, in dorsal view forcipate, wide and robust proximally, slender distally; lateral margin curving at ca 0.5 to apex, internal margin slightly curved; apexes converging; MBP positioned at basal 0.3 of cercus, in posterior view directed ventrally obliquely and projected from a dilated area of cercus; apex of VBP broadly rounded and curved inwardly. Paraproct black brown, plate-like.

***Measurements* (*mm*)**: Total length (incl. caudal appendages) 37.6; abdomen length (excl. caudal appendages) 34; head maximum width 3.3; Fw length 22.7; Hw length 21.3; Fw maximum width 3.7, Hw maximum width 3.6; Pt length on Fw and Hw 0.5; length of metathoracic femur 2.1; metathoracic tibia 1.9; length of S9+10 in lateral view 1.3; length of cercus (dorsal branch) in lateral view 0.4; length of VBP in lateral view 0.13.

Variation of male paratype. The single male paratype is very similar to the holotype. Minor differences are described below.

Wings: MP reaches anal margin at distal 0.25–0.3 on Fw, 0.40–0.45 on Hw to vein descending from subnodus, covering 2 cells on all wings; Px on Fw 12–13; Hw 11; RP2 originating at Px 8 vein on Fw, at Px 7 on Hw.

Abdomen: Epiproct reduced to membranous-like plate. L1 smooth, without any kind of special setae; VBP in lateral view perpendicular to dorsal branch, length ca 0.2 of cercus; apex of VBP broadly rounded and abruptly curved inwardly in posterior view.

***Measurements* (*mm*)**: Total length (incl. caudal appendages) 38; abdomen length (excl. caudal appendages) 32.7; head maximum width 3.2; Fw length 22; Hw length 20.4; Fw maximum width 3.4, Hw maximum width 3.3; Pt length on Fw 0.5 and Hw 0.55; no legs, length of S9+10 in lateral view 1.27; length of cercus (dorsal branch) in lateral view 0.4; length of VBP in lateral view 0.1.

Female. Unknown.

Larva. Unknown.

Ecology and behavior. The two males were collected near a small first order stream with muddy bottom under secondary forest of a typical Atlantic Forest remnant of Southeastern Brazil at 796 m a.s.l. Like other montane species in the genus, it is most likely associated with rocky seepages rather than man-made dams with muddy substrate such as the one at the type-locality (Fig. 9D).

Remarks. Interspecific *p*-distances for 16S between the paratype of *F. janeae* **sp.n.** and specimens of its genetically closest species, *F.* aff. *lucia*, was 0.6%, a value that

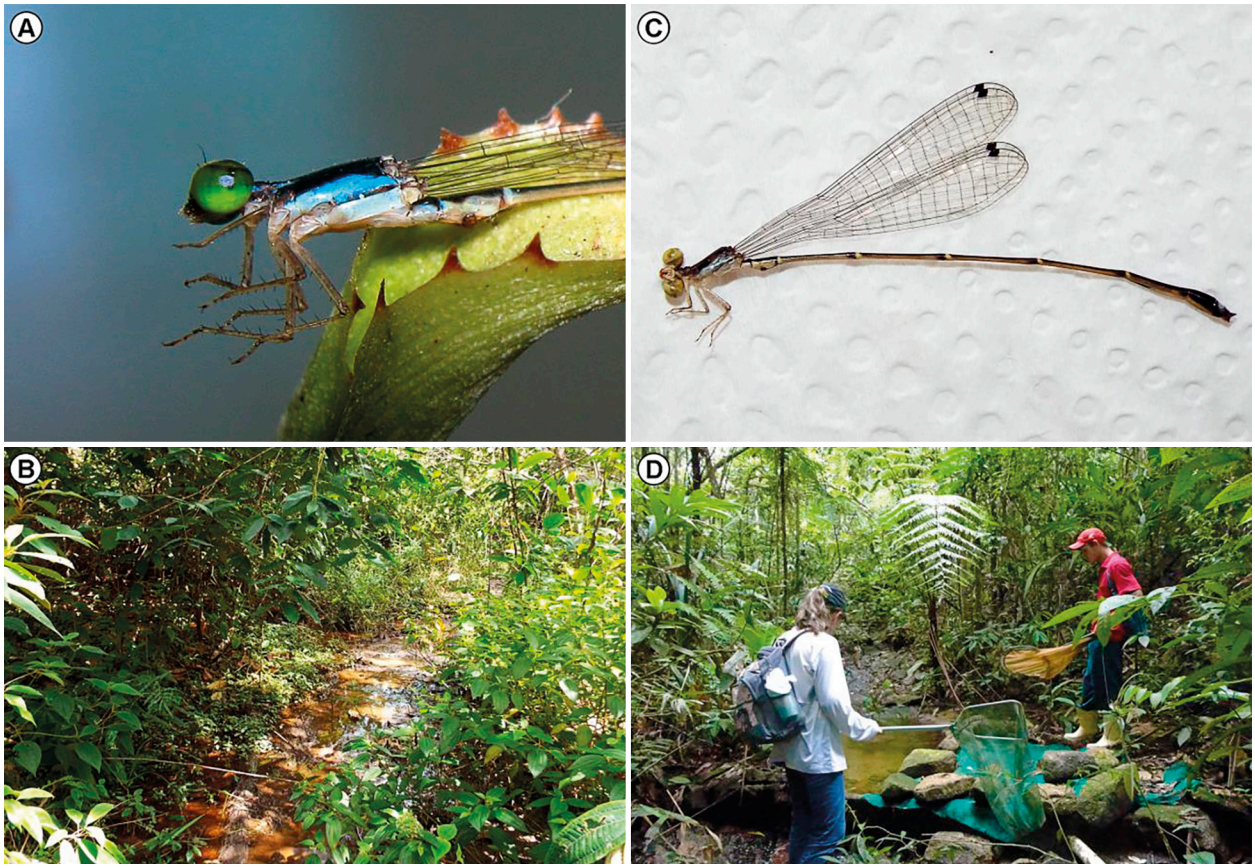

Fig. 9. Habitus and habitat of *Forcepsioneura* species. **A**–**C**: *Forcepsioneura gabriela* **sp.n.** **A**: holotype ♂ (Brazil, Bahia: Reserva Biológica de Una, DZUP 498858). **B**: type locality. **C**: paratype ♂ (same as holotype, DZRJ 3555). **D**: *Forcepsioneura janeae* **sp.n.** type locality at Brazil, Espírito Santo: Estação Biológica de Santa Lúcia. Photos: (A–C) Ângelo P. Pinto, (D) Jane P.E. Buss.

apparently supports the erection of *F. janeae* **sp.n.** as a new species based on molecular data.

The two males are in poor condition, with loss of color and poorly preserved abdominal segments, including S10, and eyes slightly to severely smashed/crushed.

3.4. Phylogeny of *Forcepsioneura*

The current analysis supports *Forcepsioneura* as a monophyletic genus based on the concatenated (Fig. 4) and individual gene (Supplementary Figs. S1–S3) Bayesian inference trees. In the concatenated tree, two main groups were recovered with moderate support: one included *F. janeae* **sp.n.** and *Forcepsioneura* aff. *lucia* (PP = 97%), and the other included all other *Forcepsioneura* species examined (PP = 50%). This subclade includes *F. serrabonita* as sister to the light blue group (PP = 96%) containing *F. sancta* (PP = 89%) and a group of species closely related to *F. garrisoni* (PP = 99%). Resulting Bayesian and NJ trees based on COI and 16S also recovered a monophyletic light blue group (*F. sancta*, *F. garrisoni*, *F. regua*, and *F. gabriela* **sp.n.**) with moderate support. A well-supported monophyletic group of three very closely related species was identified (*F. garrisoni*, *F. regua*, and *F. gabriela* **sp.n.**), despite the low support (BS = 64%) in the PRMT NJ tree.

4. Discussion

4.1. Species delimitation

4.1.1. Morphological data

Taxonomy of lower categories, hence species delimitation, in Protoneurinae is largely based on the caudal appendages and genital ligula of males (see discussion in Pinto & Kompier 2018). Often species-level is distinguished based on minor differences of caudal appendages. Several Protoneurinae genera group species with great similarity in general appearance thus making species identification a hard task (e.g., von Ellenrieder & Garrison 2008; Anjos-Santos & Pessacq 2013). The two new species herein proposed were erected after a careful study of the external morphology that allowed observation of convincing, although slight, differences used for distinction from their congeners. In *F. gabriela* we have detected minor differences in caudal appendages, wing venation, and prothorax from the two other very similar species of the light blue group, while in *F. janeae* equally minor differences in the caudal appendages and prothorax of males from the orange-black group. All these differences combined with genetic distances of COI (*F. gabriela*) and 16S rDNA (*F. janeae*) supported the erection

Table 7. Intraspecific K2P distances of COI sequences of some Zygoptera species published in the literature. (* denotes uncorrected values.)

Family	Species	# of individuals	Intraspecific distances	References
Amphipterygidae	*Pentaphlebia mangana*	7	0 – 0.009	DIJKSTRA et al. 2015
Coenagrionidae	*Xanthocnemis zealandica*	151	0–0.016*	NOLAN et al. 2007
	Xanthocnemis zealandica	51	0–0.032	MARINOV et al. 2016
	Acanthagrion aepiolum	2	0–0.003	KOROIVA et al. 2017
	Acanthagrion dorsale	5	0.006	KOROIVA et al. 2017
	Argia reclusa	4	0.007	KOROIVA et al. 2017
	Argia smithiana	2	0.0015	KOROIVA et al. 2017
	Argia tamoyo	4	0	KOROIVA et al. 2017
	Homeoura nepos	3	0.0015	KOROIVA et al. 2017
	Oxyagrion sulmatogrossense	5	0	KOROIVA et al. 2017
	Oxyagrion terminale	2	0.0154	KOROIVA et al. 2017
	Telebasis willinki	4	0.003	KOROIVA et al. 2017
	Ceriagrion banditum	6	0–0.003	DIJKSTRA et al. 2015
	Ceriagrion obfuscans	10	0–0.011	DIJKSTRA et al. 2015
	Pseudagrion aureolum	6	0–0.002	DIJKSTRA et al. 2015
	Pseudagrion dactylidium	3	0–0.005	DIJKSTRA et al. 2015
	Pseudagrion pacale	5	0–0.002	DIJKSTRA et al. 2015
	Pseudagrion tanganyicum	7	0–0.026	DIJKSTRA et al. 2015
	Nesobasis brachycera	2	0.004	BEATTY et al. 2017
	Papuagrion marijanmatoki	3	0–0.003	KALKMAN & ORR 2016
	Papuagrion occipitale	4	0.002–0.015	KALKMAN & ORR 2016
Chlorocyphidae	*Africocypha varicolor*	7	0–0.023	DIJKSTRA et al. 2015
	Chlorocypha flammea	2	0	DIJKSTRA et al. 2015
Heteragrionidae	*Heteragrion triangulare*	3	0–0.001	KOROIVA et al. 2017
Megapodagrionidae	*Rhinagrion atripes*	4	0.02	CASAS et al. 2017
	Rhinagrion flammea	2	0.013	CASAS et al. 2017
	Rhinagrion fulgifrons	3	0.006	CASAS et al. 2017
	Rhinagrion tendipes	4	0.015	CASAS et al. 2017
Platycnemididae	*Coeliccia angustior*	2	0.067	CASAS et al. 2017
	Elattoneura aurifex	5	0.003–0.012	DIJKSTRA et al. 2015
	Elattoneura lapidaria	7	0–0.011	DIJKSTRA et al. 2015
	Elattoneura tambotonorum	2	0–0.002	DIJKSTRA et al. 2015

of these species in an integrative approach due to congruence between these two sources of data.

4.1.2. Genetic distances

With the exception of *F. sancta* (K2P distances up to 5%), intraspecific COI diversity in *Forcepsioneura* species was in agreement with previous studies that reported maximum intraspecific divergence values < 2% for odonate species from the suborder Zygoptera (Table 7). However, a recent meta-analysis of the barcoding gap in 497 Zygoptera species reported a level of intraspecific variation of 0–22%, with only ca. 10% of species having divergence values > 3% (KOROIVA & KVIST 2017). The high intraspecific diversity for *F. sancta* relative to other *Forcepsioneura* species may be explained by the large sample size (14 specimens), which better captured the geographical diversity of the species and provided evidence for the possibility that some populations represent cryptic species and should be properly investigated.

Nuclear markers, including PRMT, have been successfully used to identify high levels of polymorphism in specimens of *Coenagrion mercuriale* (Charpentier, 1840) and to describe a new species of *Onychogomphus* Selys, 1854 (FERREIRA et al. 2014a,b). However, nuclear PRMT and ribosomal 16S rDNA were apparently too conservative to reveal any differences between the very similar *F. gabriela* **sp.n.**, *F. garrisoni*, and *F. regua*. Nevertheless, COI distances resulted in an intraspecific divergence value of 3.8% between *F. gabriela* **sp.n.** and *F. garrisoni*, which is well above the average intraspecific divergence for Zygoptera (Table 7). Moreover, detailed and rigorous morphological comparisons with other species (see diagnosis and Table 6) provided robust evidence to propose *F. gabriela* **sp.n.** as a new species.

Evidence for the distinction between *F. janeae* **sp.n.** and *F.* aff. *lucia*, in addition to morphological characters, was also based in a small variation (interspecific distance 0.6%) of 16S rDNA (Table 4). Although the 16S region is not widely used in species delimitation studies in insects, the distance found between these two species was higher

than no variation found between other related pair of species in *Forcepsioneura*. In insects, the 16S has a lower rate of substitution in comparison to COI, thus it has been neglected as a marker used in species delimitation studies, but Misof et al. (2002) show that 16S substitution rates are uneven across insect clades, thus, a significant interspecific distance is strongly clade dependent.

Finally, a large dataset of COI barcode sequences of Zygoptera (Koroiva & Kvist 2017) failed to reveal any differences between intraspecific and interspecific values of genetic distances (barcoding gap). This finding shows the importance of integrating morphological and molecular data in taxonomic studies, especially in highly similar and closely related species, as was the case of *F. gabriela* **sp.n.**

4.2. *Forcepsioneura* phylogeny

The current analysis recovered *Forcepsioneura* as monophyletic with high support. However, we acknowledge that we did not include probably highly related genera such as *Psaironeura* Williamson, 1915 for a more robust test of its monophyly.

Two main groups of species were recovered in the Bayesian inference analysis. However, these groups apparently do not agree with the two species groups tentatively suggested by Pinto & Kompier (2018). In our analysis, the orange-black group is not monophyletic (with low support) because *F. serrabonita* is recovered as sister group to the monophyletic light blue group. This species was expected to be more closely related to *F. janeae* **sp.n.** (herein included in the orange-black group) and *F.* aff. *lucia*, the latter of which is very similar to *F. lucia*. Even though Pinto & Kompier (2018) tentatively erected these groups and recognized them as potentially not reflecting phylogenetic relationships, the division is premature.

In all analyses conducted herein, *F. garrisoni*, *F. regua*, and *F. gabriela* **sp.n.** were recovered as a monophyletic group. Species in this group are morphologically very similar, sharing the blue coloration of the lateral portion of the synthorax and slender cercus with comparatively long VBP. Unfortunately, gaps in gene sampling and the highly conserved markers used prevented us from confidently establishing interspecific relationships among these species. As an example, *F. regua* specimens did not group together in the concatenated analysis, which is possibly a result of these factors.

5. Conclusions

This was the first attempt to establish phylogenetic relationships among species of a South American damselfly genus from the Atlantic Forest using DNA sequences. The phylogeny of *Forcepsioneura* presented herein supported the monophyly of the genus and its internal relationships. In most cases, the genetic distance method was able to distinguish intra- and interspecific divergence within *Forcepsioneura*. This study confirms the value of COI sequence variation in species-level studies, which recovered high intraspecific divergences in one case, but does not support the use of PRMT, as previously advocated, as there was very little to no variation among specimens of the same species. Additionally, very low interspecific variation was found between PRMT and 16S sequences in some closely related species pairs examined. Of the molecular markers used in a preliminary study, 16S rDNA provided the best information to resolve relationships among species with good clade support. Nevertheless, this study has some limitations, including unbalanced molecular sampling, missing data, and the use of few not very informative genes.

Finally, using an integrative approach based on morphological and molecular data, we described two new species from the states of Bahia and Espírito Santo. We suggest the proposal of new taxa in this group be based on evidence from both morphological and molecular data to accurately identify relationships among *Forcepsioneura* species, especially complexes of highly similar species.

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File 1: pimenta&al-zygopteraforcepisoneura-asp2019-electronic supplement-1.doc — **Table S1.** K2P pairwise distances of COI sequences of *Forcepsioneura* and outgroup taxa. Intraspecific distances of *F.* aff. *lucia* in gray, *F. sancta* in blue, *F. garrisoni* in red, and *F. gabriela* **sp.n.** in green. — **Table S2.** Uncorrected genetic distances (*p*-distance) of 16S sequences of *Forcepsioneura* and outgroup taxa. Intraspecific distances of *F.* aff. *lucia* in gray, *F. sancta* in blue, *F. regua* in orange, and *F. gabriela* **sp.n.** in green. — **Table S3.** Uncorrected genetic distances (*p*-distance) of the nuclear gene PRMT of *Forcepsioneura* and outgroup taxa. Intraspecific distances of *F.* aff. *lucia* in gray, *F. sancta* in blue, *F. garrisoni* in red, and *F. gabriela* **sp.n.** in green. — **Fig. S1.** Bayesian post-burn-in consensus of COI dataset of *Forcepsioneura* and outgroup taxa. Node-associated values are posterior probabilities higher than 50%. — **Fig. S2.** Bayesian post-burn-in consensus of 16S dataset of *Forcepsioneura* and outgroup taxa. Node-associated values are posterior probabilities higher than 50%. — **Fig. S3.** Bayesian post-burn-in consensus of PRMT dataset of *Forcepsioneura* and outgroup taxa. Node-associated values are posterior probabilities higher than 50%.

Authors' contributions

A.L. Pimenta gathered and analyzed the data. A.L. Pimenta and A.P. Pinto conducted the descriptions and the taxonomic study. A.P. Pinto and D.M. Takiya verified analytical methods. All authors discussed the results and contributed to the final version of the manuscript.

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