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A reappraisal of the family status of Neotropical Protoneuridae (Odonata: Zygoptera) using morphological and molecular information

Pablo Pessacq[1,2,‡], Danielle Anjos-Santos[1,2], Adolfo Cordero-Rivera[3], M. Olalla Lorenzo-Carballa[3,4,‡,*]

[1]Laboratorio de Investigaciones en Ecología y Sistemática Animal, CIEMEP, UNPSJB, CONICET-CCT-PATAGONIA NORTE, Gral. Roca 780, Esquel (9200), Chubut, Argentina
[2]Facultad de Ciencias Naturales y Ciencias de la Salud, UNPSJB, Sede Esquel (9200), Chubut, Argentina
[3]Universidade de Vigo, ECOEVO Lab, Escola de Enxeñaría Forestal, Campus A Xunqueira, 36005 Pontevedra, Galiza, Spain
[4]Department of Zoology, Genetics and Physical Anthropology, Universidade de Santiago de Compostela, Santiago de Compostela, Spain
‡These authors contributed equally.
*Corresponding author. Department of Zoology, Genetics and Physical Anthropology; Facultade de Bioloxía, University of Santiago de Compostela; R/ Lope Gómez de Marzoa s/n; CP: 15782 Santiago de Compostela, Spain. E-mail: olalla.lorenzo.carballa@usc.es

ABSTRACT

Several comprehensive studies have greatly contributed to the clarification of Odonata phylogeny, paying special attention to the damselflies (Zygoptera). Nearly half of the species of Zygoptera are included in the family Coenagrionidae, but the status of some previously recognized families is still debated. Here, we present the results of phylogenetic analyses based on nuclear and mitochondrial sequences and morphological data of 10 of the 15 Neotropical genera formerly included within the Neotropical family Protoneuridae, with the goal to test their monophyly and phylogenetic position within the Coenagrionidae *sensu lato*. Our analyses support the polyphyly of Protoneuridae, with *Proneura prolongata* and *Junix elumbis* falling within the 'core' Coenagrionidae, whereas the remaining Neotropical Protoneuridae included in our analyses form a monophyletic clade, sister to the ridged frons Coenagrionidae. Our results differ from previous analyses that suggested that the Protoneuridae were members of the ridged frons Coenagrionidae clade, most likely because our dataset has a wider coverage of the group, both in terms of taxa and data sources. We propose the redefinition of the Protoneuridae (excluding *Proneura*, *Junix*, and all the previously included Old-World genera) and its re-establishment as a redefined Protoneuridae family, which is characterized by several morphological and biological unique attributes.

Keywords: damselfly; Coenagrionoidea; morphology; DNA; phylogeny; integrative taxonomy; cladistic analysis

INTRODUCTION

With about 3000 currently described species, damselflies (suborder Zygoptera) represent nearly half of the species within the insect order Odonata (Lorenzo-Carballa and Cordero-Rivera 2014). The Zygoptera are highly diverse in the Neotropics, with nearly 2000 known species and with new species constantly being described (Neiss and Hamada 2014, Pessacq *et al.* 2018). The monophyly of the Zygoptera has been well established by several studies (Carle 1982, Bechly 1996, Rehn 2003, Bybee *et al.* 2008, 2021, Dijkstra *et al.* 2014), but the status and composition of some groups within the suborder has remained conflicting over time. The currently accepted Odonata classification recognizes four superfamilies of Zygoptera: Lestoidea, Platystictoidea, Calopterygoidea, and Coenagrionoidea; the latter including the families Isostictidae, Platycnemididae, and Coenagrionidae (Dijkstra *et al.* 2013).

The monophyly and composition of the Coenagrionoidea has been challenged in the last decades, e.g. the positions of Isostictidae and Pseudostigmatidae and the monophyly and position of Protoneuridae, have changed significantly (Bybee *et al.* 2008, 2021, Dijkstra *et al.* 2014). Regarding Coenagrionidae, the traditional arrangement of subfamilies has received little or no support (e.g. O'Grady and May 2003) and the phylogenetic analyses of this family based on molecular data (Dijkstra *et al.* 2014) supported two large groups: (i) the so-called 'core' Coenagrionidae, very likely monophyletic, and (ii) a group of species that mostly possess a ridged frons and lack postocular

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spots, with less resolved relationships (a topology first supported by Carle *et al.* 2008). Dijkstra *et al.* (2014) mentioned that if these two main groups were supported by further analyses, each could be treated as a family, or they could also be considered a subfamily each, thus retaining a very large Coenagrionidae family. The latter approach is maintained in the currently accepted classification of the Odonata (Dijkstra *et al.* 2013).

The most recent and comprehensive Odonata phylogeny published by Bybee *et al.* (2021), also supports two groups within Coenagrionidae, but it is difficult to arrive at a valid comparison between this work and the one of Dijkstra *et al.* (2014) due to the smaller number of species included in the former (12 vs. 62 species, respectively) and the differences in the sampling of taxa between both studies.

The former Protoneuridae family traditionally included all Neotropical and Palaeotropical coenagrionid damselflies that generally possess a rectangular discoidal cell and reduced venation. The first morphological cladistic analyses including protoneurids (Bechly 1996, O'Grady and May 2003, Rehn 2003), albeit comprising a limited number of species, obtained conflicting results. Bechly (1996) placed Protoneuridae (including Isostictinae) plus Platycnemididae as sister to Coenagrionidae. Rehn's (2003) results supported the Neotropical taxa as monophyletic, closely related to Isostictidae and separated from their Palaeotropical counterpart, which showed a closer relationship with Platycnemididae. However, O'Grady and May (2003) obtained a tree where the representatives of Neotropical and Palaeotropical 'Protoneuridae' nested together as a clade within Coenagrionidae.

With a similar sampling of taxa, but using molecular data, Carle *et al.* (2008) obtained results concordant with Rehn's work: the Neotropical Protoneuridae nested within Coenagrionidae, whereas the Palaeotropical Protoneuridae were included in a clade with Platycnemididae and separated from Coenagrionidae. The analysis of Bybee *et al.* (2008), based on combined molecular and morphological data, further supported the conclusions of both Rehn (2003) and Carle *et al.* (2008) regarding Protoneuridae, and found a sister-relationship between Palaeotropical Protoneuridae and Platycnemididae; whereas the Neotropical Protoneuridae were grouped in a clade related to the clade Coenagrionidae + Pseudostigmatidae. Pessacq (2008) carried out a cladistic morphological analysis, to test the monophyly of Protoneuridae, in which he included most Neotropical genera and several Old World representatives of the group. His results corroborated the split of Neotropical and Palaeotropical protoneurids, with the latter as a sister-group to Platycnemididae.

The molecular phylogeny of Zygoptera by Dijkstra *et al.* (2014) included many Coenagrionidae and Platycnemididae, four genera of Neotropical Protoneuridae, and five genera of Palaeotropical Protoneuridae. The results of this work further confirmed the polyphyly of Protoneuridae, with the Palaeotropical component recognized as a subfamily (Disparoneurinae) of Platycnemididae, and the Neotropical counterpart falling in most of the analyses within the clade of ridged frons coenagrionids. Finally, the recent molecular phylogeny of Odonata by Bybee *et al.* (2021) arrived at the same conclusion: 'Old World genera once placed in Protoneuridae are not closely related to the New World representatives' (Bybee *et al.* 2021: 9), and 'The New World taxa "represented in this study by *Neoneura* and *Protoneura*" also form a monophyletic group and are firmly established as members of the Coenagrionidae' (Bybee *et al.* 2021: 9).

Even though the results of Dijkstra *et al.* (2014) supported several distinct groups within Coenagrionidae, the authors chose to preserve former familiar names as subfamilies only in a vernacular sense and stressed that 'sampling and support are still insufficient for a comprehensive subdivision' (Dijkstra *et al.* 2014: 83). Furthermore, the classification of Odonata by Dijkstra *et al.* (2013) also merges the Neotropical Protoneuridae within Coenagrionidae, without proposing any taxonomic rank for the group.

Here, we have chosen to refer to the Neotropical Protoneuridae as 'Protoneuridae', and we use Coenagrionidae *s.s.* to refer to Coenagrionidae minus 'Protoneuridae'. We also use Coenagrionidae *s.l.* to refer to Coenagrionidae in its wider sense, i.e. the currently accepted classification of the group including 'Protoneuridae'.

So far, 'Protoneuridae' comprise 15 genera and 123 species (Garrison *et al.* 2010, Anjos-Santos and Pessacq 2013, Pessacq 2014, Lorenzo-Carballa *et al.* 2016, Tennessen 2016, Pinto and Kompier 2018, Pimenta *et al.* 2019, Mendoza-Penagos *et al.* 2022, Pessacq *et al.* 2022). To date, however, no studies have included enough representatives of both Coenagrionidae *s.s.* and 'Protoneuridae' that allow testing the monophyly of the latter, along with its taxonomic status. Here we analyse the position and test the monophyly of 'Protoneuridae' within Coenagrionidae, as well as the relationships among its members and the monophyly of some of its most species-rich genera, using both morphological and molecular data from most of the 'Protoneuridae' genera and representatives of Coenagrionidae *s.s.*.

MATERIALS AND METHODS

Sample collection

Samples of Odonata for genetic and morphological analyses were gathered from different collections (acronyms in parentheses): A. Cordero-Rivera's collection at the ECOEVO laboratory in the University of Vigo (ACR), Mark McPeek's personal collection (MMP), Rosser W. Garrison's personal collection (RWG), the Lund Museum of Zoology insect collection (MZLU), the Entomological Collection at the University of Antioquia in Colombia (CEUA), the California State Collection of Arthropods (CSCA), the entomological collection of the Museum of the Institute of Agricultural Zoology 'Francisco Fernandez Yépez' in Venezuela (MIZA), the collection at the National Museum of Rio de Janeiro in Brasil (MNRJ), the collection at the Laboratorio de Biodiversidad y Genética Ambiental in Avellaneda, Argentina (BioGEA), and the collection at Centro de Investigación Esquel de Montaña y Estepa Patagónica in Esquel, Argentina (CIEMEP).

DNA extraction, polymerase chain reaction amplification, sequencing, and molecular phylogenetic analyses

Specimens belonging to the ACR, BioGEA, CEUA, CIEMEP, CSCA, MMP, MZLU, and RWG collections were selected for molecular analyses. Samples were either preserved in ethanol (ACR, MMP, and MZLU) or dry-preserved (BioGEA, CSCA,

CEUA, RWG, and CIEMEP) prior to DNA extraction (see Supporting Information, File S2, Table S2). Total genomic DNA was extracted and purified from a single leg of each individual, using silica adsorption-based protocols implemented by different commercial kits [the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany), the Dneasy Tissue kit (Qiagen, Venlo, The Netherlands) or the GeneJet DNA extraction kit (ThermoFisher Scientific, Waltham, Massachusetts)], following the manufacturer's instructions in each case. We selected two nuclear (28S and *PRMT*) and two mitochondrial (16S and *COII*) DNA fragments for polymerase chain reaction (PCR) and Sanger sequencing. PCR amplifications were carried out using the DreamTaq Green PCR Master Mix (ThermoFisher Scientific) at specific annealing temperatures using previously published primers (see Supporting Information, File S1.1). Prior to sequencing, unincorporated primers and dNTPs were removed using Shrimp Alkaline Phosphatase and Exonuclease I (New England Biolabs, Ipswich, Massachusetts), and purified PCR products were sequenced in both directions using BigDye v.3.1 chemistry (Applied Biosystems, Foster City, California) and capillary electrophoresis on an ABI3730xl Genetic Analyzer (Applied Biosystems) at the Macrogen facilities in Madrid (Spain).

Chromatograms were visually inspected and assembled with GENEIOUS v.9.1.7 (https://www.geneious.com). For the nuclear DNA (nDNA) sequences, ambiguities with similar peak heights were considered to be heterozygous positions and re-coded with IUPAC codes. For the *PRMT* locus, there were several instances of individuals showing superimposed traces typical of heterozygous indels, which were resolved using the software INDELLIGENT v.1.2 (Dimitriev and Rakitov 2008; available at: http://dmitriev.speciesfile.org/indel.asp). BLAST searches were run for all obtained DNA sequences at the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) through GENEIOUS v.9.1.7; to ensure that they were not derived from contaminations or mitochondrial nuclear copies (*numts*; see: Lorenzo-Carballa *et al.* 2022, Ožana *et al.* 2022). All sequences generated in this study have been deposited in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). Information on accession numbers for each sequence is provided in Supporting Information, Table S2.

Previously published sequences belonging to representative genera/species were downloaded from GenBank to be added to our datasets. The final number of individuals included in the molecular phylogenetic analyses was 168. These included representatives of Coenagrionidae (68 individuals representing 41 species from 15 genera) and 'Protoneuridae' (86 individuals representing 42 species from 10 genera). The remaining 14 individuals from the dataset corresponded to two Calopterygidae and five Platycnemididae species, which were selected as outgroups for the phylogenetic analyses (see Supporting Information, Tables S1, S2).

Sequences were aligned using MAFFT (Katoh *et al.* 2002) as implemented in GENEIOUS v.9.1.7, using the *G-INS-i* algorithm, with a gap penalty of 3. Alignments were concatenated in GENEIOUS v.9.1.7 for phylogenetic analysis. Phylogenetic relationships were reconstructed using maximum likelihood (ML) and Bayesian inference (BI) approaches. ML trees were built using the algorithms implemented in IQ-TREE 1.6.12 (Nguyen *et al.* 2015). Analyses were run on the partitioned dataset under the inferred best-fitting model of nucleotide substitution (Chernomor *et al.* 2016, Kalyanamoorthy *et al.* 2017), and branch support was assessed with 10 000 ultrafast bootstrap replicates (Hoang *et al.* 2018), with the nearest neighbour interchange search option to optimize each bootstrap tree. BI analyses were carried out in MrBayes v.2.3.7 (Huelsenbeck and Ronquist 2001). Heuristic searches were run for 25 million generations in two independent runs with default priors, allowing partitions to evolve under different rates, and with the GTR+Gamma+I substitution model. The convergence of the runs was assessed by examining the estimated sample size (ESS) and the potential scale reduction factor (PSRF; Gelman and Rubin 1992) values. Burn-in samples (25%) were discarded, and the remaining were combined to produce a 50% majority rule consensus tree, with bipartition frequencies equal to posterior probability values.

The resulting trees were edited using FigTree 1.4.3 (available at http://tree.bio.ed.ac.uk/software/figtree/) and INSKCAPE 1.1.1 (Inkscape project, 2020; available at https://inkscape.org/).

Morphological character selection and codification

A total of 77 morphological characters were selected for the morphological cladistic analyses. These included 14 characters traditionally used to define families or genera, 45 characters used by other authors or by us in previous analyses and modified for this study, and 18 new characters (see Supporting Information, File S1.2, Fig. S1). Morphological character definition followed the aim of testing the monophyly and the position of the 'Protoneuridae' within Coenagrionidae, as well as to test the relationships between its genera. Out of the selected morphological characters, 14 were continuous and the remaining 63 were discrete characters. Character coding for adults was based, whenever possible, on the direct examination of specimens. In those cases where no material was available, information was retrieved from the literature and/or through the examination of specimen pictures. The terminology for wing venation follows Riek and Kukalová-Peck (1984). Due to the poor knowledge of immature stages for Neotropical Odonata (larval stages are unknown in 25% of the genera and 60% of the total number of species; Pessacq *et al.* 2018), only six larval characters were included in the morphological data matrix. All larval characters included in the data matrix were coded based on the literature (see Supporting Information, File S1.2, S1.4).

For the qualitative characters the character and its state (e.g. char. 25: 1) or the transformation sequence (e.g. char. 25: 0–1) are mentioned in the text if needed. For the quantitative characters (chars. 1–14) the character states are not included in the text.

Morphological cladistic analyses

Specimens belonging to the ACR, BioGEA, CIEMEP, MIZA, MNRJ, and RWG collections were selected for morphological cladistic analysis. The final dataset included a total of 75 terminal taxa. The ingroup included 27 species of Coenagrionidae *s.s.* belonging to 15 genera, along with all the 15 genera of 'Protoneuridae', represented by 41 species. The outgroup included two species of Calopterygidae and five species of Platycnemididae (see Supporting Information, File S2, Tables

S1, S2). The differences in the number of taxa included in the morphological cladistic analyses compared to the molecular phylogenetic analyses stem from the fact that several taxa could not be directly examined. For these, the literature did not provide enough information, which resulted in many characters not codified, particularly the continuous characters. As this set of characters was important for the definition of clades in the morphological analyses, those taxa for which no continuous characters could be codified, and which also lacked several discrete characters, were excluded from the analysis. Likewise, there were genera for which molecular information could not be obtained but were included in the morphological cladistics analysis.

Morphological cladistic analyses were carried out using TNT (tree analysis using new technology) (Goloboff *et al.* 2008). A heuristic search of the most parsimonious trees was carried out with the TBR function implemented in TNT (random seed 1, 10 replicates, saving 10 trees per replica and collapsing trees after the search). A total of 10 000 trees were saved to memory. Weighted analyses were performed to resolve conflicts between homoplastic character distribution, and a posteriori weighting was chosen, using the command *implied weighting* (IW) (Goloboff 1993, Giribet 2003). This method has proved to be effective in increasing jack-knife frequencies and generating more stable results than under equal weights (Goloboff 2008). In TNT, the constant *k* can be modified so that when the value increases, homoplastic transformations are down-weighted more mildly (Goloboff 1993, 1997), while with higher values of *k*, the method tends to equal weighting. There is not a unique true value of *k*, different values will probably support different groups; there are no well-justified criteria to choose a particular value of *k*, and this decision is probably matrix-dependent (Mirande 2009). Therefore, it is necessary to explore different *k* values to verify which groups are supported with all different values (Goloboff 2008). Following this, we explored *k* values from 5 to 15, as these have been previously used to test weighting methods (Goloboff *et al.* 2018). Jack-knife support (removal probability: 36, number of replicates: 100), along with consistency and retention indexes were calculated using TNT default commands. All the characters were considered non-additive, except for the continuous characters. The resulting trees were edited using INSKCAPE v.1.1.1 (Inkscape project, 2020; available at https://inkscape.org/).

Combined molecular and morphological phylogenetic analyses

For the analysis including both molecular and morphological data, the morphological character matrix had to be modified, as it was not possible to include continuous characters in the combined analyses. Therefore, those continuous morphological characters that showed a clear gap in their numeric values were transformed into discrete numbers. This transformation could be done with four characters (Supporting Information, File S1.3), yielding a total of 67 morphological characters that were included in the combined molecular and morphological data analyses. After selecting a representative of each species from which both DNA (preferably with information for all sequenced markers) and morphological data were available, a total of 87 species were included in the combined analysis. From these, 41 were 'Protoneuridae' (10 genera), 38 were Coenagrionidae (14 genera), two were Calopterygidae (two genera), and six were Platycnemididae (three genera) (see Supporting Information, File S2, Tables S1, S2).

Phylogenetic relationships were reconstructed using ML and BI approaches. ML trees were built using the algorithms implemented in IQ-TREE 1.6.12 (Nguyen *et al.* 2015). Analyses were run on the partitioned dataset under the inferred best-fitting substitution model (Chernomor *et al.* 2016, Kalyanamoorthy *et al.* 2017) and with the same options as described above for the ML molecular phylogenetic analyses. BI analyses were carried out in MrBayes v.2.3.7 (Huelsenbeck and Ronquist 2001) as described above, except that heuristic searches were run for 10 million generations. The GTR+Gamma+I substitution model was applied to the molecular partitions, whereas the Markov k model (Lewis 2001) was applied to the morphological partition. Resulting trees were edited using FigTree v.1.4.3 (available at http://tree.bio.ed.ac.uk/software/figtree/) and INSKCAPE v.1.1.1 (Inkscape project 2020; available at https://inkscape.org/).

RESULTS

Molecular phylogenetic analyses

The results of the molecular phylogenetic analyses were congruent between methods: both BI and ML yielded similar topologies and supported the polyphyly of 'Protoneuridae', with *Proneura prolongata* Selys, 1889 falling within the Coenagrionidae *s.s.*, closely related to *Acanthagrion* Selys, 1876 (Fig. 1). The remaining 'Protoneuridae' included in our analyses form a monophyletic clade, with high bootstrap support (BS) and posterior probability (PP) values (Fig. 1).

The Coenagrionidae *s.s.* split into three main clades (Fig. 1): the first group is comprised of the 'core' Coenagrionidae genera (*Coenagrion* Kirby, 1890, *Pseudagrion* Selys, 1876, *Agriocnemis* Selys, 1877, *Pacificagrion* Fraser, 1926, *Ischnura* Charpentier, 1840, *Proischnura* Kennedy, 1920, and *Acanthagrion*), plus the monotypic 'Protoneuridae' *Proneura prolongata*. The second group includes those genera of Coenagrionidae *s.s.*, which, in most cases, possess a ridged frons (*Telebasis* Selys, 1865, *Aeolagrion* Williamson, 1917, some species of *Phoenicagrion* von Ellenrieder, 2008, *Bromeliagrion* De Marmels in De Marmels and Garrison, 2005, *Metaleptobasis* Calvert, 1907, *Inpabasis* Santos, 1961, and *Teinobasis* Kirby, 1890). Finally, all the *Argia* Rambur, 1842 species included in our analyses were grouped in a separate clade. These three clades were all supported with high BS and PP values (Fig. 1).

The 'Protoneuridae' clade appears more closely related to the ridged frons Coenagrionidae; a relationship supported by moderate-high BS and PP values (Fig. 1). The position of *Argia* as the sister-clade to the 'Protoneuridae' + 'ridged frons' Coenagrionidae is not very well supported, as seen by the low BS and moderate PP values (Fig. 1). The 'core' Coenagrionidae appear as basal to all the other clades, with both high BS and PP values (Fig. 1). Removing *Argia* from the analyses did not change our results and the 'Protoneuridae' + 'ridged frons' Coenagrionidae relationship

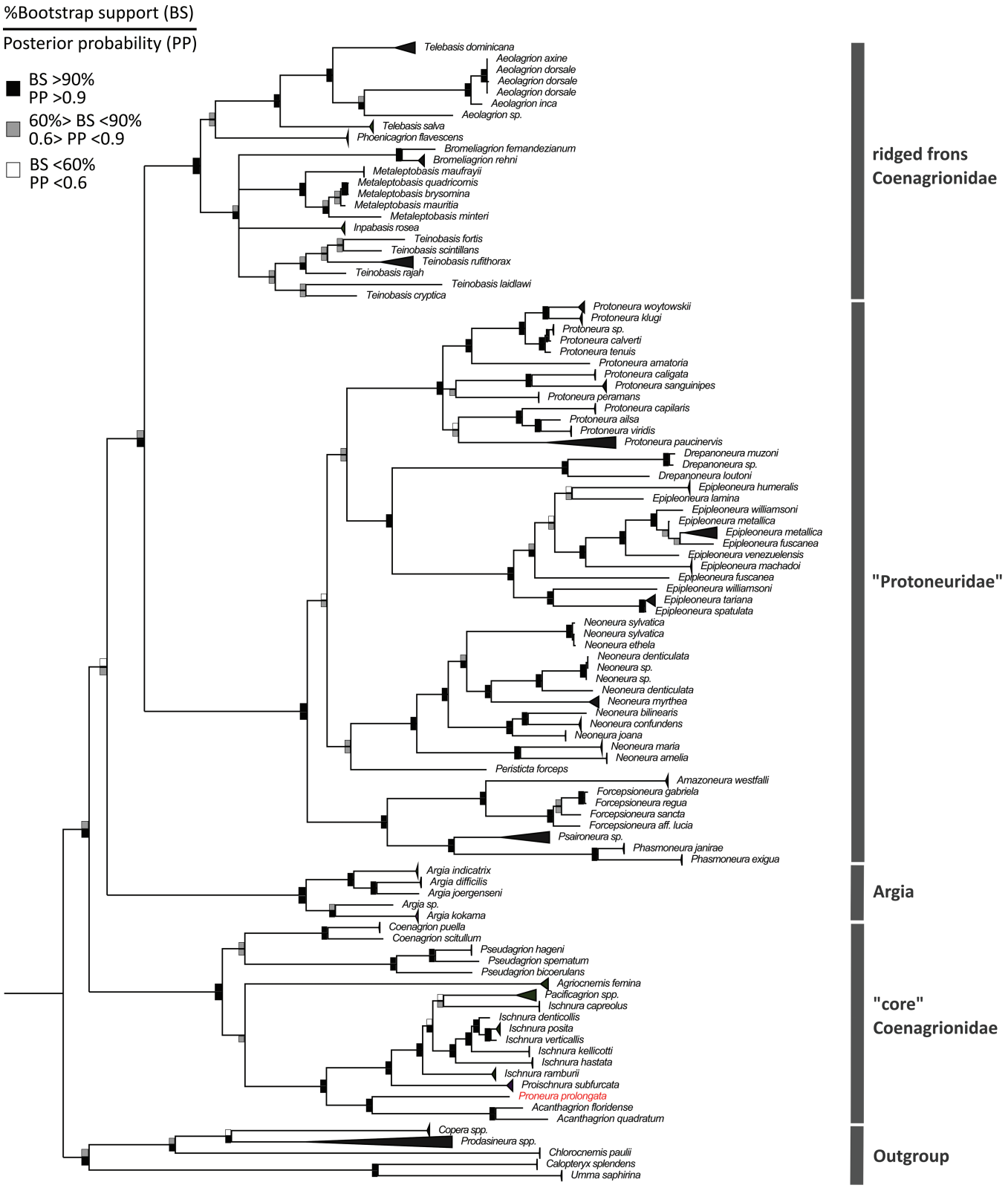

Figure 1. Phylogenetic relationships among taxa of Coenagrionoidea inferred from sequences of two nuclear (28S and *PRMT*) and two mitochondrial (16S and *COII*) DNA markers. Squares above and below branches are coloured according to maximum-likelihood bootstrap support and Bayesian inference posterior probability values, respectively, as depicted in the figure legend. The 'Protoneuridae' species *Proneura prolongata* is represented in red, as the sister species of the genus *Acanthagrion*.

was supported with high BS and PP values (see Supporting Information, File S1.7, Figs. S7 and S8).

Within the 'Protoneuridae', four well-supported clades are identified: the *Protoneura* Selys in Sagra, 1857 clade; the *Drepanoneura* von Ellenrieder and Garrison, 2005 + *Epipleoneura* Williamson, 1915 clade; the *Neoneura* Selys, 1860 + *Peristicta* Hagen *in* Selys, 1860 clade; and the clade comprised by *Amazoneura* Machado, 2004, *Forcepsioneura* Lencioni, 1999, *Psaironeura* Williamson, 1915, and *Phasmoneura* Williamson, 1916. These four clades are all supported with high BS and PP values, except for the *Neoneura* + *Peristicta* clade, which is supported by moderate BS and PP values. The *Protoneura* clade appears to be more closely related to the *Drepanoneura* + *Epipleoneura* clade, albeit with moderate support values.

Morphological cladistic analyses

As a result of the analyses using the command Implied Weighting with 11 different values of *k* (5 to 15), one tree was found in each analysis. All the trees showed consensus in the composition of 'Protoneuridae': *Proneura prolongata* and *Junix elumbis* Rácenis, 1968 always nested outside of the group, while the remaining 'Protoneuridae' are monophyletic, and relationships between their genera showed little variation among the trees (Figs 2, 3; Supporting Information, File S1.6, Figs S2–S6).

The analyses with *k* = 5 to 8 showed no congruence with any accepted classification scheme or with our molecular and combined analyses (e.g. some Platycnemididae included within the Coenagrionidae clade), which reinforces the idea that there is not a unique or true value of *k*, and that this value is matrix-dependent (see Materials and Methods section). Hereafter, we will focus only on the analyses with *k* values from 9 to 15 (see Figs 2–4; Supporting Information, File S1.6, Figs S2–S6).

The main clades of 'Protoneuridae' received little support, and consistency and retention indexes varied between 0.244–0.253 and 0.615–0.624, respectively (Figs 2, 3). Several synapomorphies support the 'Protoneuridae' (excluding the species *J. elumbis* and *P. prolongata*) in the different morphological analyses, which are useful to delimitate the clade (see Supporting Information, File S1.5). Three of these synapomorphies (chars. 5, 23, and 25) are present in all the analyses, and another three (chars. 3, 13, and 43) are present in five out of seven *k* values. These synapomorphies are: (i) paraproct relative length (char. 3, with much homoplasy); (ii) discoidal cell ratio (char. 5 = quadrangular discoidal cell, convergent with *Proneura prolongata*, *Junix elumbis*, and Disparoneurinae); (iii) relation thorax width/abdomen length (char. 14 = gracility, reverted in *Neoneura*); (iv) CuA vein reduced, not extending beyond MP-CuA (char. 23: 0–2, convergent with *J. elumbis*); (v) MP vein no longer than four cells beyond cross vein descending from subnodus (char. 25: 0–1, 2, 3, convergent with *P. prolongata*, *J. elumbis*, and Disparoneurinae); (vi) presence of accessory structures on cercus dorsal branch (char. 43: 0–1, with reversions in 'Protoneuridae' and convergent with some Coenagrionidae).

Within 'Protoneuridae', two main clades are identified: one includes the genera with non-carinated antennifer (char. 16: 0 *Amazoneura*, *Forcepsioneura*, *Phasmoneura*, *Psaironeura*, *Idioneura* Selys, 1878, *Lamproneura lucerna* De Marmels, 2003, and *Roppaneura beckerii* Santos, 1966) and another includes the genera with carinated antennifer (char. 16: 0–1, unique synapomorphy: *Drepanoneura*, *Epipleoneura*, *Epipotoneura* Williamson, 1915, *Neoneura*, *Peristicta*, and *Protoneura*). The only discrepancy to this general topology is that *Peristicta* falls within the non-carinated 'Protoneuridae' in some analyses (Fig. 3; and Supporting Information, File S1, Figs S3, S5, S6; *k*: 10–11, 14–15), and within the clade of carinated antennifer species in others (Figs 2, 4; Supporting Information, Figs S2, S4; *k*: 12, 9, 13). *Protoneura* appears as either monophyletic (Fig. 2; Supporting Information, Figs S2, S4; *k*: 9, 12, 13) or polyphyletic, with *Protoneura sanguinipes* Westfall, 1987 and *P. caligata* (Hagen in Selys, 1886) at the base of the clade of carinated antennifer species for some *k* values (Fig. 3; Supporting Information, File S1, Figs S3, S5, S6; *k*: 10, 11, 14–16).

Within the non-carinated antennifer genera, *Lamproneura lucerna* and *Phasmoneura* are always sister to *Psaironeura tenuissima* (Selys, 1886) and *Amazoneura westfalli* (Machado, 2004), forming a clade sister to *Forcepsioneura* (Figs 2–4). Within the carinated antennifer clade, all or most of the *Protoneura*, and all the *Epipleoneura*, *Epipotoneura*, and *Drepanoneura* always form a clade in which *Drepanoneura* is either sister to the remaining genera (Figs 3, 4; Supporting Information, File S1, Figures S3, S5, S6; *k*: 10, 11, 14, 15), or sister to *Epipleoneura* and *Epipotoneura nehalennia* Williamson (Fig. 2; Supporting Information, Figs S2, S4; *k*: 12, 9, 13). For the synapomorphies that support these clades see Figure 4 and Supporting Information, File S1.5.

The relationships among Coenagrionidae *s.l.* and *s.s.* showed little agreement between analyses, but two main different topologies were recovered. In the first one (Figs 2, 3; Supporting Information, File S1; *k*: 10–11–14) two or more main clades of Coenagrionidae *s.s.* are identified (including *J. elumbis* and *P. prolongata*), and the remaining 'Protoneuridae' form a separate clade. In the second topology (Supporting Information, File S1; *k*: 9 and 15) two main clades are identified: one including 'Protoneuridae' and a second clade including Coenagrionidae *s.s.* (plus *J. elumbis* and *P. prolongata*). This variation in the topologies is probably due to the fact that the morphological data matrix was built to test the monophyly and relative position of 'Protoneuridae' and to investigate the relationships among its members, rather than to solve the relationships among the Coenagrionidae *s.s.*.

Combined molecular and morphological data analyses

In agreement with the molecular and the morphological data analyses, the results obtained from the combined molecular and morphological data also suggest that 'Protoneuridae' is a polyphyletic group, with *Proneura prolongata* included within the 'core' Coenagrionidae and the remaining 'Protoneuridae' forming a monophyletic group. This topology is supported by high BS and PP values (Fig. 5).

As in the molecular analyses, Coenagrionidae *s.l.* appear as comprised by four main clades: the 'Protoneuridae', the 'core' Coenagrionidae (plus *P. prolongata*), the ridged-frons Coenagrionidae, and *Argia*. These four clades are all supported with high BS and PP values (Fig. 5). The relative positions of each of these four clades, as well as their inner relationships, are nearly the same in both the combined and molecular analyses (see Figs 1, 5).

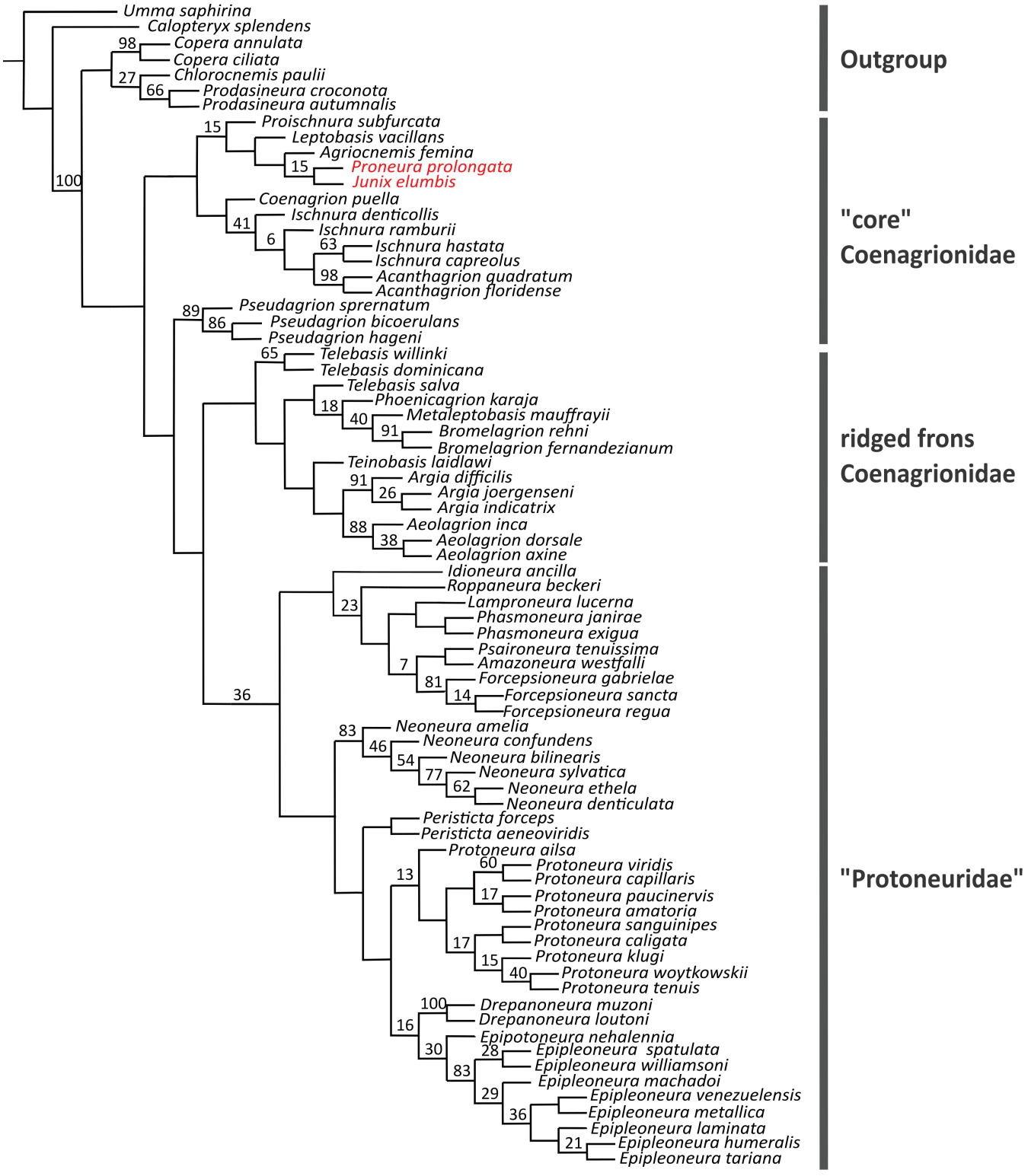

Figure 2. Phylogenetic relationships among taxa of Coenagrionidae inferred from morphological information (implied weighting, *k*: 12; consistency index: 0.245; retention index: 0.608). Numbers above the nodes correspond to relative Jack-knife support. The 'Protoneuridae' species *Proneura prolongata* and *Junix elumbis* are represented in red.

Similarly to the results of the molecular data analyses, *P. prolongata* appears within the 'core' Coenagrionidae as sister to the genus *Acanthagrion*, a relationship supported by high BS and PP values. The remaining 'Protoneuridae' form a monophyletic group that is closely related to the 'ridged frons' Coenagrionidae, a relationship supported by moderate BS and high PP values (Fig. 5). The position of the *Argia* clade is not well-supported (low BS and moderate PP values; see Fig. 5); nor did the removal of

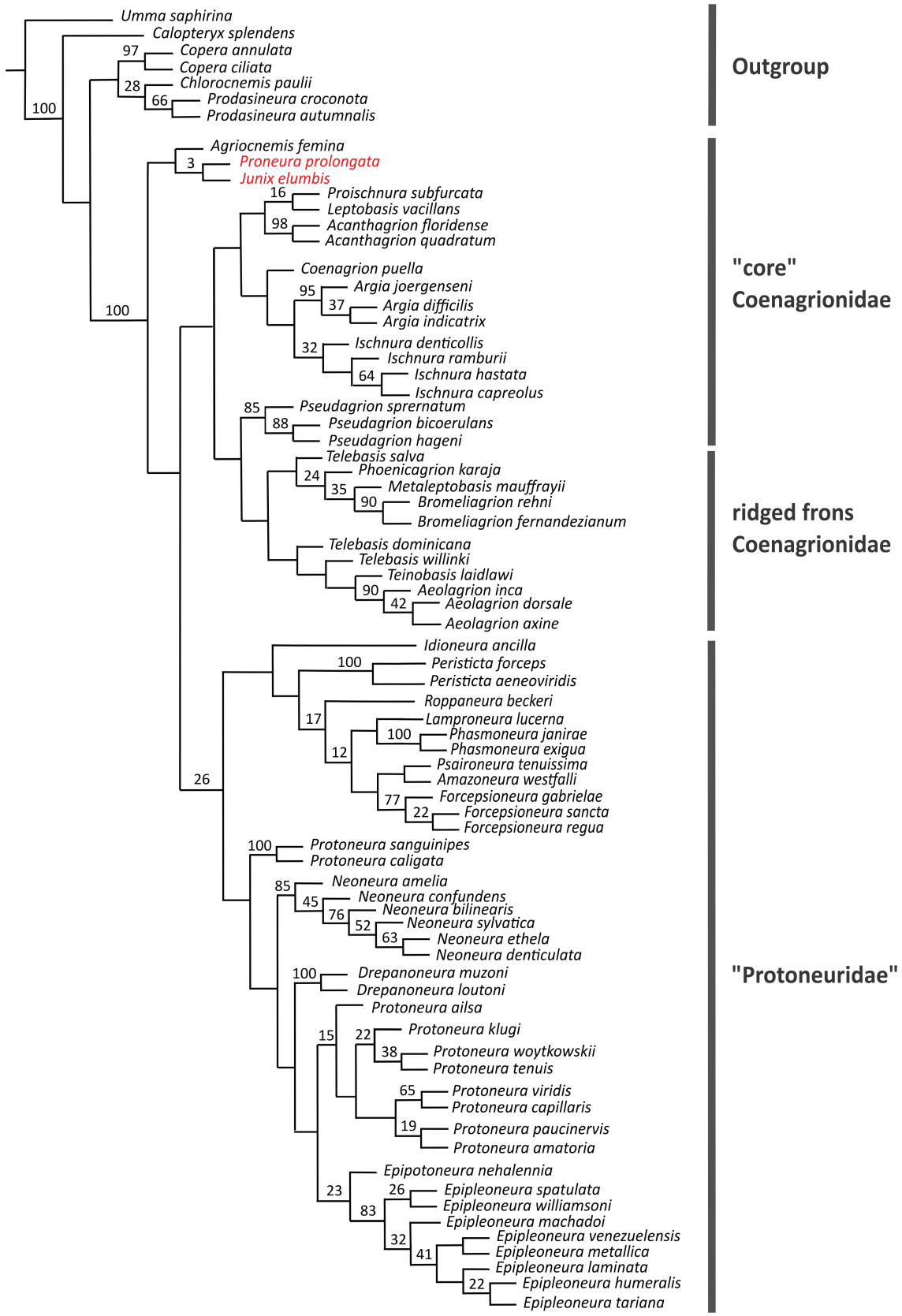

Figure 3. Phylogenetic relationships among taxa of Coenagrionidae inferred from morphological information (implied weighting, *k*: 10; consistence index: 0.245; retention index: 0.608). Numbers above the nodes correspond to relative Jack-knife support. The 'Protoneuridae' species *Proneura prolongata* and *Junix elumbis* are represented in red.

Figure 4. Phylogenetic relationships among 'Protoneuridae' species included in the morphological cladistic analyses (implied weighting, *k*: 12; consistency index: 0.245; retention index: 0.608). Black circles indicate unique apomorphies, and white circles indicate shared apomorphies. Numbers above the circles correspond to the character number, and numbers below the circles indicate character state. States are not included for quantitative characters (chars. 1–14).

this genus from the analyses changed the results (see Supporting Information, File 1.7, Figs. S9 and S10).

The 'Protoneuridae' are comprised by the same four clades identified by the molecular data analyses. Three of these clades include all the carinated antennifer genera: the *Protoneura* clade; the *Epipleoneura* + *Drepanoneura* clade (both supported with high BS and PP values); and the clade comprised by *Neoneura* and *Peristicta*, supported with moderate BS and PP values. The fourth clade includes the non-carinated antennifer genera *Amazoneura*, *Forcepsioneura*, *Phasmoneura*, and *Psaironeura*. Support for each of these clades is high, except for the *Neoneura* + *Peristicta* clade. Similarly to the molecular data analyses, the *Protoneura* clade appears as sister to the *Epipleoneura* + *Drepanoneura* clade, but in this case, the relationship is not well supported.

DISCUSSION

'Protoneuridae' and its relationships with Coenagrionidae *sensu stricto*

'Protoneuridae' appear as polyphyletic in all our analyses, with *Proneura prolongata* and *Junix elumbis* (the latter only included in the purely morphological analyses) included within

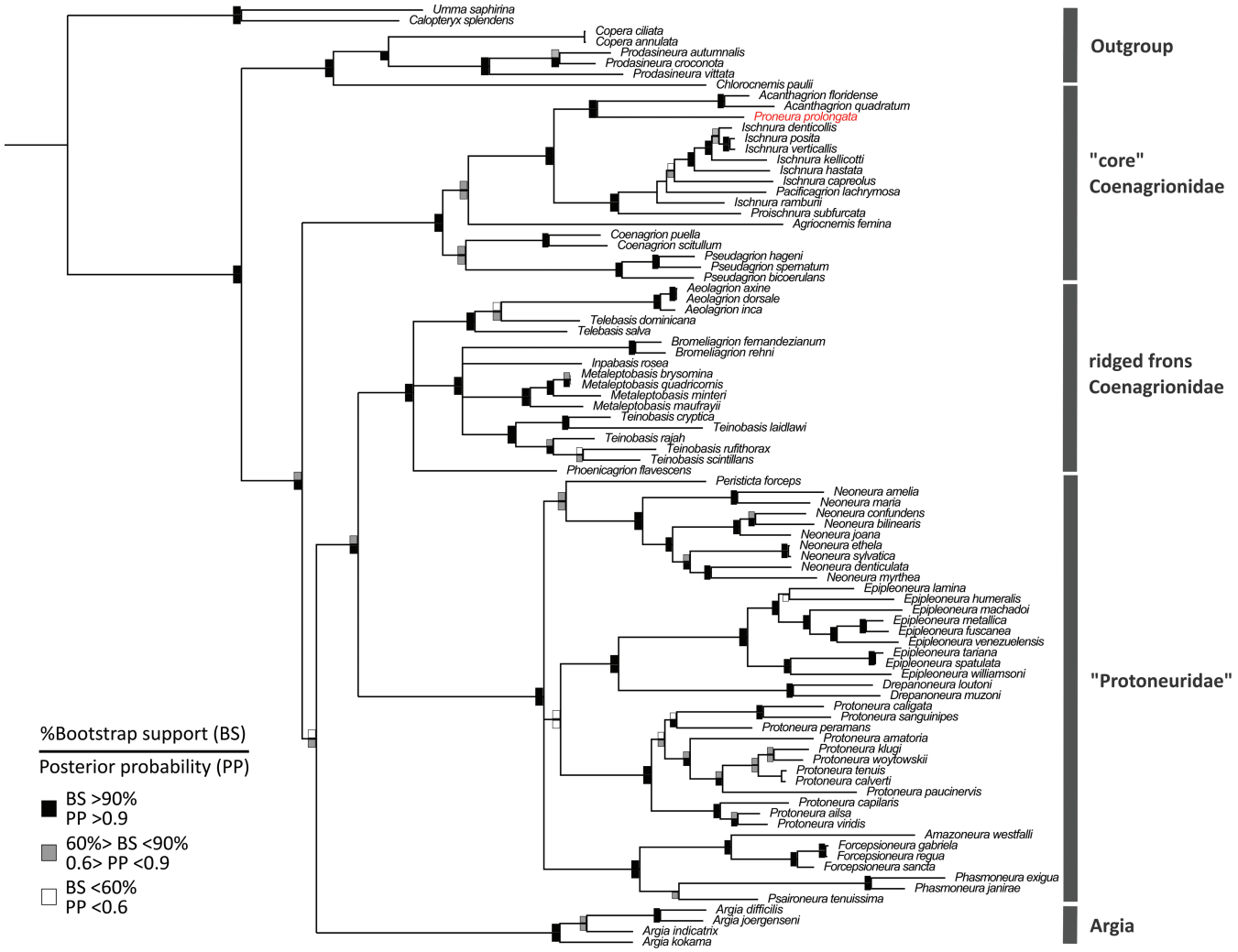

Figure 5. Phylogenetic relationships among taxa of Coenagrionidae inferred from genetic (28S + *PRMT* + 16S + *COII*) and morphological information. Squares above and below branches are coloured according to maximum-likelihood bootstrap support and Bayesian inference posterior probability values, respectively, as depicted in the figure legend. The 'Protoneuridae' species *Proneura prolongata* is represented in red.

the 'core' Coenagrionidae and separated from the remaining 'Protoneuridae' included in our analyses. The latter form a well-supported monophyletic clade in all cases (Figs 1–3, 5), which appears as sister to the 'ridged frons' Coenagrionidae in both the molecular and combined analyses and in some of the morphological analyses (*k*: 12, Fig. 3; and *k*: 11, Supporting Information, File S1).

These results have implications for the definition of 'Protoneuridae', from which the genera *Proneura* Selys, 1889 and *Junix* Rácenis, 1869 should be excluded and hereafter considered Coenagrionidae *s.s.*. Most importantly, our results also provide support for the reinstatement of the family status for 'Protoneuridae' (as newly defined, with the exclusion of the genera *Proneura* and *Junix*).

Regarding Coenagrionidae *s.l.*, Dijkstra *et al.* (2014: 83) mention that 'given the size of this group (about 30% larger than any other odonate family), applying the family rank to more manageable units might seem appropriate when better phylogenetic support is obtained'. Contrary to Dijkstra *et al.* (2014), our results indicate that 'Protoneuridae' is not merged within the 'ridged frons' Coenagrionidae, which would justify the reinstatement of this group's family status (as newly defined with the exclusion of *Proneura* and *Junix*). This would imply the need for new familial names for the remaining clades within Coenagrionidae *s.l.* (i.e. ridged frons and core Coenagrionidae). We have included in our analyses most or all of the extant 'Protoneuridae' genera. For five of these (*Epipotoneura*, *Idioneura*, *Junix*, *Lamproneura*, and *Roppaneura*) no DNA sequences could be obtained, but they were included in the morphological cladistic analyses. Previous (extensive) phylogenetic studies on Odonata (Bybee *et al.* 2008, 2021, Dijkstra *et al.* 2014) have only included between two and four genera of 'Protoneuridae' and, therefore, they did not consider most of the variability within the group. Dijkstra *et al.* (2014) included 77 species within 46 genera of Coenagrionidae *s.l.*, from which only four genera (representing six species) were 'Protoneuridae'. They included information from one nuclear (28S) and two mitochondrial (16S and *COI*) loci, and their results led to a change in the status of the group, which has been merged within Coenagrionidae (Dijkstra *et al.* 2013). In their analyses, the relationships of 'Protoneuridae' and their position

within Coenagrionidae changed depending on the information used to reconstruct the phylogenies. In the analyses based on 28S, 16S, and *COI*, 'Protoneuridae' appeared, together with *Argia*, as closely related to the 'core' Coenagrionidae. However, the analyses based solely on 28S and 16S resulted in a different phylogenetic hypothesis, in which 'Protoneuridae' nested within the 'ridged frons' Coenagrionidae, with *Aeolagrion* and *Telebasis* as the more closely related genera, albeit this hypothesis was supported by low BS values (see: Dijkstra *et al.* 2014).

The most recent phylogeny of Odonata by Bybee *et al* (2021), albeit being based on information from >400 loci, included only two species as representatives of Protoneuridae. In this study, both species appeared also within Coenagrionidae, closely related to *Argia* and the 'ridged frons' Coenagrionidae representatives analysed, but separated from the 'core' Coenagrionidae (Bybee *et al.* 2021).

Our study, based on an exhaustive 'Protoneuridae' sampling (*c.* 50 species belonging to 10–15 genera; Supporting Information, File S2, Table S1), and including information from two nuclear (*PRMT* and 28S), two mitochondrial (16S and *COII*) loci, as well as morphological data, has resulted in a change of the relative position of the group compared to that found by previous studies. With a similar sampling of 'ridged frons' Coenagrionidae to that of Dijkstra *et al.* (2014), we have recovered a distinct and well-supported monophyletic 'Protoneuridae' (Neotropical genera excluding *Proneura* and *Junix*), which is sister to the 'ridged frons' Coenagrionidae clade (see Figs 1, 2, 5) and well differentiated form the 'core' Coenagrionidae. The phylogenetic analyses carried out using either only mitochondrial or nuclear DNA sequences always recovered a monophyletic 'Protoneuridae' (Neotropical genera minus *Proneura*) with high support and well differentiated from the remaining Coenagrionidae included in our study (data not shown).

The status of *Junix elumbis* and *Proneura prolongata*

In the morphological analyses, *Junix elumbis* and *Proneura prolongata* always form a clade separated from the other 'Protoneuridae' genera included in our study (Figs 2, 3); and in the molecular and combined data analyses, *P. prolongata* falls within the core Coenagrionidae.

Both monotypic genera possess a similar genital ligula and paraprocts, and short cerci, which is reflected in the synapomorphies that sustained the clade (chars. 1–3, 38, and 49; see Supporting Information, File S1.5). Therefore, and based on morphology alone, the synonymy of these two genera could seem feasible. The only known specimen of *J. elumbis* was examined in 2009 at the MIZA by Pablo Pessacq, whereas *P. prolongata* is only known by the holotype and one additional specimen (Bota-Sierra 2012), from which a leg was used to obtain molecular information for our study. Therefore, and without direct examination of *P. prolongata* and/or the addition of molecular information on *J. elumbis*, we refrain from establishing a synonymy between both genera.

Morphological definition of 'Protoneuridae'

Six synapomorphies support 'Protoneuridae' in our analyses (chars. 3, 5, 14, 23: 2, 25: 1–3, and 43: 1; see Results). The subarculus position at, or proximal to, Rp-Ma bifurcation (char. 29: 1), a synapomorphy of 'Protoneuridae' in previous analyses (Pessacq 2008), was not recovered as such in our analyses, as it is shared with the two representatives of the family Calopterygidae used to root the tree [*Umma saphirina* Förster, 1916 and *Calopteryx splendens* (Harris, 1780)].

Three of these six synapomorphies (quadrangular discoidal cell; CuA vein reduced, not extending beyond MP-CuA; and MP vein no longer than four cells beyond cross-vein descending from subnodus) are convergent with *Proneura prolongata* and/or *Junix elumbis*, but these two genera possess a short cercus with a relatively simple morphology compared to most 'Protoneuridae' genera; and the paraprocts are at least two times longer than the cercus, filiform, and forcipate, a condition not found in any other 'Protoneuridae'. Therefore, 'Protoneuridae' can be defined and separated from other Coenagrionoidea by the following characters: the quadrangular discoidal cell (char. 5), the gracility (char. 14), the reduction of the veins MP and CuA (chars. 23 and 25), the subarculus position at, or proximal to, Rp-Ma bifurcation (char. 29), the cercus usually with accessory structures (char. 43), and the paraproct not forcipate or filiform.

Gracility (char. 14; Fig. 6A, C–G), a quantitative character that we measured as the thorax width/abdomen length relationship, was a consistent synapomorphy for 'Protoneuridae' that reverted only in *Neoneura*, a robust genus with 'coenagrionid appearance' (Fig. 6B, F). This character varied between 0.05 and 0.07 in most 'Protoneuridae' species, whereas in *Neoneura* species, gracility ranged between 0.08 and 0.1. These values were the same as for most of the Coenagrionidae species included in our analyses, except for the long and slender *Metaleptobasis* and *Bromeliagrion* genera (0.05–0.06), which is probably due to their long abdomens, adapted to laying eggs in phytothelmata.

The morphological definition of 'Protoneuridae' based solely on its venation reduction may lead to consider it as an apophyletic group, where extreme wing reduction leads to an exaggeration of its taxonomic rank. However, not only the vein reduction, but also the position of the arculus, gracility, and cercus characters, define the group.

Regarding larval characters, the reduction or absence of external teeth on the premental palp (Char. 65, state 1) is present in all known 'Protoneuridae' larvae, but it is also observed in a few other Neotropical Coenagrionoidae genera (e.g. *Telebasis* and *Nehalennia* Selys, 1850). Other larval characters, such as the shape of caudal appendages, presence of nodus, shape of the prementum, and number of premental and palpal setae (some of them included in chars. 62, 64, 65), are variable among genera and are highly homoplastic in our results, except for the larval characters that are unique for the Calopterygidae outgroup taxa (char. 61, state 0; char. 63, state 2). Despite being the most diverse group within the Zygoptera, all Coenagrionoidea larvae exhibit a remarkably uniform morphology (Lozano *et al.* 2018): the larvae of Platycnemididae and Coenagrionidae show strong similarity in the aforementioned characters; and very distantly related taxa may show similar character states, suggesting homoplasy (e.g. absence of palpal setae in *Argia*, *Isostictidae*, and *Pseudostigmatidae*).

Relationships within 'Protoneuridae'

Even though molecular information could not be obtained for some 'Protoneuridae' genera (*Epipotoneura*, *Idioneura*,

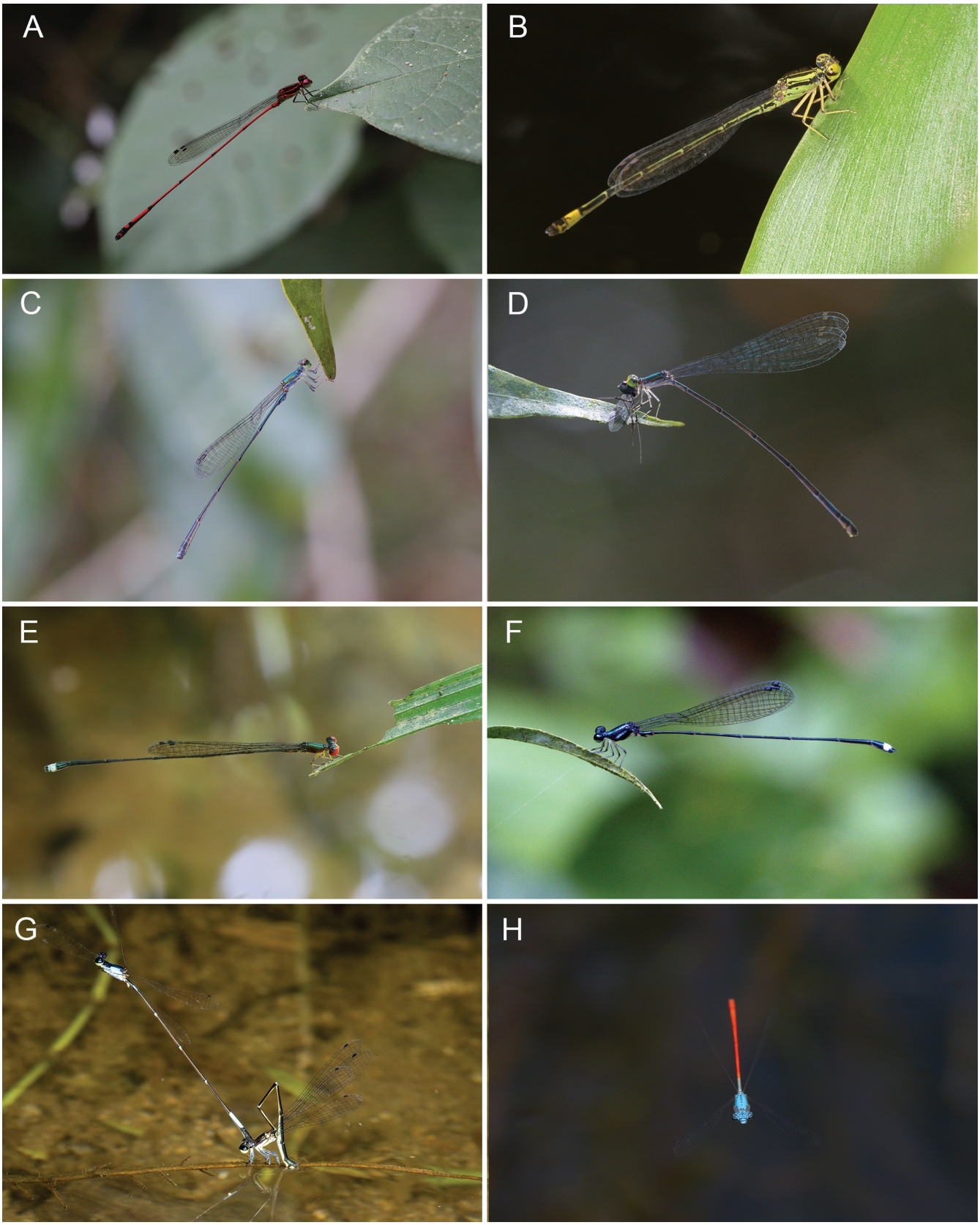

Figure 6. Habitus and behaviour of 'Protoneuridae'. A, male of *Protoneura amatoria* Calvert,1907 perching on the tip of a leaf, from the river Camarones, Manabí, Ecuador; B, male of *Neoneura confundens* Wasscher and Van't Bosch, 2013 from the National Park Pacaya-Samiria, Peru, showing a robust appearance that resembles the typical morphology of the 'core' Coenagrionidae; C, male of *Epipleoneura metallica* Rácenis, 1955 from the National Park Chapada dos Guimaraes, Brazil; D, female of *Drepanoneura muzoni* von Ellenrieder and Garrison, 2008 eating prey in the forest, from Tiputini Biodiversity Station, Ecuador; E, red-eyed male of *Psaironeura angeloi* Tennessen, 2016 from Vereda Las

Lamproneura, and *Roppaneura* Santos), which if included in the analyses could lead to a change in the relative position of other taxa, several clades were yet well supported in all or most of our analyses. The synapomorphies that sustain the main 'Protoneuridae' clades and genera in the morphological analyses with *k* 10 and 12 are summarized in the Supporting Information, File S1.5.

A clade including all the genera with non-carinated antennifer (i.e. *Amazoneura*, *Forcepsioneura*, *Phasmoneura*, and *Psaironeura*; plus *Idioneura*, *Lamproneura*, and *Roppaneura* in the purely morphological analyses; char. 16: 0), which has been previously supported by cladistic analysis (Pessacq 2008), is supported in all our analyses. The relative position of the genera within this clade differed between the morphological and the molecular and combined analyses. In the analyses based solely on morphology, the clade (*Lamproneura* + *Phasmoneura*) is sister to ((*Psaironeura* + *Amazoneura*) *Forcepsioneura*)) (Fig. 4); whereas in the combined and molecular analyses (*Amazoneura* + *Forcepsioneura*) is sister to (*Phasmoneura* + *Psaironeura*) (Figs 1, 5). This discrepancy is probably due to the fact that *L. lucerna* could not be included in the analyses based on molecular information. However, a close relationship between *Amazoneura* and *Forcepsioneura* is likely, as both genera are morphologically similar, and the former was once a synonym of *Forcepsioneura* (Lencioni, 2005; Machado, 2009).

The genera with a carinated antennifer (*Drepanoneura*, *Epipleoneura*, *Neoneura*, *Peristicta*, and *Protoneura*; plus *Epipotoneura* in the morphological analyses; char. 16: 0–1, unique synapomorphy) form a monophyletic clade in both the morphological and the molecular analyses, albeit only with moderate support in the latter (Figs 1–4). In the combined data analysis, this clade splits in two groups, resulting in a polytomy (Fig. 5). Besides the antennifer, the non-carinated genera share a similar filiform and long cercus (char. 42: 1, except *Psaironeura*) and a genital ligula with long and strongly incurved lateral lobes (char. 38:1, except *Phasmoneura*). The morphology of the carinated antennifer genera is more variable, but the clade is supported by several synapomorphies in the morphological analyses (Supporting Information, File S1.5).

The clade composed by all, or most, *Protoneura* species, *Drepanoneura*, and *Epipleoneura* is supported in all analyses, and the close relationship of *Drepanoneura* and *Epipleoneura* is supported in most analyses (except morphological analyses with *k*: 10, 11, 14, 15). In all the morphological analyses, *Epipotoneura nehalennia* is sister to *Epipleoneura*, but the first could not be included in the analyses based on molecular information.

The position of the genus *Peristicta* varied in the morphological analyses, falling either in the non-carinated or in the carinated antennifer clades (see Results), whereas in the molecular and combined analyses, this genus is sister to *Neoneura*, in a clade supported by moderate BS and PP values, probably because only a single *Peristicta* species was included in the analyses.

In most of the morphological analyses, the genus *Protoneura* is polyphyletic (see Results); and *Protoneura caligata* and *P. sanguinipes* nest in a clade separated from the remaining *Protoneura* species (see Fig. 3). The position of these species is most likely due to the cylindrical antennifer in *P. caligata*, coded as missing (?) in *P. sanguinipes* (no material could be examined). The shape of the antennifer is a character state not shared by other species of *Protoneura* or the remaining members of the carinated antennifer clade. However, the monophyly of the genus is supported by high BS and PP values in both the molecular and combined data analyses.

Our analyses do not support any taxonomic change of genera within 'Protoneuridae' included in this study, as all of them form well-defined clades. As mentioned above, the only exception could be *Protoneura*, but most of the evidence in our study justifies the monophyly of this genus. The position of certain terminal taxa, as *Epipotoneura nehalennia* as sister to *Epipleoneura* (Figs 2–5) or *Amazoneura* as sister to *Forcepsioneura*, does not justify the synonymy of either genus, as there are morphological studies that provide evidence of their status as separated genera (von Ellenrieder and Garrison 2008, Machado 2009).

Remarks for future work

Most of the Coenagrionidae genera included in this study are recovered as monophyletic, except for *Telebasis*, which is paraphyletic in all our analyses, and *Pacificagrion*, which nested within the *Ischnura* clade in both the combined and molecular analyses. Regarding *Telebasis*, we have included only three out of the *c.* 50 currently described species within the genus (Garrison 2009); two of these [*T. dominicana* Selys in Sagra, 1857 and *T. salva* (Hagen, 1861)] were included with both molecular and morphological information, whereas *T. willinki* Fraser, 1948 was included with morphological information only. The three species fall within a clade that also includes the genus *Aeolagrion*, similar to the results of Dijkstra *et al.* (2013), and which has been also suggested by Garrison (2009).

The genus *Pacificagrion*, endemic to the islands of Samoa, nested within the *Ischnura* clade in both the combined and molecular analyses. Together with *Amorphostigma* Fraser, 1925, another Samoan endemic, *Pacificagrion* is included within a 'Pacific' Ischnurinae clade 'which most likely consists of several taxa deserving of separate generic ranks' (Marinov 2022: 1). The status of *Telebasis*, as well as the Pacific Ischnurinae, requires further research in order to firmly establish their taxonomy.

The genus *Argia*, the most species-rich odonate genus of the Neotropics and probably the largest odonate genus of the world (Dijkstra *et al.* 2014), contains at least 130 species. It is represented in our study by four species, which form a monophyletic and well-supported clade in both the molecular and combined analyses, but its position as the basal clade to the 'ridged-frons' Coenagrionidae + Protoneuridae is not well supported in either analysis. In the morphological analysis the genus is recovered as belonging to either the ridged-frons or the core Coenagrionidae,

Brisas, Casanare, Colombia; F, male of *Amazoneura westfalli* from Tiputini Biodiversity Station, Ecuador; G, a tandem of *Protoneura capillaris* (Rambur, 1842) laying eggs, from Santiago de Cuba; note the extreme flexion of the abdomen of the female; H, a hovering male of *Neoneura sylvatica* Hagen in Selys, 1886 from Uberlândia, Minas Gerais, Brazil. All pictures by Adolfo Cordero-Rivera.

depending on the value of *k*. *Argia* possess unique characters in the adult stage, such as the tori in the posterodorsal margin of the 10th abdominal segment (Garrison *et al.* 2010); and larval characters such as the absence of premental setae and the caudal appendages ending in a filament, whereas not unique, are quite distinctive among the Coenagrionidae (Lozano *et al.* 2018). The position of this genus relative to other coenagrionids has remained largely unclear, as it varies depending on the type of information (morphological or molecular) or the optimality criteria used (parsimony, maximum likelihood, etc.; see: Torres-Pachón *et al.* 2017). Garrison and von Ellenrieder (2022), in their synopsis of *Argia*, have addressed some of the conclusions of Torres-Pachón *et al.* (2017), and they consider that there is great uncertainty about which group or genus would be more closely related to *Argia*, as none of the different hypotheses seems convincing (Garrison and von Ellenrieder 2022; R. Garrison, personal communication). Future studies should address this question by increasing the sampling both in terms of species and type of information included in the analyses.

The genera *Bromeliagrion*, *Inpabasis*, *Metaleptobasis*, and *Teinobasis* form a monophyletic group, supported with high BS and PP values in both the molecular and combined analyses. The recovered polytomy within this clade indicates that the relationships between these genera cannot be resolved with our sampling, probably because we did not include other representatives of Teinobasinae (e.g. *Amphicnemis* Selys, 1863, *Pericnemis* Selys, 1863, *Chromagrion* Needham, 1903, and *Papuagrion* Ris, 1913) nor any 'Pseudostigmatidae' genera in our analyses, which were included by Dijkstra *et al.* (2014) and which would improve the resolution for this clade. Future work is needed to disentangle the relationships within this particular clade, as well as the status of Pseudostigmatidae', a group that has been also recovered as monophyletic when including both molecular and morphological data in the analyses (Soldati 2021).

CONCLUSION

Previous analyses (Carle *et al.* 2008, Dijkstra *et al.* 2014, Bybee *et al.* 2021) have split Coenagrionidae *s.l.* into two main clades: the 'core' Coenagrionidae and the 'ridged frons' Coenagrionidae, with the Neotropical components of the traditionally defined family 'Protoneuridae' and the family 'Pseudostigmatidae' merged within the latter.

Our results support the redefinition of Coenagrionidae *s.s.*, which should hereafter include the genera *Proneura* and *Junix*. 'Protoneuridae', as newly defined with the exclusion of *Junix elumbis*, *P. prolongata*, and the Palaeotropical genera recently transferred to the subfamily Disparoneurinae of Platycnemididae, form a well-supported monophyletic group in all our analyses, which appears as sister to the 'ridged frons' Coenagrionidae.

With an increased number of species compared to previous analyses, which cover most of all described genera and including morphological and molecular data, our results support the redefinition and re-establishment of the 'Protoneuridae' family. An alternative option would be to maintain a large Coenagrionidae family, composed of several subfamilies. The first option would preserve the significance of the Protoneuridae name in South America for the last century. Also, the behaviour of 'Protoneuridae', including patrolling while hovering (e.g. González Soriano 2001; Fig. 6H), their habit to perch in the tip of leaves (e.g. Tennessen 2016; Fig. 6A, C–F) and the extreme flexing of the abdomen of the females while ovipositing (e.g. Trapero-Quintana *et al.* 2005; Fig. 6G) constitute further independent lines of evidence to support the maintenance of the family status for these genera, which form a clearly distinct group based not only on genetics and morphology, but also on their ecology and behaviour. Future studies, including greater taxa coverage for some genera, together with information from different sources (i.e. DNA and morphology), are needed to resolve the relationships within Coenagrionidae *s.l.*, a group that, according to our results, appears to be non-monophyletic.

According to our and previous works (e.g. Carle *et al.* 2008, Dijkstra *et al.* 2014, Bybee *et al.* 2021, Soldati 2021), at least four main and well-differentiated clades can be identified within Coenagrionidae: the 'core' Coenagrionidae, the 'ridged frons' Coenagrionidae, 'Pseudostigmatidae', and 'Protoneuridae'. We believe these clades represent different families, which should be granted familial names; something that falls outside the scope of our work.

SUPPLEMENTARY DATA

Supplementary data are available at *Zoological Journal of the Linnean Society* online.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

All sequences generated in this study have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/), where they are available for download (see Supporting Information, File S2, Table S2 for details on accession numbers).

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