Phylogeny and classification of Leptophlebiidae (Ephemeroptera) with an emphasis on Neotropical fauna

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Abstract. Mayflies from the family Leptophlebiidae are cosmopolitan and highly diverse morphologically; they are also the largest family in numbers of genera and the second in number of species in the order Ephemeroptera. In spite of their broad diversity and the efforts employed to understand the evolution of this group, the internal classification of Leptophlebiidae remains controversial at all levels. More recently, important changes have been incorporated into the systematics of the family, increasing the number of subfamilies (from two to six) and recognizing several tribes. We present a phylogeny of the family based on 153 taxa (53 genera) and two molecular markers, representing 1655 bp, and verify the taxonomic status of the subfamilies, tribes and complexes. Based on these results, the number of subfamilies has been increased from six to eight and one new tribes and two new subtribes have been added. In addition, new ranks are proposed and the concept of Atalophlebiinae revised, including genera with distributions in the Australasian and Neotropical regions.

Introduction

Mayflies (Ephemeroptera) represent what used to be a very diverse group of ancient flying insects (Ephemerida). Their origin dates from the Late Carboniferous, or from the Early Permian (*c*. 280 Ma), being consequently one of the oldest lineages among Pterygota (Sartori & Brittain, 2015). Currently, Ephemeroptera is composed of *c*. 3500 species, 400 genera and 42 families, occupying almost all freshwater environments worldwide, except for Antarctica (Barber-James *et al.*, 2013). Mayflies from the family Leptophlebiidae represent one of the most diverse and widespread groups of the order. Leptophlebiids are cosmopolitan and highly diversified morphologically; they are also the largest family in numbers of genera and the second in number of species, with *c*. 140 genera and 640 species (Sartori & Brittain, 2015).

In spite of their broad diversity and the efforts employed to understand the evolution of this group, the internal classification of Leptophlebiidae remains controversial at all taxonomic levels. Peters (1980) was the first to propose an internal division

of the family based on morphological characteristics, such as differences in the mouthparts of nymphs and characteristics of the wings, genitalia, and compound eyes in adults. He proposed a division into two subfamilies: Leptophlebiinae Banks, composed of eight genera with a distribution restricted to the northern hemisphere, and Atalophlebiinae, in which all other genera were included. Compared with Leptophlebiinae, Atalophlebiinae is highly diversified with a worldwide distribution, and displays greatest diversity in the southern hemisphere (Peters, 1980). Kluge (1994) erected Habrophlebiinae based on morphological data which included the genera *Habrophlebia* Eaton and *Habroleptoides* Schoenemund, previously placed in the Leptophlebiinae.

Based on morphological data and applying a hierarchy-based nomenclature (Kluge, 2000), Kluge (2009) restricted the concept of Atalophlebiinae (or Atalophlebia/fg5 = Atalophlebolinguata) and created three new subfamilies: Calliarcyinae, Terpidinae and Castanophlebiinae. Such division was further corroborated by Godunko *et al.* (2015) through a phylogenetic analysis also based on morphological data. O'Donnell & Jockusch (2008) proposed the first molecular phylogeny for the family based on two nuclear molecular markers. However, they did not recover Leptophlebiidae as monophyletic, although they did recover some internal monophyletic groups. They also did

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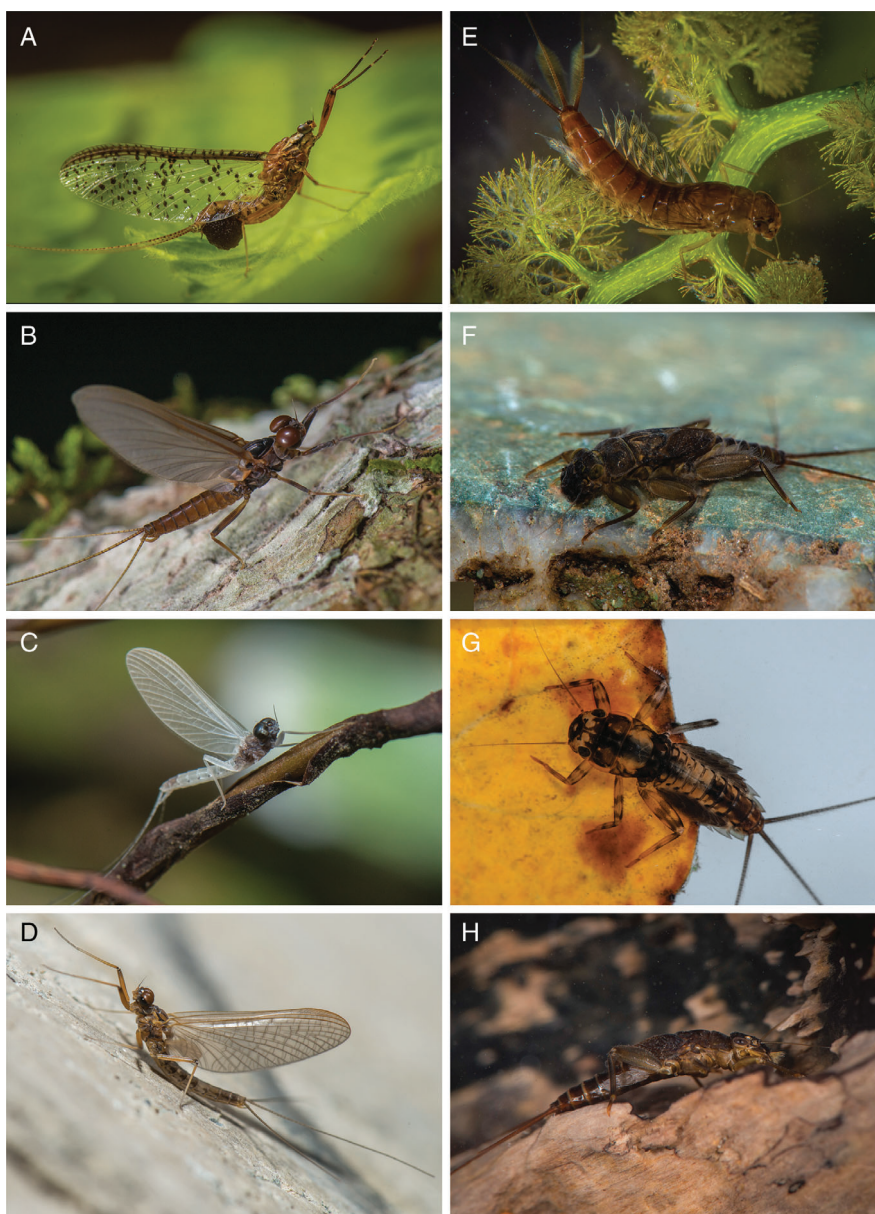
Fig. 1. Living specimens of Leptophlebiidae from the Neotropics. (A) Female imago of *Ulmeritus saopaulensis* (Traver); (B) male subimago of *Needhamella ehrhardti* (Ulmer); (C) male imago of *Hagenulopsis esmeralda* Domínguez, Molineri & Bersoza; (D) male subimago of *Penaphlebia flavidula* Pescador & Peters; (E) female nymph of *Terpides guyanensis* Demoulin; (F) female nymph of *Thraulodes consortis* Domínguez; (G) female nymph of an undescribed species of *Massartella* Lestage; (H) female nymph of *Hylister obliquus* Nascimento & Salles. All photographs are by FFS. [Colour figure can be viewed at wileyonlinelibrary.com].

not recover Atalophlebiinae as monophyletic, but their genera were grouped into four main clades (Malagasy, Paleoaustral, Choroterpes and Thraulodes groups), with the Thraulodes group being exclusively composed of three Neotropical genera [*Thraulodes* Ulmer (Fig. 1F), *Farrodes* Peters and *Traverella* Edmunds]. However, their analyses did not include the majority of the Neotropical genera. In addition, Leptophlebiinae and Habrophlebiinae were recovered as independent and monophyletic.

Taxonomic and phylogenetic history of Neotropical Leptophlebiidae

In the 19th century, Alfred Russel Wallace had already observed the incredible biodiversity of the Neotropical region. The naturalist proposed that diversity and isolation were the main factors related to the fauna observed in that region, and no other biogeographic region was so rich in endemic families and genera (Wallace, 1876). In the same book, Wallace reiterates

that the peculiarities of the southern South America fauna, together with the Andean plateaus in relation to the fauna of the tropical plains, formed well-defined subregions in the Neotropical region.

Following this reasoning, the author proposed four subregions for the Neotropical region, with two of them located in South America: Tropical South America/Brazilian subregion (Guiana and Brazilian shields, proposed by Harrington, 1962) and South Temperate America/Chilean subregion (Patagonian Shield proposed by Harrington, 1962). In addition, the following subregions have also been proposed: Tropical North America/Mexican subregion and West Indian islands/Antillean subregion. Recent authors also recognize similar subregionalization in the Neotropical region (Harrington, 1962; Holt *et al.*, 2013; Morrone, 2014).

The diversity of Leptophlebiidae in the Neotropical region is the greatest, with 40% of all species from the family (Sartori & Brittain, 2015). Here, Leptophlebiids are dominant in many river basins, in terms of both taxonomic diversity and abundance, and show high levels of endemism (Peters, 1980; Sartori & Brittain, 2015). It is estimated that 60% of the genera and 80% of the species are endemic to this region (Pescador *et al.*, 2001). Currently, there are more than 40 genera (Dominguez *et al.*, 2013) and 250 species (Sartori & Brittain, 2015). Despite the wide variety of taxa, knowledge on family diversity in the region continues to grow, making them one of the most studied Ephemeroptera groups in this region with regard to taxonomy (e.g. Peters, 1969; Savage, 1982; Flowers & Dominguez, 1991; Mariano, 2011), systematics (e.g. Savage, 1983; Pescador & Peters, 1990; Dominguez, 1995, 2009; Dominguez *et al.*, 2001; Sartori, 2005; Gonçalves *et al.*, 2012; Salles & Dominguez, 2012; Salles & Boldrini, 2019) and biogeography (e.g. Savage, 1987; Dominguez, 1999; Savage *et al.*, 2005).

Since the 1980s, the Leptophlebiidae from the Neotropical region was divided into several clusters of genera called complexes or lineages, supposedly monophyletic. Savage (1987), using the classification by Peters (1980) and the geological nomenclature proposed by Harrington (1962), divided the Neotropical Atalophlebiinae into two large groups: the Patagonian Shield, a nonmonophyletic group, composed of 10 genera adapted to the cold climate, and with lineages related to the Oriental and Australian regions; and the Guiana and Brazilian shields, a monophyletic group composed of 24 genera adapted to warmer climates, with endemic lineages reflecting the isolation caused by the formation of the Atlantic (80 Ma) until the formation of Panama isthmus (5 Ma). The genera from the Patagonian Shield were subdivided into five lineages or complexes. While one of them [Penaphlebia lineage: *Penaphlebia* Peters and Edmunds (Fig. 1D) and *Massartella* Lestage (Fig. 1G)] is restricted to the South American continent, the others contained genera from the Neotropics and other continents (Hapsiphlebia lineage, Nousia lineage, Meridialaris lineage and Dactylophlebia lineage; Savage, 1987). The genera of the Guiana and Brazilian shields were allocated to five lineages restricted to South America and supposedly monophyletic: Miroculis lineage (Savage, 1982), Terpides lineage (Savage, 1986; Fig. 1E), Farrodes lineage (Savage, 1987; subsequently

called Homothraulus), Hagenulopsis lineage (Savage, 1987; Fig. 1C), and Hermanella complex (Flowers & Dominguez, 1991; Sartori, 2005; Lima *et al.*, 2012; Fig. 1H). *Atopophlebia* Flowers, *Ulmeritus* Traver (Fig. 1A), *Perissophlebioides* Savage and *Thraulodes* Ulmer (Fig. 1F) remained indeterminate at that time. Subsequently, two new complexes were proposed: the Ulmeritus/Ulmeritoides complex (Flowers & Dominguez, 1991; Dominguez, 1995; Salles & Dominguez, 2012) and the Perissophlebioides lineage (Salles & Boldrini, 2019).

Savage (1987) proposed that all genera from complexes that occur in the Neotropical region emerged there and denominated them as Neotropical Atalophlebiinae. The Choroterpes complex (Atalophlebiinae) also occurs in the Neotropical region. This complex contains two genera: *Choroterpes* Eaton, which is divided into three subgenera (*Choroterpes* Eaton, *Euthraulus* Barnard and *Cryptopenella* Gillies); and *Neochoroterpes* Allen, which was raised to genus without any explanation by Henry (1993) (Flowers, 2009). Peters (1988) proposed that the origin of the Choroterpes complex could be Gondwanan with further expansion, while Flowers (2009) did not suggest the origin but indicated that the group diversification occurred through the Sea of Tethys in the Mesozoic Era.

As previously, the systematics of Leptophlebiidae is mainly based on morphological characteristics, and few studies tested the monophyly of the individual complexes in the Neotropical region based on a systematic methodology. In other complexes, such as the Homothraulus complex, phylogenies with morphological data suggested that the group, at least as it was initially proposed, is paraphyletic (e.g. Dominguez, 2009; Gonçalves *et al.*, 2012). Here we present the first comprehensive phylogenetic study for Neotropical Leptophlebiids using molecular data and the largest family phylogeny investigation. The goal is to provide a more thorough test of the monophyly of Neotropical groups relative to the world fauna, as well to provide the basis for a revised higher-level classification of Leptophlebiidae, and the family monophyly as a whole.

Materials and methods

Taxon sampling

Specimens from 53 genera of Leptophlebiidae from all biogeographic regions were sampled for this study, including all subfamilies except for Calliarcyinae Kluge (Appendix S1). A total of 153 specimens were sampled, with 106 obtained in this study, and vouchers are deposited at the Coleção Zoológica Norte Capixaba from the Federal University of Espírito Santo (CZNC), Brazil. Additional sequences were obtained from GenBank. The accession numbers corresponding to the sequence data obtained in this study and previously are listed in Appendix S2. Outgroups comprised five representative species of three other Ephemeroptera families: Oligoneuriidae (*Oligoneuria amazonica* Demoulin, and *Homoeoneuria watu* Salles, Francischetti & Soares), Ephemerellidae (*Serratella ignita* Poda and *Drunella ishiyamana* Matsumura) and Neoephemeridae (*Neoephemera youngi* Berner). The

phylogenetic trees were rooted with Oligoneuriidae, following previous results indicating that the family belongs to the sister lineage of Furcatergalia in which the other families are grouped (Ogden *et al.*, 2009). O'Donnell & Jockusch (2008) found that Leptophlebiidae was not monophyletic, as a specimen of Ephemerellidae (*Ephemerella* sp.) was recovered as belonging to the family. Therefore, other specimens from Ephemerellidae were sampled to evaluate the monophyly of Leptophlebiidae. The zoogeographic distribution nomenclature used followed Wallace's zoogeographical realms (Wallace, 1876). For the Neotropical subdivisions, the nomenclature proposed by Harrington (1962) was used.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from three legs, using Wizard® SV Genomic DNA Purification System (Promega, Madison, WI, U.S.A.) Tissue kit following the protocol, with a final suspension volume of $100\,\mu L$ in ddH_2O. The sampled specimens had been preserved int 95% ethanol.

Two gene regions were amplified, the mitochondrial *cytochrome oxidase* I (COI) and nuclear *28S rRNA* (28S: D2–D5 regions). Amplifications using previously described primers (COI: Folmer *et al.*, 1994; and 28S: Gillespie *et al.*, 2004, 2005) were performed in $25\,\mu L$ using the Platinum® Taq DNA Polymerase (Invitrogen, Waltham, MA, U.S.A.) kit. The PCR MasterMix contained the following: H_2O, $18.475\,\mu L$; $100\,mM$ DNTP, $0.8\,\mu L$; $50\,mM$ $MgCl_2$, $2.0\,\mu L$; Buffer -$MgCl_2$ $10\times$, $2.5\,\mu L$; primers F and R ($5\,mM$), $0.3\,\mu L$; TAQ, $0.125\,\mu L$; and $1.0\,\mu L$ of template DNA. The thermocycler programmes used for amplification were as follows: for COI, denaturation at $94°C$ for 5 min, 40 cycles of sequence amplification ($94°C$ for 45 s, $47°C$ for 45 s, $72°C$ for 45 s) and final extension of $72°C$ for 5 min; and for 28S, denaturation at $94°C$ for 5 min, 35 cycles of sequence amplification ($94°C$ for 1 min, $52°C$ for 1 min 30s, $72°C$ for 1 min), and final extension of $72°C$ for 5 min. To optimize the reactions, $1.0\,\mu L$ dimethyl sulfoxide was used in 28S amplifications. Successful bands were detected on 1% TBE agarose gel. The PCR products were purified using an ExoSAP-IT® kit (USB Corporation, Waltham, MA, U.S.A.) and sequenced at the 'Núcleo de Genética Aplicada à Conservação da Biodiversidade' (NGACB) at the Federal University of Espírito Santo using an automatic sequencer 3500 Genetic Analyzer (Applied Biosystems, Waltham, MA, U.S.A.).

Alignment and phylogenetic analyses

Sequences were checked for the correct loci amplification and taxonomy using BLAST (Altschul *et al.*, 1990) from the GenBank database. Subsequently, the COI sequences were aligned in the GENEIOUS 7.1.3 software using the CLUSTAL W algorithm with default parameters. Alignment of the 28S ribosomal loci was done using the MAFFT v.7 (Katoh *et al.*, 2017), through the strategy E-INS-I algorithm (Katoh *et al.*, 2017).

Phylogenetic reconstruction was performed after the level of saturation of sequences was verified using the test by Xia *et al.* (2003) and the DAMBE 6.0.48 software. The model $GTR + I + G$ was chosen as the best-fit model for all genes (each used as a single partition) by JMODELTEST 0.1 (Posada, 2008). The phylogenetic analysis of the concatenated genes was inferred using Bayesian inference (BI) and maximum likelihood (ML). Bayesian inference trees were generated using the software MRBAYES 3.2.2 (Ronquist *et al.*, 2012) on the CIPRES portal (Miller, Pfeiffer & Schwartz, 2010). Two chains of Markov chain Monte Carlo) were run independently with 30 000 000 generations, with sampling trees every 1000 generations and 25% of sampled trees discarded. Convergence among independent analyses was assessed by monitoring the standard deviation values of the split frequencies (< 0.05) in the MRBAYES 3.2.2 software, and parameter sampling was assessed with TRACER v.1.6 (Rambaut *et al.*, 2014). Statistical branch support was obtained through posterior probabilities (PPs), and the reliability of the clades was accepted according to the proposal by Hillis & Bull (1993), as follows: strong (> 0.95) and moderate ($0.85–0.95$). The ML trees were generated using ML RAXML (Stamatakis, 2014) on the CIPRES portal (Miller, Pfeiffer & Schwartz, 2010). Branch support was obtained through bootstrapping (BT), and the reliability of the clades was accepted following Hillis & Bull (1993) as follows: strong ($> 70\%$) and moderate ($50–70\%$). The phylogenetic tree was edited in the FIGTREE v.1.4.2 software (Rambaut *et al.*, 2014).

Characteristics of the amplified loci

Out of the 481 specimens initially selected, 295 (61%) were amplified for COI and 136 (28%) for 28S, contemplating 29 Neotropical genera and one non-Neotropical. Twenty-three genera from other biogeographic regions were added to the sample with sequences obtained from GenBank. The final sample had 158 taxa, in addition to the samples from the external group (Appendix S2). As the third position of COI codon was determined to be saturated, it was excluded, and the final alignment was 394 bp. For the 28S, after the alignment, the sequences generated a matrix of 1261 bp. The matrix of concatenated data comprised 1655 bp.

Results

Optimality criteria

The trees generated by different optimization criteria (ML and BI) showed similar topologies, and we chose to demonstrate the BI tree. The ML tree can be viewed in Appendix S3, and the original BI tree in Appendix S4.

Monophyly of Leptophlebiidae

Leptophlebiidae was recovered as a well-supported monophyletic group under all analytical parameters (BT = 91, PP = 1.0) (Fig. 2). The families Ephemerellidae +
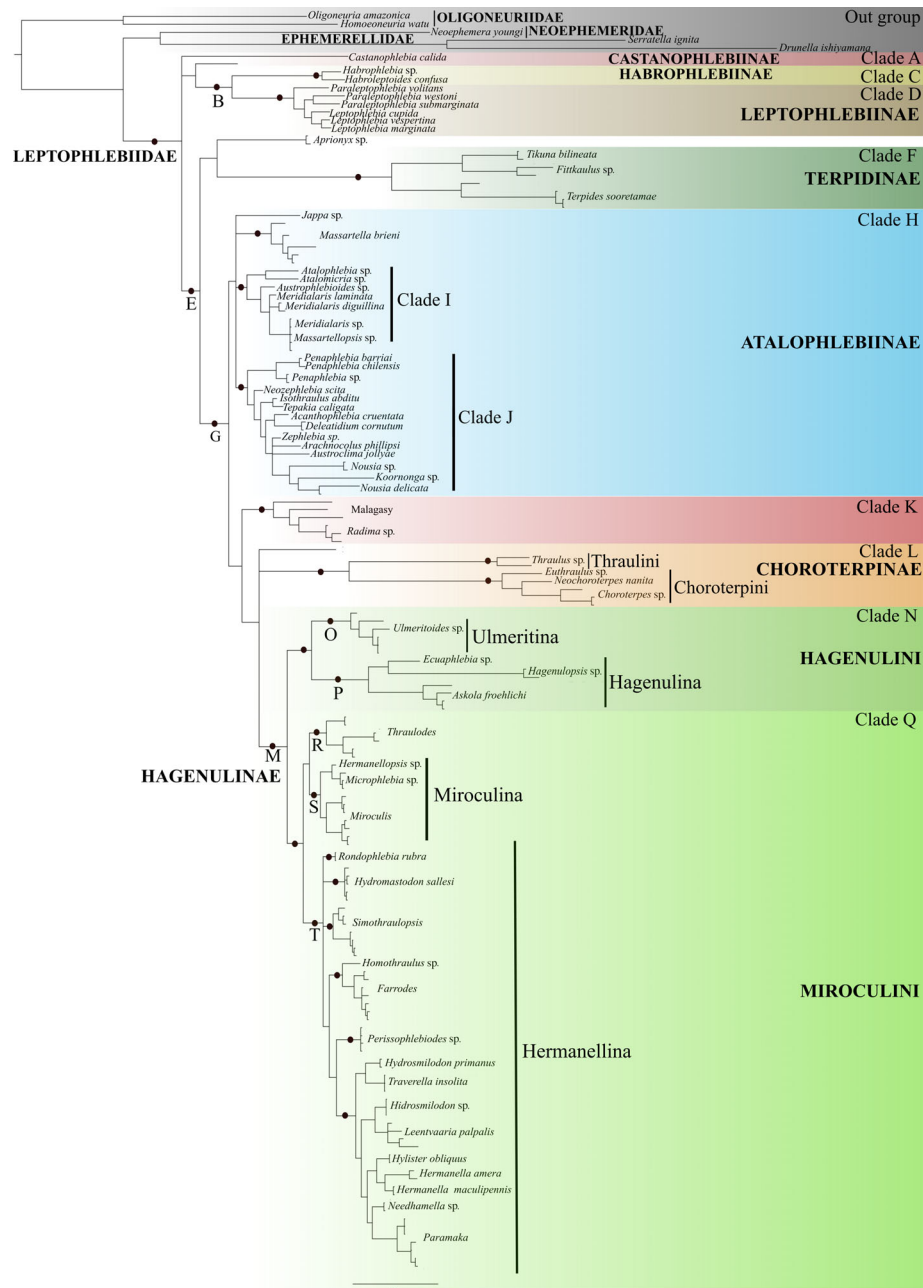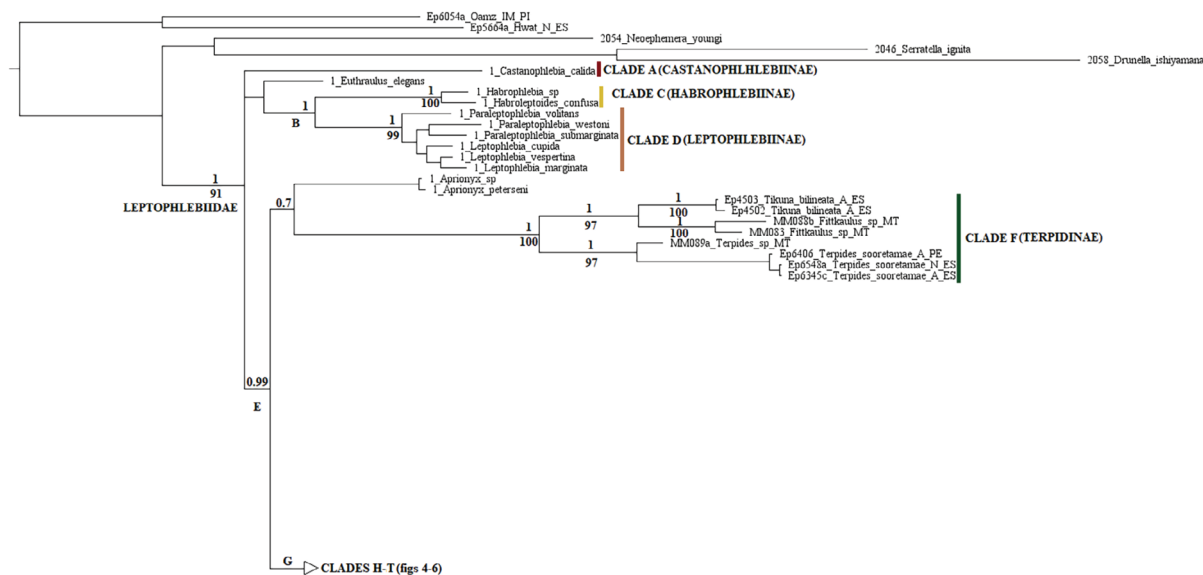
Fig. 2. Phylogeny by Bayesian inference showing Leptophlebiidae as monophyletic and major internal clades with high support with the combined dataset (COI + 28S). The black dots in the main branches correspond to a well-supported clade. [Colour figure can be viewed at wileyonlinelibrary.com].

Neoephemerellidae were clustered as a sister group of Leptophlebiidae (BT = 100, PP = 1.0). Oligoneuriidae diverged earliest from the other families, showing itself as a sister group of the other families (Fig. 2).

Phylogenetic relationships within Leptophlebiidae

Several clades were recovered through the phylogeny obtained from the concatenated data (Fig. 2): clade A represented by

the genus *Castanophlebia*; clade C (BT = 99, PP = 1.0) with representatives of genera *Leptophlebia* and *Paraleptophlebia*; and clade D (BT = 100, PP = 1.0) with representatives of genera *Habrophlebia* and *Habroleptoides*. Clades C and D were recovered as a sister group (clade B, PP = 1.0) (Fig. 3). All remaining genera were recovered in clade E (PP = 0.99) (Fig. 2). Clade F (BT = 100, PP = 1.0) was recovered including genera *Terpides* + (*Tikuna* + *Fittkaulus*) (Fig. 3); genera belonging to the Patagonian Shield (Neotropical region) were recovered with

Fig. 3. Phylogeny by Bayesian inference of Leptophlebiidae with concatenated genes (COI + 28S, 1625 bp). Numbers above branches correspond to posterior probabilities and numbers below branches correspond to bootstrap values. Partial tree from Fig. 2 showing clades A–F. [Colour figure can be viewed at wileyonlinelibrary.com].

genera from the Australian region in clade H in two distinct clades (clade I, PP = 0.92; and clade J, PP = 1.0), except for *Massartella* (Patagonian Shield) and *Jappa* (Australian region), which were recovered as incertae sedis in clade H (Fig. 4). Clade K (Malagasy group of O'Donnell & Jockusch, 2008) was recovered as monophyletic with genera exclusively from Madagascar (BT = 77, PP = 0.94) (Figs 2,5). Clade L (BT = 97, PP = 1.0) was recovered as monophyletic with genera *Thraulus* + [*Euthraulus* + (*Neochoreterpes* + *Choroterpes*)] (Fig. 5). All the genera belonging to the Guiana and Brazilian shields (Neotropical region), except for *Terpides* + (*Tikuna* + *Fittkaulus*), were recovered in a single clade (clade M, BT = 92, PP = 1.0) (Fig. 6). Two main clades were recovered in clade M (N and Q), which were divided into five subsequent clades: O and P (both on clade N); and R, S and T (on clade Q) (Fig. 6). Clade O included representatives of the genus *Ulmeritoides* (BT = 100, PP = 1.0), clade P clustered the genera *Askola* + (*Hagenulopsis* + *Ecuaphlebia*) (BT = 98, PP = 1.0), clade R included representatives of the genus *Thraulodes* (BT = 86, PP = 1.0), clade S clustered the genera *Miroculis* + (*Microphlebia* + *Hermanellopsis*) (BT = 84, PP = 1.0) and clade T clustered the genera *Hydromastodon*, *Simothraulopsis*, *Farrodes*, *Perissophlebiodes*, *Hydrosmilodon*, *Traverella*, *Leentvaaria*, *Hylister*, *Hermanella*, *Paramaka*, *Needhamella* and *Rondophlebia* (BT = 91, PP = 1.0).

Discussion

Leptophlebiidae

The family was found to be a well-supported monophyletic group (Fig. 2). Our data do not agree with those found in a previous study based on molecular data, where Leptophlebiidae was rendered as paraphyletic relative to Ephemerellidae (O'Donnell & Jockusch, 2008). In our analysis, other specimens of Ephemerellidae were used, the genera *Serratella* and *Drunella*, as well as another representative of Neoephemeridae (Pannota). Despite that, Leptophlebiidae remained monophyletic, which is compatible with previous studies based on molecular and morphological data (e.g. Ogden & Whiting, 2005; Ogden *et al.*, 2009). The same authors found Leptophlebiidae and Ephemerellidae to be phylogenetically distant, while, in this study, Leptophebiidae and Pannota were closely related, probably due to the composition of the outgroups used in our analysis.

Our phylogeny shows many internal Leptophebiidae lineages well supported as monophyletic, while certain relations remain somewhat ambiguous (Fig. 2). In addition to the monophyly of the subfamilies proposed in recent literature, new clades were found in this study and will be discussed in the following sections. Changes in the classification of Leptophlebiidae over time, including those proposed by this research, are found in Appendix S5, while the cladograms with the new proposal for Leptophlebiidae are found in Appendices S6–S8.

Leptophlebiinae (clade C) and Habrophlebiinae (clade D)

Leptophlebia Westwood and *Paraleptophlebia* Lestage were recovered in clade C (Figs 2,3). *Paraleptophlebia* was recovered as paraphyletic, as previously found by O'Donnell & Jockusch (2008) in their molecular phylogeny analysis and proposed by Peters & Edmunds (1970) based on morphological data. This clade represents Leptophlebiinae, although *Habrophlebiodes*
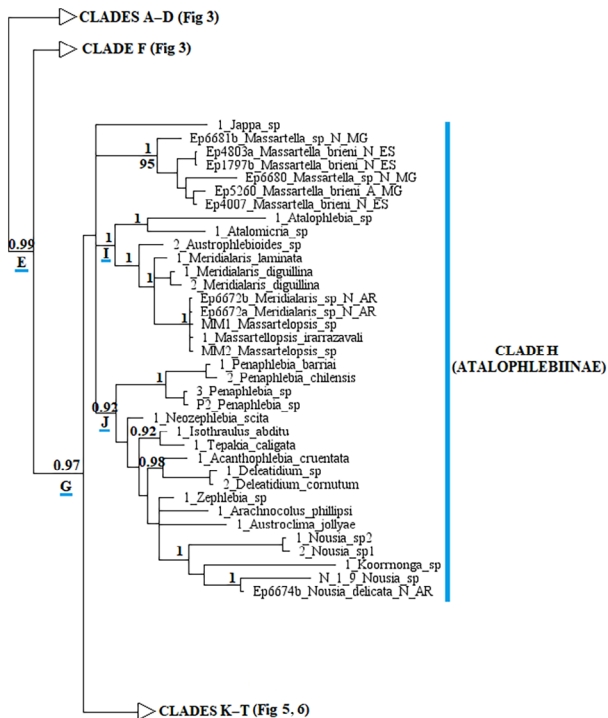
Fig. 4. Phylogeny by Bayesian inference of Leptophlebiidae with concatenated genes (COI + 28S, 1625 bp). Numbers above branches correspond to posterior probabilities and numbers below branches correspond to bootstrap values. Partial tree from Fig. 2 showing clades E–J. [Colour figure can be viewed at wileyonlinelibrary.com].

Ulmer, *Dipterophlebiodes* Demoulin and *Gilliesia* Peters & Edmunds were not included in the analysis.

Habrophlebia and *Habroleptoides* were strongly supported as sister taxa (Figs 2,3). This arrangement was proposed by Kluge (1994) as the subfamily Habrophlebiinae based on some aspects of larval and adult morphology. Consequently, the clade C found in this study corresponds to this subfamily. Kluge (2009) and Godunko *et al.* (2015) suggested that Habrophlebiinae would be closely related to the clade Atalophleboculata (Atalophlebia/fg3), which is represented in this study as clade E. However, this topology was not recovered; rather, our molecular data strongly suggest the close relationship between Habrophlebiinae + Leptophlebiinae (Clade B) (Figs 1,2). According to our results, therefore, *Habrophlebia* and *Habroleptoides* could be reintegrated to Leptophlebiinae. However, due to the limited support found in the Bayesian result and strength of morphological evidence proposed by Kluge (2009) and Godunko *et al.* (2015), we decided to keep them as distinct subfamilies.

Clade A: Castanophlebiinae

According to Peters (1997) the genus *Castanophlebia* is closely related to the Terpides lineage within Atalophlebiinae. Kluge (2009) instead proposed a new subfamily

Castanophlebiinae (composed exclusively of *Castanophlebia*) as the sister to Atalophlebolinguata. In our analyses, *Castanophlebia* (clade A; Figs 2,3) was not recovered in any of the clades mentioned earlier (cf. O'Donnell & Jockusch, 2008) and agrees with the subfamilial status suggested by Kluge (2009), but not with the close relationships between Castanophlebiinae and Atalophlebolinguata (clade G; Figs 2,3), as previously proposed by Kluge (2009), or between *Castanophlebia* and Terpidinae, as proposed by Peters (1997). The subfamily was recovered in a polytomy with the main clades of Leptophlebiidae: Castanophlebiinae + (Habrophlebiinae + Leptophlebiinae) + clade E (Figs 2,3). Given our results, the loss of the distal dentiseta would have occurred independently in Castanophlebia and Atalophlebolinguata.

Terpidinae (clade F) and Aprionyx Barnard

Terpides Demoulin (Fig. 1E), *Tikuna* Savage, Flowers & Porras and *Fittkaulus* Savage & Peters were first grouped by Savage (1986), who designated them as the Terpides complex. When compared with the other genera of Leptophlebiidae, the representatives of this complex have peculiar characteristics, as both nymphs and adults (Savage, 1986; Savage *et al.*, 2005). The combination of hypognathous mouthparts and the rows of bristles present in the caudal filaments make them unique among Neotropical leptophlebiids, resulting in nymphs with similar characteristics to Baetidae and Siphlonuridae (Savage, 1986). In that same study, the author proposed that the complex was a part of Atalophlebiinae and would be the sister group of all other genera of the subfamily due to their morphological characteristics. Later, on account of the restricted distribution of the group to the Guiana and Brazilian shields, Savage (1987) proposed that the complex, among others, would form a monophyletic group with endemic lineages belonging to the Guiana and Brazilian shields, reflecting the isolation by formation of the Atlantic from 80 Ma to the formation of the Panama isthmus around 5 Ma.

In this study, *Terpides*, *Tikuna* and *Fittkaulus* were clustered in clade F (Figs 2,3). Unlike the classification proposed by Savage (1987), this clade was not grouped with other complexes of the Guiana and Brazilian shields (clade L; Figs 2,6), or with *Castanophlebia* as proposed by Peters (1997). Instead, our results corroborate the subfamily-level category for this lineage as proposed by Kluge (2009), but not the relationships [i.e. Terpidinae + (Castanophlebiinae + Atalophlebolinguata)]. On the other hand, our data recovered the relationship between these genera and *Aprionyx*, a genus distributed exclusively in the Afrotropical region, and the first internal phylogeny is proposed for this group, *Terpides* + (*Tikuna* + *Fittkaulus*) (Figs 2,3). The systematic position of *Aprionyx* is controversial: Peters (1980) proposed that it was part of the Hapsiphlebia complex, whereas Kluge (2009), due to the presence of a pair of lateral processes in the hypopharynx, included it in Atalophlebolinguata. Later, however, Kluge (2012) noted that the nymphs of *Aprionyx* retain the distal dentiseta, a characteristic that is lost in Atalophlebolinguata but retained in Terpidinae. Given the low support for this
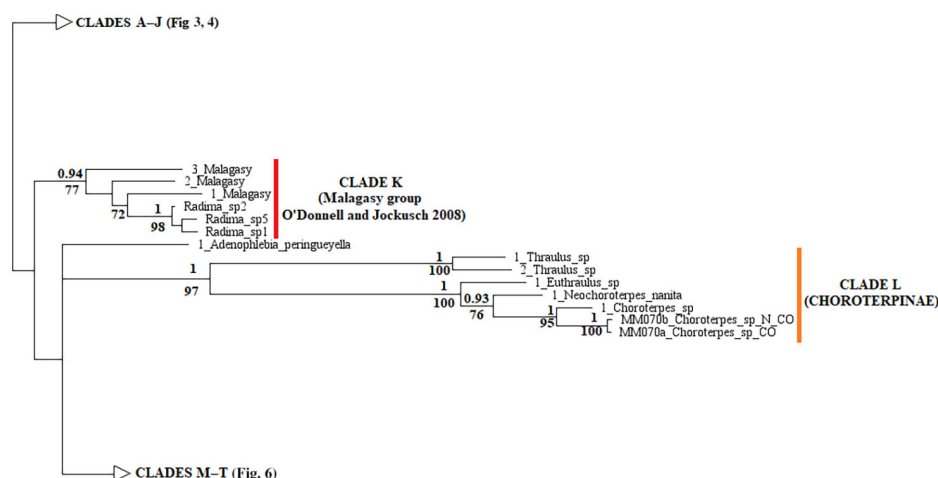
Fig. 5. Phylogeny by Bayesian inference of Leptophlebiidae with concatenated genes (COI + 28S, 1625 bp). Numbers above branches correspond to posterior probabilities and numbers below branches correspond to bootstrap values. Partial tree from Fig. 2 showing clades K and L. [Colour figure can be viewed at wileyonlinelibrary.com].

clade, further studies are necessary in order to investigate this relationship more profoundly.

Our data showed the clade formed by (*Aprionyx* + Terpidinae) + clade G with strong statistical support in the Bayesian analysis. Therefore, the absence of the pair of lateral processes in the hypopharynx in Terpidinae may have been a reversal in the family. We suggest that studying these characters (i.e. pair of lateral processes in the hypopharynx and the presence of distal dentiseta) is interesting for understanding the evolution of the group. Despite the poor statistical support, the clade formed with Terpidinae + *Aprionyx* may suggest that the ancestor's lineage began to isolate during the separation between the two regions through the movement of tectonic plates in an event distinct from the other Neotropical genera of the Guiana and Brazilian shields.

Clade H: Patagonian Shield (Savage, 1987)

This clade exclusively includes genera from the Patagonian Shield and Australian region. Based on geological data, Harrington (1962) proposed that South America has a long evolutionary history and is composed of shields formed between the Precambrian and the Lower Cambrian. Based on this perspective, Savage (1987) proposed that Patagonia Shield taxa are more related to taxa from the Australian, Madagascar and Afrotropical regions.

Our data clustered all genera sampled from the Patagonian Shield (Neotropical region) and Australian region in a single clade H (Figs 2,4), although with limited statistical support. As this clade, among others, includes *Atalophlebia* Eaton and related genera (clade I), we are restricting it to the former widely distributed and well diversified Atalophlebiinae. Even representing a small fraction of the original Atalophlebiinae, this clade is probably one of the most diverse after Hagenulinae (see below). Inclusion of additional taxa, especially from Australia,

is necessary in order to achieve a better understanding of its evolution and composition. For example, our data did not recover any previously proposed lineage as monophyletic. Our study recovered two main clades (I and J) in clade H, both containing genera of the two zoogeographic regions (Patagonian Shield and Australian region; Figs 3,5). The genera *Massartella* (Patagonian Shield; Fig. 1G) and *Jappa* Harker (Australian region) were recovered in clade H, but in a polytomy with the remaining two clades (Figs 2,4). Our data could not recover these relationships, although *Massartella*, for example, is strikingly similar to the Australian genus *Garinjuga* Campbell & Suter.

Clade J

This clade contains taxa from the Australian region and Patagonian Shield (Figs 2,4). The genera included in this clade were from the Nousia lineage (*Nousia* Navas, *Koorrnonga* Campbell & Suter, *Neozephlebia* Penniket and *Zephlebia* Penniket), the Dactylophlebia lineage (*Austroclima* Towns & Peters), the Penaphlebia lineage (*Penaphlebia*; Fig. 1D), and the Meridialaris lineage (*Deleatidium* Eaton). The following genera were also included in this clade: *Aracnocolus* Towns & Peters, *Acantophlebia* Towns, *Isothraulus* Towns & Peters and *Tepakia* Towns & Peters. *Neozephlebia* has been classified as a subgenus of *Zephlebia*. However, this was not confirmed by our analyses. These genera were not recovered as a monophyletic group, so we propose that the subgeneric status of *Neozephlebia* should be re-evaluated. *Nousia* is distributed in both regions, while *Koorrnonga* is distributed only in the Australian region. The clade *Nousia* + *Koorrnonga* (clade I; Fig. 4) is again evidence of the relationship between the Neotropical (EP) and Australian faunas (Fig. 4). Our data recovered *Nousia* paraphyletic relative to *Koorrnonga* (Fig. 4); more studies are necessary to understand the relationship between these two genera and their taxonomic status.
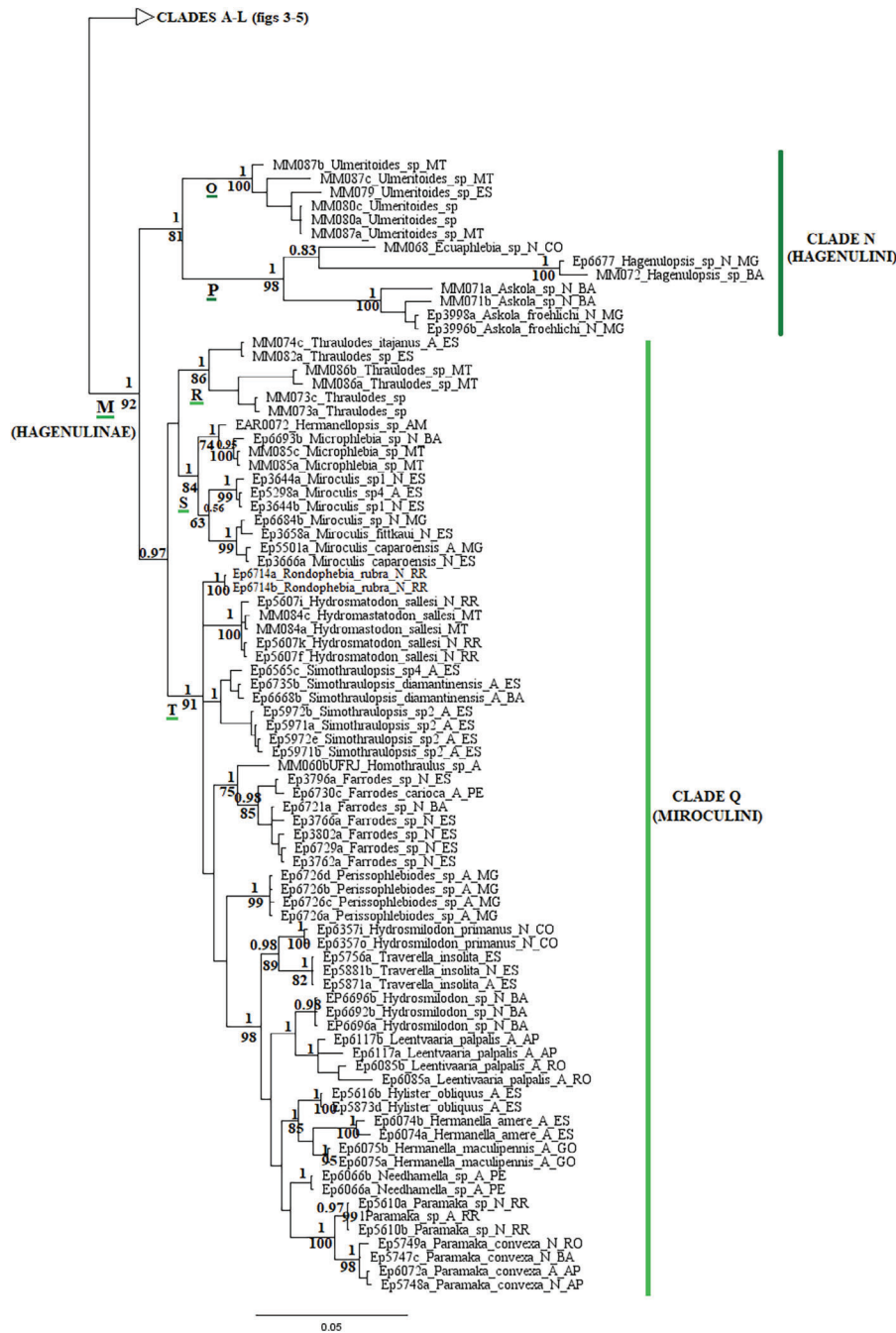
Fig. 6. Phylogeny by Bayesian inference of Leptophlebiidae with concatenated genes (COI + 28S, 1625 bp). Numbers above branches correspond to posterior probabilities and numbers below branches correspond to bootstrap values. Partial tree from Fig. 2 showing clades M–T. [Colour figure can be viewed at wileyonlinelibrary.com].

Clade I

This clade contains taxa from the Australian region and Patagonian Shield (Figs 2,4) from the Hapsiphlebia lineage (*Atalophlebia* Eaton and *Atalomicria* Harker) and Meridialaris lineage (*Meridialaris* Peters & Edmunds, *Massartelopsis* Demoulin and *Austrophlebioides* Campbell & Suter).

Clade K: Madagascar

This is a small group of exclusively Malagasy taxa (Figs 2,5). Evidence for the monophyly of this group was previously shown in the molecular data by O'Donnell & Jockusch (2008). This clade probably deserves subfamilial status, but we refrain from doing so given the low number of taxa included in the analyses.

Clade L: Choroterpinae

This is a relatively small but widely distributed group world-wide (Figs 2,5). The monophyly of this clade was established previously using both molecular (O'Donnell & Jockusch, 2008; Choroterpes group) and morphological data (Kluge, 2012; tribe Choroterpini). This clade includes the genera *Choroterpes*, *Neochoroterpes* and *Thraulus*, a classification corroborated by this analysis. Based on the morphological differences proposed by Kluge (2012), and on the well-supported clade recovered here and by O'Donnell & Jockusch (2008), we propose that Choroterpini (Fig. 5) be raised to the subfamily status. Similar to Kluge (2012), we also recovered the same two major clades in the group and propose that they be raised to tribal status (Fig. 5), i.e. Thraulini and Choroterpini. All morphological characteristics that support the subfamily and the tribes have been proposed previously by Kluge (2012). However, Boonsoong & Sartori (2016) described *Sangpradubina* with intermediate characters between these two clades. Inclusion of this genus in a phylogenetic analysis is essential for understanding its systematic position in the clade.

Clade M: Hagenulinae: Guiana and Brazilian shields (Savage, 1987)

While the Patagonian Shield fauna suggests intense interchange between the Gondwanan regions, it was proposed that the Guiana and Brazilian shields evolved in isolation (Savage, 1987). As previously mentioned, the genera belonging to the Guiana and Brazilian shields appear to form a monophyletic group. Dominguez (2009) did not recover the shield fauna as monophyletic despite a heavily sampled phylogeny for this region. We recovered representatives of the Guiana and Brazilian shields in two clades, with strong support in both analyses: Terpidinae (clade F; Figs 2,3) and clade M (Figs 2,6). This is an extremely diverse lineage, including most of the species of the Guiana and Brazilian shields, and was consistently recovered in all analyses (Figs 2,6). This group seems to have evolved in isolation in the Guiana and Brazilian shields, with subsequent dispersal to the Caribbean Islands and North America (McCafferty, 1998).

Kluge (1994) was the first to group the genera *Farrodes*, *Homothraulus* Demoulin, *Hagenulus* Eaton, *Borinquena* Traver, *Hagenulopsis* Ulmer (Fig. 1C), *Neohagenulus* Traver, *Traverina* Peters and *Careospina* Peters in the tribe Hagenulini. In this study, the author considered the genera *Borinquena*, *Hagenulopsis*, *Neohagenulus*, *Traverina* and *Careospina* as subgenera of *Hagenulus*. Kluge (2008) added other genera to Hagenulini (or Hagenulus/fg1): *Hermanella* Needham & Murphy (Hermanella/fg1), *Ulmeritus/Ulmeritoides* (Ulmeritus/g1) *Miroculis* Edmunds (Miroculus/g1) and *Thraulodes* Ulmer (Thraulodes/g1). He also suggested that *Askola* Peters and *Atopophlebia* Flowers were probably part of the group. The main characteristics for the tribe are the loss of patella-tibial suture on all legs of larvae and adults.

Our data revealed that all Neotropical genera from the Guiana and Brazilian shields, except for Terpidinae, were recovered in clade M (Figs 2,6). This agrees with Kluge (1994, 2008), except that in our dataset we were not able to add any of the endemic Caribbean genera or *Hagenulus*. Despite the absence of these genera, it is clear based on morphological characters that they belong to clade M and to clade P. We propose that to this clade be elevated to the subfamily Hagenulinae. This subfamily is composed exclusively of Neotropical genera, with few genera, such as *Farrodes*, *Thraulodes* and *Traverella*, extending to North America. Overall, we found the same five groups proposed by Kluge (2008) in Hagenulinae (see Figs 2,6).

Clade N: Hagenulini

This study was the first to recover this clade with strong support (Figs 1,5). The genera included are from the Ulmeritus/Ulmeritoides and Hagenulopsis lineages (Figs 2,6; clade N). We proposed that Hagenulini should be restricted to clade N. Representatives of *Ulmeritoides* Traver were recovered in clade O (Figs 2,6) whose monophyly and taxonomy had been hypothesized previously based on morphological and molecular data (Flowers & Dominguez, 1991; Dominguez, 1995; Salles & Dominguez, 2012). Salles *et al.* (2019) proposed a phylogeny for this complex, including a new genus called Diamantina. We propose that the complex should be classified as the subtribe Ulmeritina.

The genera *Askola*, *Hagenulopsis* (Hagenulus/fg2, *sensu* Kluge, 2008) and *Ecuaphlebia* Domínguez were recovered in clade P (Figs 2,6). Despite the absence of *Hagenulus* in our analyses, we are confident that it belongs to this clade based on several unique characteristics, such as a sagged MA2, IRP1-2 connected to IRP, presence of an egg guide and hindwings reduced or absent. We propose that this clade be classified as subtribe Hagenulina. Dominguez (1988) suggested that *Ecuaplebia* was more closely related to *Hagenulopsis* than any other genera based on morphology, although in a subsequent study of the phylogenetic relationships among dipterous leptophlebiids from South America, Dominguez (2009) did not recover a close relationship between *Ecuaphlebia* and *Hagenulopsis*.

Clade Q: Miroculini

Clade Q was recovered here with strong support and includes genera from the Miroculis, Homothraulus, Perissophlebiodes and Hermanella lineages (Figs 2,6; clade Q). We propose that this clade (Fig. 6) be classified as tribe Miroculini. The Miroculis lineage was proposed by Savage (1983), who presented a phylogeny suggesting a close relationship among *Miroculis* Edmunds, *Miroculitus* Savage & Peters, *Hermanellopsis* Demoulin and *Microphlebia* Savage & Peters. Kluge (2008) recognized this group as Miroculis/g1. Our analyses recovered the same topology (clade S; Fig. 6) as previous authors except that we did not sample the genus *Miroculitus*.

We propose that the lineage should be classified as subtribe Miroculina.

Thraulodes Eaton (Figs 1F, 6; clade R) was recovered in Miroculini. This result is partially in agreement with Kluge (2008), who considered *Thraulodes* as a member of Hagenulus/fg1 (= Hagenulinae) but without positing any relationship to other members of the group. Dominguez (2009) classified *Thraulodes* with *Meridialaris*, a genus belonging to the Patagonian Shield. Based on our results we propose that the genus be placed in the tribe Miroculini.

Clade T: Hermanellina

Kluge (2008) established the subtribe Hermanellina (Hermanellota - Hermanella/fg1), composed of genera of the Homothraulus and Hermanella complexes, and characterized it by two unique autapomorphies in the structure of male genitalia and subimaginal mesotonum. The same grouping was confirmed in this analysis (Figs 2,6; clade T), although *Perissophlebiodes* Savage also was recovered within Hermanellina. Dominguez (2009) recovered Hermanellina (with *Perissophlebiodes*) in his clade 'O'. Our data are congruent with Salles & Boldrini (2019) who also recovered *Perissophlebiodes* and two related genera in the Perissophlebiodes lineage within Hermanellina; they found additional synapomorphies for the group, such as the telopenis directed towards the base of penis. Although they were grouped in Hermanellina, our data do not allow us to conclude if the complexes mentioned are monophyletic, as a polytomy was recovered in that part of the tree (Fig. 6).

In the Hermanella complex, the most species-rich of the clade, relationships among their members at various taxonomic levels are still highly contentious (Sartori, 2005; Kluge, 2008). Similar to Sartori (2005) we recovered *Hydrosmilodon* Flowers & Dominguez as polyphyletic (Fig. 6), while *Hylister obliquus* Nascimento & Salles, (Fig. 1H) was placed within *Hermanella*. In this regard, the taxonomic status of the aforementioned genera remains somewhat doubtful. Kluge (2008) considered that the variability of species within the Hermanella complex reflects the poor current classification (Flowers & Dominguez, 1991; Sartori, 2005), and therefore classified the complex as a single genus *Hermanella*, divided into five subgenera: *Hermanella*, *Needhamella* Dominguez & Flowers (Fig. 1B), *Leentvaaria* Demoulin, *Traverella* Edmunds and *Hylister* Dominguez & Flowers (*Hydrosmilodon* and *Paramaka* Savage & Dominguez were considered as junior synonyms of *Needhamella*). Despite it supposedly being part of the Hermanella complex (Polegatto & Batista, 2007), *Hydromastodon* Polegatto & Batista was not recovered along with other genera of the complex. However, due to poor statistical support and a polytomy in this part of the tree (Fig. 6), we cannot confirm its position reliably. Morphological data, however, display an affinity with the complex.

Nomenclatural changes

(The genera used in this study are marked with an asterisk in this section)

Atalophlebiinae Peters sensu n.
Type genus. *Atalophlebia* Eaton 1881:193.
Included genera. *Acantophlebia* Towns*; *Aracnocolus* Towns & Peters*; *Atalomicria* Harker*; *Atalophlebia* Eaton*; *Austroclima* Towns & Peters*; *Austrophlebiodes* Campbell & Suter*; *Deleatidium* Eaton*; *Garinjuga* Campbell & Suter; *Isothraulus* Towns & Peters*; *Koorrnonga* Campbell & Suter*; *Massartella* Lestage*; *Massartelopsis* Demoulin*; *Meridialaris* Peters & Edmunds*; *Neozephlebia* Penniket*; *Nousia* Navas*; *Penaphlebia* Peters & Edmunds*; *Tepakia* Towns & Peters*; and *Zephlebia* Penniket*.
Distribution. Australian and Neotropical regions.

Calliarcyinae Kluge
Type genus. *Calliarcys* Eaton 1881: 12.
Include genus. *Calliarcys* Eaton.
Diagnosis. See morphological characteristics in Kluge (2009).
Distribution. Ethiopian region.

Castanophlebiinae Kluge
Type genus. *Castanophlebia* Barnard, 1932: 244.
Include genus. *Castanophlebia* Barnard*.
Diagnosis. See morphological characteristics in Kluge (2009).
Distribution. Ethiopian region.

Choroterpinae Kluge stat.n.
Choroterpini Kluge.
Type genus. *Choroterpes* Eaton, 1881: 194.
Included genera. *Choroterpes* Eaton, *; *Dilatognathus* Kluge, *Euthraulus* Barnard; *Monochoroterpes* Kluge; *Neochoroterpes* Allen*; *Nonnullidens* Grant & Peters; *Sangpradubina* Boonsoong & Sartori (2016); and *Thraulus* Eaton*.
Diagnosis. See morphological characteristics in Kluge (2012) and Boonsoong & Sartori (2016).
Distribution. Worldwide.

Choroterpini Kluge
Type genus. *Choroterpes* Eaton, 1881: 194.
Included genera. *Choroterpes* Eaton*; *Euthraulus* Barnard*; and *Neochoroterpes* Allen*.
Diagnosis. See morphological characteristics in Kluge (2012).
Distribution. Ethiopian, Nearctic, Neotropical, Palaearctic and Oriental regions.

Thraulini Kluge
Type genus. *Thraulus* Eaton, 1881: 194.
Included genus. *Thraulus* Eaton*.
Diagnosis. See morphological characteristics in Kluge (2012).
Distribution. Australian, Ethiopian, Palaearctic and Oriental regions.

Habrophlebiinae Kluge
Type genus. *Habrophlebia* Eaton, 1881: 195.
Genera include. *Habrophlebia* Eaton* and *Habroleptoides* Schoenemund*.
Diagnosis. See morphological characteristics in Kluge (1994).
Distribution. Palaearctic, Nearctic and Ethiopian regions.

Hagenulinae Kluge stat. n.
Hagenulini Kluge.
Type genus. *Hagenulus* Eaton, 1882: 207.

Included genera. *Askola* Peters*; *Atopophlebia* Flowers; *Bessierus* Thomas & Orth, *Borinquena* Traver; *Careospina* Peters; *Ecuaphlebia* Dominguez*; *Farrodes* Peters*; *Guayakia* Dominguez & Flowers; *Hagenulopsis*, Ulmer*; *Hagenulus* Eaton; *Hermanella* Needham & Murphy*; *Hermanellopsis*, Demoulin*; *Homothraulus* Demoulin*; *Hydromastodon* Savage & Peters*; *Hydrosmilodon* Flowers & Dominguez*; *Hylister* Dominguez & Flowers*; *Leentvaaria* Demoulin*; *Microphlebia* Savage & Peters*; *Miroculis* Edmunds*; *Miroculitus* Savage & Peters; *Needhamella* Dominguez & Flowers*; *Neohagenulus* Traver; *Paramaka* Savage & Dominguez*; *Perissophlebiodes* Savage*; *Rondophlebia* Salles & Boldrini*; *Simothraulopsis* Demoulin*; *Thraulodes* Ulmer*; *Traverella* Edmunds*; *Traverina* Peters; *Ulmeritoides* Traver*; and *Ulmeritus* Traver.

Diagnosis. Loss of patella-tibial suture on all legs of larva and adults. For other morphological characteristics, see Kluge (1994, 2008).

Distribution. Neotropical region.

Hagenulini sensu n.

Type genus. *Hagenulus* Eaton 1882: 207.

Included genera. *Askola* Peters*; *Atopophlebia* Flowers; *Borinquena* Traver; *Careospina* Peters; *Ecuaphlebia* Dominguez*; *Hagenulopsis*, Ulmer*; *Hagenulus* Eaton; *Neohagenulus* Traver; *Traverina* Peters; *Ulmeritoides* Traver*; and *Ulmeritus* Traver.

Diagnosis. Currently there is no morphological evidence for this tribe.

Distribution. Neotropical region.

Ulmeritina subtribe n.

Ulmeritus/*Ulmeritoides* complex sensu (Dominguez, 1991).

Type genus. *Ulmeritus* Traver 1956: 2.

Included genera. *Atopophlebia* Flowers; *Ulmeritoides* Traver*; and *Ulmeritus* Traver.

Diagnosis. See morphological characteristics in Dominguez (1991, 1995), Salles & Dominguez (2012) and Salles *et al.* (2019).

Distribution. Neotropical region.

Hagenulina stat.n.

Hagenulus/fg3 sensu Kluge (2008).

Type genus. *Hagenulus* Eaton, 1882: 207.

Included genera. *Askola* Peters*; *Borinquena* Traver; *Careospina* Peters; *Ecuaphlebia* Dominguez*; *Hagenulopsis*, Ulmer*; *Hagenulus* Eaton; *Neohagenulus* Traver; and *Traverina* Peters.

Diagnosis. See morphological characteristics in Kluge (1994, 2008).

Distribution. Neotropical region.

Miroculini tribe n.

Type genus. *Miroculis* Edmunds, 1963: 34.

Included genera. *Bessierus* Thomas & Orth; *Farrodes* Peters*; *Hermanella* Needham & Murphy*; *Hermanellopsis*, Demoulin*; *Homothraulus* Demoulin*; *Hydromastodon* Savage & Peters*; *Hydrosmilodon* Flowers & Dominguez*; *Hylister* Dominguez & Flowers*; *Leentvaaria* Demoulin*;

Microphlebia Savage & Peters*; *Miroculis* Edmunds*; *Miroculitus* Savage & Peters; *Needhamella* Dominguez & Flowers*; *Paramaka* Savage & Dominguez*; *Perissophlebiodes* Savage*; *Rondophlebia* Salles & Boldrini*; *Simothraulopsis* Demoulin*; *Thraulodes* Ulmer*; and *Traverella* Edmunds*.

Diagnosis. Currently there is no morphological evidence for this tribe.

Distribution. Neotropical region.

Hermanellina Kluge

Type genus. *Hermanella* Needham & Murphy, 1924: 39.

Included genera. *Bessierus* Thomas & Orth; *Farrodes* Peters*; *Hermanella* Needham & Murphy*; *Homothraulus* Demoulin*; *Hydromastodon* Savage & Peters*; *Hydrosmilodon* Flowers & Dominguez*; *Hylister* Dominguez & Flowers*; *Leentvaaria* Demoulin*; *Needhamella* Dominguez & Flowers*; *Paramaka* Savage & Dominguez*; *Perissophlebiodes* Savage*; *Rondophlebia* Salles & Boldrini*; *Simothraulopsis* Demoulin*; and *Traverella* Edmunds*.

Diagnosis. See morphological characteristics in Kluge (2008) and Salles & Boldrini (2019).

Distribution. Neotropical region.

Miroculina subtribe n.

Miroculis complex sensu (Savage, 1982).

Type genus. *Miroculis* Edmunds, 1963: 34.

Included genera. *Hermanellopsis*, Demoulin*; *Microphlebia* Savage & Peters*; *Miroculis* Edmunds*; and *Miroculitus* Savage & Peters.

Diagnosis. See morphological characteristics in Savage (1982).

Distribution. Neotropical region.

Leptophlebiinae Banks

Type genus. *Leptophlebia* Westwood, 1840: 31.

Genera include. *Leptophlebia* Westwood*; *Paraleptophlebia* Lestage*; *Habrophlebiodes* Ulmer; *Dipterophlebiodes* Demoulin; and *Gilliesia* Peters & Edmunds.

Diagnosis. See morphological characteristics in Peters (1980).

Distribution. Palaearctic, Nearctic, Oriental and Ethiopian.

Terpidinae Kluge

Type genus. *Terpides* Demoulin 1966: 15.

Genera include. *Terpides* Demoulin*; *Tikuna* Savage, Flowers & Porras*; and *Fittkaulus* Savage & Peters*.

Diagnosis. See morphological characteristics in Kluge (2009).

Distribution. Neotropical region.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Genera of Leptophlebiidae used in this study with indications of source and biogeographic region for the examined specimens.

Appendix S2. Taxonomic sampling and data coverage for the phylogenetic analyses.

Appendix S3. Phylogenetic inference of maximum likelihood of concatenated genes (COI + 28S, 1655 bp). The bootstrap values are shown above the branches.

Appendix S4. Phylogenetic inference of Bayesian inference of concatenated genes (COI + 28S, 1655 bp). A posteriori probability values are shown above the branches.

Appendix S5. Changes in the classification of Leptophlebiidae over time, including those of this study.

Appendix S6. Cladogram with the proposed classification for the subfamilies of Leptophlebiidae.

Appendix S7. Cladogram with the proposed classification for the tribes.

Appendix S8. Cladogram with the proposed classification for Hagenulinae.

Appendix S9. Cladogram with evidence in the genera without hindwing.

Acknowledgements

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