



Research paper

Phylogeny of *Tricorythodes* Ulmer (Leptohyphidae: Ephemeroptera) based on molecular and morphological evidenceLucimar G. Dias ^{a, **}, Carlos Molineri ^{b, *}, Daniela Takiya ^c, Pablo Benavides ^d, Tito Bacca ^e^a Grupo de Investigación Bionat, Universidad de Caldas, Caldas, Colombia^b Instituto de Biodiversidad Neotropical, CONICET (National Council of Scientific Research), Universidad Nacional de Tucumán, Argentina^c Laboratório de Entomologia, Departamento de Zoologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil^d Disciplina de Entomología, Cenicafé, Chinchiná, Caldas, Colombia^e Universidad del Tolima, Ibagué, Colombia

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ABSTRACT

The first phylogeny of *Tricorythodes* Ulmer (Ephemeroptera: Leptohyphidae) based on molecular and morphological evidence is presented. A parsimony analysis was conducted with 56 morphological (24 continuous and 32 discrete) characters of 48 species, 39 belonging to *Tricorythodes* sensu lato and nine additional groups. Bayesian and maximum likelihood analyses of molecular and combined molecular and morphological (32 discrete) data included a total of 22 taxa for which it was possible to extract DNA. DNA sequences used correspond to fragments of the nuclear 18S rDNA and the mitochondrial COI and 16S rDNA (total of 1804 bp). *Tricorythodes* sensu lato monophyly was tested, as well as validity of some recently proposed related genera. Results of this work fully support the synonymy of *Epiphrales* and *Tricorythodes*, as suggested by previous authors. Part of the analyses also support the synonymy of *Asioplax* and *Homoleptohyphes* with *Tricorythodes*, but they change position radically when morphology alone (parsimony) and the combined data (Bayesian and maximum likelihood) are analyzed. Furthermore, *Tricorythodes* was recovered as a polyphyletic group and *Cabecar* and *Loricyphe* appeared nested well within *Tricorythodes* sensu lato. Monophyletic groups of species inside this large Panamerican genus were found. A phylogenetic framework for the group is much needed to better understand the evolution of disparate nymphal body form, ecology and biogeography inside this genus.

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1. Introduction

Tricorythodes Ulmer (Leptohyphidae) is a species-rich and morphologically diverse Pan American genus of mayflies (Fig. 1A–F). Several taxonomists have contributed to the study of the group, and currently *Tricorythodes* sensu lato is represented by 69 species (Traver 1959; Allen 1967; Allen & Murvosh 1987; Kluge & Naranjo 1990; Wiersema & McCafferty 2000; Wiersema et al. 2001; Molineri 2001; Molineri 2002; Baumgardner et al. 2006; Dias & Salles 2006; Molineri & Zúñiga 2006; Emmerich 2007; Baumgardner 2007; Baumgardner 2008; Dias et al. 2009a; Dias et al. 2009b; Dias et al. 2011; Gonçalves et al. 2010; Belmont et al. 2011; Belmont et al. 2012; Souto et al. 2017; Granados et al. 2018).

The genus described by Ulmer (1920) has had a complex systematic history in the last decades. In 1987, Allen and Murvosh presented the first revision of the genus and proposed three subgenera: *Tricorythodes* Ulmer, *Tricorythodes* Allen & Murvosh, and *Homoleptohyphes* Allen & Murvosh. Later, Wiersema & McCafferty (2000) carried out a generic revision of the Leptohyphidae of North and Central America. These authors, elevated the three subgenera proposed by Allen & Murvosh (1987) to genus level, and proposed two other genera, *Asioplax* Wiersema & McCafferty and *Epiphrales* Wiersema & McCafferty, to include other species of the group. Molineri (2002) in a cladistic revision of the South American species of *Tricorythodes* sensu lato showed that *Tricorythodes* was polyphyletic and the genera proposed by Wiersema & McCafferty (2000), were nested within a paraphyletic *Tricorythodes* and suggested maintaining the later name for the entire group. In 2005, Wiersema & McCafferty (2005) reviewed *Asioplax*, including some South American species previously treated formally as *Tricorythodes* by Molineri (2002). Additionally, Dias et al. (2005) proposed

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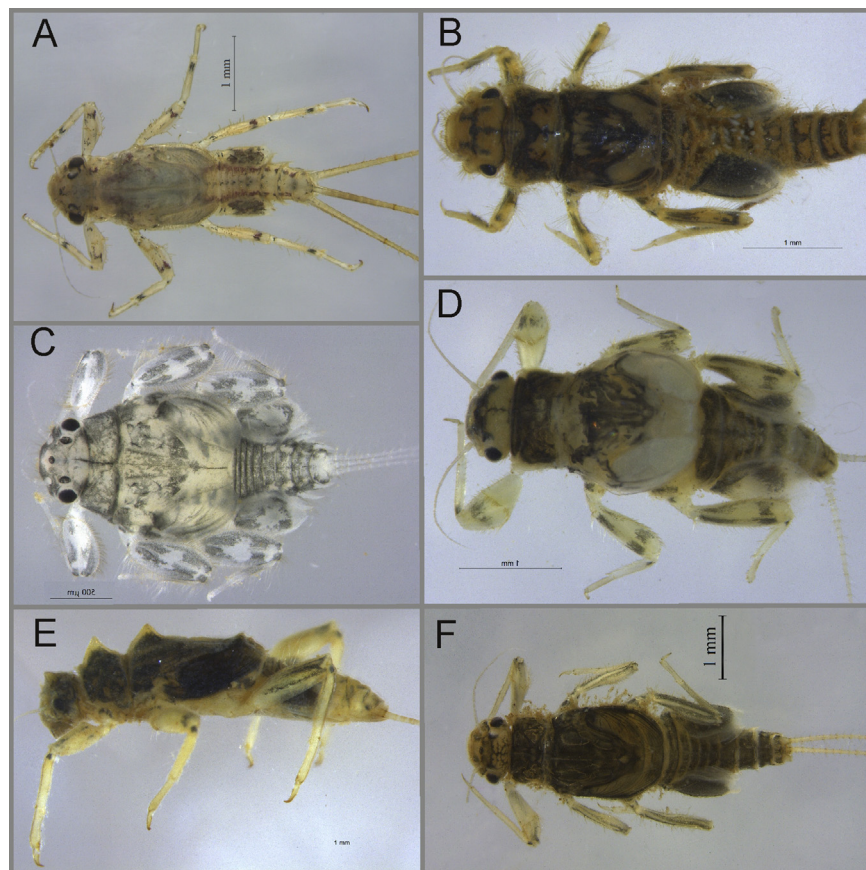


Fig. 1. Nymphal habitus. A) *Tricorythodes* sp. (Tolima) from undatus group; B) *Tricorythodes barbatus* from condylus group; C) *Tricorythodes* sp. from the “Asioplax” group; D) *Tricorythodes cubensis* from cubensis group; E) *Tricorythodes bullus* from bullus group; and F) *Tricorythodes* sp. (Sandona) from popayanicus group.

the genus *Macunahyphes* Dias, Salles & Molineri to include *Tricorythodes australis* Traver, describing for the first time nymphs of this species. These authors recognized *Macunahyphes* based on morphological characters of the nymphs, mainly mouthparts, and atypical forceps and penis shape of the male imago.

With an increased taxon and character sampling, Molineri (2006) presented a morphological phylogeny for the South American Leptohiphidae, which corroborated previous results where representatives of *Epiphrales*, *Asioplax*, and *Macunahyphes* were recovered within *Tricorythodes*. However, no nomenclatural changes were proposed as the author felt that the inclusion of Central and North American representatives in future studies would be desirable for a more stable classification. Later, Domínguez et al. (2006) preferred to treat the group as a unit and included in the *Tricorythodes* species list, type species of *Homo-leptohiphes*, *Tricorythodes*, *Asioplax*, and *Epiphrales*, formalizing the synonymy. Finally, Baumgardner & Ávila (2006) described the genus *Cabecar* Baumgardner based on nymphs and reared adults from Central America. According to these authors, *Cabecar* shares several characters with *Tricorythodes*, its only distinctive feature being the shape of femoral setae in the nymphs. Similarly, Baumgardner & Ávila (2006) commented that a phylogenetic study would be needed to assess the proper position of *Cabecar* within Leptohiphidae. Thus, the valid genera up to now are: *Tricorythodes*, *Macunahyphes* and *Cabecar*.

Loricophyes Molineri & Mariano is known from nymphs and eggs, most characters indicate a basal splitting in Leptohiphidae (Molineri & Mariano 2015). Since *Loricophyes* shares with *Tricorythodes* some characters (e.g., the number of lamellae in gills III–VI), it is relevant to include it in this revision.

After so many efforts to improve the classification of *Tricorythodes*, we present for the first time phylogenetic hypotheses based on molecular evidence for the group. DNA sequences were also combined with novel and previously used morphological characters in systematic studies of the group (Kluge 1992; Molineri 2002; Molineri 2006; Wiersema & McCafferty 2000; Baumgardner & Ávila 2006). Molecular markers (18S rDNA, 16S rDNA, and COI) used in this research are commonly used for phylogenetic analyses and other taxonomic works of mayflies (Ogden & Whiting 2003; Ogden & Whiting 2005; Ball et al. 2005; Sun et al. 2006; Hoyos et al. 2014; Massariol et al. 2016; Gonçalves et al. 2017). We believe this improved character sampling will yield more robust phylogenetic hypotheses to test the monophyly of *Tricorythodes* and the other genera proposed during the last years.

2. Material and methods

2.1. Taxon sampling

From a total of 48 species included in the morphological matrix, 39 species of *Tricorythodes sensu lato* were analyzed, representing seven of the genus—names proposed in the last decades for the group (*Tricorythodes*, *Epiphrales*, *Asioplax*, *Macunahyphes*, *Homo-leptohiphes*, *Tricorythodes*, and *Cabecar*). Additionally, one species of *Leptohiphodes*, one of *Loricophyes*, two of *Leptohiphes*, and four of *Haplohiphes* were also included in the morphological analysis (see matrix on Appendix S1). All trees were rooted in *Leptohiphes*.

A total of 22 taxa were included in the molecular analysis, details about taxon sampling are given in Table 1. Because specimens used for DNA extraction were completely macerated, another

Table 1

Taxon sampling for the phylogeny of *Tricorythodes sensu lato* with DNA voucher specimen number, collecting locality, depository institution, and GenBank accession numbers for the molecular markers sequenced (COI, 16S rDNA, and 18S rDNA).

Species	Material Examined (locality):	Voucher	Institutional Collection	GenBank accession numbers		
				18S	16S	COI
Outgroup						
<i>Haplohyphes baritu</i> Domínguez, 1984	Argentina, Tucumán, Río la Hoyada, 985 m, S26°45'32"–W65°29'28.5", 12/IX/06, Domínguez, E. et al. cols.	A-Eph001PhT	CEBUC	MK059826	MK059807	MK059792
<i>Haplohyphes aquilonius</i> Lugo-Ortiz & McCafferty, 1995	Colombia, Nariño, Sandona, Quebrada La Honda, 18/II/07, Dias, L. G. & Bacca, T. cols.	A-Eph002PhT	CEBUC	MK059829	MK059810	MK059795
<i>Leptohyphes ecuador</i> Mayo, 1968	Colombia, Nariño, Sandona, Quebrada La Honda, 18/II/07, Dias, L.G. & Bacca, T. cols.	A-Eph003PhT	CEBUC	MK059825	MK059806	MK059791
<i>Leptohyphodes inanis</i> (Pictet, 1843)	Brazil, Minas Gerais State, Araponga, Serra do Brigadeiro, Vale das Luas, VII/05, Dias, L.G. Col.	A-Eph004PhT	CEBUC		MK059812	
Ingroup						
<i>Cabecar</i> sp. (Caldas)	COLOMBIA. Caldas. Norcasia. Reserva Natural del Río Manso. 21/XI/2014 5°39'51.3"N 74°46'56.6"W. Dias, L.G. & Cardenas, T.	A-Eph005PhT	CEBUC	MK059828	MK059809	MK059794
<i>Cabecar serratus</i> Baumgardner & Avila, 2006	Costa Rica	—	—			1
<i>Macunahyphes australis</i> (Bank, 1913)	Brazil, Roraima State, Caracará, Rio Branco, C. do Bem Querer, 18–21/ii/02, A.M.O. Pes, N. Hamada, cols.	A-Eph006PhT	CEBUC	MK059830		
<i>Tricorythodes arequita</i> Traver, 1959	Argentina, Misiones, P.P. Uruguai, A° Uruzu, 7–11/XII/99, Molineri, C. col.	A-Eph016PhT	CEBUC		MK059814	
<i>Tricorythodes barbuis</i> Allen, 1967	Argentina, Misiones, INTA San Vicente, Km 274, 29/xi/86, Domínguez, E. col.	A-Eph007PhT	CEBUC		MK059815	
<i>Tricorythodes</i> aff. <i>bullus</i>	Brazil, Minas Gerais, Paula Cândido, C. Airões, 13/VII/04, Dias, L. G.	A-Eph008PhT	CEBUC	MK059834	MK059818	MK059798
<i>Tricorythodes caunapi</i> Dias, 2009	Colombia, Nariño, Tumaco, Tangareal, Río Caunapi, 06/xi/07, Dias, L. G., Ângulo, D., Estácio, J. and Bacca, T. cols.	A-Eph009PhT	CEBUC	MK059832	MK059816	MK059797
<i>Tricorythodes cubensis</i> Kluge & Naranjo, 1990	Cuba, Los Morones, 19/iv/07, González-Lazo, D. col.	A-Eph010PhT	CEBUC	MK059833	MK059817	
<i>Tricorythodes dimorphus</i> Allen, 1967	USA, New Mexico, Conton Co. Wilow Cr, 7400 ft., N33°29'37" – 108°34'20", Baumgardner.	A-Eph017PhT	CEBUC		MK059811	
<i>Tricorythodes hiemalis</i> Molineri, 2001	Argentina, Salta, PN El Rey, A° Los Noques, 905m, S24°44'44"– W64°38'11", 11/xi/05, Molineri, C. col.	A-Eph018PhT	CEBUC	MK059835	MK059819	MK059799
<i>Tricorythodes molinerii</i> Dias & Salles, 2006	Brazil, Minas Gerais State, Campos Altos, Rio da Prata, 09/viii/2001, Salles, F. F. & Lugo-Ortiz, C. cols.	A-Eph011PhT	CEBUC		MK059820	MK059800
<i>Tricorythodes montanus</i> Kluge & Naranjo, 1990	Cuba, Los Morones, 19/vi/07, González-Lazo, D. col.	A-Eph012PhT	CEBUC	MK059836	MK059821	MK059801
<i>Tricorythodes popayanicus</i> Domínguez, 1982	Argentina, Tucumán, Acheral, Río Araniillas, 366 m, S27°06'59.9"– W65°27'43.9", 5/viii/06, Molineri, C. and Nieto, C. cols.	A-Eph019PhT	CEBUC	MK059837	MK059822	MK059802
<i>Tricorythodes quizeri</i> Molineri, 2002	Argentina, Tucumán, Achera, Río Aramillas, 28/vii/06, Molineri, C. et al. cols.	A-Eph020PhT	CEBUC	MK059838	MK059823	MK059803
<i>Tricorythodes sallesi</i> Dias, Cabette & Souza, 2009	Brazil, Mato Grosso State, Nova Xavantina, Córrego do Papagaio, vii/2005, Cabette, H. S. R. et al. cols.	A-Eph013PhT	CEBUC	MK059839		MK059804
<i>Tricorythodes santarita</i> Traver, 1959	Argentina, Misiones, Aristóbulo del Valle, Río Cuñá –Pirú, 19/xi/1998, Domínguez et al. Cols.	A-Eph014PhT	CEBUC	MK059827	MK059808	
<i>Tricorythodes sierramaestrae</i> Kluge & Naranjo, 1990	Cuba, Río Turquino, 22/vi/07, González-Lazo, D. col.	A-Eph015PhT	CEBUC	MK059840	MK059824	MK059805
<i>Tricorythodes yura</i> Molineri, 2002	Bolivia, La Paz, A° between Caranavi and Guanai, 500 m, 27/XI/00, Domínguez, E. and Nieto, C. cols.	A-Eph021PhT	CEBUC	MK059841		
<i>Tricorythodes</i> sp. (Sandona)	Colombia, Nariño, Sandona, Quebrada La Honda, 18/ii/07, Dias, L. G. & Bacca, T. cols.	A-Eph016PhT	CEBUC	MK059831	MK059813	MK059796

1: Sequence donated by Dr. Wills Flowers.

conspecific specimen from the same collecting event was elected as voucher and their information listed in Table 1. All examined material are deposited in the following institutions: Instituto de Biodiversidad Neotropical (IBN, Tucumán, Argentina) and Colección Entomológica del Programa de Biología de la Universidad de Caldas (CEBUC, Colombia).

2.2. Morphological characters

Thirty-two discrete and 24 continuous characters were coded from adults and immature specimens (Appendix S1). Some characters are newly proposed here, but the majority was selected from Molineri (2002, 2006), Wiersema & McCafferty (2000). Characters are given in Appendix S1 (supplementary data). Some characters of type species of *Tricorythodes* (*Tricorythodes explicatus*) were obtained from Baumgardner (2009).

2.3. DNA extraction and fragment amplification

Genomic DNA was isolated from macerated specimens previously preserved in absolute ethanol with the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer instructions and stored at -20°C . Three PCR primer sets were used in order to amplify the markers COI with LCO1490 (5'–GGTCAACAAATCATAAAGATATTGG) and HCO2198 (5'–TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al., 1994), 18S rDNA with 18Sf (5'–AGGGCAAGTCTGGTCCAGC) and 18Sr (5'–TTTCTAGCTTTGCAACCATAC) (Whiting 2002) and 16S rDNA with 16Sa (5'–GCCTGTTTATCAAAAACAT) and 16Sb (5'–CTCCGGTTTGAATCAGATCA) (Ogden & Whiting 2005). PCR reactions were carried out in a final volume of 50 μL . Each reaction consisted of approximately 100 ng of total DNA, 0.5 μM of each primer (forward and reverse), 0.2 mM of each d (AGCT)TP, 1X of 5X Taq PCR buffer, 1.25U of Taq polymerase (Go Taq flexi Promega), and 2 mM of MgCl_2 for 16S and 18S, but 1.5 mM of MgCl_2 for COI. PCR reactions consisted of an initial 5-min denaturation step at 95°C , 35 cycles of 30 s at 94°C , 30 s at the annealing temperature (50°C for 18S and 45°C for 16S), 45 s at 72°C , and then a final elongation step for 5 min at 72°C . For COI, the procedure reported by Ball et al. (2005) was followed. PCR products were visualized on 1% agarose gels using ethidium bromide. All PCR products were then purified using the QIAquick PCR purification kit (Qiagen) and sequenced by Macrogen (South Korea). Electropherograms of complementary strands were aligned and checked manually in Geneious 9.1.8 (Biomatters Ltd.), in order to generate consensus sequences. GenBank® accession numbers to sequences obtained are given in Table 1.

2.4. Sequence alignment

COI sequences alignments (658 bp) were conducted in Geneious 9.1.8 (Biomatters Ltd.) and aminoacid translations were checked. Alignments of 16S (520 bp) and 18S (620 bp) sequences were done using the Q-INS-I method (Katoh & Toh 2008) implemented in MAFFT version 7 (Katoh & Standley 2013).

2.5. Phylogenetic analyses

Analysis of morphological data (24 continuous and 32 discrete characters—see Appendix S1) were conducted in TNT (Goloboff et al. 2008) under implied weights ($k=7$), 100 Wagner trees were generated and then submitted to TBR (Tree Bisection Reconnection). Implied weighting was suggested to ameliorate problems of scaling in continuous characters (Goloboff et al. 2006). All characters were treated as non-additive except for continuous characters (chars. 0 to 23). Relative Bremer support was calculated with 3000 suboptimal trees (up to 20 steps or 70% longer than

shortest tree). Frequency difference (GC, Goloboff et al. 2003), using 250 replications of symmetric jackknifing, was also calculated as a measure of group support. Trees were visualized and optimizations of individual continuous characters were studied with TNT.

Phylogenetic reconstructions were conducted using Bayesian Inference on MrBayes 3.2.6 (Ronquist et al. 2012) based on the molecular and on the combined matrices (Appendix S3) (morphology with only discrete characters), with four independent runs of 4 MCMC chains for 5M generations, sampling every 500 trees on CIPRES (Miller et al. 2010). Convergence of independent runs was assessed by values of the standard deviation of split frequencies <0.05 at the last generation and visually inspecting combined sampled distributions of parameters using Tracer v. 1.5 (Rambaut & Drummond 2009), as well as adequate mixing of sampled parameters assessed by effective sampling size (ESS) values >200 . Both molecular and combined matrices were also analyzed under maximum likelihood in Garli 2.01 (Zwickl 2006) with 500 independent search replicates with automatic termination with a generation threshold of 20,000 and score threshold of 0.05. In both analyses, each molecular partition was independently modeled according to the following evolutionary models selected with the Akaike Information Criterion in jMODELTEST 2.1.4 (Posada 2008; Darriba et al. 2012): GTR + I + G was selected for COI, GTR + G for 16S, and K2P + I for 18S sequences. Additionally, morphological data was modeled using the Mk model (Lewis 2001). Clade support was estimated by Bayesian posterior probabilities (BPP) and bootstrap frequencies for maximum likelihood (MLB), calculated over 1000 and 500 matrix pseudoreplicates, respectively. Clade support was estimated by Bayesian posterior probabilities (BPP) and bootstrap frequencies for maximum likelihood (MLB) calculated over 500 matrix pseudoreplicates.

3. Results

3.1. Genera nested within *Tricorythodes sensu lato*

In this work, we present for the first time a phylogeny of *Tricorythodes sensu lato* based on molecular and morphological data. Here, parsimony (morphology data, Fig. 2) and Bayesian (molecular + morphology data, Fig. 3 and molecular data, Fig. S1 supplementary data) analyses coincided in many points. In particular, we find that members of *Cabecar* and *undatus* group are consistently nested within *Tricorythodes* (i.e., the large monophyletic group that includes *T. explicatus*, type species of the genus, Fig. 2).

Cabecar was represented by *Cabecar serratus* and an undescribed species (*Cabecar* sp. from Caldas), while *Epiphrales* was represented by *Tricorythodes undatus*, *T. sp.* from Tolima, *Tricorythodes bullus* and *T. aff. bullus* (the first two only analyzed in the morphological dataset) (Table 1). Here, in all analyses, *Epiphrales* species are nested within *Tricorythodes*, besides, in parsimony analysis, *Epiphrales* resulted as a polyphyletic group (Fig. 2). *T. undatus* (type-species of *Epiphrales*) and *Tricorythodes* sp. from Tolima were recovered as sister group of *Cabecar*, while *T. bullus* and *T. aff. bullus* are not related to this clade. Synapomorphies shared by this group (*Cabecar* + *undatus* group) are (Supplementary Appendix S2): pronotum with anterolateral projection, mesonotum with posterior tubercle, abdominal margin undulate, and shape and coloration of operculate gill (Fig. 1A). Additionally, *Loricyphe* was recovered inside the *bullus* group (Fig. 1), since this taxon shows large dorsal tubercles on the body (see list of shared characters in Appendix S2).

3.2. Genera controversially nested within *Tricorythodes sensu lato*

The genus *Tricorythyphes*, represented in this study by *Tricorythodes condylus*, *Tricorythodes barbus* (Fig. 1B), *Tricorythodes*

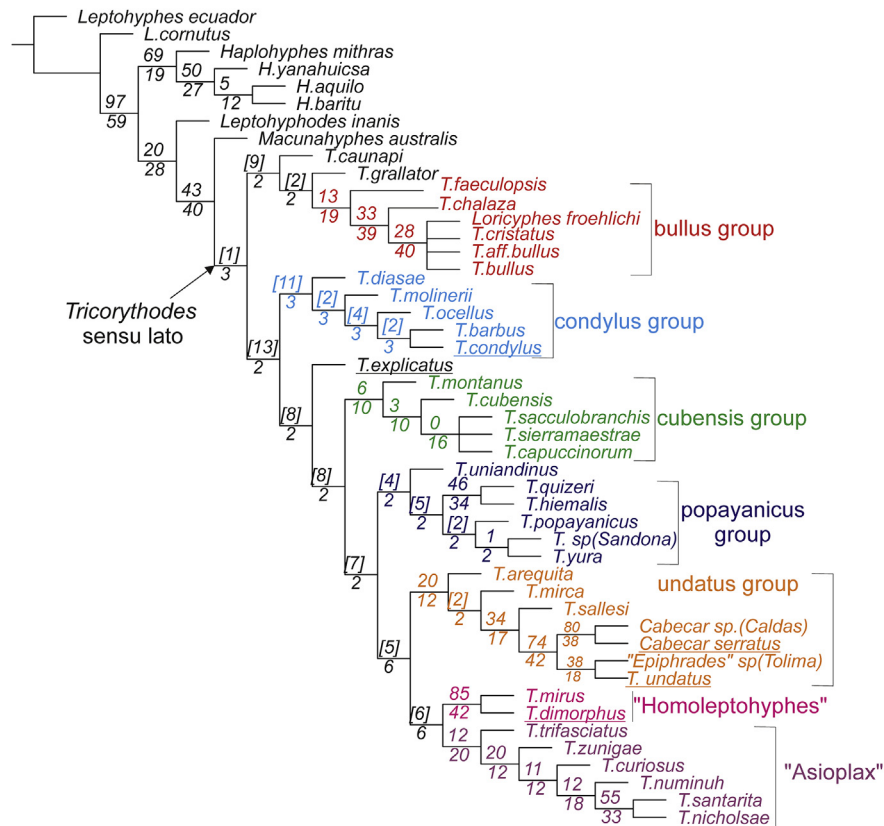


Fig. 2. Strict consensus of three shortest trees (adjusted homoplasy = 13.51) obtained by parsimony analysis with implied weights ($k = 7$) based on 32 discrete and 24 continuous morphological characters. Numbers above branches indicate GC values and those below are relative Bremer supports (number in brackets indicate that contradictory group was more frequent during resampling). Underlined are the type-species of different generic or subgeneric groupings proposed by previous authors.

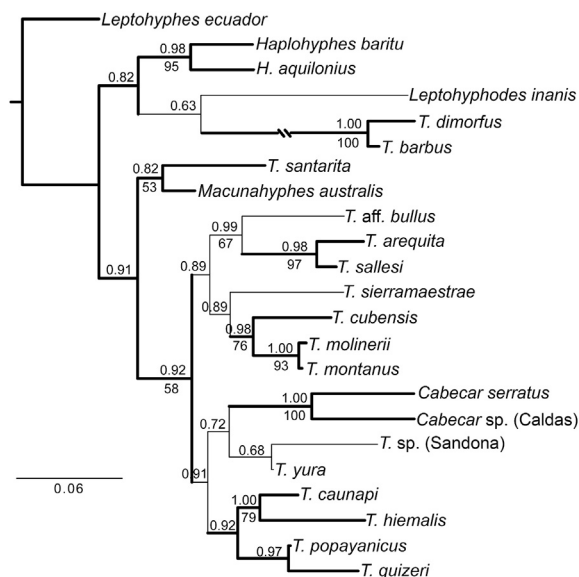


Fig. 3. Bayesian consensus resulting from a mixed-model analysis based on a combined dataset of 18S, 16S, COI, and 32 discrete morphological characters of *Tricorythodes* sensu lato. Thickened branches are also recovered in the maximum likelihood analysis of the same dataset. Numbers above branches indicate posterior probabilities and those below are maximum likelihood bootstrap frequencies. Type-species are underlined.

popayanicus, and *Tricorythodes ocellus* (the later for morphology only), was not recovered as a natural group in our analyses, and all species were found nested within *Tricorythodes sensu lato* in the parsimony analysis of morphological data (Fig. 2). However, in the combined Bayesian analysis, *T. barbus* was not recovered nested in *Tricorythodes sensu lato*, while *T. popayanicus* was so (Fig. 3).

Position of both *Asioplax* (Fig. 1C) and *Homoleptohyphes* varied depending on the type of data analyzed. Morphology-based parsimony analysis (Fig. 2) supports *Asioplax* (represented by 5 species) as a monophyletic group nested well within *Tricorythodes*. On the other hand, in Bayesian and maximum likelihood analyses of combined (molecular and morphology) dataset, the position of *Asioplax* is different, with the single species represented grouping together with *Macunahyphes*, as sister to the remaining species of *Tricorythodes* (Fig. 3). The maximum likelihood analysis of molecular dataset alone recovers *Tricorythodes santarita* (a clear representative of "Asioplax") as sister to a clade containing sampled *Macunahyphes*, *Haplohyphes*, *Leptohyphodes*, *Homoleptohyphes*, and *Tricorythodes*. Similarly, the parsimony analysis recovered *Homoleptohyphes* (with 2 species, Fig. 2) nested within *Tricorythodes*, but molecular and combined Bayesian and maximum likelihood analyses (with only one species represented, Fig. 3 and Fig S1) suggest its close relationship with *T. barbus* (in *Tricorythodes*).

3.3. Genera recovered independently of *Tricorythodes sensu lato*

In all analyses conducted (Figs. 1 and 2, and Fig. S1), *Haplohyphes* resulted in a monophyletic group of Leptohyphidae, and this genus together with *Leptohyphodes* and *Macunahyphes* were recovered as independent lineages from *Tricorythodes*. Penis with a dorsal

projection covered with spines is an autapomorphy recovered for *Macunahyphes* (Appendix S2).

3.4. Defining *Tricorythodes*

Based on results of this analysis, *Tricorythodes* can be defined by the following synapomorphies: forceps with basal swelling on second segment, row of thick setae and dentisetae of maxilla directed apicomediaally and galealacinia with apical portion getting thinner toward the canines. Several interesting clades within this genus were recovered and are discussed below. Some species–groups are highlighted in Fig. 2.

In parsimony (morphology) and combined Bayesian analyses, the Cuban species (*Tricorythodes cubensis* (Fig. 1D), *Tricorythodes montanus*, *Tricorythodes sacculobranchis*, and *Tricorythodes sierra-maestrae*) appear related (Figs. 2 and 3), although in the former they also are related to *Tricorythodes capuccinorum* (Fig. 2) and in the later, to *Tricorythodes molinerii* (Fig. 3).

Another interesting relationship recovered only under parsimony was the grouping of *Tricorythodes arequita*, *Tricorythodes sallesi*, *Tricorythodes mirca*, *Cabecar* (Fig. 1A), and *undatus* group, which all share the body and operculate gill with a particular coloration pattern (heterogeneous pigments, small spots widely distributed in the body). This clade (Fig. 2) was not recovered in any of the analysis including the molecular dataset.

Tricorythodes diasae, *T. molinerii*, *T. barbus* (Fig. 1B), and *T. ocellus*, based on morphological data (parsimony analysis), form a monophyletic group nested within *Tricorythodes* (condylus group, Fig. 2). However, the position of *T. molinerii* and *T. barbus* change with molecular data, because *T. barbus* appears as sister to *Homo-leptohyphes* (Fig. 3 and Fig S1), while *T. molinerii* appears as sister to *T. cubensis* with high support (Fig. 3), as previously mentioned.

In this study, the close relationship between *Tricorythodes chalaza*, *Tricorythodes cristatus*, *Tricorythodes faeculopsis*, *T. bullus* (Fig. 1F), *T. aff. bullus* and *Loricyphe* was recovered in parsimony analysis of morphological data (Fig. 2, bullus group), and these species share many morphological similarities (nymphs with tubercles on head or thorax, stout body, slender legs, and penis shape and reduced CuP in adults).

Finally, parsimony analysis of morphological data found the clade formed by: *Tricorythodes uniandinus*, *Tricorythodes yura*, *Tricorythodes* sp. (Sandona) (Fig. 1F), *Tricorythodes hiemalis*, *Tricorythodes quizeri*, and *T. popayanicus* (Fig. 1, popayanicus group) supported by a single synapomorphy: CuP strongly curved towards vein A. However, analysis including the molecular data (Fig. 2 and Fig S1) recovered a strongly supported clade including the three later species and *Tricorythodes caunapi*. Unfortunately, we do not have molecular information for *T. uniandinus* and for *T. yura* we only have information from a much conserved gene (18S).

4. Discussion

Based on the results of this study, *Cabecar* and *Loricyphe* are not supported as valid genera. We consider that *Cabecar* should be treated as a junior synonym of *Tricorythodes*, because this relationship was supported in both analyzes (morphology and molecules). Nevertheless we doubt about the synonymy of *Loricyphe* and *Tricorythodes*. A broader phylogenetic context, including other families (e.g., Coryphoridae, Melanemerellidae, Tricorythidae, etc.) would be important to attain a more supported hypothesis of its relationships, besides molecular data. Our results also support the synonymy of *Epiphraodes* and *Tricorythyphes* proposed by Domínguez et al. (2006). In addition, these data suggest that the genus *Epiphraodes* (*T. undatus*, *T. bullus* and *E. cristatus*) proposed by Wiersema & McCafferty (2000) is a polyphyletic group, since *T. undatus* (its

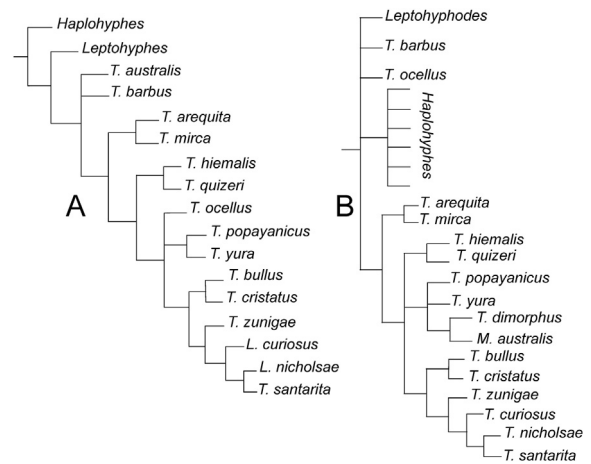


Fig. 4. Previous phylogenetic hypotheses of *Tricorythodes* based on morphological data. A, Molineri (2002) and B, Molineri (2006).

type–species) appears as sister to *Cabecar* (including its type–species *C. serratus*) and not closely related to *T. bullus* and *T. cristatus*. A similar case occurs with *Tricorythyphes*, genus initially proposed by Allen & Murvosh (1987) as subgenus of *Tricorythodes*, treated at the genus level by Wiersema & McCafferty (2000) and including: *T. barbus*, *T. condylus*, *Tricorythodes mulaiki*, *T. ocellus*, and *T. popayanicus*. According to all analyses in our study and also with trees reconstructed by other authors (Fig. 4) (Molineri 2002; Molineri 2006; Baumgardner 2008), *T. condylus*, *T. barbus*, and *T. popayanicus* were recovered in different lineages as a polyphyletic group.

On the other hand, *Macunahyphes*, based on morphology and molecular characters, was found to be an independent genus, the sister genus to *Tricorythodes* sensu lato in the parsimony analysis of morphological characters or included in the sister clade (*Macunahyphes australis* + *T. santarita*) in the combined Bayesian analysis. But, in this study, we only included *M. australis* (type–species) and it would be interesting in the future to include other species recently described for this genus (Molineri et al. 2011; Souto & Salles 2016). These species have some egg characteristics and genitalia different from the type–species of *Macunahyphes*, and recent findings of the nymphal stage of two of them indicate that they should be excluded from this genus.

The conflicting position of *Asioplax* in analyses based on morphology alone and in those including molecular data deserves further study. The position of this genus and others could be elucidated with the addition of molecular data to a greater number of species or complementing the morphological characters, for example, egg ultrastructure and geometric morphometrics to quantify the morphological variation of body form, gills, and legs (Fig. 4C). Here, with morphology alone, the results for *Asioplax* are similar to the phylogeny of Molineri (2006), where this genus forms a group of derived species in *Tricorythodes* (Fig. 3). It is clear that morphological data merge species of *Asioplax* in a well–supported group, but within *Tricorythodes*, as found by Baumgardner (2008), who propose a subgenus for this group.

The closeness between *T. arequita*, *T. mirca*, *T. sallesi*, *Cabecar*, and *T. undatus* in the parsimony analysis of morphological data, is very interesting, since part of their phenotypical similarities could not be scored in the data matrix (general aspect of nymphs, Fig. 4A). A species recently published of *Tricorythodes* from Brazil (Souto et al. 2017), *Tricorythodes tragoedia* is morphologically very similar to these species, and in future and more comprehensive studies all these species may form a well–supported group.

Tricorythodes molinerii, *T. barbus*, and *T. ocellus* are species known only by peculiar nymphs (Fig. 4B) (with large rounded tongue-like anterolateral corners of pronotum and ventral lamellae of the gills 3 to 5 with a strongly developed dorsal extension). Molineri (2002) found *T. barbus* as the first species splitting from *Tricorythodes*, while in Molineri (2006), *T. barbus* appears outside *Tricorythodes*, in a polytomy with *Leptohyphodes*, *Haplohyphes*, and *Tricorythodes sensu lato* (Fig. 4), similar to our bayesian results. In the same way, the relationship found between *T. molinerii* and the cubensis group with molecular data is puzzling, because Cuban species form a group defined by numerous morphological features (Fig. 4D) including the shape of gills, legs, and femoral setae (Kluge & Naranjo 1990) and other continuous characters found here.

Based on morphology, *T. uniandinus*, *T. hiemalis*, *T. quizeri*, *T. popayanicus*, *T. yura*, and *T. sp.* (Sandona) constitute a monophyletic group (Fig. 4F), the close relationship of *T. hiemalis* and *T. quizeri* had already been documented by Molineri (2002, 2006). On the other hand, the close relationship between *T. chalaza*, *T. cristatus*, *T. faeculopsis*, *T. bullus*, and *T. aff. bullus* recovered in this study has been discussed (in part) by Molineri et al. (2014). These species have many morphological similarities (Fig. 4E): penis shape and reduced CuP in adults, form of legs, gills, and tendency to present dorsal tubercles in the nymphs (Gonçalves et al., 2010; Molineri et al., 2014).

Our morphological analysis found *Homoleptohyphes* as monophyletic, but well within *Tricorythodes* species, while the molecular data suggests a different position for *Tricorythodes dimorphus*. However, we could amplify only 16S for this species, thus it would be important to amplify additional markers to get a stronger hypothesis about this group.

Unfortunately, some species sampled herein were only represented by a single molecular marker (*C. serratus*, *T. yura*, *T. dimorphus*, *T. barbus*, *M. australis*, and *T. arequita*), so the additional markers would probably give more support to some nodes of the tree. This situation especially applies to *T. yura* and *Macunahyphes*, because the amplified fragment of the 18S gene is too conserved and was not very informative to study relationships at a specific level. In addition, regarding the morphological characters, the shape of the opercular gill was not used in this work, because it is a subjective character, however, we recommended in a future study the inclusion of a geometrical morphometric configuration to quantify the variation more accurately.

In relation to problems with PCR amplification of the material used in the molecular analysis, we stress that their preservation in 75% ethanol, and the age of the samples (more than 3 years old) was inadequate and possibly affected the integrity of the DNA. Srinivasan et al. (2002) discussed extensively the effect of fixatives and tissue processing on the content and integrity of nucleic acids, which agrees with our study. Thus, despite the value of this contribution, it would be interesting that results presented here can be complemented with new data, such as the additional sequence of some species or the use of other markers, for the taxonomic formalization of the clades (i.e., subgenera or genera).

5. Conclusions

Among the proposed genera of Leptohyphidae closely related with *Tricorythodes* (*Asioplax*, *Cabecar*, *Epiphrades*, *Homoleptohyphes*, *Loricyphe*, *Tricoryhyphes*, *Tricorythodes*, and *Macunahyphes*), only *Macunahyphes* is consistently supported as an independent lineage by both morphological and molecular data. On the other hand, *Loricyphe*, *Cabecar* and *Epiphrades* are consistently recovered nested within *Tricorythodes*. Both *Asioplax* and *Homoleptohyphes* were also recovered as independent lineages in analyses including molecular data, but nested within species of *Tricorythodes sensu stricto* in the parsimony analysis of morphological data.

Tricoryhyphes and *Tricorythodes sensu stricto* (Wiersema & McCafferty 2000) were recovered as para- or polyphyletic groupings as was mentioned by other authors (Molineri 2002; Molineri 2006; Baumgardner 2008).

Due to the morphological diversity of *Tricorythodes* together with its wide distribution, it was largely suspected to be formed by many different lineages (Kluge 1992; Wiersema & McCafferty 2000; Baumgardner 2008). Here, some lineages (cubensis group, condylus group, bullus group, popayanicus group, undatus group, “Homoleptohyphes” group and “Asioplax” group) are recovered but their support values are not high, thus, their taxonomic formalization as subgenera must wait until addition of new data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcz.2018.10.008>.

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