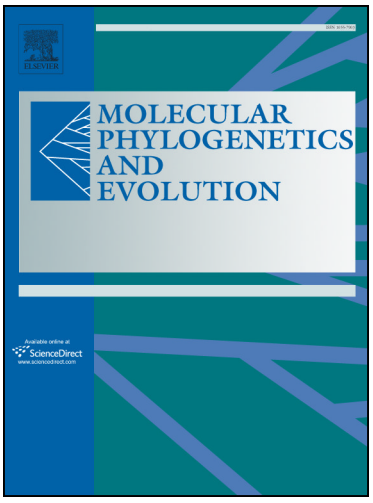

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Multilocus analysis of the catfish family Trichomycteridae (Teleostei: Ostariophysi: Siluriformes) supporting a monophyletic Trichomycterinae

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Abstract

Trichomycteridae is the second most diverse family of the order Siluriformes, its members are widely distributed through the freshwaters of Central and South America, exhibiting an exceptional ecological and phenotypic disparity. The most diverse subfamily, Trichomycterinae, represented mainly by the genus *Trichomycterus*, historically has been recognized as non-monophyletic and various characters used to unite or divide its constituents are repeatedly called into question. No comprehensive molecular phylogenetic hypothesis regarding relationships of trichomycterids has been produced, and the present study is the first extensive phylogeny for the family Trichomycteridae, based on a multilocus dataset of three mitochondrial loci and two nuclear markers (3284 bp total). Our analysis has the most comprehensive taxon-sampling of the Trichomycteridae published so far, including members of all subfamilies and a vast representation of *Trichomycterus* diversity. Analysis of these data showed a phylogenetic hypothesis with broad agreement between the Bayesian (BI) and maximum-likelihood (ML) trees. The results provided overwhelming support for the monophyletic status of Copionodontinae, Stegophilinae, Trichomycterinae, and Vandelliinae, but not Sarcoglanidinae and Glanapteryginae. A major feature of our results including current conceptualization of

Trichomycterinae, which includes *Ituglanis* and *Scleronema* but exclude the *"Trichomycterus" hasemani* group. Divergence time analysis based on DNA substitution rates suggested a Lower Cretaceous origin of the family and the divergence events at subfamilial level shaped by Paleogene events in the geohistory of South America. This hypothesis lays a foundation for an array of future studies of evolution and biogeography of the family.

Keywords:
Freshwater fishes, molecular phylogeny, Neotropical Region, Systematics

1. Introduction

Siluriformes (catfishes) is the third most speciose order of extant fishes with more than 3700 valid species (Eschmeyer et al., 2017) widely distributed in freshwaters of all continents (except Antarctica) and estuarine and marine habitats of continental shelves (de Pinna, 1998). Catfishes present an unparalleled diversity of morphological, ecological, and behavioral traits (Adriaens et al., 2010). Within the order, Trichomycteridae is the second richest family and includes species commonly known as pencil and parasitic catfishes. With about 300 valid species (Eschmeyer et al., 2017), trichomycterids are currently divided into eight subfamilies (Copionodontinae, Glanapteryginae, Sarcoglanidinae, Stegophilinae, Trichogeninae, Trichomycterinae, Tridentinae, and Vandelliinae) and 41 genera (Eschmeyer et al., 2017). Members of the family are distributed through continental freshwaters from Costa Rica to Patagonia (de Pinna and Wosiacki, 2003), with a single species occurring on the small island Gorgona island, off the Pacific coast in Colombia (Fernández and Schaefer, 2005). Species of Trichomycteridae exhibit a remarkable variety of feeding strategies, including semiparasitic hematophagy (Vandelliinae), lepidophagy and mucophagy (Stegophilinae), and occupy a wide range of habitats from subterranean waters to Andean streams and lakes up to 4500 m asl (Arratia and Menu-Marque, 1984; de Pinna and Wosiacki, 2003; Rizzato et al., 2011).

Eigenmann (1918) proposed an evolutionary tree depicting relationships among the 18 genera of trichomycterine genera known at the time, including *Nematogenys* as the most basal taxon. Baskin (1973) was the first to provide explicit cladistic support for the monophyly of the Trichomycteridae and divided all then-known trichomycterids into the Trichomycterinae-group (Glanapteryginae, Sarcoglanidinae and Trichomycterinae) and the Vandelliinae-group

(Stegophilinae, Tridentinae and Vandelliinae). Later discoveries added a clade composed of two subfamilies, Copionodontinae and Trichogeninae, as the sister to all other trichomycterids (de Pinna, 1992; de Pinna, 1998; Datovo and Bockmann, 2010). Costa and Bockmann (1994a) realigned the Glanapteryginae and Sarcoglanidinae with Baskin's Vandelliinae-group to form the so-called TSVSG clade. Subsequent studies upheld the TSVSG clade with both morphological (Datovo and Bockmann, 2010) and molecular (Fernández and Schaefer, 2009) evidence.

The monophyly of seven of the eight subfamilies of the Trichomycteridae is well supported by morphology: Copionodontinae (de Pinna, 1992), Glanapteryginae (de Pinna, 1989b), Sarcoglanidinae (de Pinna, 1989a; Costa 1994; Costa and Bockmann, 1994), Stegophilinae (Baskin, 1973; de Pinna and Britski, 1991; DoNascimiento, 2015), Trichogeninae (de Pinna et al., 2010), Tridentinae (Baskin, 1973) and Vandelliinae (Baskin, 1973; de Pinna, 1998).

Trichomycterinae is the most speciose subfamily of the Trichomycteridae with about 200 species distributed in eight genera: *Bullockia* (1 species), *Eremophilus* (1), *Hatcheria* (1), *Ituglanis* (26), *Rhizosomichthys* (1), *Scleronema* (3), *Silvinichthys* (7), and *Trichomycterus* (160+) (Eschmeyer et al., 2017). The monophyly of this subfamily remains ambiguous, as well as the synapomorphies repeatedly called into question (de Pinna, 1989a, Datovo and Bockmann, 2010; García-Melo et al., 2016). The main obstacle to understanding the relationships within Trichomycteridae is the most diverse genus, *Trichomycterus*, which has a complex taxonomic history and is a non-monophyletic assemblage that basically includes those species lacking the diagnostic characters of other trichomycterine genera (Baskin, 1973; de Pinna, 1989, 1998; Datovo and Bockmann, 2010). Despite the description of more than 70 new species in the last two decades (Eschmeyer et al., 2017), *Trichomycterus* still includes a large number of undescribed taxa. The limits and phyletic status of the whole Trichomycterinae is also controversial. While most studies agree with the exclusion of the so-called "*Trichomycterus*" *hasemani* group from the subfamily, alternative hypotheses of placement and inclusion or not of *Scleronema* and *Ituglanis* has been proposed (de Pinna, 1989; Arratia, 1990; Costa and Bockmann, 1992; 1998; Datovo and Bockmann, 2010; Dutra et al., 2012; DoNascimiento, 2015). All these issues contribute to make the phylogenetic revision of the Trichomycterinae one of the greatest challenges of catfish systematics.

Although recent work using morphology and molecules has shed much light on the

phylogenetic relationships of the Trichomycteridae, disagreements persist, especially on the monophyly and composition of the two key taxa that concentrate the vast majority of the family diversity: Trichomycterinae and *Trichomycterus*. We present here a multilocus analysis of the Trichomycteridae based on the most comprehensive taxon sampling to date that includes members of all subfamilies and a vast representation of *Trichomycterus* diversity. Also, a time-calibrated molecular tree analysis was performed to hypothesize diversification dates relative to the evolutionary history of this important Neotropical lineage and key to our understanding of catfish relationships.

2. Material and Methods

2.1 Taxon sampling

Analysis was based on a total of 94 terminals representing 18 genera of all eight subfamilies of the Trichomycteridae (Copionodontinae, Glanapteryginae, Sarcoglanidinae, Stegophilinae, Trichogeninae, Trichomycterinae, Tridentinae, and Vandelliinae). We included samples of cis- and trans-Andean species of *Trichomycterus*, the former better represented by species from the Atlantic coast of Brazil. *Nematogenys inermis*, the sole extant member of the Nematogenyidae, was chosen as the outgroup as this taxon is often hypothesized to be the sister group of the Trichomycteridae (Eigenmann, 1918, 1927; de Pinna, 1992, 1998) and is widely recognized as retaining the most primitive morphology within Loricarioidei (Eigenmann, 1918, 1927; Baskin 1973; Arratia and Huaquín, 1995; de Pinna, 1992, 1998). Data sequences were obtained from tissue samples collected by the authors. Vouchers of samples are deposited in the ichthyological collections of the Academy of Natural Sciences of Philadelphia, USA (ANSP) and the Laboratório de Biologia e Genética de Peixes, Botucatu, Brazil (LBP). Taxonomic identification of voucher specimens was validated by direct examination. Catalog numbers of vouchers and tissues used in this study are given in supplementary Table S1.

2.2 DNA extraction and sequencing

DNA was extracted from tissues preserved in 95% EtOH using the DNeasy Tissue kit (Qiagen Inc.; http://www.qiagen.com) following the manufacturer's instructions. Partial sequences of three mitochondrial (16S rRNA, cytochrome C oxidase subunit I - coi and cytochrome B - cytb) and two nuclear (myosin heavy chain 6, cardiac muscle, alpha gene - myh6

and recombination activating gene 2 - rag2) genes were amplified by polymerase chain reaction (PCR) with the primers described in Table S2. Amplifications were performed in a total volume of 12.5 µl with 1.25 µl of 10X buffer (10 mM Tris-HCl+15 mM MgCl2), 0.5 µl dNTPs (200 nM of each), 0.5 µl each 5 mM primer, 0.05 µl Platinum® Taq Polymerase (Invitrogen), 1 µl genomic DNA (10-50 ng), and 8.7 µl ddH2O. The thermo-cycler profile consisted of an initial denaturation (4 min at 95ºC) followed by 30 cycles of chain denaturation (30 s at 95ºC), primer hybridization (30-60 s at 52-54 ºC) and nucleotide extension (30-60 s at 72 ºC). All PCR products were first visually identified on 1% agarose gel and then purified using ExoSap-IT® (USB Corporation) following manufacturer instructions. The purified PCR products were sequenced using the "Big DyeTM Terminator v 3.1 Cycle Sequencing Ready Reaction Kit" (Applied Biosystems), purified again by ethanol precipitation and loaded onto an automatic sequencer 3130-Genetic Analyzer (Applied Biosystems) in the Instituto de Biociências, Universidade Estadual Paulista - UNESP, Botucatu, São Paulo, Brazil. All sequences were read twice (forward and reverse).

2.3 Sequences assembly and alignment

The consensus sequences for each individual gene were assembled from chromatograms for forward and reverse sequences using Geneious software v7.1.7 (Biomatters Ltd., Auckland, New Zealand). Initially we built matrixes for every gene and they were independently aligned using the MUSCLE algorithm under default parameters (Edgar, 2004). The alignments were inspected by eye for any obvious misalignments that were then corrected. To detect potential cases of sequencing errors due to contamination or paralogy, the alignment for each gene was analyzed by maximum likelihood (ML) and rapid bootstrapping using RAxML (Stamatakis, 2006). Sequences that were found misplaced in relation to putative congeneric or conspecific specimens in the resulting gene tree were re-sequenced or eliminated from subsequent analysis. To evaluate the occurrence of substitution saturation, we used the index of substitution saturation (Iss) test as described by Xia et al. (2003) and Xia and Lemey (2009) implemented in the software Dambe 5.3.38 (Xia, 2013). Only unambiguously alignable regions were included; hypervariable, unalignable loop regions were excluded. Alignments of all loci were concatenated into a single matrix consisting of 3284 bp of the 93 terminals plus the outgroup *Nematogenys inermis*. Each gene except 16S was partitioned by gene and codon positions to determine codon-

specific models of molecular evolution in PartitionFinder v1.1.1 (Lanfear et al., 2012). The best models were chosen under the best value for the Bayesian Information Criterion (BIC) index as detailed in Table S3.

2.4 Phylogenetic analysis

The phylogenetic hypotheses were inferred from two reconstruction methods using the partitioned data. First, Bayesian Inference (BI) was conducted in MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) via the CIPRES web portal (Miller et al., 2010). MrBayes was programmed to run for 15 million generations, with two runs of four independent MCMC chains (three heated, one cold), sampling one tree every one thousand generation. After a graphical analysis of the evolution of the likelihood scores, and checking for the stationarity of all model parameters, the first 4 million generations (25%) were discarded as burn-in. The remaining trees were used to calculate the consensus tree. The maximum likelihood (ML) phylogenetic reconstructions were performed using RAxML (Stamatakis, 2006) via command line with the "a" algorithm to rapid bootstrapping analysis (ML search and bootstrapping) in one step and specifying a random number seed for the parsimony estimation. The number of alternative runs was 100 and the analyses were performed under the model GTR+G.

Measures of branch support are given as posterior probability (P) and non-parametric bootstrap percentage (BS) separated by a common slash (/); asterisks represent values <0.5 (P) or <50% (BS). A topology test was performed to evaluate the degree of support for the resulting molecular phylogeny versus the previously published morphological hypothesis. We compared the ML scores of an unconstrained tree (our resulting topology) with a constrained tree enforcing the interfamilial relationships proposed by Datovo and Bockmann (2010). A Constraint tree was constructed in Mesquite (Maddison and Maddison, 2011), the confidence in the comparison of ML scores to every topology was evaluated with the Shimodaira-Hasegawa test (SH) (Shimodaira and Hasegawa, 1999), Kishino and Hasegawa test (KH) and Unbiased test (AU) using the CONSEL package.

2.5 Time calibrated tree

The uncorrelated relaxed molecular clock (lognormal) was estimated using BEAST v.1.7 (Drummond et al., 2012) and all clade-age inferences are presented as 95% highest posterior

density (HPD). We included two calibration points to constrain divergence dates for the 93 species of trichomycterids included in our phylogenetic tree. The first calibration point was implemented in the root of the phylogeny for the origin of Trichomycteridae about 106 million years ago (Mya) as estimated by Betancur-R et al. (2015). We implemented a normally distributed prior with mean of 106 and standard deviation of seven. The search was conducted among the interval of 92.28 - 119.7 Mya using the lower and upper quantiles of 2.5%, respectively.

The second calibration point was implemented using a log-normal prior offset and mean of 4.5 Mya and standard deviation of 1.5 for the origin of the subfamily Trichomycterinae. The only known fossil for the family Trichomycteridae was described from the Monte Hermoso Formation in Argentina by Bogan and Agnolin (2009). Based on biostratigraphy, Tomassini et al. (2013) estimated the upper and lower boundaries of the Monte Hermoso Formation to be 4.5/5 and 5.3 Mya, respectively. The search for the second calibration point was conducted within the interval of 4.4 - 32.13 Mya using the lower and upper quantiles of 2.5%, respectively. We used a macroevolutionary Birth-Death model for the diversification likelihood values and a starting tree obtained from the Bayesian inference. The analyses were conducted under different models of molecular evolution for each partition of the data matrix as evaluated by the software PartitionFinder v1.1.1 (Lanfear et al., 2012) (Table S3). The analysis was run for 10 million generations and sampled every 1000 generation. Stationarity and sufficient mixing of parameters (ESS.200) were checked using Tracer v1.5 (Rambaut and Drummond, 2007a). A consensus tree was built using TreeAnnotator v1.8.2 (Rambaut and Drummond, 2007b).

3. Results

3.1 Overall aspects of the matrix

The concatenated matrix of the three mitochondrial and two nuclear genes consist of 3284 bp after alignment (466 for 16S; 524 for COI; 859 for Cytb; 544 for Myh6; 891 for Rag2). In the total matrix, 1278 sites were variable, 1010 were parsimony informative and 2008 were invariant (I). The nucleotide composition of the concatenated matrix was of 28.1% thymine, 22.6% cytosine, 25.5% adenine and 23.7% guanine.

The Iss index indicated no saturation considering that the Iss.c value is greater than the Iss. For each gene, the number and percent of sequences obtained, size in base pairs (bp), number

of variable and invariant sites (I), number of informative characters under parsimony, nucleotide frequency and overall mean genetic distance (S.E.) are presented in Table 1. The matrix was partitioned by gene and coding positions, except by 16S, into 13 sections. The partition scheme consisted of eight subsets and 250 parameters, and the evolutionary model for each gene and codon position evaluated in PartitionFinder are showed in the Table 2.

3.2 Phylogenetic hypothesis

The tree topologies estimated by the BI and ML analyses were very similar with exception of the relationships of *Eremophilus mutisii* that in ML analysis was hypothesized as an independent lineage with a low statistical support. Although sampling at genus level was incomplete, statistical support for the monophyly of four subfamilies was high: P = 1.00, BS = 100 for Copionodontinae (2 of 2 genera sampled; clade B); P = 0.99, BS = 23 for Trichomycterinae (5 of 8 genera; clade D); P = 1.00, BS = 86 for Stegophilinae (4 of 11 genera, clade TSVSG); and P = 1.00, BS = 100 for Vandelliinae (2 of 4 genera, clade TSVSG) (Figure 2). The monophyly of the Trichogeninae (1 genus), Tridentinae (4 genera) and Glanapteryginae (4 genera) could not be tested due to the low taxonomic representation that included one genus from each subfamily, *Trichogenes longipinnis*, *Tridens* sp n2 and two species of genus *Listrura*, respectively. Our results did not support the monophyly of Sarcoglanidinae (6 genera) based on the two genera analyzed (*Sarcoglanis* and *Stauroglanis*).

The sister group relationship between Trichogenes and Copionodontinae (Fig. 2; P = 1.00, BS = 98, clade B) is strongly supported (Figure 2), as well as the placement of this clade as sister to remaining trichomycterids (Fig. 2; P = 1.00, BS = 100). The TSVSG clade was recovered as monophyletic, but with relatively low statistical support (Fig. 2; P = 0.58, BS = 57). Within the TSVSG clade, the clade composed by two glanapterygine species, *Listrura camposi* and *L. picinguabae* is the first group to diverge. The two genera of sarcoglanidines analyzed, the monotypic *Sarcoglanis* and *Stauroglanis*, were not found closely related to each other within the TSVSG clade. *Sarcoglanis* grouped with "*Trichomycterus*" *hasemani, Tridens melanops* (Tridentinae) and members of the Stegophilinae, whereas *Stauroglanis* grouped with two genera of the Vandelliinae (*Paravandellia* and *Vandellia*).

Our analyses supported the monophyly of the Trichomycterinae exclusive of "*Trichomycterus" hasemani* with high BI support (Fig. 2; P = 0.99, BS < 50). Species of five

genera (*Bullockia*, *Eremophilus*, *Ituglanis*, *Scleronema* and *Trichomycterus*) constitute a large "Clade D" (sensu Datovo and Bockmann, 2010) divided into two subclades and six main lineages. The first clade to diverge within subclade D1+D2+E+D3 (D1, P = 0.54, BS < 50; Fig. 2) clusters three trans-Andean taxa, the troglomorphic *Trichomycterus sandovali* (Magdalena basin), *T. punctulatus* (Pacific versant of Peru), and *Eremophilus mutisii* (Magdalena basin), with a species from the Meta River (Orinoco basin), *Trichomycterus* cf. *knerii*. These relationships were different in ML analysis, with *E. mutisii* as an independent lineage with a low support (BS < 50).

Clade D2 (Fig. 2; P = 1.00, BS < 50) groups species of *Trichomycterus* from the Magdalena basin (*T. banneaui*, *T. cachiraensis, T. ruitoquensis, T. straminius*, *Trichomycterus* cf. *trasandianus*) with *T* cf. *guianensis* (Essequibo basin, Guyana) and *Trichomycterus* aff. *spilosoma* (Pacific versant of Ecuador). The third clade (Fig. 2; P = 0.94, BS = 86) partially corresponds to "Clade E" of Datovo and Bockmann (2010) and grouped two trans-Andean species from Chile, *Bullockia maldonadoi* and *T. areolatus*, as sister to an undescribed cis-Andean species, *Trichomycterus* sp. 2, from upper Paraguay River basin. Clade D3 (Fig. 2; P = 1.00, BS = 100) correspond to analyzed species of *Ituglanis* from the Amazon basin (Jari, Madeira, Tapajós and Tocantins Rivers), La Plata system (Paraguay and Paraná Rivers) and smaller Atlantic Coast drainages of southern (Jacuí and Ribeira de Iguape Rivers) and southeastern (Macabú River) Brazil.

The sister group relationship between clades D4 and D5 is strongly supported (Fig. 2; P = 0.99, BS = 87) and ties *Scleronema* (lower La Plata system and Atlantic coastal drainages in Southern Brazil and Uruguay) with species of *Trichomycterus* distributed in Atlantic coastal drainages from the São Francisco River in the north to the Paraná and Uruguay rivers in the south. Clade D4 has high nodal support in both the BI and ML analysis (Fig. 2; P = 0.99, BS = 100) and places the clade formed by *S. minutum* and *Scleronema* cf. *angustirostre* (from tributaries of Laguna dos Patos system) sister of a group of species of *Trichomycterus* distributed in the Atlantic coastal drainages, including the upper São Francisco basin, Itapocu, Jacuí, Ribeira de Iguape, Uruguay, and tributaries to the Paraná drainages (Paranapanema and Tietê Rivers).

Clade D5 (Fig. 2; P = 0.99, BS = 100) also includes species of *Trichomycterus* from Atlantic coastal drainages of eastern and southern Brazil. In this clade, the first group to diverge includes *T. itatiayae* (Paraiba do Sul, River), *T. piratymbara* (Grande-Paraná, River system), and

T. reinhardti (Paraopeba-São Francisco River system). The second group to diverge includes *T. florensis* (Paraiba do Sul River) sister to specimens of *T. nigroauratus* from the same basin and the Grande River (Paraná basin). The third group to diverge is represented by *T. albinotatus* (Paraiba do Sul River), *T. alternatus* and *T. immaculatus* (Doce River), and two species of *Trichomycterus* from the Jequitinhonha River, *T. pradensis* and *T.* cf. *mimosensis*. The third group is sister to a clade partially corresponding to the so-called *T. brasiliensis* complex by Barbosa and Costa (2010), including species distributed in the Grande River (Paraná basin) and upper São Francisco basin.

Our topology test using a constraint tree for subfamilial relationships proposed by Datovo and Bockmann (2010), resulted in a hypothesis with low likelihood value (Table 3). However, the difference between the constraint tree and the herein proposed tree was minimal, and consequently, the topology test failed to reject the null hypothesis of morphology-based relationships within Trichomycteridae.

3.3 Time calibrated tree

The mean substitution rate for the dataset estimated in BEAST was 0.0163% per My. The Trichomycteridae was estimated to have originated near the end of the Lower Cretaceous about 103.2 Mya (54.5-109.7 Mya, 95% HPD) and around the time of the continental separation between Africa and South America (ca. 100 Mya; Torsvik et al. 2008). The first split is estimated during the Middle of the Upper Cretaceous diverging in two larger groups, Copionodontinae + Trichogeninae and the clade of the remaining trichomycterids. The next split is estimated at 66.8 Mya, just before the K-T boundary, and established ancestral of the clade TSVSG clade and the subfamily Trichomycterinae. Within the TSVSG clade, the oldest glanapterygine genus *Listrura* was originated in the Paleocene and the remaining subfamilies arose in the Eocene (Sarcoglanidinae, Tridentinae, Vandelliinae) and Oligocene (Stegophilinae). Oligocene diversification within Trichomycterinae established the three major clades composed mostly of trans-Andean taxa (D1-2, E), and the three remaining strictly of cis-Andean clades (D3-5). Much of the diversification within those major clades appears to have occurred during the Miocene (Figure 3).

4. Discussion

4.1 Phylogenetic relationships among Trichomycteridae subfamilies

The molecular phylogenetic hypothesis supported the monophyletic status of the Copionodontinae, Stegophilinae, Trichomycterinae, and Vandelliinae, but not Sarcoglanidinae. The current conceptualization of the Trichomycterinae, which includes *Ituglanis* and *Scleronema* but excludes the *"Trichomycterus" hasemani* group (Datovo and Bockmann, 2010; see item 4.2 below), was confirmed as a monophyletic unit with high support both in both, BI and ML analyses. The topology corroborated the most recent morphological tree of the family (Datovo and Bockmann, 2010) in several important ways: Copionodontinae and Trichogenis form a monophyletic group that is sister to remaining trichomycterids, and this latter clade is divided into two main linages, the TSVSG clade and Trichomycterinae. Internal relationships for the TSVSG clade, however, differs in some significant aspects from the traditional phylogenetic hypotheses, mainly due to the recovered non-monophyly of the Sarcoglanidinae, although not all genera herein represented. The systematic positions of the two sarcoglanidines herein analyzed yielded a novel topology that remained consistent between the two analytical methods (BI and ML) with considerable statistical support. Nevertheless, the topologies advanced by previous morphological studies of inter-subfamilial relationships could not be rejected by the present molecular analysis according to the topology test (Table 3).

The monophyletic assemblage of the subfamilies Copionodontinae and Trichogeninae, identified as Clade B in Datovo and Bockmann (2010), was originally suggested by de Pinna (1998) and corroborated by Bichuette et al. (2008). These subfamilies share the derived presence of an anterior process at the anterolateral corner of hypobranchial 1, the endopterygoid ankylosed to the ventral surface of the autopalatine, and an enlarged subtemporal fossa (de Pinna, 1998). Datovo and Bockmann (2010) additionally found a myological synapomorphy for the clade consisting in the presence of the *adductor hyomandibulae* muscle, a morphological character that is functionally linked to the hollow ventral surface of the pterotic (subtemporal fossa). This clade is the sister group to all other trichomycterids and exhibits the plesiomorphic condition for several characters uniquely derived in the remaining trichomycterids (de Pinna, 1998; Datovo and Bockmann, 2010). Therefore, the monophyly of this clade and its phylogenetic placement at the base of the Trichomycteridae is supported by both morphology and molecules hypotheses.

The group comprising all subfamilies of the Trichomycteridae with the exception of Copionodontinae and Trichogeninae (identified as Clade C in Datovo and Bockmann, 2010), was

also recovered in the present molecular analysis, despite some disagreement in the composition. Results from both inferences herein analyzed, divided Clade C into two subclades, the TSVSG (Costa and Bockman, 1993) and the Trichomycterinae as redefined by Datovo and Bockmann (2010). Our hypothesis of a monophyletic group including all trichomycterines and members of the TSVSG clade corroborates previous morphological studies (de Pinna, 1992, 1998; Bockmann et al., 2004; Datovo and Bockmann, 2010) based on a wealth of anatomical support: anterior cranial fontanel partially or completely closed; sphenotic, prootic, and pterosphenoid fused; Weberian capsule with a small opening, much smaller than its lateral profile; interhyal absent; five or fewer pelvic-fin rays; dorsal caudal-fin plate with six or fewer rays; dorsal caudal-fin lobe with five or fewer branched rays; ventral caudal-fin plate with eight or fewer rays; ventral caudal-fin lobe with six or fewer branched rays; incomplete infraorbital branch of the laterosensory canal system; presence of a *protractor operculi* muscle; and *levator operculi* muscle with fibers posterodorsally oriented towards its origin.

Despite the low support values (P=0.58, BS= 57) the monophyly of the TSVSG clade is corroborated by previous studies (Costa and Bockmann, 1994; Fernández and Schaefer, 2009) and it is supported by four shared derived morphological characters: absence of a posterior process of the parasphenoid; extreme reduction or absence of the metapterygoid, interopercular patch of odontodes reduced, being nearly as long as deep; and primary section of the *dilatator operculi* passing dorsolateral to *the levator arcus palatini* (Costa and Bockmann, 1993; de Pinna, 1998; Datovo and Bockmann, 2010). A major source of incongruence between our hypothesis and previous phylogenetic arrangements lay in the internal relationships of the TSVSG clade. The main departures in the genetic analysis are the non-monophyly of the Sarcoglanidinae, with *Sarcoglanis* being most closely related to *"Trichomycterus" hasemani*, and *Stauroglanis* most closely related to Vandelliinae (*Paravandellia* and *Vandellia*). This internal arrangement of the TSVSG clade (Fig. 2) is highly supported in BI but not in ML analysis. Most morphological studies (Baskin, 1973; Costa and Bockmann, 1994; de Pinna, 1998; Datovo and Bockmann, 2010) and previous molecular analyses (Fernández and Schaefer, 2009) supported a sister group relationship between the Sarcoglanidinae and Glanapteryginae, with the following shared derived morphological features: reduced vomer; reduced number of premaxillary teeth; quadrate with a posteriorly-directed anterodorsal process; anterior portion of the hyomandibula modified

into a long process; seven or fewer anal-fin rays; and insertion of the *stegalis* (*sensu* Datovo and Vari, 2013, 2014; =A3) section of the *adductor mandibulae* onto the buccopalatal membrane.

The Sarcoglanidinae was established by Myers and Weitzman (1966) to include *Sarcoglanis simplex* and *Malacoglanis gelatinosus*. New genera were subsequently described and added: *Stauroglanis* (de Pinna, 1989), *Stenolicmus* (de Pinna and Starnes, 1990), *Microcambeva* (Costa and Bockmann, 1994), and *Ammoglanis* (Costa, 1994). The monophyletic status of this subfamily has been successively reexamined during the last two decades (Datovo and Bockmann, 2010) and only the inclusion of *Ammoglanis pulex* has been questioned (de Pinna and Winemiller, 2000). The monophyly of the Sarcoglanidinae is supported by a large set of osteological (de Pinna, 1989; Costa, 1994) and myological (Datovo and Bockmann, 2010) synapomorphies. The sister group relationship between *Sarcoglanis simplex* and "*Trichomycterus" hasemani* is unexpected and novel, since this is the first molecular analysis to include both taxa. Morphological studies have placed "*T." hasemani* sister to a clade formed by the Tridentinae, Stegophilinae, and Vandelliinae (= Vandelliinae-group), either analyzing it alone (DoNascimiento, 2015) or as part of the "*T." hasemani* group, which also includes "*T." johnsoni* and "*T." anhinga*, and *"T". wapixana* (Dutra et al., 2012; see next section). In our analysis, long-branch attraction and likely the incomplete lineage sorting may be responsible for the non-monophyly of Sarcoglanidinae, as well as the lack of support for the clade Sarcoglanidinae + Glanapteryginae. Our sampling of both subfamilies was incomplete (two of six sarcoglanidine genera and only one of four glanapterygine genera). The two sarcoglanidine genera in our analysis (*Sarcoglanis* and *Stauroglanis*) occupy rather distal positions in the subfamilial phylogeny inferred from morphology (summarized in Costa, 1994) and *Sarcoglanis simplex* has a remarkable highly specialized morphology.

Previous morphological (Baskin, 1973; de Pinna, 1998; Datovo and Bockmann, 2010) and molecular (Fernández and Schaefer, 2009) analyses hypothesized the monophyly of the Vandelliinae-group, a clade formed by the Tridentinae, Stegophilinae, and Vandelliinae. Such an arrangement is not recovered in the present study, with the Vandelliinae appearing more closely related to the Glanapteryginae and the sarcoglanidine *Stauroglanis* than to tridentines and stegophilines. Another point of disagreement corresponds to the relationship between the semiparasitic subfamilies Stegophilinae and Vandelliinae. Most studies (Baskin, 1973; de Pinna, 1998; Fernández and Schaefer, 2009; DoNascimiento, 2015) have supported a sister relationship

between those two subfamilies. Datovo and Bockmann (2010) provided myological evidence favoring and alternative hypothesis in which Tridentinae and Stegophilinae are sister taxa. These authors, however, confirm the validity of anatomical characters supporting the traditional hypothesis and concludes that the relationships between the three subfamilies could not be decisively determined based on the morphological evidence available. Our results reinforce the myological evidence presented by Datovo and Bockmann (2010) and is the first study to provide molecular support for the hypothesis of a tridentine-stegophiline group.

4.2 The subfamily Trichomycterinae

Baskin (1973) was the first to suggest the non-monophyly of the Trichomycterinae. De Pinna (1989) corroborated his hypothesis based on the lack of synapomorphies for the subfamily and the possibly closer relationship of some species with other subfamilies: *Scleronema, Trichomycterus boylei* and *T. santaeritae* would be more closely related to the Sarcoglanidinae and "*T*". *hasemani* and "*T*". *johnsoni* more closely related to the Tridentinae. Whereas the former hypothesis has been rejected (Arratia, 1990; Costa and Bockmann 1993, 1994; de Pinna, 1998; Datovo and Bockmann, 2010), the latter was partially corroborated by subsequent morphological studies (Datovo and Bockmann, 2010; Dutra et al., 2012; DoNascimiento, 2015). The present analysis is the first molecular evidence corroborating the exclusion of "*T.*" *hasemani* from the Trichomycterinae, although in our topology this species is more closely related to *Sarcoglanis* (Sarcoglanidinae) than to members of the Vandelliinae-group. The so-called "*T.*" *hasemani* group (*"T". anhanga*, *"T". hasemani*, and *"T". johnsoni*, and *"T". wapixana*; Dutra et al., 2012) remains provisionally classified as *Trichomycterus* while its formal description as a new trichomycterid genus and subfamily is still underway (by W. Wosiacki and M. C. C. de Pinna; pres. comm.).

Arratia (1990) proposed four synapomorphies in supporting a monophyletic Trichomycterinae, none of which were present in "*Trichomycterus*" *hasemani* (de Pinna, 1998). Costa and Bockmann (1993) and de Pinna (1998), on the other hand, advanced that *Ituglanis* and *Scleronema* were more closely related to the TSVSG clade than to remaining trichomycterines, a result not corroborated by the present analysis. More recently, Datovo and Bockmann (2010) and Datovo et al. (2016) revised the characters proposed to support these two alternative hypotheses and concluded that none of them were valid. The authors, however, concluded that the

Trichomycterinae exclusive the "*T.*" *hasemani* group could form a monophyletic group on the basis of the sharing of a posterior portion of *levator internus IV* originated from the dorsal face of the posttemporo-supracleithrum. Thus, the present analysis based on representatives of five trichomycterine genera (*Eremophilus*, *Bullockia*, *Ituglanis*, *Scleronema*, and *Trichomycterus*; missing *Hatcheria*, *Rhizosomichthys* and *Silvinichthys*), supported the monophyly of the Trichomycterinae (clade D) as circumscribed by Datovo and Bockmann (2010).

In our topology, the Trichomycterinae is divided into two large clades: one including all species from Atlantic coastal drainages and Upper Paraná (*Scleronema* and part of *Trichomycterus*; clade D1+D2+E+D3) and another primarily including Amazonian and trans-Andean taxa (*Bullockia, Eremophilus, Ituglanis*, and part of *Trichomycterus*; clade D4+D5). Such a scheme contradicts previous hypotheses in which *Scleronema* and *Ituglanis* are in some way closely related (Costa and Bockmann, 1993; de Pinna 1998). The monophyletic status of genus *Ituglanis* (clade D3) is highly supported, in agreement with the morphological studies (Costa and Bockmann, 1993; Datovo and de Pinna, 2014; Datovo et al., 2016; Wosiacki et al., 2012). De Pinna and Keith (2003) tentatively proposed the existence of two monophyletic groups within the genus, one including species from the Amazon and Guiana shield and another primarily formed primarily by species from the La Plata system and Atlantic coastal drainages. This hypothesis has been challenged in light of additional anatomical evidence and newly discovered taxa (Datovo and Landim, 2005; Datovo, 2014; Datovo and de Pinna, 1994). Our analysis refuted de Pinna and Keith's (2003) proposal, with the clustering of species from the Amazon and Paraguay in two occasions: in one clade, including *I. herberti* and *I. parkoi*, and a second lineage that includes *I. amazonicus*, *I. eichhorniarum*. It is worth mentioning, however, that identity and limits of the three latter species are poorly understood and some terminals are only tentatively assigned to these taxa (*I. amazonicus*, *I.* cf. *eichhorniarum*, and *I.* cf. *parkoi*). Interestingly, all analyzed species from the Atlantic coastal drainages (*I. boitata*, *I. parahybae*, and *Ituglanis.* sp. n. 1) are herein highly supported as forming a monophyletic lineage. A fourth major clade of *Ituglanis* grouped the epigean *I. goya* and the hypogean *I.* cf. *ramiroi*, both from the lower Tocantins basin.

The sister group of *Ituglanis* is Clade E composed of *Bullockia*, *Trichomycterus areolatus* and an undescribed *Trichomycterus* from Sepotuba River, upper Paraguay River basin. A previous study of DoNascimiento (2015) clustered *Bullockia*, *Hatcheria*, and *Trichomycterus*

areolatus. Datovo and Bockmann (2010) included to this clade another Chilean species, *T. chiltoni* and other two from southern Brazil to this clade, *Trichomycterus immaculatus* and *T. zonatus*, but our molecular results nested those species in different clades, D5 and D4 respectively.

Our molecular results corroborated the non-monophyletic status of *Trichomycterus* as previously hypothesized by several studies (Baskin, 1973; de Pinna, 1989, 1998; Arratia, 1998; Datovo and Bockmann, 2010). Identity of the type species of *Trichomycterus*, *T. nigricans*, is surrounded by uncertainties and conflicting information (A. Datovo, in prep.), but its type locality is certainly an Atlantic coastal drainage in Brazil. In our analysis, species of *Trichomycterus* from this area are restricted to sister clades D4 and D5. Clade D4 also includes two species of *Scleronema*, *S. minutum* and *Scleronema* cf. *operculatum*.

In spite of the present study to be the most comprehensive phylogenetic analysis of the Trichomycterinae published to date, including 70 representative terminals, taxonomic changes at this moment are premature considering the constant increase in the description of new species assigned to the genus *Trichomycterus*, as well as, the incomplete genera representation of some subfamilies. Nevertheless, our results constitute an important evidence for the necessary nomenclatural changes within *Trichomycterus*, which should be ideally associated with the geographical distribution of the clades herein identified. Based on our results, clades containing species of the *Trichomycterus* from trans-Andean basins (D1, D2, E) are more closely related to *Ituglanis* (Clade D3) than to clades containing species of *Trichomycterus* from Atlantic coastal drainages, which probably are more related with the type species of this genus. Finally, we suggest that revisionary studies of key taxa at the generic level, an increase in the molecular information and its integration with morphological data are crucial to confidently to perform accurate nomenclatural decisions in the subfamily.

4.3 Timing of diversification

Molecular-clock methods provide neontological tools for estimating the temporal origins of clades (Hipsley and Müller, 2014) and linking cladogenesis to major events in geohistory. For example, Lundberg et al. (2007) used a fossil-calibrated, relaxed-clock molecular analysis to estimate the divergence of the Neotropical freshwater catfish, *Lacantunia enigmatica*, from its closest relatives which are endemic to Africa. Based on the estimated age of *Lacantunia* (83-86.5

Mya), its occurrence in the New World is more likely attributable to dispersal via Holarctic land bridges from the Late Cretaceous to Late Miocene than to older vicariant events associated with the breakup of Pangea and subsequently Gondwana. Employing similar methods, Sullivan et al. (2013) estimated that the Neotropical superfamily Pimelodoidea (long-whiskered catfishes) originated between 110 and 95 Mya, and that its five major lineages (family-level clades) split off soon afterward during a period of explosive diversification. Based on the fossil records of pimelodoids and other Neotropical fishes, the freshwater fauna of South America appears to have been essentially modern by the mid-Miocene (Lundberg, 1998; Lundberg et al., 2010; Sullivan et al., 2013).

Trichomycteridae is one of six family-level groups in Loricariodei, a suborder that is endemic to the Neotropic and sister to all other catfishes (Sullivan et al., 2006). Our estimate of the origin of Trichomycteridae (103.2 Mya) is approximated the time of the rifting caused the connection between the Central and South Atlantic (ca. 100 Mya; Torsvik et al. 2008) which severed the last continental connection between Africa and South America. The oldest split within Trichomycteridae is estimated at 84.66 Mya and established the clade Copionodontinae + Trichogeninae. Extant members of this clade are restricted to a few, relatively small Atlantic tributaries in eastern and southeastern Brazil. Ribeiro (2006) used this split to exemplify the initial (Cretaceous) phase of diversification contributing to an ancient fish fauna endemic to Brazilian coastal rivers (his "Pattern A"). He linked Pattern A cladogenesis to the original Atlantic coastal drainages that were probably structurally oriented by megadomes, large faults and grabens. The second major split within the Trichomycteridae appears to have established two large clades, TSVSG and Trichomycterinae, just before the K-T Boundary (ca. 65.5 Mya).

The TSVSG and Trichomycterinae clades provide more examples of cladogenesis involving Atlantic coastal drainages. The first group to diverge in the TSVSG clade is *Listrura* (60.8 Mya), a genus restricted to coastal streams of southern and southeastern Brazil. The remaining members of the TSVSG clade are restricted to larger cis and trans-Andean basins that were variously interconnected throughout the Cenozoic. Within the Trichomycterinae, there are two major clades containing Atlantic coastal taxa, D3 and D4+D5. By our estimates, Clade D4+5 split from the remaining trichomycterines 33.3 Mya (Lower Oligocene). This clade is composed exclusively of taxa from Atlantic coastal drainages and tributaries to the Paraná-Paraguay basin. Clade D3 is younger, established ~24 Mya (Upper Oligocene), and includes species of the genus

Ituglanis from both Atlantic coastal drainages and the major cis-Andean drainages (Amazonas, Paraná-Paraguay system, Tocantins, and Amazonas River basin). Our results therefore suggested that separate geological events are responsible for the modern-day fauna of Atlantic coastal trichomycterines.

The distribution and diversification of fishes in Atlantic coastal tributaries are commonly attributed to sea level changes during the Late Pleistocene (Weitzman et al., 1988; Thomaz et al., 2015). Ribeiro (2006) argued that changes in sea level fail to explain the occurrence of closely related taxa in both coastal drainages and upland rivers draining the Brazilian Shield inland towards much larger basins such as the Paraná-Paraguay system. Alternatively, he hypothesized megadome uplifts, rifting, vertical movements between rifted blocks and the erosive retreat of the eastern continental margin of South America to be the main geological forces controlling the biogeography of Atlantic coastal fishes. Those forces gave rise to taphrogenic (rift related) basins along the Brazilian coast that repeatedly captured adjacent upland drainages, providing a one-way conduit for introducing upland taxa to Atlantic coastal basins. Ribeiro (2006) asserted that only significant tectonic deformations could account for dispersal in the opposite direction whereby fishes common to coastal basins are introduced to upland rivers draining inland. Our results point to the Miocene as an active period for such geological events in concert with the diversification of trichomycterins in clades D3 and D4+5.

Of equal interest are dates of origin estimated for trichomycterine clades D1, D2 and E. Together with clade D3 (*Ituglanis*), those groups are one of the first two clades to diverge within Trichomycterinae 33.3 Mya. Clades D1, D2 and E are dominated by taxa from trans-Andean drainages (e.g., Magdalena, Pacific coastal drainages). According to our analysis, clade D1 was the first to split off 30.1 Mya, followed by D2 at 27.2 Mya. The last split between clade E (*Bullockia* and two species of *Trichomycterus*) and clade D3 (*Ituglanis*) is estimated at 24 Mya. Those major cladogenetic events pointed to the Oligocene as an active time for the impact of the Andean uplift on local drainage patterns. Of special interest is the placement of *Trichomycterus* cf *guianensis* within clade D2. *Trichomycterus* cf *guianensis* is a species complex endemic to upland left-bank tributaries of the Essequibo River which drain a portion of the Guiana Shield into the Atlantic Ocean. Our results nested *T.*cf. *guianensis* within clade D2 as sister to a group composed of *Trichomycterus* from the Magdalena and a Pacific versant in Ecuador. As one would expected, the timing of that split, 19.2 Mya (Lower Miocene), predates the estimated

isolation of the Magdalena River via the uplift of the Northern Andes ~7-11 Mya (plate 15 in Hoorn and Wesselingh, 2010).

Overall, the tempo of diversification within the Trichomycteridae is consistent with that described by molecular-clock analyses of other Neotropical catfishes (Lundberg et al., 2007; Sullivan et al. 2013). Likewise, trichomycterid diversity appears to have been essentially modern by the mid-Miocene and largely shaped by Paleogene events in the geohistory of South America.

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Figures

Figure 1. Previous phylogenetic hypothesis for the family Trichomycteridae based in **A)** de Pinna (1998); and **B** Datovo and Bockmann (2010).

Figure 2. Phylogenetic relationships of Trichomycteridae. Node numbers correspond to Bayesian Posterior Probability (P) and Maximum Likelihood (BS) support values. * indicate P and BS values <0.50 and <50 respectively.

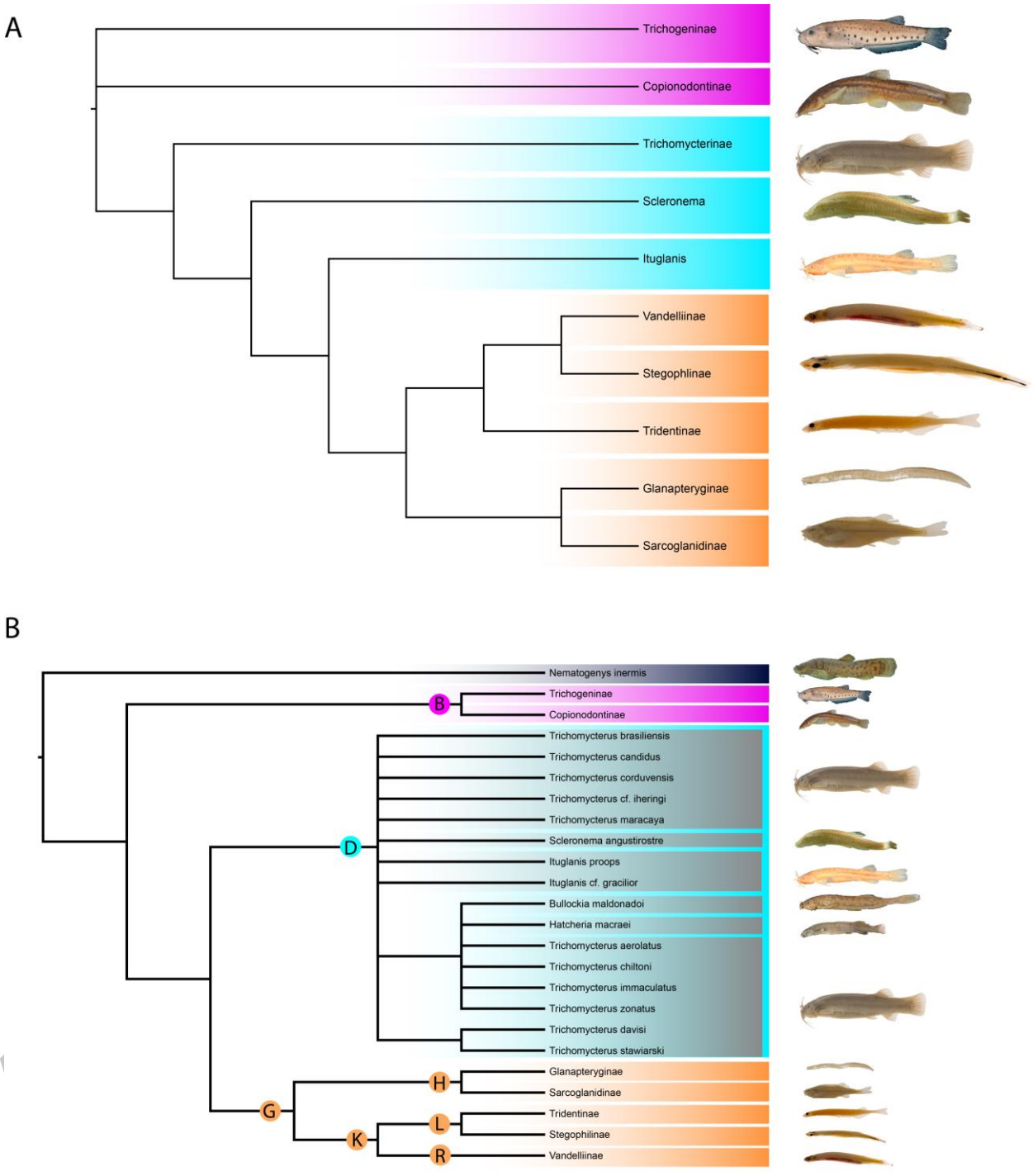
Figure 3. Maximum clade credibility (MCC) tree of Trichomycteridae obtained from BEAST analysis.

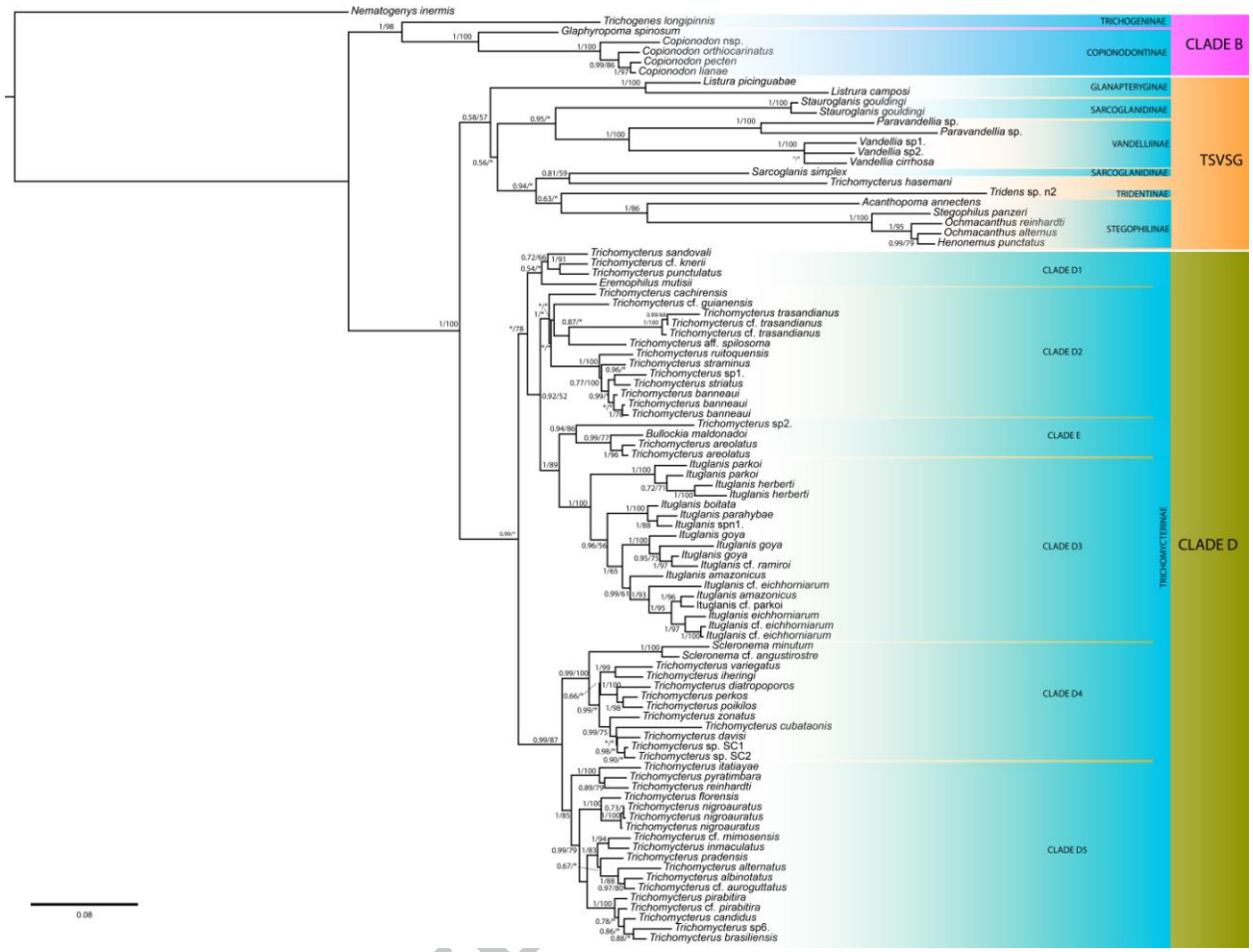
Tables

Table 1. Characteristics of the matrix for each gene and total matrix.

Table 2. Evolutionary models by gene and codon position found in PartitionFinder.

Table 3. Results from topology test in Consel, assessing the significance between the unconstrained RAxML best tree and a tree constrained to the subfamilial relationships proposed by Datovo and Bockmann (2010).





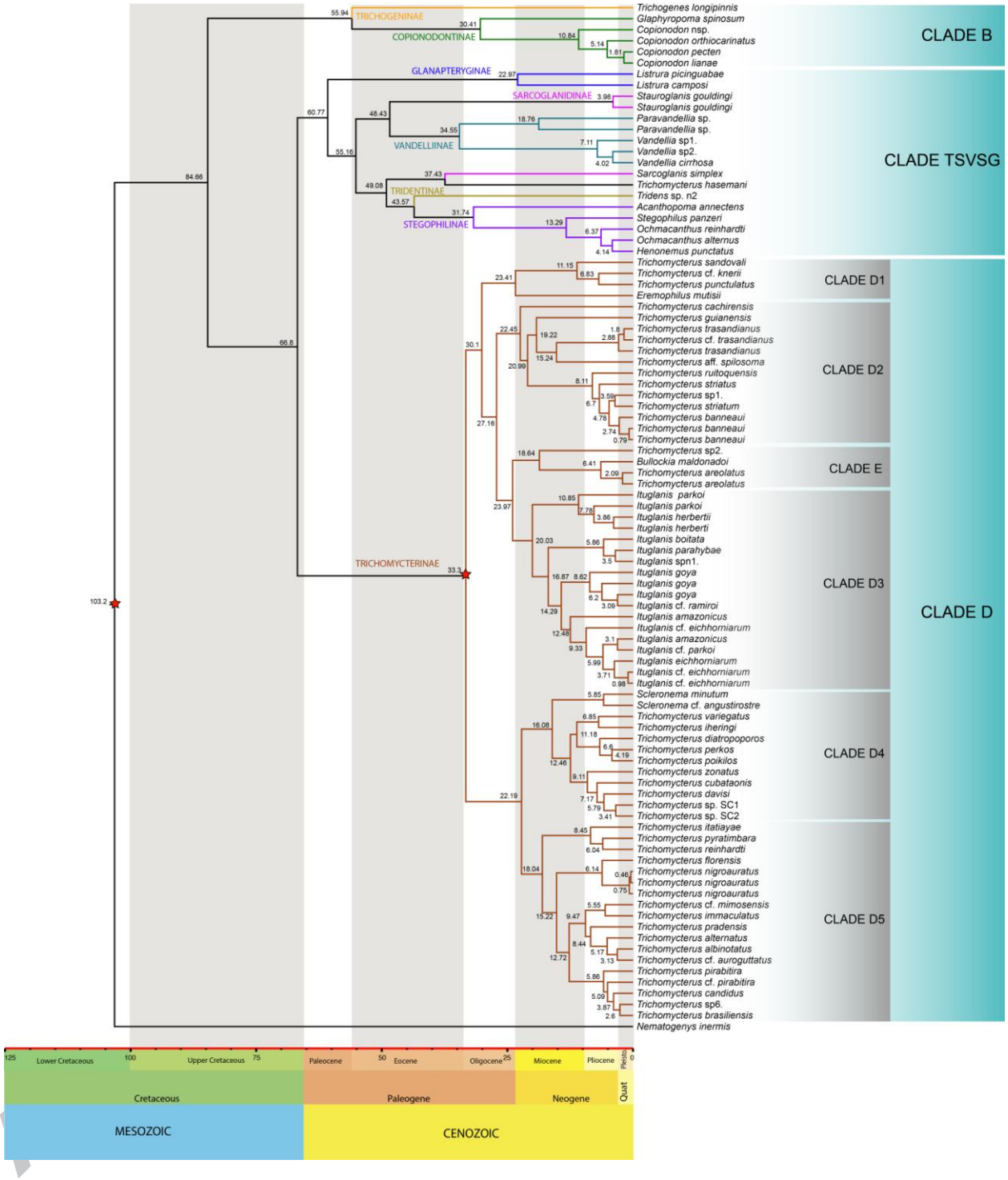


Table 1. Characteristics of the matrix for each gene and total matrix.

	Mitochondrial			Nuclear		
	16S	**COI**	**Cytb**	**Myh6**	**Rag2**	**Concatenated matrix**
Total number of sequences	92	89	66	86	62	93
Base-pairs (bp) after alignment	466	524	859	544	891	3284
Number of variable sites	139	216	403	193	329	1278
Number of invariants (I) sites	327	308	456	351	566	2006
Number of informative characters under parsimony	95	205	360	140	214	1010
Nucleotide frequency						
A	22.0	25.0	28.0	25.4	26.4	25.5
C	24.2	28.2	15.2	22.3	24.2	22.6
G	23.7	17.4	29.0	22.2	25.2	23.7
T	30.1	29.4	27.7	30.1	24.2	28.1
Overall mean genetic distance (S.E.)	0.042±0.004	0.147±0.009	0.151±0.007	0.046±0.004	0.050±0.003	0.090±0.003

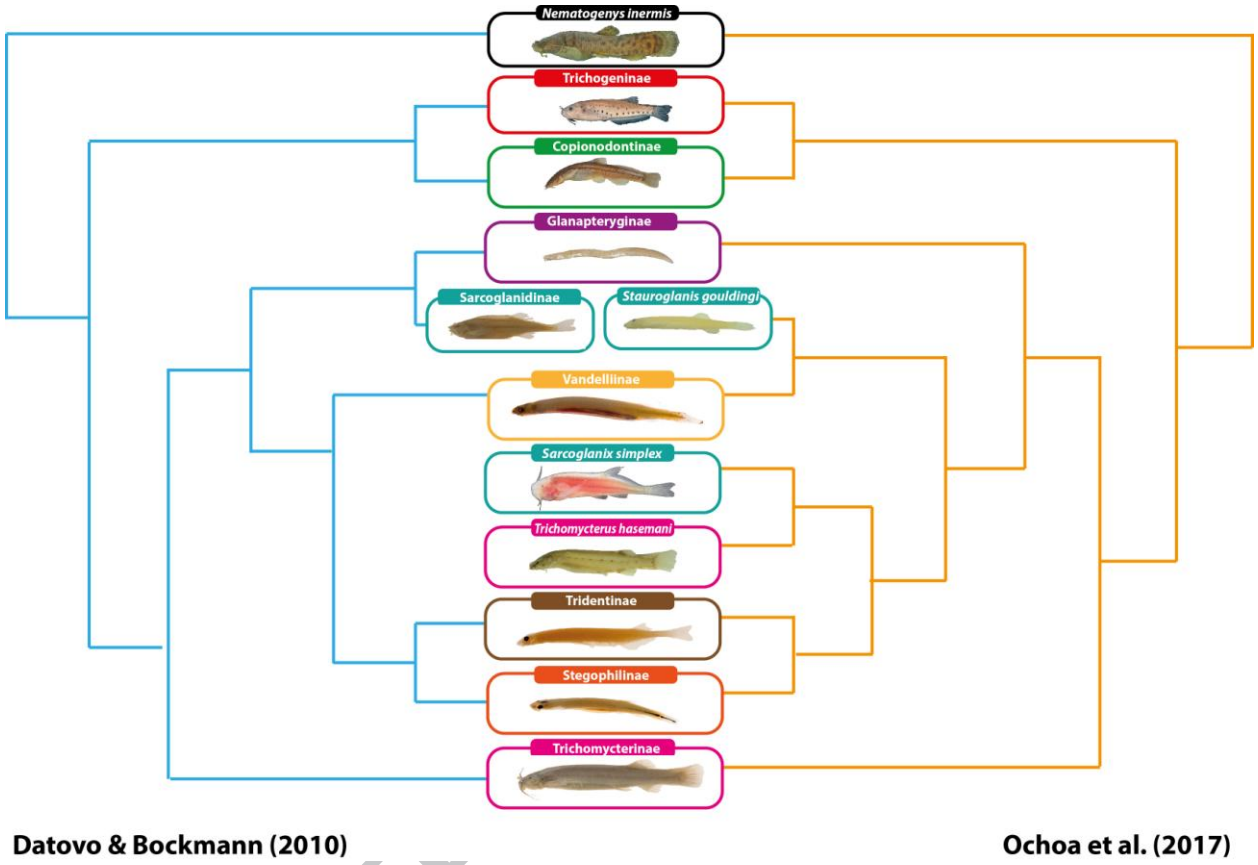
Table 2. Evolutionary models by gene and codon position found in PartitionFinder.

Subset	Scheme of partition	Partitions	Best-fit model
1	16S	1-466	GTR+I+G
2	COI 1st position	467-990\3	K81+I+G
	Cytb 1st position	991-1849\3	
3	COI 2nd position	468-990\3	F81+I
4	COI 3rd position	469-990\3	GTR+I+G
5	Cytb 2nd position	992-1849\3	GTR+G
6	Cytb 3rd position	993-1849\3	HKY+I+G
	Myh6 2nd position	1851-2393\3	
7	Myh6 1st position	1850-2393\3	TVMEF+G
	Rag2 1st position	2394-3284\3	
8	Myh6 3rd position	1852-2393\3	TRNEF+I+G
	Rag2 2nd position	2395-3284\3	
	Rag2 3rd position	2396-3284\3	
lnL: -36058.22		Number of parameters: 250	

Table 3. Results from topology test in Consel, assessing the significance between the unconstrained RAxML best tree and a tree constrained to the subfamilial relationships proposed by Datovo and Bockmann (2010).

Analysis	lnL score	Rank	Delta lnL	AU- P value	SH- P value	KH - P value
Unconstrained tree	-36106.04	1	-26.6	0.976	0.964	0.964
Constrained tree	-36133.43	2	26.6	0.024	0.036	0.036

Graphical abstract



Highlights

- The monophyletic status of Trichomycterinae is supported, which includes *Ituglanis* and *Scleronema.*
- Molecular evidence for exclusion of *"T" hasemani* from Trichomycterinae.
- *Trichomycterus* is corroborated as non-monophyletic group.
- The diversification time to Trichomycteridae is associated with the last continental connection between Africa and South America
- Geological events during the Miocene contributed in the diversification of trichomycterins