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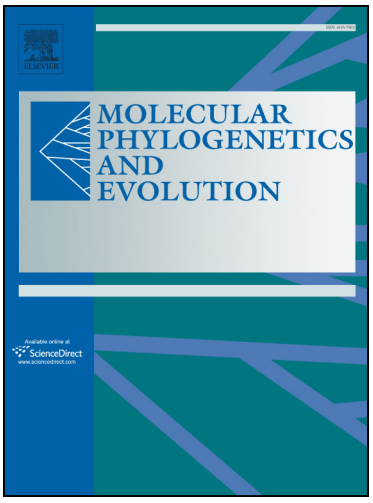

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PII: S1055-7903(19)30305-7
DOI: https://doi.org/10.1016/j.ympev.2019.106711
Reference: YMPEV 106711

To appear in: *Molecular Phylogenetics and Evolution*

Received Date: 22 May 2019
Revised Date: 20 October 2019
Accepted Date: 14 December 2019

Please cite this article as: Jardim de Queiroz, L., Cardoso, Y., Jacot-des-Combes, C., Anne Bahechar, I., Alberto Lucena, C., Rapp Py-Daniel, L., Maria Sarmento Soares, L., Nylinder, S., Oliveira, C., Estevam Parente, T., Torrente-Vilara, G., Covain, R., Buckup, P., Montoya-Burgos, J.I., Evolutionary units delimitation and continental multilocus phylogeny of the hyperdiverse catfish genus *Hypostomus*, *Molecular Phylogenetics and Evolution* (2019), doi: https://doi.org/10.1016/j.ympev.2019.106711

Evolutionary units delimitation and continental multilocus phylogeny of the hyperdiverse catfish genus *Hypostomus*

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Highlights

- We inferred a comprehensive phylogeny of the species-rich and widespread catfish genus *Hypostomus*.
- Morphology together with multispecies coalescent analyses unraveled 108 species and 118 clusters.
- We present the Cluster Credibility index that allows testing alternative cluster delimitations
- To delimit species, the *COI* pairwise divergence threshold of 2% is not applicable in *Hypostomus*.
- Our phylogeny reveals four *Hypostomus* super-groups for which we give diagnostic characters

Abstract

With 149 currently recognized species, *Hypostomus* is one of the most species-rich catfish genera in the world, widely distributed over most of the Neotropical region. To clarify the evolutionary history of this genus, we reconstructed a comprehensive phylogeny of *Hypostomus* based on four nuclear and two mitochondrial markers. A total of 206 specimens collected from the main Neotropical rivers were included in the present study. Combining morphology and a Bayesian multispecies coalescent (MSC) approach, we recovered 85 previously recognized species plus 23 putative new species, organized into 118 'clusters'. We presented the Cluster Credibility (CC) index that provides numerical support for every hypothesis of cluster delimitation, facilitating delimitation decisions. We then examined the correspondence between the morphologically identified species and their inter-specific *COI* barcode pairwise divergence. The mean *COI* barcode divergence between morphological sisters species was 1.3 ± 1.2%, and only in 11% of the comparisons the divergence was ≥ 2%. This indicates that the *COI* barcode threshold of 2% classically used to delimit fish species would seriously underestimate the number of species in *Hypostomus*, advocating for a taxon-specific *COI*-based inter-specific divergence threshold to be used only when approximations of species richness are needed. The phylogeny of the 108 *Hypostomus* species, together with 35 additional outgroup species, confirms the monophyly of the genus. Four well-supported main lineages were retrieved,

hereinafter called super-groups: *Hypostomus cochliodon, H. hemiurus, H. auroguttatus*, and *H. plecostomus* super-groups. We present a compilation of diagnostic characters for each super-group. Our phylogeny lays the foundation for future studies on biogeography and on macroevolution to better understand the successful radiation of this Neotropical fish genus.

Keywords: Hypostominae; multispecies coalescent approach; species delimitation; DNA barcode; cluster credibility index; hidden diversity

1 Introduction

The genus *Hypostomus*, member of the family Loricariidae, is composed of 149 recognized species (Eschmeyer et al., 2018), placing it among the most species-rich catfish genera. This genus includes essentially bottom-dwelling fishes (Boeseman, 1968) found across a wide range of environments, including floodplain lakes, deep river channels, lowland and upland small streams, rapids and waterfalls and even estuarine brackish waters (Casatti et al., 2005; Weber, 2003). In addition to their high abundance and species richness in the Amazon and La Plata System, representatives of *Hypostomus* can be found in the majority of Neotropical basins, from the coastal rivers of Costa Rica to central Argentina, including some Caribbean islands. This wide distribution is the result of an outstanding continental radiation experienced by *Hypostomus* throughout its evolutionary history (Montoya-Burgos, 2003; Silva et al., 2016).

Not surprisingly, along with the high species diversity and transcontinental radiation, the genus *Hypostomus* is characterized by high levels of intra-specific variation in morphology and color patterns, especially in species with a wide distribution range (Silva et al., 2016; Zawadzki et al., 2012). This characteristic has made challenging to properly delimit species on a morphological basis throughout the long and rich history of *Hypostomus* descriptions. Modern molecular phylogenetic methods have been very useful to solve taxonomic issues and unravel hidden diversity within *Hypostomus* (Cardoso et al., 2019, 2016, 2012, 2011; Montoya-Burgos, 2003; Weber and Montoya-Burgos, 2002).

The history of *Hypostomus* descriptions started in 1758, with the description of *H. plecostomus* by Linnaeus. 70 years later, Hancock (1828) described the second species, *H. watwata*. During the 19th century, approximately one *Hypostomus* species was described every three years (~ 0.3 species/year; Fig. 1), mainly by European descriptors, with the prominence of French (e.g., Achille Valenciennes and Francis de Laporte de Castelnau) and Austrian naturalists (e.g., Rudolf Kner and Franz Steindachner), but also with the contribution of the North American

Carl and Rosa Eigenmann. In the 20th century, the rate of species description increased to one species every 15 months (~ 0.8 species/year), mainly thought the work of North American naturalists, with punctual contributions by Europeans like the Dutch taxonomist Marinus Boeseman and the Swiss Claude Weber, and the first contributions by South Americans like Rodolpho von Ihering and Alípio de Miranda-Ribeiro. A more substantial increase in *Hypostomus* species description occurred during the 21st century, with one species described every five months (~ 2.3 species/year). Most of these descriptions were headed by South American taxonomists, who contributed the most to the exponential trend in new *Hypostomus* species descriptions (Fig. 1) and more generally to the knowledge of Neotropical fish biodiversity. Today, the curve of Hypostomus species description is far from plateauing (Fig. 1), suggesting that there are still many species to be described.

DNA-barcoding has become the most popular tool for rapid species identification based on DNA sequences. For fish taxonomy, a standardized region of the mitochondrial *Cytochrome oxidase I* (*COI)* of an unknown specimen is compared against a fish *COI* sequence database. Based on the sequence similarity, it is possible to identify a species or to find evidence suggesting that the species is new to science (or absent from the database) (Ward, 2012; Ward et al., 2009). It is suggested that a pairwise sequence divergence ≥ 2% with the closest relative would be an indication of distinct species, while a divergence < 2% would correspond to intra-specific variation (Ward et al., 2009). The effectiveness of this approach has been demonstrated in studies on both marine (Ribeiro et al., 2012; Ward et al., 2005) and freshwater fishes (Bhattacharjee et al., 2012; Lara et al., 2010). However, strong criticisms have been raised against the use of a single mitochondrial gene to delineate recently radiated species, which may show lower inter-specific pairwise divergences. The 2% *COI* divergence threshold is probably not appropriate for all fish groups, as the inter-specific divergence can often be much lower, at least among Neotropical fish (Pereira et al., 2013). The opposite situation is also true, since species with substantial phylogeographic structure can show more than 2% intra-specific *COI* pairwise divergence (Hickerson et al., 2006).

Alternative methods of species delimitation relying on multiple genes (multilocus) and based on the coalescence theory may cope with part of the limitations of the barcode divergence threshold approach (Jones, 2017; Jones et al., 2015; Rannala and Yang, 2013; Yang and Rannala, 2010). Multispecies coalescent (MSC) methods integrate explicitly ancestral coalescent processes, taking into consideration incomplete lineage sorting, to infer species delimitations or to identify independent evolutionary units (Jones, 2017; Lim et al., 2012). The MSC methods may also be useful to detect hidden diversity and recent speciation in some species complexes

with wide geographical distribution range and or high intraspecific variation. In general, correct species delimitation, or at least the delimitation of independent evolutionary units, is central for addressing questions about the evolution and biodiversity of any group.

Hypostomus has been shown to be a very interesting model to unravel the role played by hydrogeological history in shaping fish diversity and distribution in South America (Cardoso et al., 2012; Montoya-Burgos, 2003) as well as the nature of continental fish radiations in the Tropics (Silva et al., 2016). However, these studies were based on a limited taxonomic sampling, especially with a few species from the wide Amazon Basin and Guianese rivers. Thus, a more comprehensive phylogeny of this genus is required for deeper understanding of its extraordinary diversification and its use as a model taxon.

In this work, we present the most complete phylogeny of *Hypostomus* in terms of taxon sampling and geographical distribution, with 206 representatives coming from all of the main basins of the Neotropics. We analyzed two mitochondrial and four nuclear markers. To delineate independent evolutionary units, often corresponding to species, we combined conventional taxonomy and the MSC approach. We present an extension of the MSC approach, the Cluster Credibility (CC) index, which gives support to every alternative hypothesis of cluster delimitation, allowing an informed decision about the best delimitation of independent evolutionary units. Together, these methods allowed us to provide an assessment of the amount of hidden putatively new species in this genus. We also critically examine the validity of the classical *COI* barcode threshold of 2% pairwise divergence to delineate closely related *Hypostomus* species. Further, we present a broad view of the main *Hypostomus* lineages (super-groups) that have been inferred in our phylogeny, we discuss their interrelationships, and we provide diagnostic characters.

2 Material and Methods

2.1 Sampling, DNA extraction, amplification and sequencing

This study includes 206 samples of *Hypostomus* species and 49 additional samples of closely related genera. The samples were obtained from field expeditions or donations from scientific collections. Complementary sequence data were obtained from open access (OA) online databases (Appendix A: Supplementary material 1).

To extract the whole-genomic DNA from muscle or fin samples, which were preserved in 80–100% ethanol, we used either the commercial peqGOLD Tissue DNA kit (Peqlab Ltd., Darmstadt, Germany) or the saline method (Miller et al., 1988). We optimized PCR conditions to

amplify partial sequences of two mitochondrial and four nuclear genes (Appendix A: Supplementary material 2) according to the following criteria:

(*I*) Mitochondrial Cytochrome c oxidase subunit I protein-coding gene (*COI*): a fragment of 888 bp, including the classical barcode region for fish (Bucklin et al., 2011; Ward et al., 2009, 2005).

(*II*) Mitochondrial *D-loop*: we amplified a fragment of 620 bp of this hypervariable non-coding region. The *D-loop* shows an impressive accumulation of substitutions and indels (Brown et al., 1986; Saccone et al., 1987), which has been considered informative for phylogenetic studies in fish (Cardoso et al., 2016, 2012; Cardoso and Montoya-Burgos, 2009; Montoya-Burgos, 2003; Montoya-Burgos et al., 2002).

(*III*) Gene encoding Teneurin transmembrane protein 3: four genes of the teneurin group have been reported in vertebrates (Tucker and Chiquet-Ehrismann, 2006). These genes play an important role in the nervous system, being directly implicated in neuronal migration and development, and axonal guidance (Kenzelmann et al., 2007). For this study, we amplified an 1104 bp-fragment of the *Hypostomus*-homologous Teneurin transmembrane protein 3 (*Hodz3*, hereinafter, with the 'H' standing for *Hypostomus*). This fragment covers the exons 25 (partial), 26 and 27 (partial), including the introns 25 and 26.

(*IV*) A *Hypostomus* anonymous marker, possible gene *ZBTB10* intron 3 (HAM-*ZBTB10*-3): this marker is most probably a fragment of the intron number 3 of the gene *ZBTB10* (zinc finger and BTB domain containing 10), as identified in the genome of the catfish *Ictalurus punctatus* (Source: NCBI gene; Acc: 108257261). According to SINE Base (Vassetzky and Kramerov, 2013), it includes a sequence of 313 bp that is a putative SINE of the Ras1 type (Ogiwara et al., 2002). The complete marker included 1375 bp.

(*V*) *Recombination activating gene 1 (RAG1)*: the *RAG1* gene encodes the RAG1 protein. The final portion of the exon 1, the complete intron 1, and the initial part of the exon 2 were sequenced, totalizing 1359 bp.

(*VI*) Fish *Reticulon 4* gene: this gene is one of the members of the conserved reticulon (*RTN*) gene family (Pinzón-Olejua et al., 2014), composed of four paralogs (*RTN1*, *RTN2*, *RTN3*, and *RTN4*) (Diekmann et al., 2005) in vertebrates. The nuclear fish *reticulon 4* (*RTN4*) codes for neurite outgrowth inhibitor (Nogo-A), a membrane-bound protein that is widely expressed in the central nervous system (Berry et al., 2018). In the present work, we used a fragment of 1104 bp, which includes part of the first intron and the second exon of this gene.

PCR reactions were run in a volume of 25 µl, consisting of 5 µl 5× reaction buffer, 0.5 µl dNTP at 10 mM each, 0.5 µl of each primer at 10 µM, 0.5 µl $MgCl_2$ at 25 mM, 0.13 µl *Taq* DNA

polymerase equivalent to 1 U of polymerase per reaction, 1–5 µl DNA (depending on the DNA quality of the sample). DEPC-treated water was used to complete the solution to 25 µl. PCR profiles were as follows: (i) 2 min at 94 °C (initial denaturation); (ii) 30 s at 94 °C (denaturation); (iii) 30 s at 53 °C (annealing temperature for COI), 52 °C (*D-loop*, *RAG1* and *RTN4*) or 55 °C (*HAM-ZBTB10-3* and *Hodz3*); (iv) 60 s (*COI* and *RTN4*), 45 s (*D-loop*), 120 s (*HAM-ZBTB10-3*), 80 s (*Hodz3*) or 90 s (*RAG1*) at 72 °C (extension); and (v) 5 min at 72 °C. Steps ii–iv were repeated 38–40 and 40–42 times for the mitochondrial and nuclear genes, respectively.

All PCRs products were visualized in 1.7% agarose gel. The PCR products were purified either by using the commercial High Pure PCR Product Purification Kit (Roche, Penzberg, Germany) or the ExoSAP-IT™ Express reagent (Affymetrix, Santa Clara, CA, USA). Sequencing was performed at the sequencing facility RPT01A of the Oswaldo Cruz Foundation (Fiocruz is the Portuguese acronym; Rio de Janeiro, Brazil), Fasteris SA (Geneva, Switzerland) and Macrogen Inc. (Amsterdam, Netherlands). The sequences were manually aligned using BioEdit 7.2.5 (Hall, 1999). For downstream analyses, markers were concatenated in a single alignment using Sequence Matrix 1.8 (Vaidya et al., 2011). Details about the sequence lengths, missing data and other information can be found in Appendix A (Supplementary material 3).

2.2 Bayesian multispecies coalescent approach to define evolutionary units

Hypostomus is a taxonomically complex genus, composed of 149 recognized species (Eschmeyer et al., 2018; Froese and Pauly, 2018). The intraspecific range of morphological and color pattern variations can be very wide (Dias and Zawadzki, 2018). Therefore, the alpha taxonomy of many species still remains a challenge, and further revision of this group using modern methods is required (Cardoso et al., 2012). Previous studies have suggested that *Hypostomus* diverged from its closest lineage in the Mid Miocene, and many species have emerged only in the recent time (Montoya-Burgos, 2003; Silva et al., 2016). Consequently, incomplete lineage sorting may be a challenge when using molecular phylogenetic methods, as it typically affects recently diverged species (Maddison and Knowles, 2006). To cope with this issue we used STACEY (Jones, 2017), a multispecies coalescent (MSC) approach that identifies independent evolutionary units, which is implemented in BEAST 2 (Bouckaert et al., 2014). To avoid the complexity of the species concept, Jones (2017) recommend the use of the term ‘cluster’ when referring to the independent evolutionary units delimited by STACEY.

The cluster delimitation provided by STACEY must be interpreted with caution, as a cluster does not necessarily correspond to a species recognized by another species concept, or

from the perspectives of different fields, such as taxonomy, evolutionary biology, ecology, and others. Species delimitation methods have been criticized because they may overestimate the number of species by considering structured populations as species (Carstens et al., 2013; Chambers and Hillis, 2019; Sukumaran and Knowles, 2017; Yang et al., 2019). Yet, recent studies have suggested that STACEY is one of the most efficient methods to delimit species based on molecular data due to the strong congruence between the species it delineates and the morphologically recognized species (e.g. Klimov et al., 2019; Matos-Maraví, 2016; Sproul and Maddison, 2018; Vitecek et al., 2017). Although assessing species status from multispecies coalescent approaches may be controversial, it has been recognized as a valuable tool for evaluating species richness from genomic data (Leaché et al., 2019).

To optimize the STACEY's implementation, we first performed a maximum likelihood phylogenetic analysis with RAxML 7.2.8 (Stamatakis, 2006), based on the full set of 206 specimens of *Hypostomus*. As outgroups, we included 49 specimens of various genera that are closely related to *Hypostomus*, namely: *Hemiancistrus*, *Pterygoplichthys*, *Isorineloricaria, Aphanotorulus*, *Peckoltia, Hypancistrus, Panaqolus, Scobinancistrus, Panaque, Baryancistrus* and *Pseudacanthicus*. We followed the classification of Ray and Armbruster (2016), who consider *Aphanotorulus* as a synonym-senior of *Squaliforma*. Finally, one species of *Ancistrus*, a more distantly related genus, was used to root the tree. We used the rapid hill-climbing algorithm, setting the random tree search to 25. GTR+G was chosen as the nucleotide substitution model based on a search scheme using PartitionFinder 2 (Lanfear et al., 2016). The remaining parameters were set to the default values. Using the same parameters, we also ran a rapid bootstrap analysis with 1000 replicates (Stamatakis et al., 2008). This preliminary phylogenetic analysis served to identify the main clades with high support within *Hypostomus*, disregarding their inter-relationships and internal topologies (Appendix A: Supplementary material 4).

To infer cluster delimitation, we analyzed independently each well-supported main clade obtained in this preliminary RAxML analysis (Appendix A: Supplementary material 4–5). In some cases, we grouped more than one well-supported main clade and analyze them together to optimize informatic resources (Appendix A: Supplementary material 4–5). We applied the Bayesian multispecies coalescent method as implemented in the package STACEY 1.2.4 (Jones, 2017) for BEAST2 (Bouckaert et al., 2014). As STACEY does not require the samples to be a priori assigned to clusters, each sample in our analyses was treated as a potentially unique cluster. In order to reduce model overparameterization, to ensure computational feasibility and to reach convergence, we used six partitions, assigning each marker to a single partition.

BEAUTi 2.4.8 (Bouckaert et al., 2014) was used to set the XML file for running STACEY. Site models and clock models were unlinked among the partitions. Although *COI* and *D-loop* are both mitochondrial markers and often share similar evolutionary history, preliminary runs to calibrate the priors also showed very low convergence when the trees of the mitochondrial markers were linked. Since incongruence among mtDNA markers may arise from multiple reasons (Grechko, 2013), we unlinked the trees for all the makers. Moreover, rather than assigning specific substitution models for each partition, we used the model averaging tool bModelTest (Bouckaert and Drummond, 2017). This Bayesian method extracts from the data the information on which model is the most appropriate (Bouckaert and Drummond, 2017). For this, we used the default transition/transversion split option. We assumed a lognormal relaxed clock, which had the rate set to 1 for the *COI* and was estimated for all the remaining markers. Collapse height was fixed to 1×10^{-4}, while growth rate, relative death rate, collapse weight and origin height were estimated. We followed the recommendations of Jones (2017), and used a lognormal distribution with: mean equal to 4.6 ± 2 for the growth rate; a beta distribution [1,1] for the collapse weight; a uniform distribution (-0.5–0.5) for the relative death rate; and a lognormal (-7.0, 2.0) for the population size parameter.

Five independent runs of 10^7 to 10^8 generations, depending on the number of samples in each clade, were performed, sampling trees in order to obtain 10,000 trees *per* run. We confirmed the convergence and stationarity of parameter values by examining the effective sample size (ESS) values and likelihood plots, as well as the convergence among runs using Tracer 1.6 (Drummond and Rambaut, 2007). By visually inspecting the likelihood curve in Tracer 1.6, we also established the burn-in (10%). Since all the independent runs showed good mixing and convergence, we combined only three independent runs for performing downstream analyses. A tree file and a log file were combined using LogCombiner 2.4.7 (Bouckaert et al., 2014). The results from STACEY were processed in SpeciesDelimitationAnalyser (Jones et al., 2015), setting the collapsing height to 1×10^{-4}. The output was then summarized in R (R Development Core Team, 2017), using the script provided by Jones et al. (2015), but with custom modifications to automate some steps (Appendix A: Supplementary material 6).

During the STACEY MCMC runs, each sampled tree provides a hypothesis of clustering, i.e., a hypothesis of cluster delimitation. We took advantage of these data to develop an index that gives numerical support to every hypothesis of cluster delimitation. The index is the relative frequency of finding all the individuals of a given cluster grouped together and exclusively in the same cluster across all the trees sampled. We call this index ‘cluster credibility’ (CC). A script to

calculate the CC index for any combination of specimens is presented in Appendix A (Supplementary material 6).

In a few cases, group of specimens previously identified as the same species were found to be non-monophyletic lineages. In such cases, the species name was given to the lineage that included specimens from the type-locality.

2.3 Barcode divergence

We also aimed at assessing the validity of the popular *COI* barcode pairwise divergence threshold to discriminate species, the so-called 'barcode gap'. In fishes, a ~650 bp region of *COI* has been traditionally used for such a purpose, and a genetic divergence threshold of 2% has been proposed to discriminate inter-specific from intra-specific genetic divergence (Ward, 2012; Ward et al., 2009). Therefore, we calculated the pairwise genetic divergence between *Hypostomus* species, within species and between clusters, using the Kimura-2-parameter (K2P) model in Mega 6.06 (Tamura et al., 2013).

2.4 Molecular phylogenetic reconstructions

To reconstruct the phylogenetic relationships within *Hypostomus*, we first used RAxML 7.2.8 (Stamatakis, 2006), using 206 *Hypostomus* specimens and 49 samples of closely related genera, as listed before (Appendix A: Supplementary material 1). The monophylies of the clusters obtained from STACEY were constrained (flag *–g*). We added a single sample of *Ancistrus* sp. into the alignment for rooting the tree *a posteriori*. The concatenated alignment was partitioned by marker (*–q*) and the GTR+GAMMA model (*–m GTRGAMMA*) was set to four gamma rate categories (*–c 4*). We ran 100 iterations (*–# 100*) to search for the most likely tree based on the rapid hill-climbing algorithm (*–f d*) (Stamatakis et al., 2007). In parallel, we ran a bootstrap analysis with 1000 replicates (*–# 1000*) using the same parameters before.

As an alternative to the unrooted time-free model of phylogenetic inference provided by the RAxML software, we opted to apply a relaxed clock model of phylogenetic inference as implemented in BEAST 1.8.1 (Drummond et al., 2012; Drummond and Rambaut, 2007). Drummond et al. (2006) highlighted that time-free models may bias phylogeny estimations, thus decreasing the probability to find the true tree. These authors recommend that, even if phylogenetic relationships are of primary concern rather than dating, phylogenetic approaches with the explicit implementation of molecular clocks are the most appropriate (but see Werheim et al. 2010). To run BEAST, BEAUTI 1.8.1 (Drummond et al., 2012) was used to construct the XML file. We also constrained the monophyly of the clusters found by STACEY. The

concatenated alignment was partitioned by marker and the following substitution models were set based on PartitionFinder 2 results: *COI*: TRN+I+G; *D-loop*: TRN+I+G; *Hodz3*: GTR+G; HamZBTB10-3: GTR+G; *RAG1*: GTR+G; *RTN4*: GTR+I+G.

We used a lognormal relaxed clock for each partition, and set the Yule process as the tree prior, using a random starting tree. We used an exponential distribution prior on the clock rates, with mean of 0.01. Five independent runs were performed with 2×10^8 generations, sampling trees and log information every 20,000th generation. Tracer 1.6, Logcombiner 2.4.8 and Treeannotator 2.4.7 were used, as previously described in the section on STACEY analyses. Given that no calibration point was included in the phylogenetic reconstruction, the scale of the tree was expressed as an average number of substitutions per 'time' unit.

3 Results

Our morphological identifications, performed before running the molecular delimitations of evolutionary units (STACEY's clusters), resulted in 65 morphological species with valid names, which were hypothesized as monophyletic in the preliminary maximum likelihood tree reconstruction (Appendix A: Supplementary material 4). For another nine species, the morphologically identified specimens led to non-monophyletic lineages. Among them, the nine following species were split into two independent non-sister lineages: *H. ericae, H. nigromaculatus, H. regani, H. ancistroides, H.* cf. *borelli, H. weberi, H. plecostomus*. Three independent non-sister lineages were found for the two following taxa: *H. pyrineusi* and *H. affinis*. We gave the species name to the lineage with samples coming from the type-locality, while the remaining lineages were named differently and were considered as new cryptic species. As an example, the lineage of *H. plecostomus* from the Guianese rivers, where the types specimens were collected, was considered as *H. plecostomus*, while the Amazonian lineage was named *H.* sp. 1 'aff. *plecostomus*'. *H. ancistroides* was a special case, as none of the specimens of the two lineages morphologically identified as *H. ancistroides* were sampled in the type-locality. These two lineages were consequently called *H.* sp. 'gr. *ancistroides* 2' and *H.* sp. 'gr. *ancistroides* 4'. In our preliminary phylogenetic tree, two more non-identified *Hypostomus* species were intermingled among *H.* sp. 'gr. *ancistroides* 2' and *H.* sp. 'gr. *ancistroides* 4', and we called them *H.* sp. 'gr. *ancistroides* 1' and *H.* sp. 'gr. *ancistroides* 3' (Fig. 7; Appendix A: Supplementary material 5). This clade, composed of these four putative species, is named here the *H. ancistroides* species-complex. Finally, the nine non-monophyletic morphological species were reassigned to nine monophyletic morphological species plus 11 new cryptic species (7 + 4), totalizing 20 species.

A total of 68 specimens were not identified a priori and were named '*Hypostomus* sp.' as the specimens were still too young or, if adult, they could not be assigned to a species. Among these 68 specimens, 26 were found nested to one of the well-identified morphological species lineages of our preliminary phylogenetic tree, which allowed us to give them a species name. The remaining 42 specimens that could not be identified were organized into approximately 18 different terminal clades in our preliminary tree (Appendix A: Supplementary material 4).

3.1 STACEY Cluster delimitation

We implemented STACEY and interpreted the results of cluster delimitation following the standard procedure of visual identification of sudden color change (intensity of color being related to the probability of belonging to the same cluster). We then calculated our cluster credibility (CC) index for the clusters delineated in this classical way. In cases where the standard STACEY procedure did not provide clear delimitation of the clusters, different hypotheses of clustering were tested using the CC index (Appendix A: Supplementary material 5). The alternative with the best CC support was kept. With this approach, we identified 118 clusters, which showed CC values ranging from 0.14 to 1 (80% of the clusters had a CC index ≥ 0.8; CC values can vary from 0 to 1, with 1 being the maximum credibility). Among the 42 specimens that were not identified morphologically, STACEY results complemented with our CC index hypothesized 23 clusters, which are considered in the present work as putative species. By adding these 23 putative species to the 85 species identified previously, we reach a total of 108 species + putative species.

Nevertheless, we found that the specimens of some well-identified morphological species, although forming a monophyletic lineage, were organized into more than one STACEY cluster. For instance, the clade composed of *Hypostomus plecostomus* specimens (CC = 0.07) was split into two clusters: *H. plecostomus* 'A' (CC = 0.47) from the eastern Guianese rivers (Kaw River, in French Guiana, to Coppename River, Suriname) and *H. plecostomus* 'B' (CC = 0.40) from the western Guianese rivers (Corantijn and Berbice rivers). The clade grouping the specimens of *H. gymnorhynchus*, which had no support given by the CC index (CC = 0.0), included three clusters: *H. gymnorhynchus* 'A' (CC = 0.64) from the Oyapock River, *H. gymnorhynchus* 'B' (CC = 0.75) from the Opprouague River, and *H. gymnorhynchus* 'C' (CC = 0.68) from the Suriname River. The clade grouping the specimens of *H. hoplonites* (CC = 0.03) was divided into two clusters: *H. hoplonites* 'A' (CC = 0.77) from the Madeira River and *H. hoplonites* 'B' (CC = 0.97) from the Purus River. Distinct clusters were also found within *H. carinatus*, *H. uruguayensis*, *H. watwata, H. scabriceps, H. topavae* and *H. oculeus* (Appendix A:

Supplementary material 5). To be conservative, we did not consider such distinct clusters as putative species.

3.2 Barcode divergence

The *COI* barcode pairwise divergence between non-sister *Hypostomus* species varied from 0.1% to 17.5% (average = 4.2% ± 1.6%), and approximately 94% of these comparisons resulted in divergences higher than 2% (Fig. 2). If we restrict the comparisons to sister-species pairs that include at least one morphological species, the genetic divergence ranged from 0.1% to 4.3% (average = 1.3% ± 1.2%). In turn, intraspecific divergences ranged from 0% to 2% (average = 0.3% ± 0.5%).

We further determined that in approximately 82% of the sister-species comparisons (considering only comparisons between sister-species pairs that included at least one morphological species), the *COI* barcode pairwise divergence was ≥ 0.5%, while in about 78% of the intraspecific comparison, the divergence was < 0.5% (Fig. 2). Therefore, if we had to determine an adapted *COI* barcode divergence threshold to discriminate between closely related *Hypostomus* species, the value of 0.5% pairwise divergence may be a new tentative threshold.

To help clarifying the taxonomic status of morphological species composed of more than one cluster, we calculated the *COI* barcode pairwise divergence between clusters belonging to a same morphological species. Divergence ranged from 0.1% to 2% (average = 1% ± 0.6%), with only 1 case out of 28 being equal to 2% (*H. plecostomus* 'A' vs. *H. plecostomus* 'B'). Applying a 0.5% threshold, approximately 86% (24 out of 28) of the cases showed *COI* barcode divergence ≥ 0.5%.

3.3 Phylogenetic relationships

In our Bayesian (BEAST) phylogenetic inference (Fig. 3; Appendix A: Supplementary materials 8, 12 and 13), *Hypostomus* was strongly supported as a natural group (posterior probability, PP = 1). Among all the close genera used as outgroups in the phylogenetic reconstructions, *Hemiancistrus fuliginosus* plus *H. punctulatus* were found to form the sister-group of *Hypostomus* (PP = 1; Appendix A: Supplementary materials 8, 12 and 13). The genus *Pterygoplichthys* was retrieved as the sister group of *Hypostomus* + the two *Hemiancistrus* species (PP = 0.97; Appendix A: Supplementary materials 8, 12 and 13).

Within *Hypostomus,* we found four major lineages called super-groups, whose monophyly was well supported by BEAST analysis (Figs. 3–7). We named the super-groups after the first species described in the respective lineage: (i) *Hypostomus cochliodon* super-group (PP = 1), (ii) *H. hemiurus* super-group (PP = 1), (iii) *H. auroguttatus* super-group (PP = 1), (iv)

H. plecostomus super-group (PP = 1). *H. cochliodon* super-group is retrieved as the sister-lineage of all the remaining *Hypostomus*, while *H. hemiurus* super-group appears as the sister-lineage of *H. auroguttatus* + *H. plecostomus* super-groups (Fig. 3A).

3.4 Hypostomus cochliodon *super-group*

The *H. cochliodon* super-group was represented in our phylogeny by 26 morphological species (Fig. 4). Assuming the Tocantins-Araguaia River as part of the Amazon System, most of the species in this group are Amazonian (19 out of 26) (Appendix A: Supplementary material 9). A few species are distributed in the La Plata System (which includes Paraguay, Paraná and Uruguay rivers), such as *H. cochliodon* and *H. basilisko*. Three species are distributed in the Orinoco Basin (*H. plecostomoides*, *H. hemicochliodon* and *H. sculpodon*), three species in the Guianas (*H.* aff. *weberi, H. macushi* and *H. taphorni*), and one species in the Maracaibo and Magdalena basins (*H. hondae*).

The species *H. sculpodon* was retrieved as the sister-species of all the remaining species in the *H. cochliodon* super-group (Fig. 4). Six additional lineages compose the *H. cochliodon* super-group: (i) a clade comprising two non-identified species, *H.* sp. 'gr. *cochliodon*-Xin2' from Xingu River and *H.* sp. 'gr. *cochliodon*-Tar' from Tocantins-Araguaia (PP = 1), which is the sister-clade of the remaining five clades of the *H. cochliodon* super-group; (ii) *H. hondae* + *H. plecostomoides* (PP = 1), from the Magdalena/Maracaibo and Orinoco system, respectively, as sister-clade of the other four clades of the *H. cochliodon* super-group; (iii) a clade formed by Amazonian species, *H. oculeus* + *H. weberi* + *H. kopeyaka* + *H.* sp. 'gr. *cochliodon*-Xin' + *H. hemicochliodon* (the last is also present in the Orinoco River) + the Guianese *H. taphorni*, (PP = 1); (iv) a group composed of the pair of species from the Upper Amazon Basin *H. fonchii* + *H.* sp. 'PE08700' (PP = 1); (v) in a weakly supported group (PP = 0.59), *H. cochliodon* (Paraguay River) was found as the sister-species of a well-supported lineage (PP = 0.99) composed of the Amazonian *H. pyrineusi* + *H. soniae* + *H.* aff. *pyrineusi* + *H.* sp. 'gr. *cochliodon*-Tap' and the Paraguayan *H. basilisko*; (vi) and a last lineage, sister-clade of the group *v*, composed of *H. paucipunctatus* + *H. ericae* +*H.* sp. 'MNLM2640' + *H.* aff. *weberi* + *H. macushi* + *H.* sp. 'Huallaga' + *H.* sp. 'CA003' (PP = 0.91).

3.5 Hypostomus hemiurus *super-group*

The *H. hemiurus* super-group is a small clade, represented in our study by five species (Fig. 5). All of these species are endemic to the Guyanas coastal rivers, except *Hypostomus* sp. 'Paru', a potential new species that is distributed in some Amazonian tributaries draining the Guiana Shield. *Hypostomus* sp. 'Paru' is retrieved here as the sister-species to all remaining

species in this super-group. The next split concerns *H. hemiurus* as sister-species of *H. micromaculatus* + (*H. crassicauda* + *H. saramaccensis*) (PP = 1; Fig. 5).

3.6 Hypostomus auroguttatus *super-group*

The *H. auroguttatus* super-group is composed of a high number of species, with 39 included in this work (Fig. 6). It can be divided into three groups: *H. asperatus* (PP = 1), *H. auroguttatus* sensu stricto (PP = 1) and *H. regani* (PP = 1) groups. However, the *H. auroguttatus* and *H. regani* groups showed low bootstrap support (Fig. 6), indicating that the species composition of such groups is not stable. The monotypic lineage *H. nematopterus* is retrieved as the sister-species to the *H. auroguttatus* super-group (Fig. 3).

The *Hypostomus asperatus* group is the sister-clade to *H. regani* group + *H. auroguttatus* sensu stricto group (Figs. 3 and 6). The *H. asperatus* group is a relatively small lineage, represented in our phylogeny by seven species (Fig. 6). Members of this group cover a relatively wide geographical area: *H. francisci* and *H.* aff. *francisci* are present in the Rio São Francisco; the unidentified species *H.* sp. 'Contas' and *H.* sp. 'AZ4' are known only for the Rio Jequitinhonha and Rio de Contas, respectively, drainages of Bahia state; and *H. asperatus*, *H.* sp. 'Maranhão' and *H.* sp. 'BR1100' are distributed in the Tocantins-Araguaia River.

The *H. regani* and *H. auroguttatus* sensu stricto groups hold higher species diversity, with 17 and 14 species, respectively, included in our study (Fig. 6). The *H. auroguttatus* sensu stricto group is mostly distributed in the La Plata River System (Appendix A: Supplementary material 9), except for *H. auroguttatus* itself (Rio Paraíba do Sul) and *H. faveolus* (Xingu and Tocantins-Araguaia rivers). The *H. regani* group is also a lineage from the La Plata System, with the exception of *H. goyazensis* (Tocantins-Araguaia River).

3.7 Hypostomus plecostomus *super-group*

The *H. plecostomus* super-group is a species-rich lineage. In our phylogeny, 38 morphological species were included (Fig. 7). This group is distributed all over the main basins of the Neotropics. Five groups were identified within this super-group: *H. carinatus* group (PP = 1), *H. watwata* (PP = 1), *H. robinii* (PP = 1), *H. plecostomus* sensu stricto (PP = 1) and *H. punctatus* groups (PP = 0.99) (Figs. 3 and 7). The species *H. niceferoi* was not assigned to any specific group because it was found as sister-species of *H. plecostomus* s. str. + *H. punctatus* group (PP = 1).

The *H. carinatus* group was found as the sister-clade to the remaining *H. plecostomus* lineages (Fig 3). The two morphological species of this clade, *H. carinatus* and *H. hoplonites* (Fig. 7), are both strictly Amazonian (Appendix A: Supplementary material 9).

Concerning the *H. watwata* group, two sub-lineages were identified: one composed of three non-identified Amazonian species (*H.* sp. 'Cris' + *H.* sp. 'Aripuanã' + *H.* sp. 'Teotônio'; PP = 1), all of them known only from the Madeira River Basin; and another group that is endemic to the Guyanese rivers, including *H. watwata*, *H. gymnorhynchus, H. coppenamensis*, *H. corantijni* and *H. paucimaculatus* (PP = 1) (Fig. 7).

The *H. robinii* group is retrieved as sister-group of the *H. plecostomus* s. str. + *H. punctatus* groups + the species *H. niceforoi* (Fig. 7). This is a small lineage composed of one species from the island of Trinidad (*H. robinii*), one species from the Orinoco (*H. ranthos*) and another species from the Magdalena System (*H. holostictus*) (Appendix A: Supplementary material 9).

The *H. plecostomus* s. str. group includes *H. plecostomus* from the Guianas coastal rivers, the Amazonian *H. plecostomus*-like (referred here as *Hypostomus* sp. 1 'aff. *plecostomus'*), *H.* sp. 'L231' (also from the Amazon Basin), *H. formosae* (Paraguay River), *H.* sp. 'Pindaré' (Brazilian Atlantic coastal rivers, including Rio Pindaré and Rio Itapicuru), *H.* sp. 'MNRJ35628' (Tapajós River) and *H. pusarum* (Brazilian Atlantic coastal rivers).

The last clade, the *H. punctatus* group, includes species inhabiting mostly southern Neotropical basins. The *H. ancistroides* species-complex (distributed in the Upper Paraná River) appears as the sister clade of all the remaining species in this group (Fig. 7). We also retrieved a small lineage that is endemic to the Southeastern Atlantic costal drainages (*H. interruptus* + *H. affinis* + *H.* cf. *punctatus* + *H. scabriceps*), while the remaining species are distributed over the La Plata System (e.g., *H. derbyi, H. cordovae* and *H. commersoni*). However, a single species from the Upper Madeira River was also nested in this part of the phylogeny, *H.* sp. 'Rio Grande', and it was found as sister-species of *H.* cf. *borellii* (Fig. 7), which inhabits the Upper Pilcomayo (PP = 1).

4 Discussion

4.1 Evolutionary units, species delimitation and hidden diversity

Species are fundamental units in biology, hence their correct delimitation is essential in many different fields (Fujita et al., 2012). However, the concept of species has been defined in several ways and remains controversial. With the fast growing acquisition of genetic data and the rapid development of bioinformatics tools, a variety of methods have been proposed and popularized to delimit independent evolutionary units, or clusters, often assigned as species (e.g., Ence and Carstens 2011; Fujisawa and Barraclough 2013; Jones 2017; Jones et al. 2015; Masters et al. 2011; O'Meara 2010; Puillandre et al. 2012; Yang and Rannala 2010; Zhang et al. 2013).

In the present work, we used the Bayesian multispecies coalescent STACEY approach (Jones, 2017) to delimit evolutionary units within *Hypostomus.* STACEY has many advantages over other methods, such as the non-dependence on a guide tree, no requirement to assign individuals to species or clusters a priori, and it converges much faster than similar Bayesian approaches (Jones, 2017). Moreover, STACEY takes into consideration incomplete lineage sorting by making use of multilocus sequence data. This aspect is particularly important because, as speciation is a continuous process (De Queiroz, 2007) and establishing species limits may be a very difficult task depending on its position in the 'spectrum' of speciation (sensu Shapiro et al. 2016). Thus, STACEY may detect recent speciation events even though reciprocal monophyly has not been reached in all genomic loci (Lim et al., 2012).

Nonetheless, defining the limits of clusters based on STACEY output is sometimes not straightforward. In fact, STACEY does not propose explicit cluster hypotheses, but provides a general pairwise matrix showing the probability that two samples belong to the same cluster (or species). Thus, the researcher must often make subjective decisions. To help resolving this issue, we proposed an index that expresses the posterior support for each cluster, calculated as the relative frequency at which a given cluster was sampled during the MCMC runs (after the burn-in). We call it the cluster credibility (CC) index. Such index allows the user to assess *a posteriori* as many cluster hypotheses as desired, each one with its CC support. Thus, it is possible to compare distinct scenarios, including clusters made of a single individual, and to identify in an informed manner the one with the highest CC support.

We found the CC to be particularly useful when species delimitation was unclear using the standard STACEY procedure. An example involves the sister-species *H. arecuta* and *H. meleagris*. Based on the STACEY output, the single sample that was identified morphologically as *H. meleagris* showed approximately 39% probability to belong to the *H. arecuta* cluster. However, we had morphological and biogeographical evidence that they were different species. Therefore, we used the CC index to clarify this situation. When we assumed that all the samples belonged to the same cluster, the CC value of this unique cluster was relatively low (~0.37). On the contrary, the CC values were substantially higher when splitting the samples into two clusters, *H. arecuta* with CC = 0.51, and *H. meleagris* with CC = 0.48. Thus, the CC values clearly showed more support for the hypothesis of two distinct clusters (and species in this case) (Appendix A: Supplementary material 5).

Our study is the first to include such an amount of *Hypostomus* species: 74 morphologically identified valid species + 11 morphologically cryptic evolutionary units + 23 clusters which are putative new species. Thus, our results highlight an important fraction of

hidden diversity, since 34 out of 108 evolutionary units (~31%) are most probably new species to science.

Most of the clusters proposed by STACEY were in agreement with the morphological species. However, STACEY results suggested that some morphological species were in fact composed of distinct clusters. Some examples are *H. plecostomus*, *H. gymnorhynchus*, *H. carinatus*, and *H. hoplonites*, among others (Appendix A: Supplementary material 5). Within all these examples, the clusters are always distributed in allopatry. These groups could be considered as species-complexes but, for conservative reasons, we assumed them in this work as the outcome of structured allopatric populations belonging to a same species.

Coincidently, *H. plecostomus* used to be split into more species in the past. One of them, *Hypostomus ventromaculatus*, was described by Boeseman (1968). Le bail et al. (1996) even recognized a third morphotype, *H.* cf. *ventromaculatus.* However, at a later stage, *H. ventromaculatus* was considered as a synonymous-junior of *H. plecostomus* (Weber et al., 2012). A similar case is found in *H. gymnorhynchus.* Boeseman (1969) suggested the presence of three different subspecies within *H. gymnorhynchus*. Their geographical distributions matches with the distribution of the three clusters of *H. gymnorhynchus* identified in this work. Thus, the clusters within *H. plecostomus* and *H. gymnorhynchus* are probably in agreement with past taxonomic rearrangements in this group, and suggest that a deeper taxonomic investigation may corroborate the presence of distinct species. However, these clusters may well represent structured populations instead of species or subspecies, as the method we used to delineate clusters has been criticized in this same respect (e.g. Carstens et al., 2013; Chambers and Hillis, 2019; Sukumaran and Knowles, 2017; Yang et al., 2019). Moreover, as some of our independent evolutionary units (or clusters) show no pattern of morphological differentiation, there are no sufficient indications that these clusters are potential cryptic species.

4.2 Validity of the COI barcode to discriminate species

We also aimed to assess the validity of the 2% threshold of *COI* barcode pairwise divergence widely employed to discriminate species, the so-called 'barcode gap'. The mitochondrial *COI* gene has been widely used as a DNA tool for the recognition and identification of animal species (Hajibabaei et al., 2006; Hebert et al., 2004; Meyer and Paulay, 2005; Nagy et al., 2012). In fishes, a ~650 bp region of *COI* has been traditionally used for such purpose, and a pairwise divergence threshold of 2% has been proposed to discriminate inter-specific from intra-specific genetic divergence (Ward, 2012; Ward et al., 2009).

Our results showed that pairwise divergence between non-sister species varied from 0.1% to 17.5% (average = 4.2% ± 1.6%). However, about 94% of these comparisons resulted in divergences equal or higher than 2%, indicating that this *COI* divergence threshold can be used to distinguish non-closely related species within the genus *Hypostomus*. Regarding sister-species pairs that involved at least one morphological species, 82% showed divergence values lower than 2% (range: 0.1–4%; average = 1.3% ± 1.2%). Furthermore, many well-recognized species in terms of morphology, ecology and color patterns showed low *COI* barcode pairwise divergence with their closest relatives, such as *H. coppenamensis* vs *H. corantijni* (0.6–0.8%), *H. hondae* vs *H. plecostomoides* (0.8%), *H. regani* vs *H. luteomaculatus* (0.6%), *H. hermanni* vs *H. margaritifer* (0.8%), *H. auroguttatus* vs *H. luteus* (1.2%) and *H. myersi* vs *H. paulinus* (1.6%). Thus, the standard threshold of 2% pairwise divergence to discriminate between closely related species cannot be applied to *Hypostomus* species.

Pereira et al. (2013) emphasized that the threshold of 2% may be used as a starting point, but should not be applied to all species of Neotropical fishes, as they demonstrated that many congeneric fish species may have divergent values lower than 2%. In the present work, we showed that in approximately 82% of the sister-species pairs including at least one morphological species, the *COI* barcode divergence was ≥ 0.5%, whereas in 78% of the intraspecific comparisons, the *COI* barcode divergence was < 0.5%. As a consequence, if we had to provide an adapted value for our genus, we would suggest that 0.5% is a better threshold to delimit species within *Hypostomus*. According to this threshold, five out of nine species that were morphologically identified, but were composed of different clusters, should be split into more than one species, as the *COI* barcode divergence was ≥ 0.5%: *H. carinatus* (*COI* barcode divergence: 0.8–1.2%), *H. hoplonites* (1.4%), *H. oculeus* (0.9–1.1%), *H. plecostomus* (1.4–2%) and *H. topavae* (0.9%). This exemplifies that our adapted 0.5% threshold may result in delimitations that partly disagree with current taxonomy. Therefore, we do not recommend the use of a standard DNA-barcode divergence threshold if there is availability of other diagnostic characters such as morphology, anatomy, ecology or biogeography.

4.3 The monophyly of Hypostomus

Since the removal of *Aphanotorulus* (=*Squaliforma* sensu Ray and Armbruster, 2016) and *Isorineloricaria* from the genus *Hypostomus* (Lujan et al., 2015) and the proposition of *Cochliodon* as synonymous-junior of *Hypostomus* (Weber and Montoya-Burgos, 2002), the monophyly of *Hypostomus* seemed to be established, and has been hypothesized with strong support in molecular phylogenies (e.g. Cardoso et al., 2019, 2012; Silva et al., 2016). The only

exception is a recent publication that founds '*Hemiancistrus' cerrado* nested in the *Hypostomus sensu stricto* clade (Roxo et al., 2014), indicating that this species is in fact a *Hypostomus* as well. In the present work, we also retrieved *Hypostomus* as a monophyletic group, well supported in both maximum likelihood and Bayesian phylogenetic approaches.

However, different sister-groups of *Hypostomus* have been suggested in the literature. The hypotheses of *Pterygoplichthys* (Montoya-Burgos et al., 1998; Silva et al., 2016) or *Pterygoplichthys* + *'Hemiancistrus*' *annectens* group (Armbruster, 2008, 2004) seem to lack taxonomic sampling among the close outgroups. In fact, the species of *'Hemiancistrus' annectens* group have been recognized to belong to the *Hypostomus* genus (Lujan et al., 2015), and our single species representing this lineage, *Hyp. holostictus*, corroborates this finding.

Phylogenetic studies covering a wider range of closely-related genera have indeed pointed to a specific sub-group of '*Hemiancistrus'* as the closest group to *Hypostomus* (Cardoso et al., 2012; Lujan et al., 2015; Montoya-Burgos, 2003; Montoya-Burgos et al., 2002; Roxo et al., 2019), as also observed in the present work. *Hemiancistrus medians* has also been found as sister group of *Hypostomus* (Covain and Fisch-Muller, 2012), but according to the phylogeny published by Lujan et al. (2015), *Hem. medians* is not related to other taxa currently placed in the genus *Hemiancistrus*, and is positioned far from the *Hypostomus* clade. The genus *Hemiancistrus* has been shown to be paraphyletic, and it is very likely that *Hemiancistrus* might be monotypic, including only *Hem. medians* (Armbruster et al., 2015; Lujan et al., 2015). Thus, the two *Hemiancistrus* species found to be the closest relatives to *Hypostomus* in the present work, *Hem. fuliginosus* and *Hem. punctulatus*, are expected to be placed in a different genus in future studies.

Our phylogenetic tree revealed new lineages within *Hypostomus*, in particular, the *H. hemiurus* super-group. In general, the taxonomic composition of the super-groups and groups we have found is compatible with previous studies (Cardoso et al., 2012; Lujan et al., 2015; Montoya-Burgos, 2003; Montoya-Burgos et al., 2002; Silva et al., 2016), but disparities in the relationships among such lineages are observed (Fig. 3).

4.4 Hypostomus cochliodon *super-group*

In our study, the *H. cochliodon* super-group is strongly supported as the sister-lineage of all remaining *Hypostomus* (Fig. 3). Silva et al. (2016) have also found a very strong support for this relationship (Fig. 3E). Nevertheless, the topologies of Montoya-Burgos et al. (2002), Montoya-Burgos (2003), Cardoso et al. (2012) and Lujan et al. (2015) showed that the *H. cochliodon* super-group is nested within *Hypostomus*, placed as sister-lineage of the *H.*

plecostomus super-group (Fig. 3B–D). This relationship hypothesis was used by Montoya-Burgos et al. (2002) to propose '*Cochliodon*' as a junior synonym of *Hypostomus*. Moreover, the discovery of *H. fonchii, H. sculpodon* and *H. hemicochliodon*, characterized by teeth with intermediate structures between *Hypostomus* and *'Cochliodon'*, has been a strong argument for the synonymization (Armbruster, 2004, 2003; Weber and Montoya-Burgos, 2002).

The *H. cochliodon* super-group is one of the easiest lineages to be diagnosed (Table 1), supported by the following synapomorphies: notch loss between the metapterygoid and hyomandibula, a strongly curved maxilla, and a notable spoon-shaped teeth (Armbruster, 2004). The teeth morphology is used to scratch wood, the main substrate where these species are found (Lujan et al., 2011; Nelson et al., 1999). The xylophagous diet of these wood-eating catfishes is suggested as an adaptive specialization rather than a simple facultative foraging strategy (Lujan et al., 2011), and it is employed as a diagnose character of the *H. cochliodon* super-group (Armbruster, 2003).

The geographical distribution of the *H. cochliodon* super-group is not homogeneous across the continent. The highest diversity is concentrated in the Amazonian Basin (Armbruster, 2003). In our phylogenetic reconstruction, approximately 75% of the species belonging to this super-group were distributed in the Amazon System, including the Tocantins-Araguaia Basin (Appendix A: Supplementary material 9). The remaining species are distributed over the Guianese coastal rivers, Orinoco Basin, Maracaibo-Magdalena System, and Paraguay River. To our knowledge and to date, no species of the *H. cochliodon* super-group have been found on the Atlantic Brazilian coast or in the São Francisco Basin.

Table 1. Compilation of relevant diagnostic characters for the super-groups of *Hypostomus* retrieved in the present work. The characters are not always exclusive to each group.

Clade	Proposed diagnostic characters	Source	Montoya-Burgos (2003)'s codes
Hypostomus cochliodon super-group	Dentary rami angled to each other less than 80°. Preoperculo-hyomandibular ridge deflected posterior to the main body of the hyomandibula. Presence of large spoon-shaped teeth (cochleariform teeth). Diet specialized in wood. Area of the preoperculo- hyomandibular ridge deflected posteriorly. Presence of a highly curved maxilla. Loss of the buccal papilla. Keels along the lateral series of plates.	(Armbruster, 2004, 2003; Weber and Montoya-Burgos, 2002; Zawadzki et al., 2013)	D1
Hypostomus hemiurus super-group	A combination of the following characteristics: endemic to the Guiana Shield drainages; depth of caudal peduncle 1.35–1.7 in interdorsal length (shared with *H. plecostomus* s. str. group); reophilic biotopes. Species of this super-group show a larger	(Boeseman, 1968; Weber et al., 2012); this study for geographical distribution, and mouth size.	–

	mouth compared to the other Guianese species,.		
Hypostomus auroguttatus super-group	Large size of the mandible and high number of teeth (25–125), characterized by a long crown. Pale blotches over the body and fins. Abdominal area often naked or partially naked. Distribution: Tocantins-Araguaia, São Francisco, Northeastern Brazilian coastal rivers, La Plata System, Eastern Brazilian Atlantic coast. Only *H. faveolus* is found in the Amazon Basin.	(Muller and Weber, 1992; Zawadzki et al., 2008); this study for geographical distribution	D3 = *H. asperatus* and *regani* groups D4 = *H. auroguttatus s. str*. group
Hypostomus nematopterus	In young individuals, extremely elongated dorsal-fin spine and anterior branch of its first dorsal fin ray, extending beyond the base of the caudal fin. Adult individuals are very similar to *H. gymnorhynchus*, but can be distinguished by round dark dots (*vs.* horizontally elongated) dispersed over the body (vs. concentrated).	(Isbrücker and Nijssen, 1984; Weber et al., 2012)	–
Hypostomus plecostomus super-group	Small to intermediate size of the mandible with a low number of teeth (6–41). The teeth are characterized by a short crown. Abdominal area completely plated. Dark blotches over the body and fins. Moderate to well-developed rows of odontodes on keels.	(Montoya-Burgos et al., 2002; Muller and Weber, 1992; Weber et al., 2012; Zawadzki et al., 2008)	D2

4.5 Hypostomus auroguttatus *super-group*

The *H. auroguttatus* super-group includes three groups: the *H. auroguttatus s. str*., the *H. regani* and the *H. asperatus* groups. The *H. asperatus* group is found only in the Tocantins-Araguaia River, São Francisco River and Northeastern Brazilian coastal rivers (Appendix A: Supplementary material 9). The *H. auroguttatus s. str.* and *H. regani* groups, on the other hand, are mostly distributed in the Southern basins of South America, especially in the La Plata System. However, some species from these two lineages are distributed in the Amazon System, São Francisco River and in the Eastern Brazilian Atlantic coast. Within these groups, the monophyly of the numerous species formed *in situ* in the Paraná River and their wide morphological and color diversity, suggested that *Hypostomus* underwent an adaptive radiation in this region (Silva et al., 2016).

The only Amazonian species within the *H. auroguttatus* super-group is *H. faveolus* (except for *H. goyazensis* from the Tocantins-Araguaia River). Initially, *H. faveolus* was assigned to the *H. plecostomus* super-group, based on the number and morphology of teeth, presence of keels over the body and a completely plated abdominal area (Zawadzki et al., 2008). However, *H. faveolus* was also diagnosed by the presence of pale blotches over the body and fins, a characteristic known only for the *H. auroguttatus* super-group. Instead, species of *H. cochliodon* and *H. plecostomus* super-groups often show dark blotches (Zawadzki et al., 2008). Therefore, this color pattern seems to be consistent across species from the *H. auroguttatus*

super-group, and supports the genetic placement of *H. faveolus* with the *H. auroguttatus* super-group.

Our composition of the *H. auroguttatus* super-group is compatible with all the *Hypostomus* phylogenies published to date. However, the sister-group relationship of *H. auroguttatus s. str.* and *H. regani* groups has not been proposed before. In the phylogenies of Montoya-Burgos et al. (2002), Montoya-Burgos (2003) (Fig. 3B), and Cardoso et al. (2012) (Fig. 3C), the *H. asperatus* group is found as the sister-clade of the *H. regani* group.

The *H. auroguttatus* super-group has also been characterized by the presence of wide dentaries, in opposition to species from the *H. cochliodon* and *H. plecostomus* super-groups (Muller and Weber, 1992). Furthermore, the wide mandibles reflect directly in the number of teeth: species from the *H. auroguttatus* super-group were suggested to show a higher number of teeth (25–125) compared to the *H. plecostomus* super-group, which would present a lower number (6–41) (Muller and Weber, 1992; Weber et al., 2012).

4.6 Hypostomus nematopterus*: a potential relictual species*

This is the first time, to our knowledge, that the species *H. nematopterus* is included in the phylogenetic inference of *Hypostomus*. This rare species seems to have a very limited geographical distribution. It is only known from its type-locality, the Oyapoke River (Isbrücker and Nijssen, 1984; Weber et al., 2012) and only three specimens have been sampled to date: the holotype and the paratype used in the original description (Isbrücker and Nijssen, 1984) and the individual used in the present work, which was also reported in a study of *Hypostomus* species from the Guianas (Weber et al., 2012). Besides the low abundance and restrict distribution, *H. nematopterus* is a morphologically conspicuous species. Young specimens can be easily recognized by an extremely elongated dorsal-fin spine, extending beyond the base of the caudal fin (Table 1) (Isbrücker and Nijssen, 1984; Weber et al., 2012). On the other hand, adults *H. nematopterus* are very similar in morphology to *H. gymnorhynchus*, but they differ in color pattern: *H. gymnorhynchus* shows horizontally-elongated dark dots covering the body, while in *H. nematopterus* they are more rounded and more dispersed.

Even if most of the Guianese *Hypostomus* species are closely related to each other (notably *H. hemiurus* super-group and *H. watwata* group), *H. nematopterus* shows no close relationship with species from the Guianas coastal rivers (Figures 1 and 4). This species appears as sister-group of the *H. auroguttatus* super-group*,* a clade mostly distributed in Southern basins of South America, suggesting that *H. nematopterus* originated from an old and independent colonization of the Oyapock River, probably by an Amazonian ancestor.

4.7 Hypostomus plecostomus *super-group*

In our work, the *H. plecostomus* super-group is composed of five groups: the *H. plecostomus s. str.,* the *H. punctatus*, the *H. robinii,* the *H. watwata* and the *H. carinatus* groups (Figs. 1 and 5). The species *H. niceferoi* was not assigned to any of these groups, since it appears as the sister-species of *H. plecostomus s. str*. + *H. punctatus* groups. In previous publications, the *H. plecostomus* super-group was represented by a lower number of internal lineages. Only the *H. plecostomus s. str., H. punctatus* and *H. watwata* groups appeared in the phylogenies of Montoya-Burgos et al. (2002), Montoya-Burgos (2003) and Cardoso et al. (2012), whereas Lujan et al. (2015) and Silva et al. (2016) included only the *H. punctatus* and the *H. robinii* groups (Fig. 3).

The *H. plecostomus* super-group is a well-supported lineage and its monophyly has been supported in previous studies. Only Silva et al. (2016) did not infer the monophyly of this super-group as conceived in our study (Fig. 3E), since these authors found the *H. robinii* group, a internal lineage of the *H. plecostomus* super-group, as sister-lineage of the *H. auroguttatus* super-group. The *H. plecostomus* super-group has been tentatively diagnosed by the small size of the mandible accompanied by a low number of teeth (Montoya-Burgos et al., 2002; Muller and Weber, 1992; Weber et al., 2012) when compared to the *H. auroguttatus* super-group (Table 1). Moreover, the colour pattern seems to be different between these two groups, as *H. plecostomus* super-group species present often dark blotches over the body and fins (Zawadzki et al., 2008).

We found that the *H. plecostomus s. str*. group is the sister-clade of the *H. punctatus* group. An identical result was found by Montoya-Burgos et al. (2002) and Montoya-Burgos (2003). Lujan et al. (2015) were the first authors to include the *H. robinii* group in a phylogeny. They found it as the sister-clade of the *H. punctatus* group, but they did not include any species of the *H. plecostomus s. str.* group in their study. However, the *H. robinii* group was hypothesized to be the sister-clade of the *H. auroguttatus* super-group in the topology of Silva et al. (2016) (Fig. 3).

Our *H. robinii* group contains *H. holostictus*, a species that was originally placed in the genus *Hemiancistrus*. Armbruster (2008, 2004) found *H. holostictus* to belong to a group of '*Hemiancistrus*' species that he called the '*Hemiancistrus' annectens* group, suggesting that this group could be a new genus. Interestingly, Armbruster's *annectens* group was hypothesized to be the sister-lineage of *Pterygoplichthys* based on morphological analyses (Armbruster, 2008, 2004), but the present study corroborates the hypothesis proposed by Armbruster et al. (2015), which places the *annectens* group within *Hypostomus sensu stricto.*

The *H. plecostomus* super-group is a very species-rich group distributed in all the main basins of Tropical South America. However, the lineages of this super-group show a marked geographical organization with clear patterns of distribution (Appendix A: Supplementary material 9). For instance, the *H. watwata* group is mainly a Guianese lineage, with the exception of a small monophyletic lineage of Amazonian species (*H.* sp. 'Cris' + *H.* sp. 'Aripuanã' + *H.* sp. 'Teotônio'). The *H. carinatus* group is also Amazonian, while the *H. robinii* group seems to represent an ancient connection between the island of Trinidad, Orinoco River, and Magdalena and Maracaibo systems. Further, within the *H. punctatus* group, there is also a monophyletic group that is exclusive to the Southeastern Brazilian Atlantic coast, which includes *H. scabriceps, H. affinis*, *H. interruptus* and *H.* cf. *punctatus* (Fig. 5). The *H. punctatus* group may represent a very recent colonization and radiation into the Atlantic coastal rivers.

4.8 Hypostomus hemiurus *super-group*

Based on body measurements, Boeseman (1968) considered *H. saramaccensis* + *H. crassicauda* as a sub-group of the *H. plecostomus* super-group. These two species were indeed found to be closely related in our work, but they were placed in another clade, the *H. hemiurus* super-group. In our phylogeny, the *H. hemiurus* super-group is the sister-group of a clade comprising the *H. plecostomus* and the *H. auroguttatus* super-groups (Fig. 3). As all the species of the *H. hemiurus* super-group are endemic to the Guianas coastal rivers, except for *H.* sp. 'Paru' (Appendix A: Supplementary material 9), their phylogenetic placement indicates a very early colonization of this region. The other species inhabiting Guianas belong to the *H. watwata* and *H. plecostomus* s. str. groups, and their ancestors had probably colonized the Guianas independently and later in time.

5 Conclusions

In this work, we showed that applying a multispecies coalescent approach to delimit evolutionary units, or clusters, has a great potential to help in the identification of internal lineages within *Hypostomus* species, especially among those with an obscure taxonomic status, such as *H. plecostomus*, *H. gymnorhynchus*, *H. affinis*, *H. ancistroides* and *H. scabriceps*. Moreover, this approach allowed us to unravel an important fraction of the hidden *Hypostomus* diversity, since about 31% of the *Hypostomus* species (34 out of the 108) included in our work have the potential to be new to science.

Considering the limits of the coalescent-based cluster delimitation, in particular the somewhat subjective delimitation decision, we proposed a new tool, the cluster credibility (CC) index. The CC gives a support for any hypothesis of cluster delimitation based on STACEY

output, allowing to evaluate competing hypotheses. Our index has also the advantage of testing the hypothesis of a cluster being represented by a single specimen.

We assessed the efficiency of the *COI* barcode region to discriminate between closely related species of *Hypostomus* using the 2% pairwise divergence threshold. We have shown that this traditional threshold may be useful to identify non-closely related *Hypostomus* species, but it fails to distinguish sister-species. Instead, with an error rate of about 20%, a tentative threshold to discriminate between closely related *Hypostomus* species would be 0.5% of *COI* barcode pairwise divergence. However, we do not recommend the use of a standard pairwise divergence threshold of any value if additional diagnostic characters are available.

We then presented the largest hypothesis of phylogenetic relationships among species of *Hypostomus,* including 108 morphological species plus new evolutionary units covering all the main basins of South America, based on six molecular markers. The inclusion of a great diversity of closely related genera provides additional and convincing support for the monophyly of *Hypostomus*. Our phylogenetic results revealed four main *Hypostomus* lineages, namely *H. cochliodon, H. hemiurus, H. auroguttatus* and *H. plecostomus* super-groups. We have compiled diagnostic characteristics and distribution for the super-groups.

In the future, our comprehensive phylogeny of *Hypostomus* can be utilized as the baseline for inferring the biogeographical history of this genus across the Neotropics. Our results will also be valuable to motivate macroevolutionary studies, providing crucial information to understand the timing and modes of diversification, and also the evolutionary patterns of *Hypostomus* traits. We expect that the on-going use of *Hypostomus* as a biogeographic and macroevolutionary model organism will shed more light on the complex processes that have driven the extraordinary freshwater fish diversification in the Neotropics.

Funding

This work was supported by the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq–Program Sciences without Borders, 229237/2013-4, granted to LJQ; 306054/2006-0, granted to CO; 307775/2018-6 and 424668/2018-1, granted to TEP), the Brazilian–Swiss Joint Research Programme (S18794 BSJRP, granted to JIMB and GTV), the Swiss Seed Money Grants Latin America 2015 (granted to JIMB and YPC), the Swiss National Science Foundation (SNSF 3100A0-104005, granted to JIMB), the *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP, 2014/26508-3 and 2016/09204-6, granted to CO), the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES, Finance Code 001, granted to TEP), and the Claraz Foundation (granted to JIMB).

Acknowledgments

For sample donation, we thank: Dr. Camila Ribas (INPA, Manaus, Brazil), Dr. Sonia Fisch-Müller (MHNG, Geneva, Switzerland), Dr. Ronaldo Pinheiro (*Museu Mello Leitão*, Santa Teresa, Brazil), Dr. Ângela Zanata (UFBA, Salvador, Brazil), Dr. Fabrício Barreto Teresa (UEG, Anápolis, Brazil), Dr. Nivaldo Piorski (UFMA, São Luís, Brazil), Dr. José Luís Costa Novaes (UFERSA, Mossoró, Brazil), Dr. Igor David da Costa (UNIR, Presidente Médice, Brasil), Dr. Pablo Lehmann (UNISINOS, São Leopoldo, Brazil), Dr. Nathan Lujan (American Museum of Natural History, New York, USA), Dr. Margaret Zur and Dr. Mary Burridge (Royal Ontario Museum, Canada), and Dr. Jonathan Armbruster and Dr. David Werneke (Auburn University, USA). We are also grateful to the team of the sequencing facility RPT01A of the *Fundação Oswaldo Cruz* (Fiocruz; Rio de Janeiro, Brazil). Dr. Yamama Naciri (*Conservatoire et Jardin botaniques*, Geneva, Switzerland) helped with advices about species delimitation methods. We also thank Luigi Manuelli for the manuscript proofreading. Part of the analyses was performed on BAOBAB, a high performance-computing cluster of the University of Geneva.

Appendix A. Supplementary material

Supplementary material has been uploaded in Mendeley Data (http://dx.doi.org/10.17632/wccvm8p5gx.1), but the activation is still pending. The referees may download the data from our professional web page, where the data is temporarily available: https://genev.unige.ch/research/laboratory/Juan-Montoya

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Figures captions

Fig. 1. *Hypostomus* species descriptions through time, taking into consideration only the species that are currently valid. The plot includes the total number of descriptions per decade, the total accumulated, and the accumulated number of descriptions per geographical (continent) origin of the principal descriptor.

Fig. 2. *COI* barcode pairwise divergence (K2P-corrected) between pairs of *Hypostomus* species. The histogram shows three groups of data: pairwise divergences only between individuals of the same morphological species (intraspecific), only between sister-species, containing at least one morphological species, and only between non-sister species. The frequency was calculated independently for each group of data. The continuous vertical line represents the traditional 2% threshold of *COI* divergence, which suggests interspecific divergence between congeneric fish species. Note that for *Hypostomus* species, the threshold of 2% of divergence cannot be used, and it underestimates the diversity of species within the genus. The dashed vertical line represents the 0.5% threshold, a new tentative threshold specific to *Hypostomus*. Genetic distance higher than 0.08 not shown.

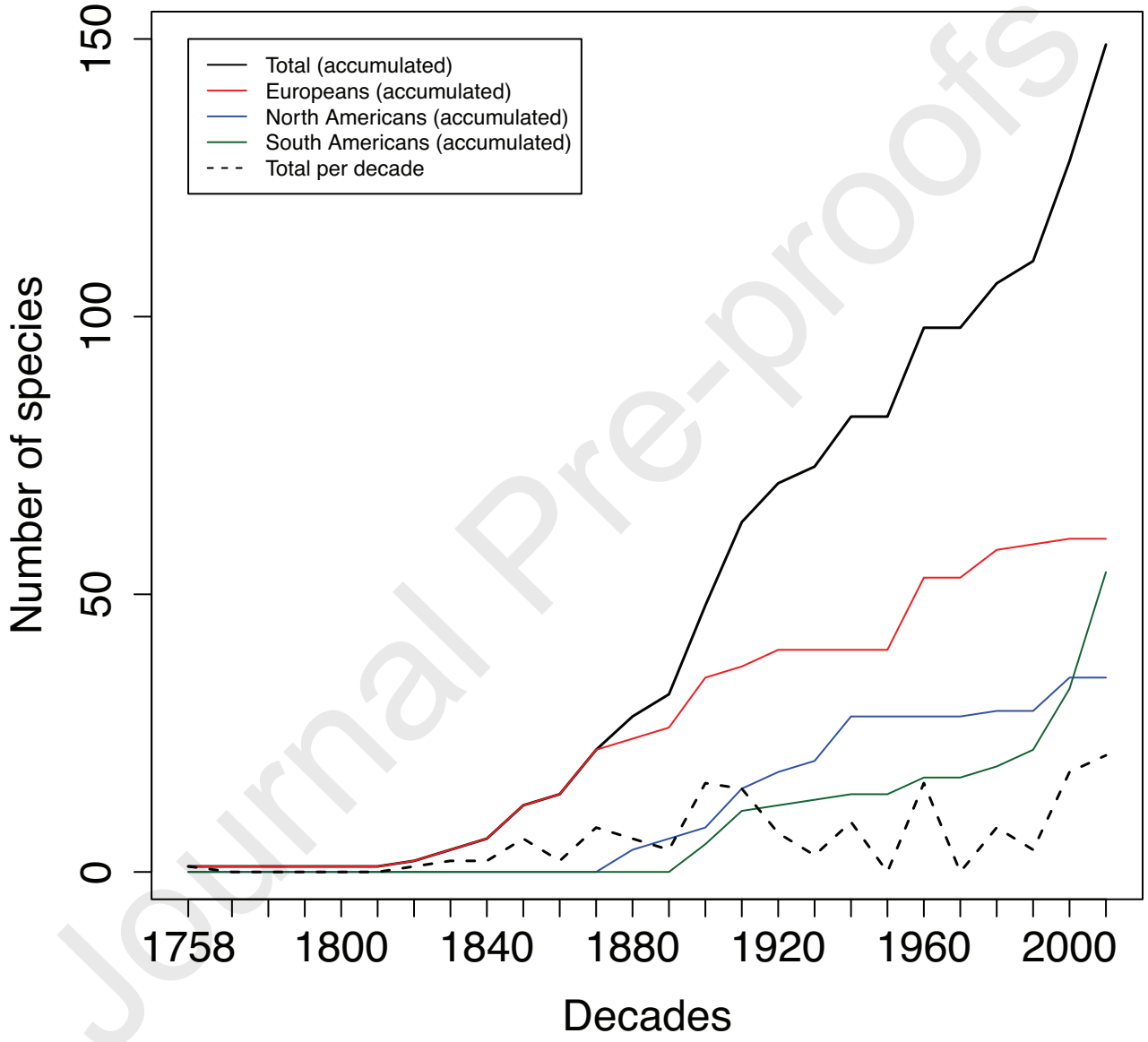
Fig. 3. Hypotheses of phylogenetic relationships among the main lineages of *Hypostomus* proposed in A) the present work; B) Montoya-Burgos (2003) and Montoya-Burgos et al. (2002); C) Cardoso et al. (2012); D) Lujan et al. (2015); and E) Silva et al. (2016). Circles at the nodes represent the support of the nodes, either the posterior probability or the bootstrap support: green = very high support (0.9–1/90–100), yellow = high support (0.7–0.89/70–89), grey = low support (0.5–0.69/50–69), white = no support < 0.5/50. Lineages represented by a single species are shown with a single branch.

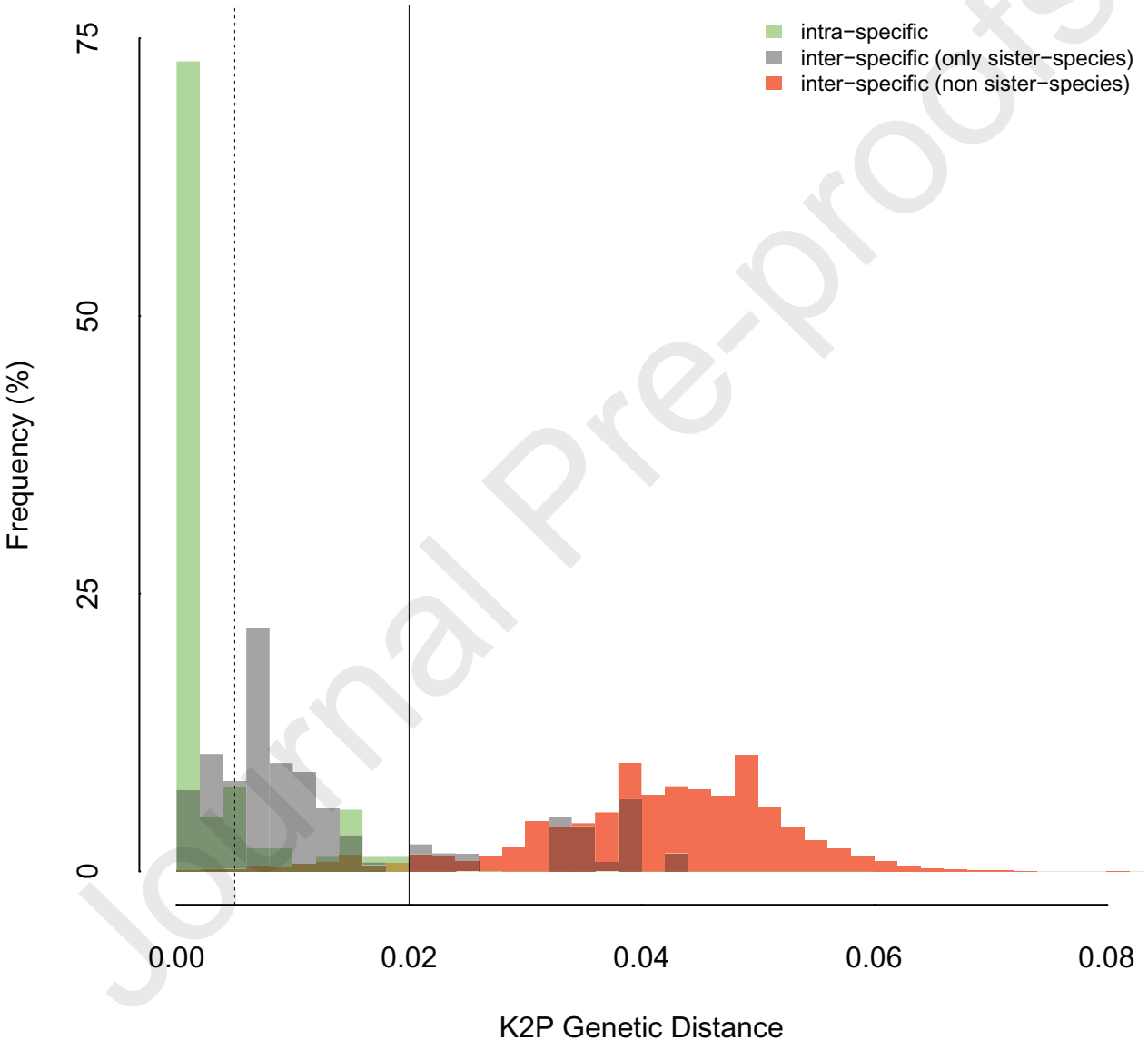
Fig. 4. BEAST tree showing the phylogenetic relationship hypotheses within the *Hypostomus cochliodon* super-group. Numbers represent the support of the nodes: posterior probability and rapid bootstrap, respectively. Node supports smaller than 0.5 or 50 (PP and bootstrap, respectively) are noted with '--'. Sample names are shown between parentheses. For the position of this super-group in the full *Hypostomus* tree, refer to Fig. 3.

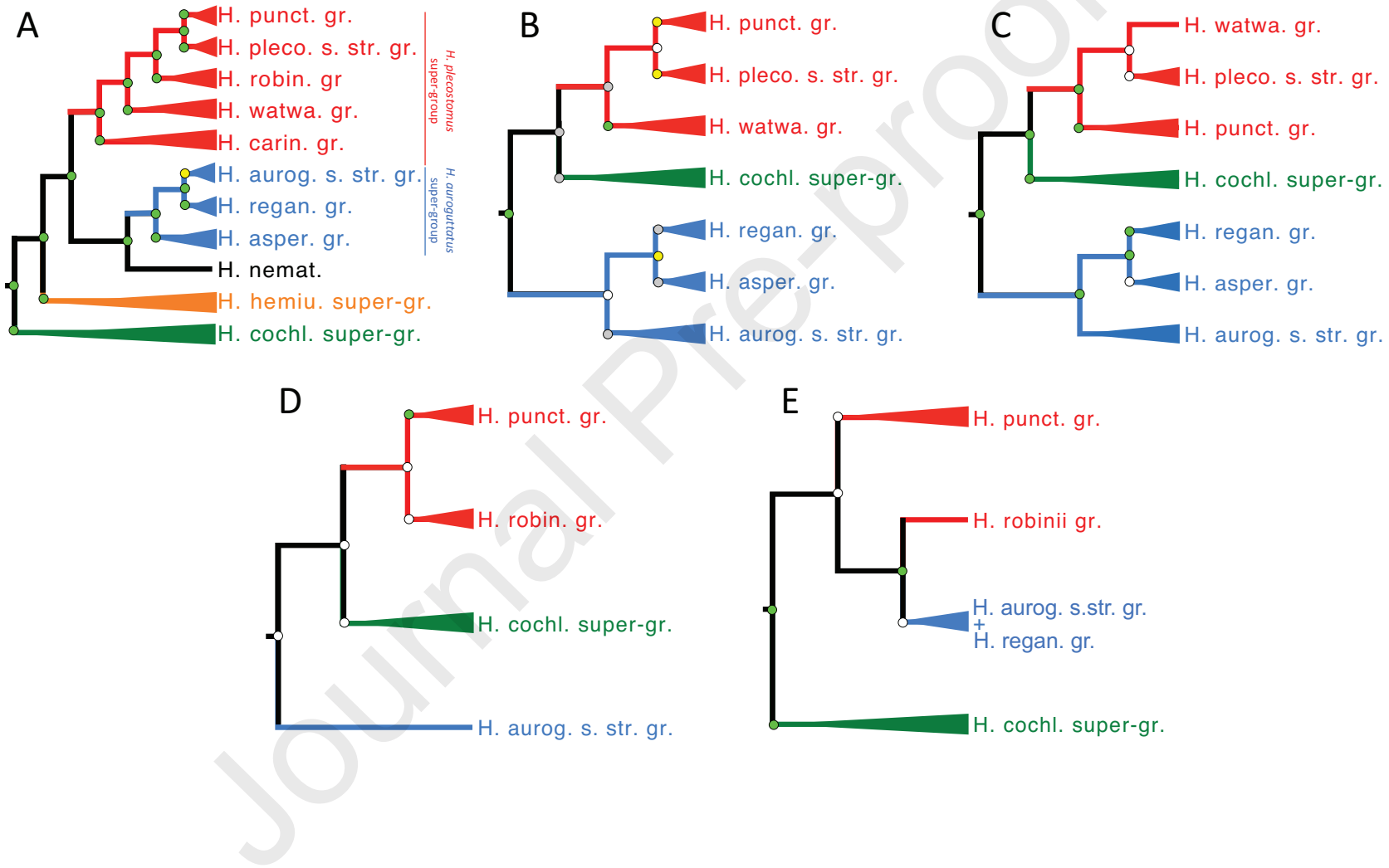
Fig. 5. BEAST tree showing the phylogenetic relationship hypotheses within the *Hypostomus hemiurus* super-group and *H. nematopterus*. Numbers represent the support of the nodes: posterior probability and rapid bootstrap, respectively. Node supports smaller than 0.5 or 50 (PP and bootstrap, respectively) are noted with '--'. Sample names are shown in parentheses. For the position of this super-group in the *Hypostomus* tree, see Fig. 3.

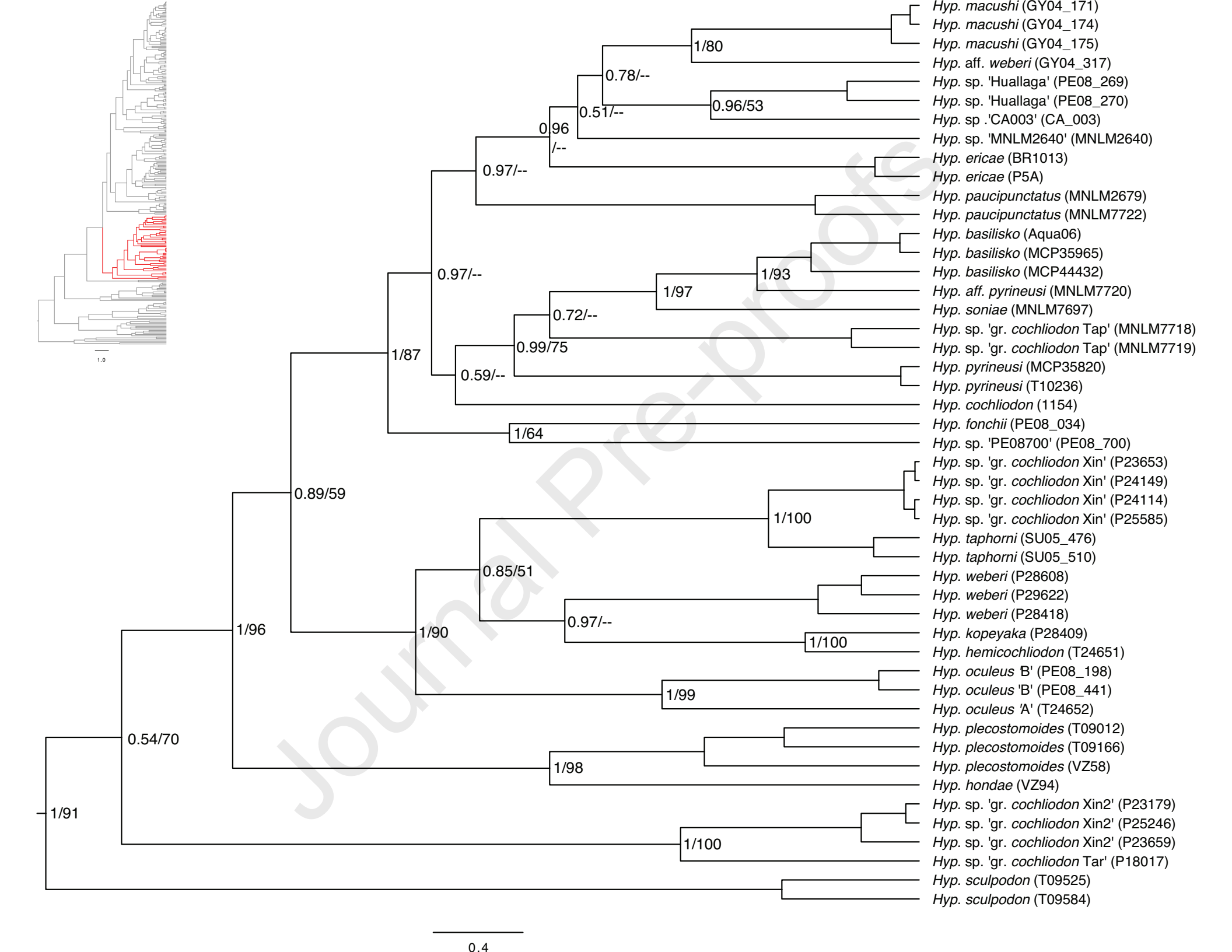
Fig. 6. BEAST tree showing the phylogenetic relationships within the *Hypostomus auroguttatus* super-group. Numbers represent the support of the nodes: posterior probability and rapid bootstrap, respectively. Node supports smaller than 0.5 or 50 (PP and bootstrap, respectively) are noted with '--'. Sample names are shown between parentheses. For the position of this super-group in the *Hypostomus* tree, refer to Fig. 3.

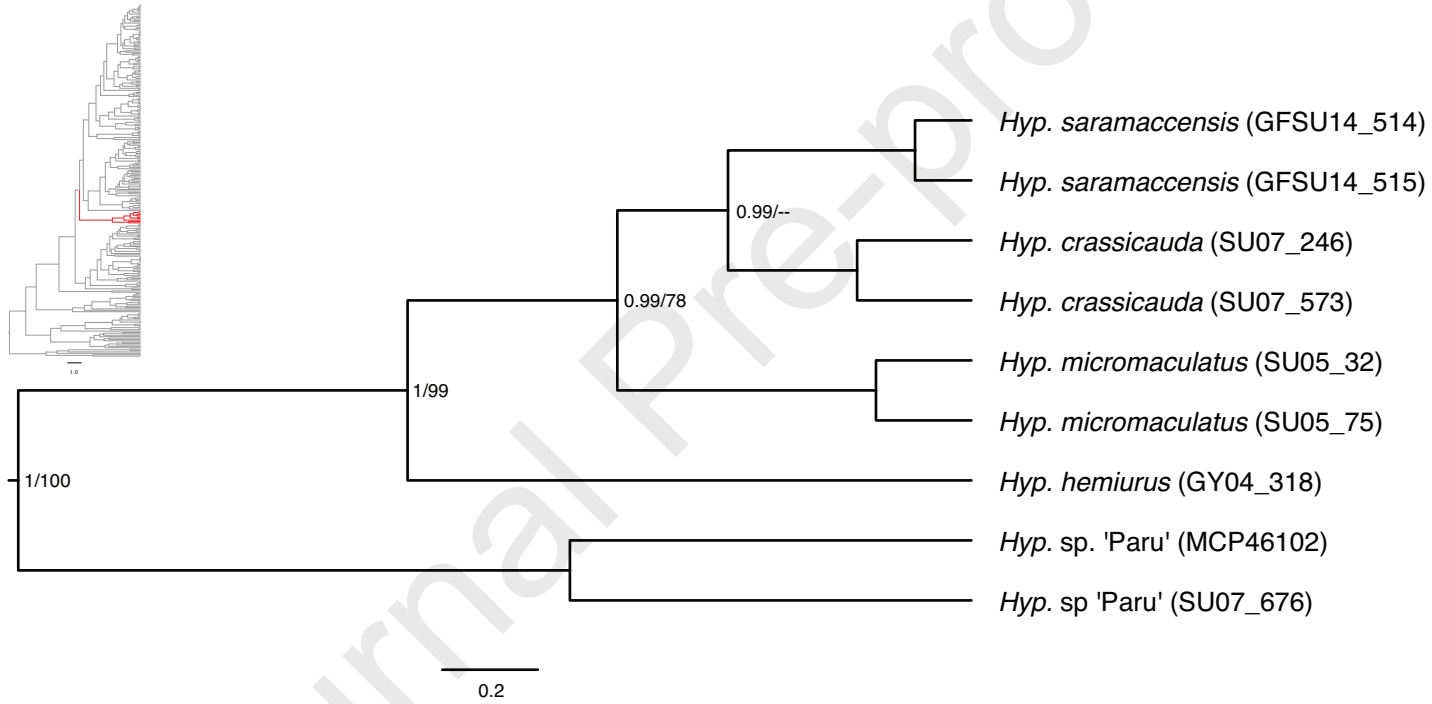
Fig. 7. BEAST tree showing the phylogenetic relationships within the *Hypostomus plecostomus* super-group. Numbers represent the support of the nodes: posterior probability and rapid bootstrap, respectively. Node supports smaller than 0.5 or 50 (PP and bootstrap, respectively) are noted with '--'. Sample names are shown between parentheses. For the position of this super-group at the *Hypostomus* tree, refer to Fig. 3.

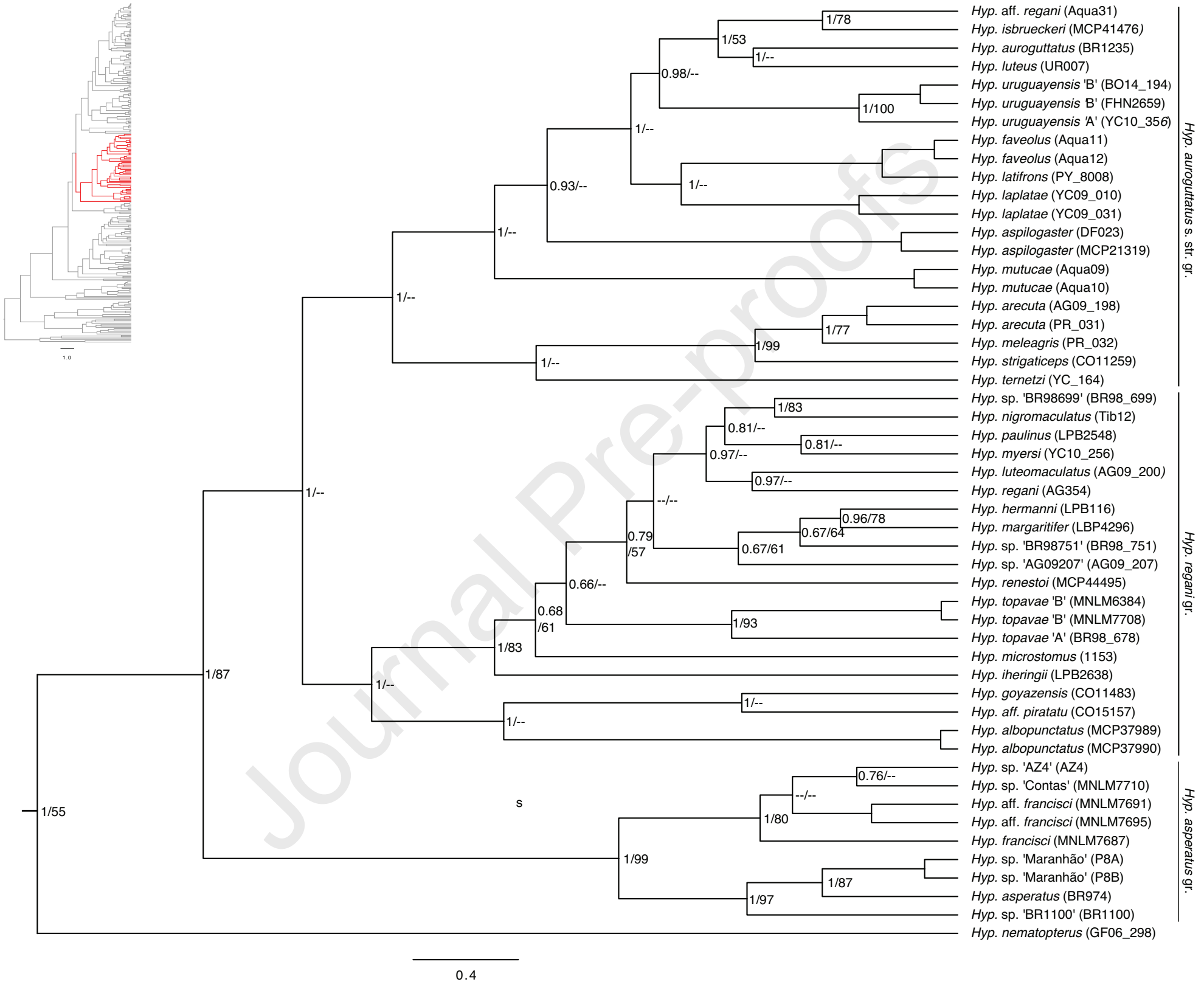


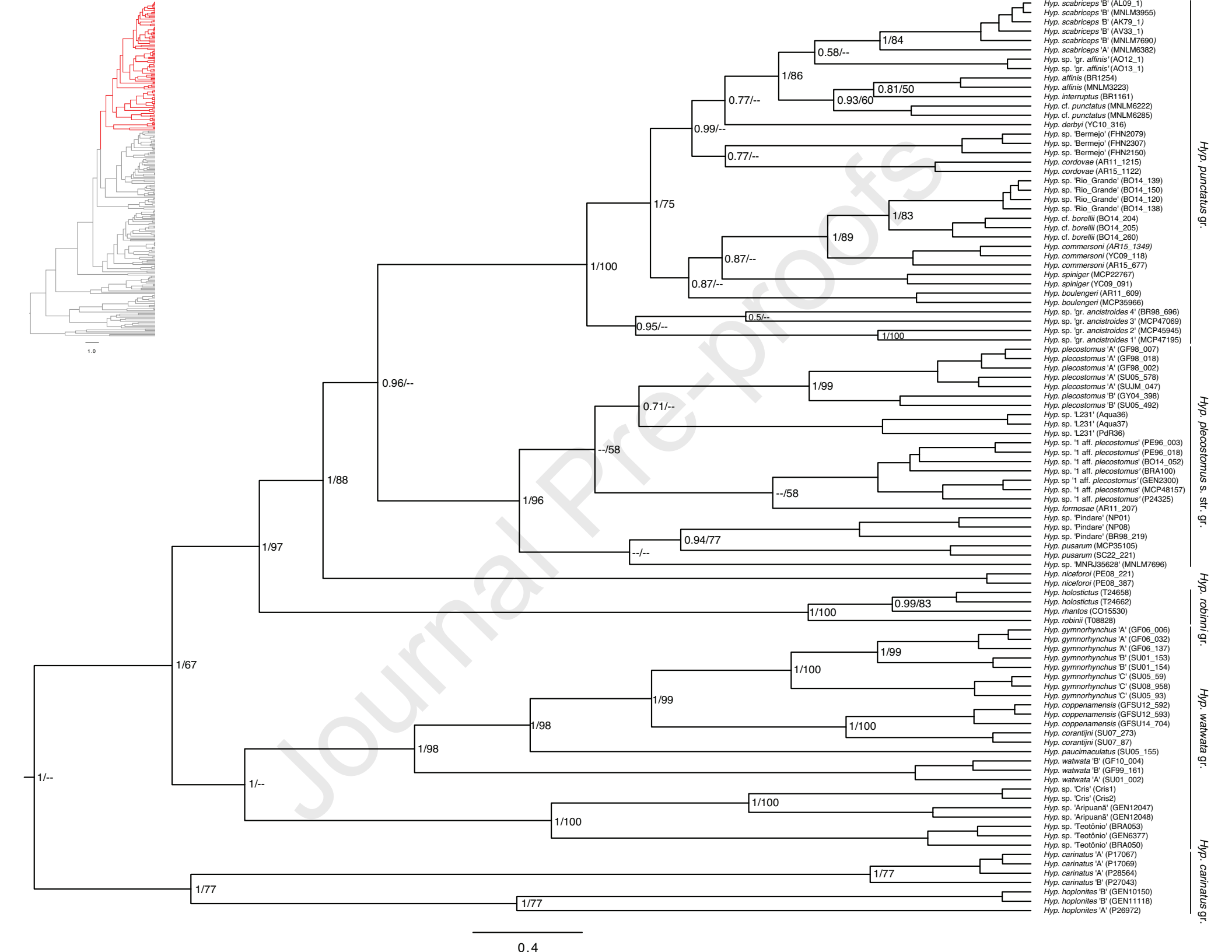




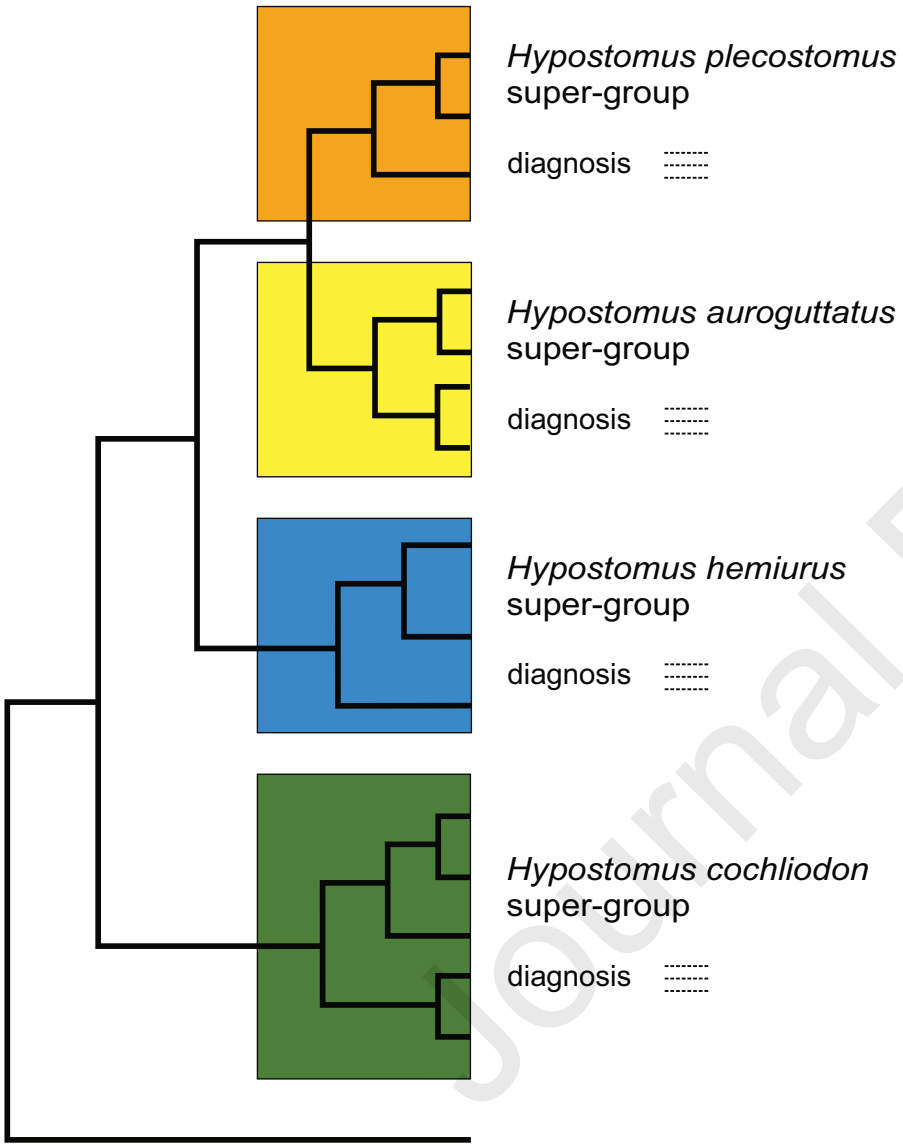




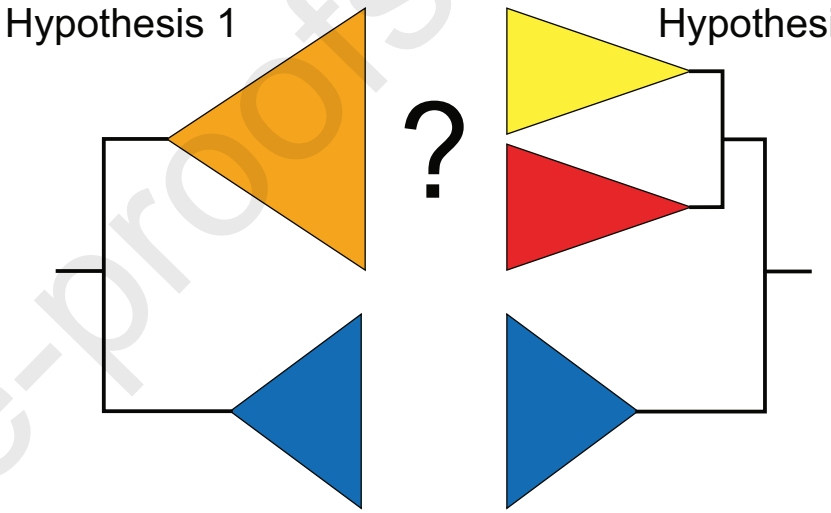


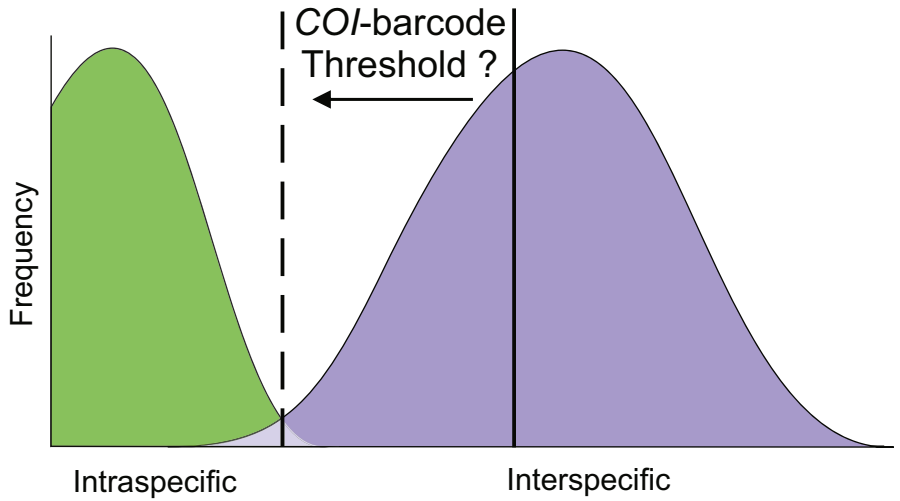


Hypostomus phylogeny



Cluster Credibility index: a new decision tool





Highlights

- We inferred a comprehensive phylogeny of the species-rich and widespread catfish genus *Hypostomus*.
- Morphology together with multispecies coalescent analyses unraveled 108 species and 118 clusters.
- We present the Cluster Credibility index that allows testing alternative cluster delimitations
- To delimit species, the *COI* pairwise divergence threshold of 2% is not applicable in *Hypostomus*.
- Our phylogeny reveals four *Hypostomus* super-groups for which we give diagnostic characters