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PRIMARY RESEARCH PAPER

Species delimitation based on integrative approach suggests reallocation of genus in Hypostomini catfish (Siluriformes, Loricariidae)

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Abstract Integrative approaches are particularly useful to resolve taxonomic uncertainties in species-rich groups that have undergone explosive radiation, such as Hypostomini (suckermouth armored catfishes). This tribe encompasses the genera *Hypostomus* and *Pterygoplichthys*, but the actual number of species in each genus and the intergeneric interrelationships are confusing or unknown for some ecoregions in the Neotropics. Here, we analyzed three endemic species *Hypostomus chrysostiktos*, *H. jaguar*

and *H. unae* from Northeastern Mata Atlântica in northeastern Brazil based on meristic characters, geometric morphometrics, chromosomal data, DNA barcode and species delimitation algorithms. The current taxonomic status of these catfishes is challenged. While *H. unae* and *H. jaguar* revealed a close evolutionary relationship similar to those observed within *Hypostomus* from other basins, *H. chrysostiktos* was herein more closely related to *Pterygoplichthys*, being invariably recovered as a highly distinctive taxonomic unit than to *Hypostomus*. Therefore, we recommend the reallocation of *H. chrysostiktos* as *Pterygoplichthys chrysostiktos* and a thorough systematic review of *Pterygoplichthys*, particularly focusing on putative convergence traits in relation to *Hypostomus*.

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Introduction

Ever since the validation of *Hypostomus* Lacepède 1803, the identification of suckermouth armored catfish species of the family Loricariidae has been intensively debated, including several cases of synonyms, redescriptions and reallocations (Eschmeyer et al., 2019). In fact, taxonomic and phylogenetic uncertainties in this and other genera of Loricariidae (e.g., poor definition of type-locality and high overlapping of meristic traits) remain to be solved to validate the actual number of species and the interrelationships from this speciose group of fishes (Lujan et al., 2015; Roxo et al., 2019). Overall, the lack of conspicuous species-specific patterns in suckermouth catfishes is particularly related to their remarkable adaptive radiation (Silva et al., 2016) and morphological convergence (Roxo et al., 2017), thus, the incorporation of other analytical methods can be useful (Padiál et al., 2010; Carstens et al., 2013).

Accordingly, genetic studies have detected cryptic diversity in loricariids from several Neotropical riverine systems (Zawadzki et al., 2008; Bitencourt et al., 2011a, b; Puggedo et al., 2016; Rubert et al., 2016). Furthermore, molecular analyses confirmed that the subfamily Hypostominae is monophyletic but the relationships among tribes diverged from those formerly proposed by morphological studies (Lujan et al., 2015). For instance, the tribes Hypostomini and Pterygoplichthyini (sensu Armbruster, 2004) were recovered as a single clade comprising both *Hypostomus* and *Pterygoplichthys* (named tribe Hypostomini) with nearly 170 valid species (Lujan et al., 2015).

The abovementioned scenario is clearly exemplified by the *Hypostomus* taxa reported for coastal basins at Northeastern Mata Atlântica (NMA) ecoregion (sensu Abell et al., 2008). Seven species are morphologically recognized in these coastal drainages from northeastern Brazil: *H. breviceuda* Günther, 1864, *H. wuchereri* Günther, 1864, *H. unae* Steindachner, 1878, *H. chrysostiktos* Birindelli et al., 2007, *H. jaguar* Zanata et al., 2013, *H. leucophaeus* Zanata

& Pitanga, 2016, and *H. nigrolineatus* Zawadzki et al., 2016. Some of these species are considered endemic to a single or a few basins, such as *H. leucophaeus* (Itapicuru River Basin) and *H. nigrolineatus* (Zawadzki et al., 2016) (Jequitinhonha and Pardo River Basins) while the range of the remaining taxa is uncertain (Zanata et al., 2013). Moreover, species complexes have been indicated in some morphotypes by cytogenetic data (Bitencourt et al., 2011a, b, 2012), providing evidence of both Linnean and Wallacean shortfalls (Lomolino, 2004; Whittaker et al., 2005).

Among the *Hypostomus* described from NMA, *H. chrysostiktos* (Birindelli et al., 2007) is particularly intriguing. Originally cited as *Pterygoplichthys* sp. (Higuchi et al., 1990), this is an endemic and highly distinguishable suckermouth catfish from Paraguaçu River Basin. This species was later described within *Hypostomus*, as a putative sister group of *H. emarginatus* and *H. commersoni* (Birindelli et al., 2007), because of the absence of some diagnostic traits reported in *Pterygoplichthys* (modified stomach and 2–3 plates between the suprapreopercle and the exposed opercle) (Armbruster, 2004). Furthermore, *H. chrysostiktos* has the anterior process of the pterotic-supracleitrum passing halfway through the orbit, a hatched-shape opercle, and a pointed postcleithral process—features widely used in the diagnosis of *Hypostomus*—even though it shares a high number of dorsal fin rays (10–11) with *Pterygoplichthys* representatives (Armbruster, 1998a, b).

Based on recent molecular phylogenies (Lujan et al., 2015; Roxo et al., 2019), it is clear that morphology is very plastic, and convergence is common within the Loricariidae. The taxonomy of *Hypostomus* and related genera has been in a state of flux. Armbruster's (2004) *Hypostomus* was found to be polyphyletic and was split into *Hypostomus* sensu stricto (including *H. chrysostiktos*), *Aphanotorulus* (including *Squaliforma*), and an expanded *Isorineloricaria* (Lujan et al., 2015; Ray & Armbruster, 2016). Further, Armbruster et al. (2015) placed the trans-Andean species of Armbruster's (2004) *Hemiancistrus annectens* group into *Hypostomus*. *Hypostomus* was found to be in a clade with southern species of *Hemiancistrus* and *Pterygoplichthys* (Lujan et al., 2015). Nonetheless, molecular reports that include the endemic species of *Hypostomus* from NMA are not available.

Different from other Hypostomini (sensu Lujan et al., 2015), there are few genetic reports about *Pterygoplichthys*, except for cytogenetic and DNA barcoding analyses in a few taxa from Upper Paraná River Basin (Alves et al., 2006; Pereira et al., 2013; Fernandes et al., 2015; Bueno et al., 2018) and some studies related to their impacts as invasive exotic species (e.g. Orfinger & Goodding, 2018). So far, the genus *Pterygoplichthys* has not been recorded for NMA coastal river basins, with a single native species (*P. etentaculatus* Spix & Agassiz, 1829) being recognized in the São Francisco River Basin (Carvalho et al., 2011), an ecoregion that supposedly shares a close biogeographic history with coastal drainages in eastern Brazil (Rosa et al., 2004; Ribeiro, 2006).

Under this context, we performed a reevaluation of *H. chrysostiktos*, *H. jaguar* and *H. unae* from coastal river basins in NMA ecoregion, based on sequencing of cytochrome *c* oxidase subunit I (COI), morphology, geometric morphometrics (GMs) and cytogenetics. The present data were also compared to sympatric and allopatric species of *Hypostomus* and *Pterygoplichthys* to determine their taxonomic status and to infer the utility of the selected methods in inquiring the systematics of Hypostomini (sensu Lujan et al., 2015).

Materials and methods

Sampling

Three *Hypostomus* species from NMA were collected, totaling 17 specimens of *H. chrysostiktos*, 46 of *H. jaguar* and 19 of *H. unae*. These species were selected because *H. jaguar* occurs in sympatry with *H. chrysostiktos* in Paraguaçu River Basin in its type-locality (Zanata et al., 2013), while some populations of *H. jaguar* are also found in Recôncavo Sul Basin along with *H. unae* (present study) (Fig. 1). Voucher specimens are deposited in the ichthyologic collection of the Instituto Nacional da Mata Atlântica (INMA) in Santa Teresa, ES, Brazil.

Morphological and morphometric analysis

The morphological analyses were carried out based on counts of meristic traits and diagnostic features reported by Boeseman (1968), Armbruster & Page (1996), Bockmann & Ribeiro (2003) and Hollanda-

Carvalho & Weber (2004). The collected specimens were then compared to previously recognized species from NMA ecoregion and other basins, as follows: Paraguaçu River Basin—MBML 11016 (Paraguaçu River, Itaeté-BA), MBML 11019 (Paraguaçu River, Iaçú-BA), MBML 11017 (Paraguaçu River, Itaeté-BA), MBML 11020 (Paraguaçu River, Iaçú-BA), MUZUFBA 2787 (Paraguaçu River, Iaçú-BA: holotype) (*H. chrysostiktos* and *H. jaguar*); Recôncavo Sul Basin—MBML 11015 (Serra River, Maraú-BA), MBML 10331 (Jequiriçá River, Mutuípe-BA) (*H. jaguar* and *H. unae*); Pardo River Basin—MBML 11021 (Pardo River, Itapetinga-BA) (*Hypostomus* sp.); Itapicuru Basin—UESB 8231 to UESB 8243, UFBA 03251; São Francisco River Basin—MNRJ 703 (*P. etentaculatus*), São Francisco River; Upper Paraná River Basin—MBML 3216, MBML 3912, MBML 3918, MBML 3926 (*H. auroguttatus*); São Mateus River Basin—MBML 492, MBML 519, MBML 581, MBML 617, MBML 1057, MBML 1107, MBML 1259, MBML 3494, MBML 3501 (*H. scabriceps*); Doce River Basin—MBML 5704, MBML 5714, MBML 5760 (*Hypostomus* sp.). The morphometric and meristic data for *H. chrysostiktos*, *H. jaguar* and *H. unae* are shown in ESM_1.

The GM analysis included 80 specimens (> 112.0 mm in standard length) of the sampled *Hypostomus* species in the present study. Dorsal and left side body views were photographed using a Canon SX60HS digital camera while the ventral view was scanned individually using a multifunctional HP F4400 printer (600 dpi), with a metric scale. Images were saved as jpeg's. We used tpsutil (Rohlf, 2017a) to create a tps file for use in tpsDig2 for landmark placement (Rohlf, 2017b). A total of 20, 17, and 18 landmarks was inserted in the dorsal, left side, and ventral views, respectively (ESM_2). The tps files were analyzed in MorphoJ v 2.0 (Klingenberg, 2011). Generalized Procrustes Superimposition was performed in MorphoJ to remove the possible effects of rotation, translation, and scaling (Slice, 2007). This procedure decomposes the form into the centroid size (mean coordinates of landmarks) and shape (geometric information of organisms excluding size and position or rotation effects) (Bookstein, 1991) to create a covariance matrix.

Variance analysis (ANOVA) and pairwise Tukey's test (significance level of $P < 0.05$) were performed to evaluate the differences in the centroid size

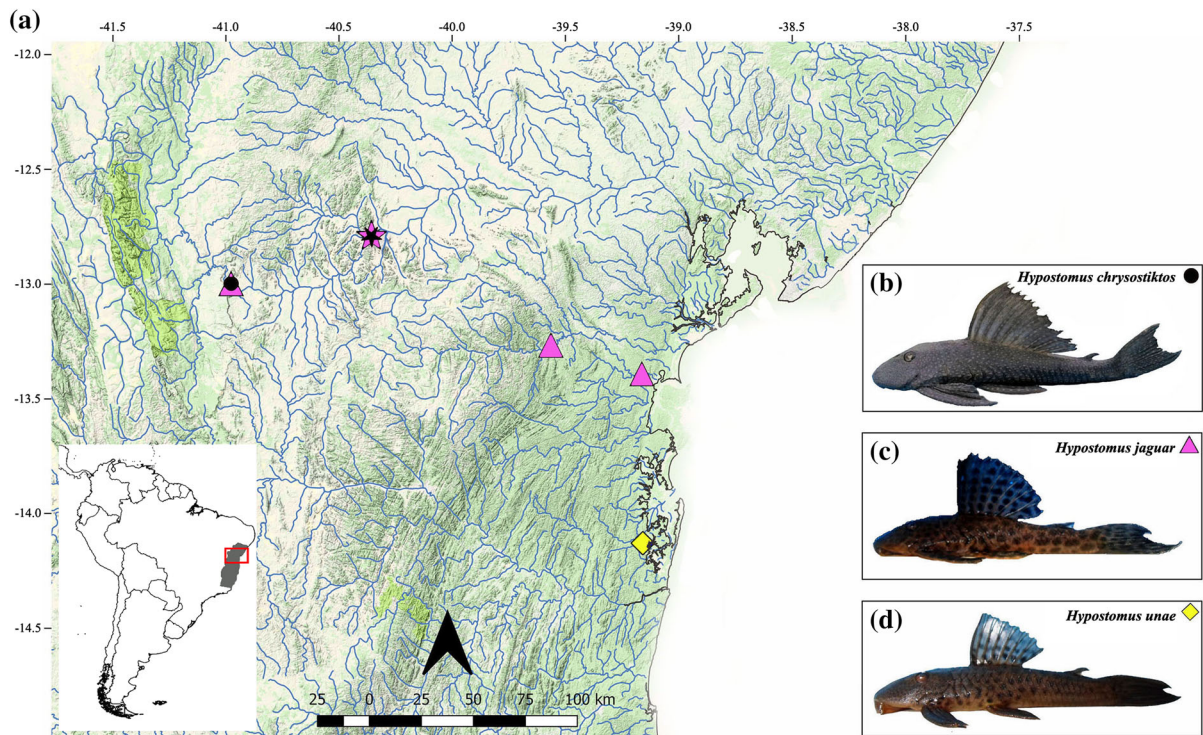


Fig. 1 Map of hydrographic basins from Northeastern Mata Atlântica (NMA) ecoregion, northeastern Brazil (a) created in Quantum GIS 3.0.3, showing the collection sites of *Hypostomus chrysostiktos* (b) in black (Paraguaçu River Basin), *H. jaguar*

(c) in pink (Paraguaçu and Recôncavo Sul Basins) and *H. unae* (d) in yellow (Recôncavo Sul Basin), with images of representative specimens on the right. The star symbols indicate the type-locality of *H. jaguar* in pink and *H. chrysostiktos* in black

considering the three views using the software PAST v 3.25 (Hammer et al., 2001). To assess the differences in the body shape among species and to exclude putative allometric effects, a regression analysis was applied from the raw data and the residues as new variables. Then, these variables were used in canonical variate analysis (CVA) based on a priori species groups ($P < 0.05$). Afterwards, the shape differences were submitted to a linear discriminant analysis to perform the cross-validation and estimate the percentage of correct classification for the three species groups. The principal component analysis (PCA) was applied to reduce the data dimension and calculate the percentage of each component to explain the shape variation ($P < 0.05$) without previous assignment of groups. The CVA, PCA and discriminant analyses were performed in the software MorphoJ v 2.0.

DNA barcode and molecular analysis

The total DNA was extracted using the Wizard[®] Genomic DNA Purification kit (Promega) according to the manufacturer's instructions. A fragment of the COI was amplified via polymerase chain reaction (PCR), using the VF1i_t1 (Ivanova et al., 2006) and VR1_t1 (Ward et al., 2005). Each PCR comprised 1.25 × buffer, 2.9 mM of MgCl₂, 0.25 mM of dNTP, 0.21 μM of each primer, 0.08 U/μl of platinum[®] Taq DNA polymerase, 4 to 8 ng/μl of template DNA and ultrapure water to a final volume of 12 μl. The PCR conditions were: initial denaturation step at 94°C for 4 min, 35 cycles at 92°C for 30 s, 54°C for 40 s and 72°C for 90 s, plus a final extension at 72°C for 10 min.

The PCR products were checked by electrophoresis in 1% agarose gel after staining with bromophenol blue and Gel Red[™] (Biotium, USA) at a ratio of 3:1. Subsequently, the successfully amplified products were purified in 20% polyethylene glycol (PEG) and

washed in 80% ethanol to perform the bidirectional sequencing reactions using the BigDye Terminator v. 3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems/Life Technologies) according to the manufacturer's instructions. The sequences were read in ABI 3500 XL Genetic Analyzer Automatic sequencer (Applied Biosystems, USA). The COI sequences were uploaded in the BOLD platform (www.barcodinglife.org) under the Project “*Hypostomus* northeastern Brazil – HYPBA” (codes HYPBA021-023, HYPBA037-039, HYPBA042-045, HYPBA048-056).

The consensus COI sequences (four of *H. chrysostiktos*, eight of *H. jaguar* and three of *H. unae*) were obtained using the software DNA Baser Sequence Assembler v. 4.16 (Heracle BioSoft, 2013). Subsequently, the sequences were aligned using CLUSTAL W (Thompson et al., 1994) in the software BioEdit Sequence Alignment Editor v. 7.1.9 (Hall, 1999) and translated in MEGA X (Kumar et al., 2018).

For comparative analyses, we added 55 COI sequences of 24 related species of *Hypostomus* and eight sequences of 4 species of *Pterygoplichthys* from other basins (*P. ambrosettii*, *P. etentaculatus*, *P. joselimaianus*, *P. punctatus*). COI data from *Ancistrus* sp., *Chaetostoma fischeri*, *Delturus carinotus*, *Harttia punctata*, *Kronichthys subteres*, *Megalancistrus parananus*, *Neoplecostomus microps*, *Otocinclus vittatus*, *Panaque nigrolineatus*, *Pareiorhina rudolphi*, *Pareiorhaphis* (*P. bahianus*, *P. nasuta* and *P. vestigipinnis*), *Parotocinclus britskii* and *Rineloricaria* sp., most of them used as comparative material in the description of *H. chrysostiktos*, were selected as outgroups, to assure the molecular comparisons with other species that are likely to be related or that could represent taxonomic misidentifications, thus totaling 80 sequences of 45 taxa (ESM_3).

Based on the COI dataset, we performed a phylogenetic reconstruction using maximum likelihood (ML) and Bayesian (BI) inferences in RAxML v. 8.2 (Stamatakis, 2014) and MrBayes 3.2.6 (Ronquist & Huelsenbeck, 2003; Ronquist et al., 2012), respectively. Both methods were carried out in CIPRES Science Gateway v 3.3 (Miller & Pfeiffer, 2010). In the case of BI, two rows of four chains (10×10^6 generations) were used with sampling at each 1,000 trees assuming 10% of burn-in. The support values on branches were calculated according to posterior probabilities (BI) and 1000 bootstrap replicates (ML). The

best evolutionary models as determined by jModelTest 2.1.10 (Darriba et al., 2012) were TPM2uf+I+G and TIM2+I+G for BI and ML trees, respectively.

In addition, seven algorithms commonly used to species delimitation based on single locus were included in the present study: automatic barcode gap discovery (ABGD; Puillandre et al., 2012), barcode index numbers (BIN; Ratnasingham & Hebert, 2013), jMOTU (Jones et al., 2011), multi-rate Poisson tree process (mPTP; Kapli et al., 2016); bPTP (Bayesian implementation of the PTP; Zhang et al., 2013), sGMYC and mGMYC (single and multiple general mixed Yule-coalescent analyses; Pons et al., 2006; Fujisawa & Barraclough, 2013).

The ABGD analysis was carried out on the online platform <http://www.abi.snv.jussieu.fr/public/abgd/abgdweb.html> using a gap width of 1.0 based on Kimura-2-parameter (K2P), Jukes–Cantor and *p*-distances obtained in interspecific comparisons. The COI sequences uploaded in BOLD platform were analyzed according to their BINs, a system that divides the COI sequences into molecular operational taxonomic units (MOTUs) independently on their predefined taxonomic classification. Therefore, the BINs represent a useful method to evaluate whether the DNA barcodes and species designation are in agreement or not. This analysis is performed by comparing the taxa present in the input files with all others that share identical BINs, including those uploaded by different users based on the refined single linkage (RESL) algorithm (Ratnasingham & Hebert, 2013). Furthermore, the number of diagnostic sites that are exclusive to each BIN is also available in the BOLD platform, providing additional information to recognize unique MOTUs. On the other hand, jMOTU (<https://www.nematodes.org/bioinformatics/jMOTU>) preclusters input sequences and further calculates the exact number of pairwise mismatches derived from Needleman–Wunsch (NW) algorithm under different distance cutoff values (we tested distances of 1%, 2%, 3%, and 4% between sequence pairs) inasmuch as the final clusters should correspond to biological reality (Jones et al., 2011).

For the bPTP (available in <https://species.h-its.org/>) and mPTP (<https://mptp.h-its.org/#/tree>) analyses, a ML tree built in the software RAxML 8.2.10 (Stamatakis, 2014) was used as input, using 100,000 Monte Carlo Markov chains (MCMC) generations, thinning = 100 and 10% of burn-in. The bPTP adds Bayesian support to the nodes from a tree considering

similar diversification rates (Zhang et al., 2013), while mPTP assumes distinct evolutionary rates and relies on Akaike information criterion (AIC) to infer the number of species in the input trees (Kapli et al., 2016). On the other hand, the sGMYC and mGMYC analyses (Pons et al., 2006; Fujisawa & Barraclough, 2013) used as input an ultrametric tree obtained in BEAST 1.10.4 (Suchard et al., 2018) with the following parameters: substitution model GTR+G+I; strict molecular clock; mutation rate of 1 substitution/site/million years; Yule prior; and 50 million generations with sampling at every 1,000 generations. In both methods, the species are identified according to the changes in the branching rate in the input tree (Fujisawa & Barraclough, 2013) based on single (sGMYC) or multiple (mGMYC) time thresholds carried out in package splits (SPecies Limits by Threshold Statistics) available in <http://r-forge.rproject.org/projects/splits> (Ezard et al., 2009).

Furthermore, after calculation of K2P distances in the software MEGA X (Kumar et al., 2018), the interspecific distances were used to generate a heatmap in the R package SPIDER (Species Identity and Evolution in R) available in <http://spider.r-forge.r-project.org/SpiderWebSite/spider.html> (Brown et al., 2012), to summarize the pairwise comparisons. In the same package, we also applied the “localMinima” function, which disregards any previous knowledge about the species identity, to indicate potential thresholds related to intra and interspecific variation levels (Brown et al., 2012) and nucDiag function to determine diagnostic nucleotides (Sarkar et al., 2008) for species delimitation in the Hypostomini dataset. Finally, a haplotype network was generated using PopArt (Leigh & Bryant, 2015) to elucidate the genealogic relationships among *H. chrysoptiktos*, *H. jaguar*, and *H. unae*.

Cytogenetic analysis

The mitotic chromosomes were obtained from kidney cells of *H. chrysoptiktos* (five specimens) and *H. jaguar* (six specimens from Paraguaçu River and Recôncavo Sul) according to Netto et al. (2007) and Molina et al. (2010). In the case of *H. unae*, the cytogenetic information was based on a previous report by Bitencourt et al. (2011b, 2012). The chromosomal morphology (m = metacentric, sm = submetacentric, st = subtelocentric,

a = acrocentric) was determined based on arm ratio (Levan et al., 1964), using the software Easydio v. 3.0 (Diniz & Xavier, 2006). The active nucleolar organizer regions (Ag-NORs) on chromosomes were visualized after silver nitrate staining (Howell & Black, 1980).

Results

External morphology

Hypostomus chrysoptiktos is an endemic species from Paraguaçu River Basin that can be promptly distinguished from congeners by the number of branched dorsal-fin rays (10–11), an uncommon feature among *Hypostomus*. In fact, all known species of *Hypostomus* have seven branched rays in dorsal-fins. Besides the unusual fin ray count, *H. chrysoptiktos* presents slightly evertible lateral plates on the head, four branched rays in the anal fin, a plate-free abdomen and snout mostly covered by plates (Birindelli et al., 2007; present study). Moreover, *H. chrysoptiktos* is also readily discriminated from the other species herein analyzed by their coloration (yellowish spots over a dark background) (Fig. 1b). On the other hand, dark spots on a brownish body are observed in *H. jaguar* (Fig. 1c) and *H. unae* (Fig. 1d) from Paraguaçu and Recôncavo Sul Basin, as also reported by Zanata et al. (2013) and Sardeiro (2012), respectively.

Therefore, *H. chrysoptiktos* is a quite distinctive species under Hypostomini. The slightly evertible cheek plates, the uncommon long dorsal fin, with more than 10 rays, the regionally contrasting body pigmentation of this species is shared with representatives of Pterygoplichthys. Nevertheless, the small maxillary barbels on lateral border of a large oral disc, smooth head and body, keels limited to mid-ventral series of plates, whitish and uniform coloration on the abdomen are morphological features present in species of *Hypostomus* from the same region (see ESM_4 for a comparative analysis).

Morphometric analysis

The centroid size among *H. chrysoptiktos*, *H. jaguar* and *H. unae* (Fig. 2a–c) differed significantly in both ANOVA ($F_{2-35} = 25.8$, $P = 0.001$) and Tukey's test ($P < 0.05$), with the widest variation observed in *H.*

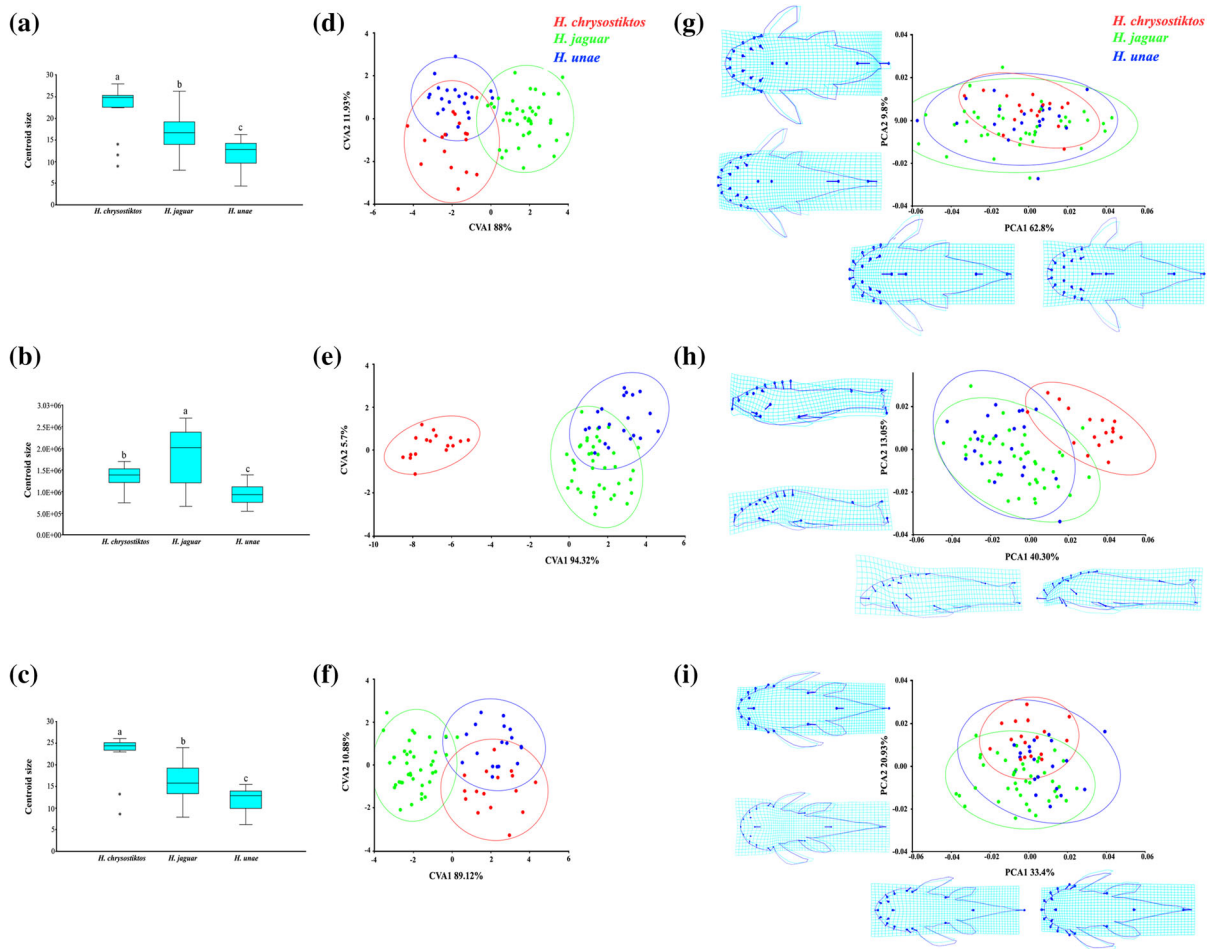


Fig. 2 Box-plot centroid size variation for the three views (dorsal, left side and ventral) per species based on analysis of variance (ANOVA). Different letters indicate significant differences in Tukey’s test ($P < 0.05$) (a–c). The asterisks indicate the outliers. Canonic variate analysis (CVA) with the ellipses ($P < 0.05$) indicating each species (d–f). Principal component

analysis (PCA) for the dorsal (g), left side (h) and ventral (i) views of *Hypostomus chrysostiktos*, *H. unae*, and *H. jaguar* with the ellipses plotted for each species. The deformation grids and shape contours demonstrate the shape variation of the morphotypes (dark blue) in relation to the mean (light blue) form from the dataset in positive and negative scores of PC1 and PC2

jaguar for the three views. As for the body shape, the first and second canonical variates accounted for 100% of variation in shape for all views (Fig. 2d–f). In spite of some overlapping, the CVA allowed discriminating the three species groups, particularly *H. chrysostiktos* in the left side view (Fig. 2d–f).

unae was highly overlapped to the other species groups (Table 1).

On the other hand, the cross-validation of samples to each species group based on discriminant analysis for the three views was low (usually below 50%), ranging from 42.8% (for the left side view in *H. unae*) to 87.8% (ventral view in *H. jaguar*). It should be pointed out that *H. jaguar* had the highest scores of correct reclassification while the body shape of *H.*

Similarly, the PCA revealed overlapped variation in body shape for all views in the three sampled species, particularly on dorsal view (Fig. 2g–i). The first two components accounted for 72.6% of variation in the dorsal view (Fig. 2g), being mostly related to the deformation grids in the head. In the left side view (Fig. 2h), the first two components accounted for 53.35% of variation, influenced by the deformation grids and vectors related to body height at the insertion of dorsal fins, head height and snout length. The first two components accounted for 54.33% of the variation

Table 1 Percentage of correct classification from linear discriminant analysis for the body shape based on the three views (dorsal, left side and ventral)

	<i>H. chrysostiktos</i>	<i>H. jaguar</i>	<i>H. unae</i>	Total
Discriminant function dorsal view: cross-validation				
<i>Hypostomus chrysostiktos</i>	8	2	7	17
<i>Hypostomus jaguar</i>	6	29	6	41
<i>Hypostomus unae</i>	9	3	9	21
Discriminant function left side view: cross-validation				
<i>Hypostomus chrysostiktos</i>	12	0	5	17
<i>Hypostomus jaguar</i>	0	25	16	41
<i>Hypostomus unae</i>	1	11	9	21
Discriminant function ventral view: cross-validation				
<i>Hypostomus chrysostiktos</i>	8	3	6	17
<i>Hypostomus jaguar</i>	2	36	3	41
<i>Hypostomus unae</i>	7	5	9	21

in the ventral view (Fig. 2i), with influence of head width and length, dentary angle and snout length over the deformation grids and vectors. In spite of the high overlapping in PCA, the left side view showed that *H. chrysostiktos* was more differentiated in relation to the other two species, as a result of the increased retraction from the region around nostrils, eyes and supraoccipital bone to the insertion of dorsal fin (Fig. 2h).

DNA analysis

In total, 15 COI sequences comprising 629 base pairs (bp) were obtained for *H. chrysostiktos*, *H. jaguar* and *H. unae* (ESM_3). The ML and BI trees based on COI data shared the same topology, differing slightly in their support values (Fig. 3, ESM_5). Both methods of phylogenetic reconstruction revealed that *H. chrysostiktos* was highly supported (bootstrap = 100% and a posteriori probability = 1) as a distinct taxon in relation to its congeners. Additionally, *H. chrysostiktos* and *P. etentaculatus* representatives from São Francisco Basin formed a cluster, indicating that *H. chrysostiktos* is more closely related to *Pterygoplichthys*.

Following this trend, the BIN analysis discriminated *H. chrysostiktos* (BIN ACZ9667), placing *P. etentaculatus* from São Francisco Basin as the nearest neighbor (NN = 2.25%). On the other hand, *H. jaguar* and *H. unae* shared the BIN ACZ7798 restricted to NMA. Similarly, the other *Pterygoplichthys* species shared a single BIN (ACK1995), a scenario also observed for several taxa in *Hypostomus* (Fig. 3), suggesting the presence of cryptic species and/or

misidentifications in the online database. Furthermore, the remaining algorithms invariably recognized *H. chrysostiktos* as an independent MOTU closely related to *Pterygoplichthys* (Fig. 3) in spite of the divergence in the total number of species suggested by each method (jMOTU, bPTP and GMYC were the most conservative ones).

The intraspecific genetic divergence using the K2P distance and based on the clusters shown in the consensus tree (Fig. 3) ranged from 0 to 1.6% (ESM_6). The interspecific genetic distance varied from 0.1 to 10% (Fig. 4a). Considering only the target species in this study, the interspecific distance values ranged from 1.5% (*H. jaguar* × *H. unae*) to ~ 7% (*H. chrysostiktos* in relation to *H. unae* and *H. jaguar*) (ESM_7). Moreover, a distance value of 0.02 was observed between *H. chrysostiktos* and *P. etentaculatus*, while it ranged from 0.03 up to 0.05 in relation to *P. ambrosettii*, *P. joselimaianus*, and *P. punctatus*. It should be pointed out that the interspecific difference among *Pterygoplichthys* species ranged from 0.1 to 5.1% (Fig. 4a, ESM_7).

In addition, the threshold potential from intra to interspecific variation in the analyzed dataset using SPIDER package was established in 1.95% (Fig. 4b). Furthermore, 30 diagnostic sites were detected in *H. chrysostiktos*, while *H. jaguar* and *H. unae* lacked diagnostic nucleotides (ESM_6). A reduced number of mutation steps (12) were observed between *Pterygoplichthys* and *H. chrysostiktos* while the latter diverged in more than 39 mutations from other *Hypostomus* species. Accordingly, the haplotype network for the *Hypostomus* species from NMA revealed species-

specific haplotypes and corroborated the remarkable genetic distinctiveness of *H. chrysostiktos* while *H. jaguar* and *H. unae* were closely related (Fig. 4c).

Cytogenetic analysis

The diploid number ($2n$) of *H. chrysostiktos* was equal to 52, with a karyotype formula composed of $22m + 20sm + 10st$ (Fig. 5a). The silver nitrate staining revealed Ag-NORs at terminal position on long arms of a single st pair, with size heteromorphism between homologous (Fig. 5a, detail). On the other hand, *H. jaguar* presented $2n = 76$, distributed into $10m + 20sm + 46st/a$ chromosomes (Fig. 5b). The same diploid number was observed in *H. unae* but their karyotype formulae were differentiated (Bitencourt et al., 2011b, 2012). Moreover, *H. jaguar* was also distinguished by the presence of multiple NORs (pairs 6 and 16) (Fig. 5b, detail) while most *Hypostomus* usually present single rDNA cistrons (ESM_8).

Discussion

As widely documented elsewhere (e.g. Lujan et al., 2011, 2015; Silva et al., 2016; Roxo et al., 2019), Loricariidae is a group of controversial systematics including several cases of misidentifications. Apparently, the phenotypic plasticity of armored catfishes could hinder the establishment of morphology-based intrafamilial interrelationships and alpha taxonomy (Armbruster, 2004; Armbruster & Page, 2006; Ferraris, 2007). In these cases, molecular analyses have been useful to detect cryptic species and to validate taxa inasmuch as MOTUs are often regarded as taxonomic entities according to the phylogenetic species concept (Jones et al., 2011; Pereira et al., 2013; Zhang et al., 2013; Souza et al., 2018).

Nonetheless, low genetic distance values have been reported among armored catfishes based on traditional molecular markers such as COI, thus leading to narrow barcode gaps (Carvalho et al., 2015). As a matter of fact, the mean interspecific genetic divergence among the *Hypostomus* representatives compiled in the present study was $\sim 3\%$, with most values ranging from 2 to 4% (Fig. 4a, b), what is surprisingly low for such a speciose and widespread group in South America. As proposed by Silva et al. (2016), the lack of conspicuous genetic divergence in *Hypostomus*

from distinct ecoregions in South America is likely to reflect their explosive diversification and fast speciation rates, particularly in Paraná River Basin.

In cases where both isolated morphology and molecular studies might fail in recognizing species, integrative methods and/or analyses are recommended (Padial et al., 2010), as currently performed. In general, the GM analyses revealed that both body size and shape vary within *H. unae*, *H. jaguar* and *H. chrysostiktos*. It should be pointed out that this pattern was more evident in *H. jaguar* (Fig. 2a–c), the only species from our study that was sampled in Paraguaçu and Recôncavo Sul Basins, representing the first report of this species in the latter.

Therefore, the significant variation in centroid size could be related to the environmental traits of each habitat since the collection site in Paraguaçu River Basin (where *H. chrysostiktos* is endemic) comprises a third-order river of rocky bottom and dark waters. On the other hand, the rivers where *H. unae* and *H. jaguar* were sampled in Recôncavo Sul Basin encompassed small drainages with sandy to rocky bottom. In fact, other studies have shown the close relationship between morphological diversity and riverscape features (e.g. Roxo et al., 2017). Moreover, the increased morphological variation in *H. jaguar* could also have been influenced by the high number of sampled individuals in this species ($n = 41$), thus representing more accurately its phenotypic range when compared to the others.

Even though this variation in shape has been probably determined by some overlapping in the GMs (Fig. 2d–i), this approach recovered the three analyzed species as distinct units (95% of confidence in the PCA and CVA ellipses). In particular, most striking differences in shape were related to changes in the head conformation, as commonly reported in species of *Hypostomus* (e.g. Silva et al., 2016) and other loricariids (Lujan & Conway, 2015; Roxo et al., 2017). Nonetheless, the morphometric analysis indicates, that *H. jaguar*, *H. unae* and *H. chrysostiktos* would represent three related species from a single genus, what was clearly refuted by the inclusion of other methods, reinforcing the importance of integrative methods to taxonomic and systematic inferences.

While the overall shape analysis placed *H. chrysostiktos* as more closely related to *H. unae* than to *H. jaguar* (Table 1; Fig. 2d, f, g, i), the meristic comparisons (ESM_4), the molecular data (Figs. 3, 4)

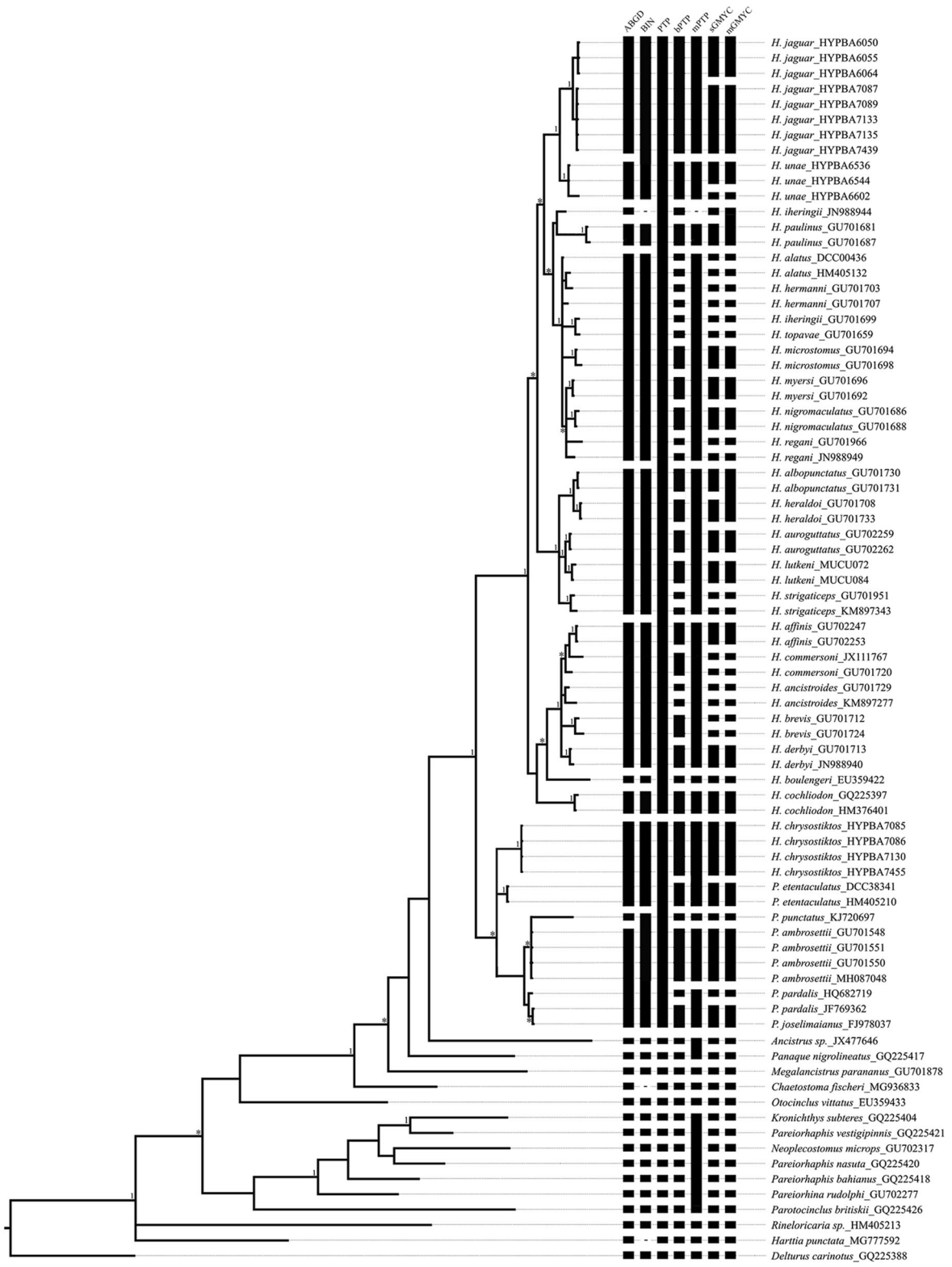


Fig. 3 Bayesian inference tree based on COI sequences of *Hypostomus* and related species with a posteriori probability (BI) values higher than 90% on each branch followed by the bars indicating the results of species delimitation algorithms and morphometric analysis. The asterisks indicate posterior probabilities of 0.9. The vertical bars indicate the species delimitation according to each algorithm

and the cytogenetic features (ESM_8) showed that *H. jaguar* and *H. unae* are very similar to each other. The divergence in COI sequences between both taxa was 1.5%, being inferior to the cutoff values traditionally used to discriminate species by DNA barcoding (Ratnasingham & Hebert, 2013) and to the optimized threshold (1.95%) inferred from the present dataset. Therefore, both species would fall into the “grey zone” of species delimitation, being indicative of lineages under speciation after recente divergence (Bittner et al., 2010). If confirmed by further studies with other *Hypostomus* populations and species from NMA, the armored catfishes from this ecoregion would expand the model of explosive diversification proposed in other Neotropical basins (Silva et al., 2016).

As a result, both taxa could not be differentiated by ABGD, BIN, and jMOTU algorithms. Nonetheless, it should be pointed out that the values of genetic distance between populations of *H. jaguar* and *H.*

unae were higher than those observed among other *Hypostomus* from highly isolated basins (Fig. 4, ESM_7). In addition, tree-based algorithms (sGMYC, mGMYC, bPTP, and mPTP) recovered *H. jaguar* and *H. unae* as distinct taxonomic units (Fig. 3). Actually, these methods are more refined than distance-based algorithms because they take the diversification time between lineages (GMYC) or the number of substitutions along the branches (bPTP, mPTP) into account (Kaplí et al., 2016).

Chromosomal analyses also discriminated both nominal taxa (Fig. 5, ESM_8). In spite of sharing the same diploid number ($2n = 76$), *H. jaguar* and *H. unae* presented distinct karyotype formulae, thus suggesting the occurrence of species-specific structural rearrangements, mainly driven by pericentric inversions combined with centric fissions in relation to the putative ancestor karyotype ($2n = 52$) (Artoni & Bertollo, 2001). In fact, the tribe Hypostomini encompasses a remarkable karyotype diversity with several species presenting distinctive diploid numbers and chromosome formulae, thus being highly informative to cytotaxonomy (Artoni & Bertollo, 2001; Bueno et al., 2012, 2018). Furthermore, *H. jaguar* was characterized by multiple Ag-NORs, a rare and putatively apomorphic condition in Loricariidae (Artoni & Bertollo, 2001), indicated as chromosomal markers

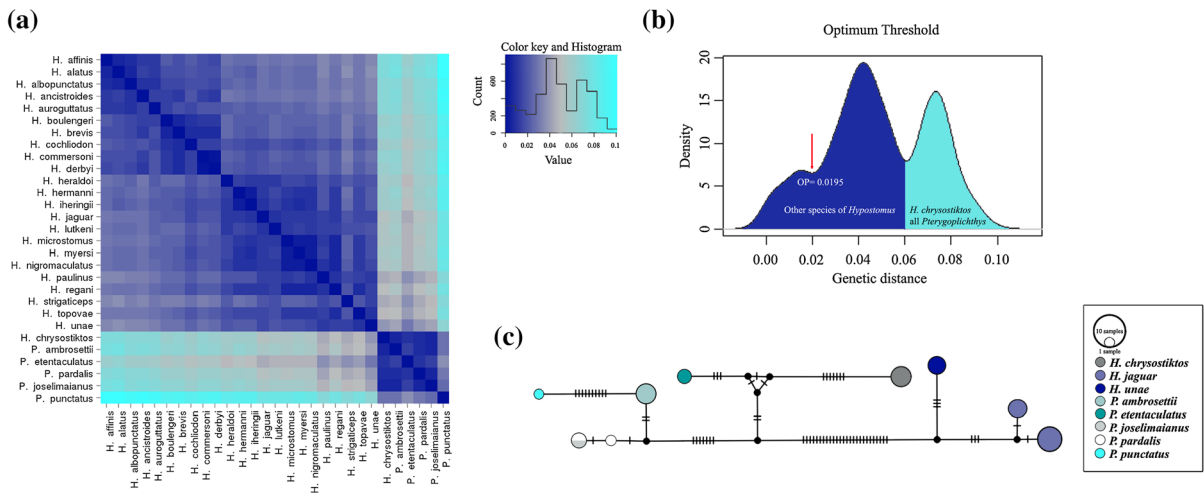
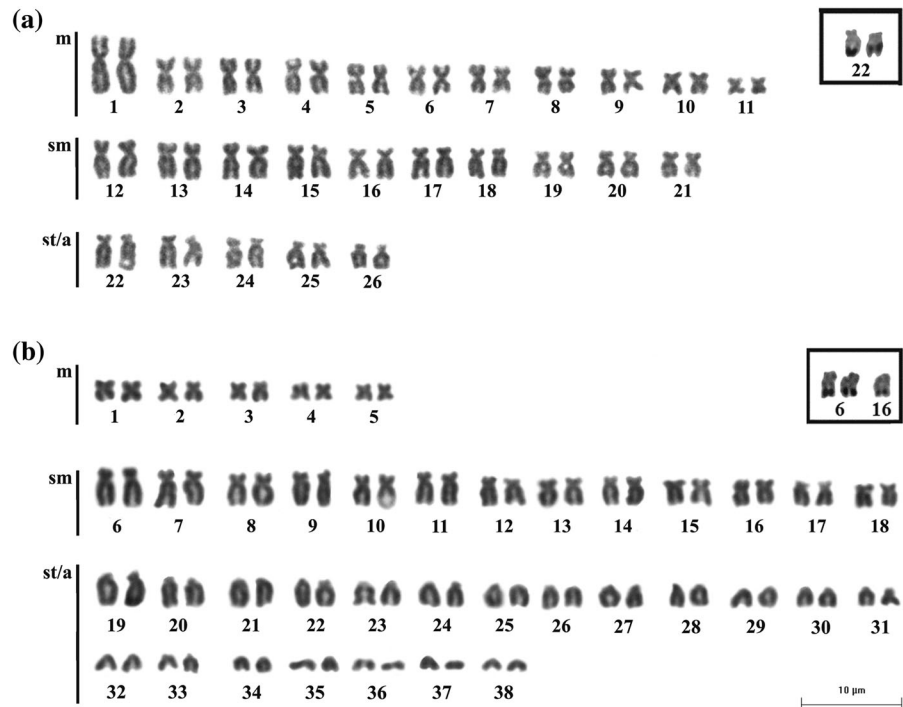


Fig. 4 Heatmap based on the interspecific distance matrix and the histogram showing the distribution frequency of genetic variation in the analyzed dataset of Hypostomini (a), density graph of the distance variation in relation to the optimized threshold established from the present dataset using SPIDER package (b), haplotype network based on COI sequences of

analyzed samples for *Hypostomus* and *Pterygoplichthys*. The color code indicates the species (see legend). Small black circles represent the median vectors corresponding to homoplasies or missing haplotypes. The size of the circles indicates the number of individuals having that particular haplotype (see legend). Dashes represent the numbers of nucleotide mutations (c)

Fig. 5 Representative karyotypes of *Hypostomus chrysostiktos* (a) and *H. jaguar* (b) after conventional Giemsa staining. In detail, the pairs bearing NORs after silver nitrate staining



in species of *Hypostomus* (Rubert et al., 2016). Thus, the distinctive karyotype pattern of *H. jaguar* combined with inferences based on tree-based algorithms supports its status as a taxonomic unit reproductively isolated from *H. unae*, particularly considering that both species are sympatric in Recôncavo Sul Basin.

On the other hand, a peculiar scenario was observed in *H. chrysostiktos*. Differently from most *Hypostomus* in which the genetic distances among species are usually low, the pairwise distance in COI sequences between *H. chrysostiktos* and congeners was much higher (6 to 8%) than the optimized threshold. This pattern was maintained even when compared to the other species from the same or adjacent basins. Indeed, 30 diagnostic sites were identified in *H. chrysostiktos* resulting in the lack of shared haplotypes separated by more than 39 mutations in relation to *H. jaguar* and *H. unae* (Fig. 4c, ESM_6, ESM_7). Consequently, *H. chrysostiktos* was placed apart from the *Hypostomus* cluster in the phylogenetic trees and discriminated from the congeners by all tested algorithms (Fig. 3).

Instead, *H. chrysostiktos* was more genetically related to the genus *Pterygoplichthys* than to *Hypostomus*, as demonstrated by the BI and ML trees and species delimitation algorithms (Fig. 3, ESM_5). In particular, a close evolutionary relationship is

indicated between *H. chrysostiktos* and *P. etentaculatus* from São Francisco River Basin (genetic distance of ~ 2%, near the optimized cutoff of 1.9%), being differentiated by four diagnostic sites. The haplotype network also showed that this taxon is separated from *Pterygoplichthys* by a lower number of mutation steps (12) when compared to *H. unae* and *H. jaguar* (Fig. 4c).

Accordingly, a comparative morphological analysis between *H. chrysostiktos* and *P. etentaculatus* (ESM_1) reveal some resemblance between *H. chrysostiktos* and *P. etentaculatus*, due to high number of dorsal-fin branched rays (10–11) and 8–11 post-dorsal vertebrae in both species, which have probably influenced the first recognition of *H. chrysostiktos* from the Diamantina Plateau as *Pterygoplichthys* sp. (Higuchi et al., 1990). In fact, several authors have described sister taxa between São Francisco and NMA ecoregions, presently separated by the Espinhaço Mountains (Camelier & Zanata, 2014; Sarmento-Soares et al., 2016; Ramirez et al., 2017) but phylogeographic studies along these areas are still incipient, hindering further species inferences.

Again, cytogenetics provided additional evidence to the distinctiveness of *H. chrysostiktos*. While the species of *Hypostomus* from coastal basins in NMA

studied so far share $2n = 76$ and a high number of one-armed chromosomes as reported in *H. unae* (Biten-court et al., 2011a, b, 2012) and presently observed in *H. jaguar* (Fig. 5b, ESM_7), *H. chrysoptiktos* presented $2n = 52$ and a high number of bi-armed chromosomes ($22m + 20sm + 10st$) (Fig. 5a). Similarly to the present molecular data, the cytogenetic features in *H. chrysoptiktos* ($2n = 52$; $22m + 20sm + 10st$) (Fig. 5a) resemble those reported in *Pterygoplichthys* since *P. ambrosettii*, *P. anisitsi*, *P. multiradiatus*, *P. gibbiceps* (Alves et al., 2006; Fernandes et al., 2015; Bueno et al., 2018), and *P. joselimaianus* (Oliveira et al., 2006) from distinct Brazilian basins are also characterized by $2n = 52$ and few acrocentric pairs, regarded as symplesiomorphic features in *Pterygoplichthini* (Bueno et al., 2018). These data also highlight the importance of cytogenetic data for the integrative taxonomy of Neotropical fish.

Based on the multiple lines of evidence presented in this paper, *H. chrysoptiktos* was artificially allocated within *Hypostomus* because putative convergent morphological traits have been used in their diagnosis, including synapomorphies previously recognized as homoplastic (Armbruster & Page, 2006). In fact, fishes from fast-water habitats usually share similar adaptations related to fusiform body, wedge-shaped head and peculiar skin structure that could account for the convergence of morphological traits in non-related species, with several examples in Loricariidae (Lujan & Conway, 2015; Roxo et al., 2017). Since *H. chrysoptiktos* and other representatives of *Hypostomus* co-occur in coastal drainages along the NMA ecoregion, they should be under similar selective pressures that could theoretically lead to the evolution of morphological convergence. As a result, phylogenetically distant groups in sympatry can resemble more to each other than to actual closely related species from distinct regions under a morphological viewpoint (Langerhans, 2008), characterizing cases of convergence as also reported by Roxo et al. (2019).

In conclusion, we recognize *H. chrysoptiktos* as *Pterygoplichthys chrysoptiktos* n. comb., representing the first description for this genus in NMA ecoregion. As exemplified in the present work, a reappraisal of the diagnostic traits in *Pterygoplichthys* should be carried out and rather combined with other sources of information (e.g. molecular and cytogenetic data) to discriminate homoplastic from homologous features

and to resolve the taxonomic uncertainties in this and other groups of Loricariidae.

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