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Molecular characterization of *Astyanax* species (Characiformes: Characidae) from the upper Paraguaçu River basin, a hydrographic system with high endemism

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Molecular tools have been employed to improve the knowledge about freshwater Neotropical fishes. Such approaches supporting studies of groups including species complexes such as *Astyanax*, one of the most diversified and taxonomically complex genus of the family Characidae. Here, we employed species delimitation analyses in four *Astyanax* species described for the upper Paraguaçu River basin, a drainage within Northeastern Mata Atlântica freshwater ecoregion with high endemism. We implemented single and multilocus approaches based on two mitochondrial and one nuclear markers. Cytochrome c Oxidase I sequences previously available for *Astyanax* species were also added to our dataset. The single locus analyses showed *A. epiagos*, *A. rupestris*, and *A.* aff. *rupestris* as different Molecular Operational Taxonomic Units (MOTUs), while *A. brucutu* and *A. lorien* were grouped. However, the multilocus approach distinguished these two species and showed congruence for the remaining single locus results. *Astyanax* aff. *rupestris* was separated into two MOTUs using both approaches, highlighting the need for an integrative taxonomic revision including *A.* aff. *rupestris*. These findings contribute to a better understanding of the diversity of this fish group in the upper Paraguaçu, identifying hidden diversity and reinforcing the relevance of this hydrographic system as a notable hotspot for ichthyofauna biodiversity endemism.

Keywords: Biodiversity, Caatinga fishes, Freshwater fish, Hidden genetic diversity, Species delimitation.

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Ferramentas moleculares têm sido empregadas para melhorar o conhecimento sobre os peixes Neotropicais. Tais abordagens apoiam estudos de grupos que incluem complexos de espécies, como *Astyanax*, um dos gêneros mais diversificados e taxonomicamente complexos dentro da família Characidae. Neste estudo, nós empregamos análises de delimitação de espécies em quatro espécies de *Astyanax* recentemente descritas da bacia do alto rio Paraguaçu, uma drenagem dentro da ecorregião Mata Atlântica Nordeste que apresenta alto endemismo. Nós realizamos abordagens de loco único e multilocos baseadas em dois marcadores mitocondriais e um nuclear. Sequências de Citocromo c Oxidase I anteriormente disponíveis para espécies de *Astyanax* foram adicionadas ao nosso conjunto de dados. As análises de loco único mostraram *A. epiagos*, *A. rupestris* e *A.* aff. *rupestris* como diferentes Unidades Taxonômicas Operacionais Moleculares (MOTUs), enquanto *A. brucutu* e *A. lorien* foram agrupadas. Entretanto, a abordagem multilocos distinguiu estas duas espécies e mostrou congruência com os demais resultados das análises de loco único. *Astyanax* aff. *rupestris* foi separada em duas MOTUs usando ambas as abordagens, sugerindo a necessidade de uma revisão taxonômica integrativa incluindo *A. rupestris* e ambas *A.* aff. *rupestris*. Esses achados contribuem para uma melhor compreensão da diversidade desse grupo de peixes na bacia do rio Paraguaçu, identificando diversidade oculta e reforçando a relevância desse sistema hidrográfico como um notável *hotspot* de endemismo da biodiversidade da ictiofauna.

Palavras-chave: Biodiversidade, Delimitação de espécies, Diversidade genética oculta, Peixe de água doce, Peixes de Caatinga.

INTRODUCTION

Astyanax Baird & Girard, 1854 is one of the most diversified and taxonomically complex genus within the Characidae family (Characiformes), including 125 valid species widespread throughout nearly the entire Neotropical region (Rossini *et al*., 2016; de Pinna *et al*., 2018; Fricke *et al*., 2022). This genus comprises small species of about 40 to 200 mm standard length (Garutti, 1998), occurring in a wide diversity of niches and aquatic environments within freshwater drainages from the southern United States to central Argentina (Eigenmann, 1921; Bertaco, Garutti, 2007).

Characterized by presenting high phenotypic plasticity and adaptation to distinct environmental conditions (Orsi *et al*., 2004), *Astyanax* species are among the most important components of the freshwater food web, with significant participation in the diet of large predator fishes (Prioli *et al*., 2002), being usually dominant in headwaters and small tributaries (Bertaco, Lucena, 2010). Within the Brazilian Shield, the genus is commonly found in large river systems (*e.g*., Amazon, La Plata, and São Francisco) and in the northeastern Brazilian coastal basins, including the Paraguaçu River basin.

The Paraguaçu River basin is considered one of the largest basins in northeastern Brazil (Higuchi *et al*., 1990) and an extremely relevant drainage of the Northeastern Mata Atlântica freshwater ecoregion (NMAF, ecoregion 328, *sensu* Abell *et al*., 2008; Camelier,

Zanata, 2014a). The fish fauna of the basin has been recognized by its high level of endemism (Buckup, 2011; Camelier, Zanata, 2014a; de Pinna *et al*., 2018). Recently, new species have been described for this basin, including different fish groups, such as *Astyanax* (*e.g*., Camelier, Zanata, 2014b; Zanata *et al*., 2017, 2018; Burger *et al*., 2019), *Characidium* Reinhardt, 1867 (*e.g*., Zanata, Camelier, 2015; Melo, Espíndola, 2016), *Copionodon* de Pinna, 1992 (*e.g*., de Pinna *et al*., 2018) *Moenkhausia* Eigenmann, 1903 (*e.g*., Benine *et al*., 2009), and *Rhamdiopsis* Haseman, 1911 (*e.g*., Bockmann, Castro, 2010).

More than ten species of *Astyanax* are currently reported as occurring in the Paraguaçu hydrographic system (Santos, Caramaschi, 2007, 2011). From this total, six species are endemic to the upper Paraguaçu course and have allopatric distribution, occurring in different tributaries: *A. brucutu* Zanata, Lima, Dario & Garhard, 2017, Pratinha River (Zanata *et al*., 2017); *A. epiagos* Zanata & Camelier, 2008, Jacuípe River (Zanata, Camelier, 2008); *A. hamatilis* Camelier & Zanata, 2014, Utinga, Una, and São José rivers (Camelier, Zanata, 2014b); *A. lorien* Zanata, Burger & Camelier, 2018, Santo Antônio River; *A. rupestris* Zanata, Burger & Camelier, 2018, Coisa Boa and Cumbuca rivers (Zanata *et al*., 2018); and *A. sincora* Burger, Carvalho & Zanata, 2019, Tremedal stream (Burger *et al*., 2019). Furthermore, according to Zanata *et al.* (2018), the Piabinha River shelters a morphotype tentatively identified by the authors as *Astyanax* aff. *rupestris*, due to divergences in some morphological characters when compared to *A. rupestris*. The high richness within *Astyanax* and the fact of being traditionally defined by a combination of non-exclusive characters (see Eigenmann, 1921), added to its recognized phenotype plasticity (Orsi *et al*., 2004), occasionally hinders accurate species identification. Consequently, some taxa are frequently identified only at the generic level or into species complexes (*e.g*., Moreira-Filho, Bertollo, 1991; Garutti, Britski, 2000). A recent integrative phylogeny (Terán *et al*., 2020) recovered species attributed to *Astyanax* in different subfamilies and genera, including the resurrected *Psalidodon* Eigenmann, 1911 and a new genus, *Andromakhe* Terán, Benitez & Mirande, 2020 (Terán *et al*., 2020). Dagosta, Marinho (2022) argue that although this study has been efficient in recovering the polyphyletic nature of *Astyanax*, it failed in providing consistent diagnosis characters for the proposed clades. None of the species evaluated here were analyzed by Terán *et al*. (2020), except *A. brucutu* that, due to the lack of molecular data, was inserted as *incertae sedis* in Gymnocharacini. In view of that, the species is herein assigned to *Astyanax*.

It is well known that, given the remarkable richness and phenotypic plasticity observed in the Neotropical freshwater ichthyofauna (Wimberger, 1992; Reis *et al*., 2016), and its high number of cryptic species (Piggott *et al*., 2011), the genetic analysis is a powerful tool for improving our knowledge on taxonomy and evolution of this group (Bellafronte *et al*., 2013; Costa-Silva *et al*., 2015; Anjos *et al*., 2020). Different DNA-based approaches, such as DNA barcode (Hebert *et al*., 2003; Ward, 2009), molecular species delimitation (Pons *et al*., 2006; Puillandre *et al*., 2012; Ratnasingham, Hebert, 2013), and molecular phylogeny analyses (Edwards, 2009), have been successfully used for defining Molecular Operational Taxonomic Units (MOTUs) and characterizing hidden biodiversity within Neotropical freshwater fish (*e.g*., Ramirez, Galetti Jr., 2015; Carvalho *et al*., 2011; Pereira *et al*., 2011, 2013; Machado *et al*., 2016; Ramirez *et al*., 2017; Silva-Santos *et al*., 2018; Souza *et al*., 2018; Lopes *et al*., 2020).

Here, we performed species delimitation analyses in four recently described species of *Astyanax* plus the morphotype *A.* aff. *rupestris*, all endemic to the upper Paraguaçu River basin. We aimed to produce a DNA barcode reference library for the focal species and to investigate the existence of hidden diversity, contributing thus to a better knowledge of this relevant fish group and its diversification. Using mitochondrial and nuclear sequences, we combined single and multilocus-based methods to carry out genetic analyses. Our sequence data were compared to those that had already been published in *Astyanax* species studies.

MATERIAL AND METHODS

Biological sampling. Biological samples of four endemic species and one morphotype of *Astyanax* were collected from 76 specimens distributed along six tributaries in the upper Paraguaçu River basin, Bahia, Brazil (Fig. 1). The material analyzed included *A. brucutu* from the Pratinha River, Iraquara (n = 3); *A. epiagos* from the Ferro Doido River, Jacobina (n = 5); *A. lorien* from the Preto River, Palmeiras (n = 15); *A. rupestris* from the Coisa Boa River, Andaraí (n = 17) and Cumbuca River, Mucugê (n = 3); and the morphotype *Astyanax* aff. *rupestris* from the Piabinha River, Mucugê (n = 33). Fin fragments were sampled from each specimen, using tweezers and scissors, and then stored in ethanol (95%) in a freezer at 4°C. Vouchers were deposited in the ichthyological collection of the Museu de História Natural da Bahia, Salvador, Bahia, Brazil. All information related to the sampling localities, specimens, and vouchers is available in Tab. **S1**.

We complemented our biological sampling by downloading 1,792 COI sequences available in the BOLD system database (http://www.boldsystems.org/, accessed on March 31, 2020) for 66 nominal *Astyanax* species of several hydrographic basins from localities informed by the database depositors (Tab. **S**2). That dataset included *A. hamatilis* from São José River (n = 7); and unidentified specimens of *Astyanax* sp. from Coité (n = 2) and Piabinha (n = 2) rivers from the upper Paraguaçu basin. Altogether we analyzed five from the six *Astyanax* species endemic to the upper Paraguaçu River, except *A. sincora*, that was not collected and was not available in the BOLD system as well.

DNA isolation, amplification, purification, and sequencing. DNA extraction was performed using buffer saline protocol (Aljanabi, 1997), and DNA was quantified using a Biophotometer (Eppendorf, Hamburg, Germany). Partial Cytochrome c Oxidase subunit I (COI), Cytochrome b (Cytb) and the first intron of the S7 ribosomal protein (S7) genes were amplified using the following oligonucleotides: COI FishF1 and COI FishR1 (Ward *et al*., 2005), AnosCytBF and AnosCytBR (Ramirez, Galetti, 2015), and S7RPEX1F and S7RPEX2R (Chow, Hazama, 1998). Polymerase Chain Reactions (PCRs) were performed according to their respective authors.

The amplified products were checked on agarose gel 1% by electrophoresis, and then purified with a polyethyleneglycol (PEG) 20% protocol (Lis, 1980). Sequencing was run on an automated sequencer ABI3730XL (Applied Biosystems, Little Chalfont, UK), and all sequences were aligned and edited with the Geneious 6.1.6c software (Kearse *et al*., 2012). The plugin "find heterozygotes" of this software was used with a 0.80

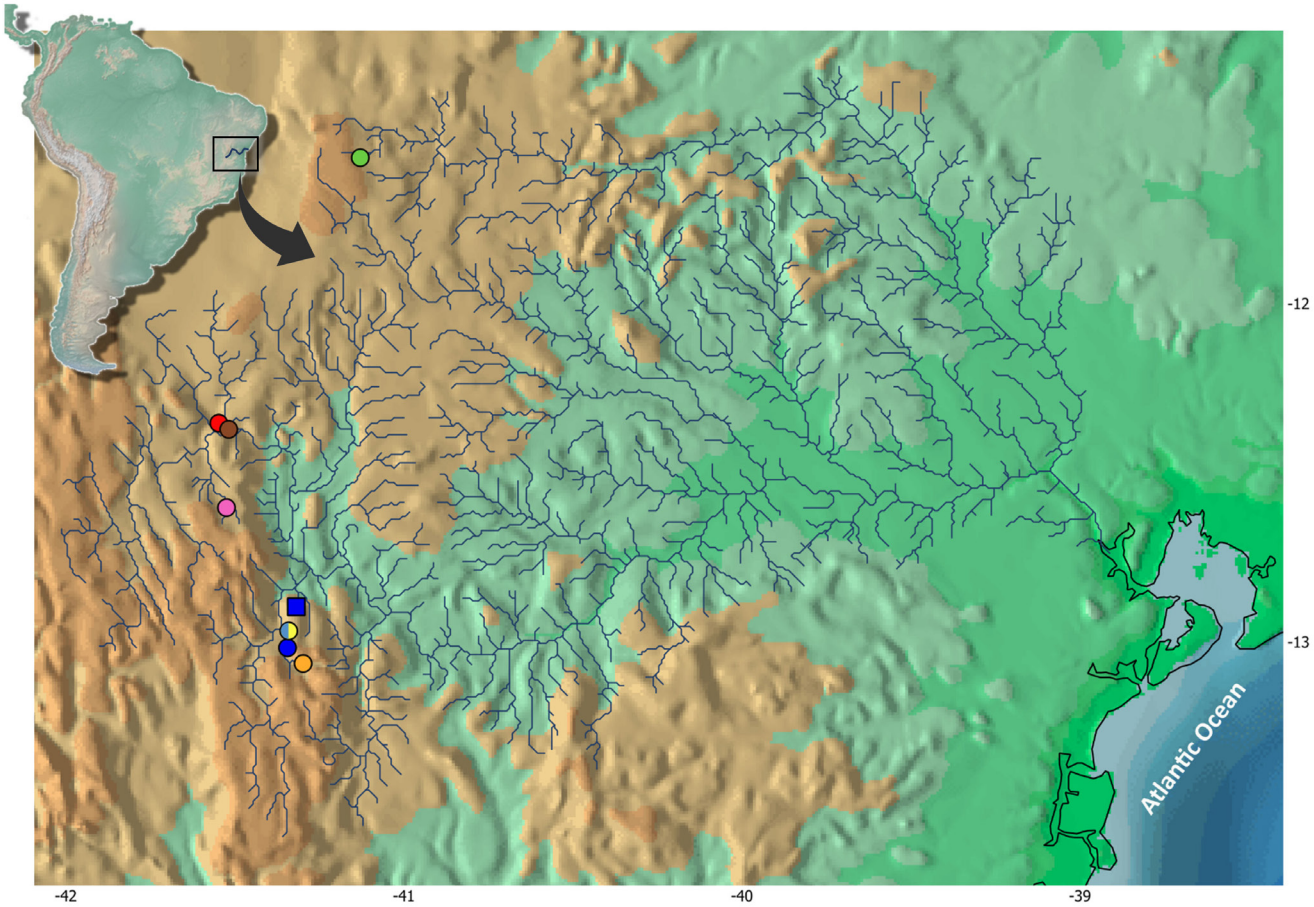

FIGURE 1 | Map of the Paraguaçu River basin, Bahia, northeastern Brazil, showing collection sites of *Astyanax* species sampled in this study and for two *Astyanax* sp. available in the BOLD system database (*), with the exception of *A. hamatilis*. *Astyanax rupestris* from the Coisa Boa River (dark blue square) and Cumbuca River (dark blue circle), *A.* aff. *rupestris* from the Piabinha River (half yellow and blue circle), *Astyanax* sp. from the Piabinha River (orange circle*), *A. lorien* from the Preto River (pink circle), *Astyanax* sp. from Coité River (brown circle*), *A. brucutu* from the Pratinha River (red circle), and *A. epiagos* from the Ferro Doido River (green circle). The colors of the symbols on the map are in accordance with Fig. 2. Scale 1:1300723.

threshold in order to identify heterozygous positions and assign ambiguity codes in the nuclear sequences, such as eventual NUMTS (nuclear mitochondrial DNA segments). The S7 haplotypes were estimated using the SEQPHASE web tool (Flot, 2010). COI sequences were deposited in the BOLD system under Project name ASTBA. Cytb and S7 sequences were deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/) under specific accession numbers as shown in the Tab. **S1**.

Single locus analyses. To obtain a general picture of genetic relationships within *Astyanax*, we first implemented a broad Bayesian inference analysis with BEAST 2.4.6 (Heled, Drummond, 2010) using a large COI sequence dataset, representing the four species studied herein and other 66 nominal *Astyanax* species obtained from BOLD. Two independent runs were performed following the parameters: 100 million generations (Markov chain Monte Carlo, MCMC), sampling every 10,000, a strict lognormal clock for all partitions, and the Yule speciation model. The best-fitting model (GTR+I+G) was

selected under the Bayesian Information Criterion (BIC) by jModeltest 2 (Darriba *et al.,* 2012). A consensus tree was combined and resampled in LogCombiner with 30% burn-in, and then summarized in TreeAnnotator using BEAST 2.4.6 (Heled, Drummond, 2010). An effective sample size (ESS) of 200 or higher was required for all parameters and checked in TRACER 1.6 (Rambaut *et al*., 2014).

Based on the COI tree, only the species recovered in a single clade, which included the four targeted *Astyanax* species, were hereafter analyzed. For this new dataset, we implemented three species delimitation approaches using the COI sequences: Barcode Index Number (BIN, Ratnasingham, Hebert, 2013), Automatic Barcode Gap Discovery (ABGD, Puillandre *et al*., 2012), and General Mixed Yule Coalescent (GMYC, Pons *et al*., 2006). The BIN analysis was performed automatically in the BOLD system. Our sampling was assembled in a preexisting BIN database or assigned to a new BIN (Ratnasingham, Hebert, 2013). For the ABGD we used the K2P (Kimura-2-parameters) modified parameters (Pmin = 0.04, Pmax = 0.1, relative value gap X = 0.1), and 100 steps. The GMYC analysis was implemented in the SPLITS package for R statistical software (R Development Core Team, 2017), using a single threshold under the standard parameters (interval = c(1,10)). This analysis uses an ultrametric tree to establish species limits based on the Yule (pure-birth) and Kingman models (coalescence), and to calculate the probability of splits in a lineage based on speciation rates (Ratnasingham, Hebert, 2013). As input, we used an ultrametric tree obtained with a lognormal relaxed clock, birth-death speciation model, HKY + G substitution model, 50 million MCMC sampling every 5,000 and burn-in of 10% in BEAST 2.4.6. Convergence was assessed by estimating the effective sampling size (ESS) using Tracer 1.7 software (Rambaut *et al*., 2014) and accepting ESS values of 200 or more.

We calculated the genetic distances among the MOTUs obtained through the three species delimitation methods using the K2P model with MEGA 7.0.26 (Kumar *et al*., 2016). We used the K2P, since this model allows us to compare the values found here with those previously reported in other *Astyanax* studies (*e.g*., Carvalho *et al*., 2011; Pereira *et al*., 2011; Rossini *et al*., 2018). Collins *et al.* (2012) tested whether the K2P is a well-fitted model at the species level by comparing it to the other models (JC, F81, TrN, HKY, HKY+C and GTR+C) using data sets from different animal groups, including fish. The results indicate that the differences in distance between K2P and other models were usually minimal, and the identification success rates were largely unaffected by model choice, even when interspecific threshold values were reassessed.

Multilocus analyses. To obtain a multilocus Bayesian species tree (ST) we considered the nominal species recognized by morphological studies and the results generated by the GMYC analysis. This analysis was performed in BEAST (Star-BEAST) using 500 million MCMC, sampling every 10,000, relaxed clock and Yule models, and a burn-in of 20%. Nucleotide substitution models were selected based on BIC (Bayesian Information Criterion) using jModeltest 2 (Darriba *et al*., 2012). The best-fitting models were HKY for COI and Cytb, and F81 + G for S7. All generations were sampled from the stationary phase. The convergence of analyses and adequate ESS (>200) were evaluated in Tracer v1.7 (Rambaut *et al*., 2014).

The Bayesian ST was used as guide tree to the Bayesian species delimitation approach using multilocus data (Yang, Rannala, 2010; Rannala, Yang, 2013) in the BP&P 3.3 software (Yang, 2015). This software uses a coalescent model, calculating the posterior

probability of potential species considering the coalescent process of lineage sorting. The basic model used by BP&P involves two types of parameters: the population sizes on the species tree (θs), and the species divergence times (τs). To evaluate the impact of these parameters on species delimitation results and consider a range of speciation histories, we tested different gamma prior configurations and some default parameters in four distinct combinations. A first one assumed relatively large ancestral population sizes and deep divergences (θ ~ G(1,10) and τ_0 ~ G(1, 10)) among species; a second combination considered small ancestral population sizes and shallow divergences among species (θ ~ G(2, 2000) and τ_0 ~ G(2, 2000)); and the other two combinations assumed either large ancestral populations sizes (θ ~ G(1, 10)) and relatively shallow divergences among species (τ_0 ~ G(2, 2000)), or small ancestral population sizes and deep divergences (θ ~ G(2, 2000), τ_0 ~ G(1, 10)).

RESULTS

From the 76 individuals sampled, we obtained 75 COI sequences, comprised of 614 bp, without stop-codons, deletions, or insertions. Our primary Bayesian tree, obtained with a total of 1,867 COI sequences (75 generate herein and 1,792 downloaded from BOLD), grouped all *Astyanax* species belonging to the upper Paraguaçu River basin in a clade named hereafter Clade 1, with 0.95 probability posterior value (Fig. 2A), except *A. hamatilis*. This latter species was joined with species from other hydrographic basins (*Astyanax taeniatus* Jenyns, 1842 from the Ribeira da Terra Firme River; *A. burgerai* Zanata & Camelier, 2009 from the Almada River; *Astyanax* sp. from the Marcanaí River, and *Astyanax* sp. from the Macacuá River) in a distant clade from Clade 1.

The Clade 1 recovered 19 nominal *Astyanax* species from 17 hydrographic basins, which are part of the Brazilian crystalline shield and the Atlantic coast drainages (Figs. 2A, B), representing 390 sequences for *A. bifasciatus* Garavello & Sampaio, 2010, *A. bockmanni* Vari & Castro, 2007, *A.* aff. *bockmanni*, *A. dissimilis* Garavello & Sampaio, 2010, *A. fasciatus* Cuvier, 1819, *A. gymnodontus* Eigenmann, 1911, *A. gymnogenys* Eigenmann, 1911, and *A. minor* Garavello & Sampaio, 2010 from Paraná River basin; *A. paranae* Eigenmann, 1914 (Paraná and Paraguay basins); *A. intermedius* Eigenmann, 1908 from Paraíba do Sul basin; *A.* aff. *intermedius* from Paraná and Paraíba do Sul basins; *A.* cf. *fasciatus* and *A. rivularis* Lutken, 1875 from Paraná and São Francisco basins; *A. scabripinnis* Jennys, 1842 from Paraná, Paraíba do Sul, Paraguay, São Francisco, and Doce basins; *A. bimaculatus* Linnaeus, 1758 from São Francisco basin; *A. laticeps* Cope, 1894 and *A. obscurus* Hensel, 1870 from Itapocu basin; *A.* aff. *fasciatus* and *A.* aff. *jequitinhonhae* Steindachner, 1877 from Jequitinhonha basin; *A. xavante* Garutti & Venere, 2009 from Araguaia basin; and *A. brucutu*, *A. epiagos*, *A. lorien*, *A. rupestris*, and *A.* aff. *rupestris* from upper Paraguaçu basin (Fig. 2B).

Specimens of *Astyanax* sp. were named following the indication of the collection site reported in the BOLD database. Among the specimens identified at the genus level only (*i.e.*, *Astyanax* sp.), two sequences belong to individuals from the Coité River and two belong to individuals collected in the Piabinha River, both rivers from the upper Paraguaçu River basin. Details about the samples are available in the Tab. **S2.**

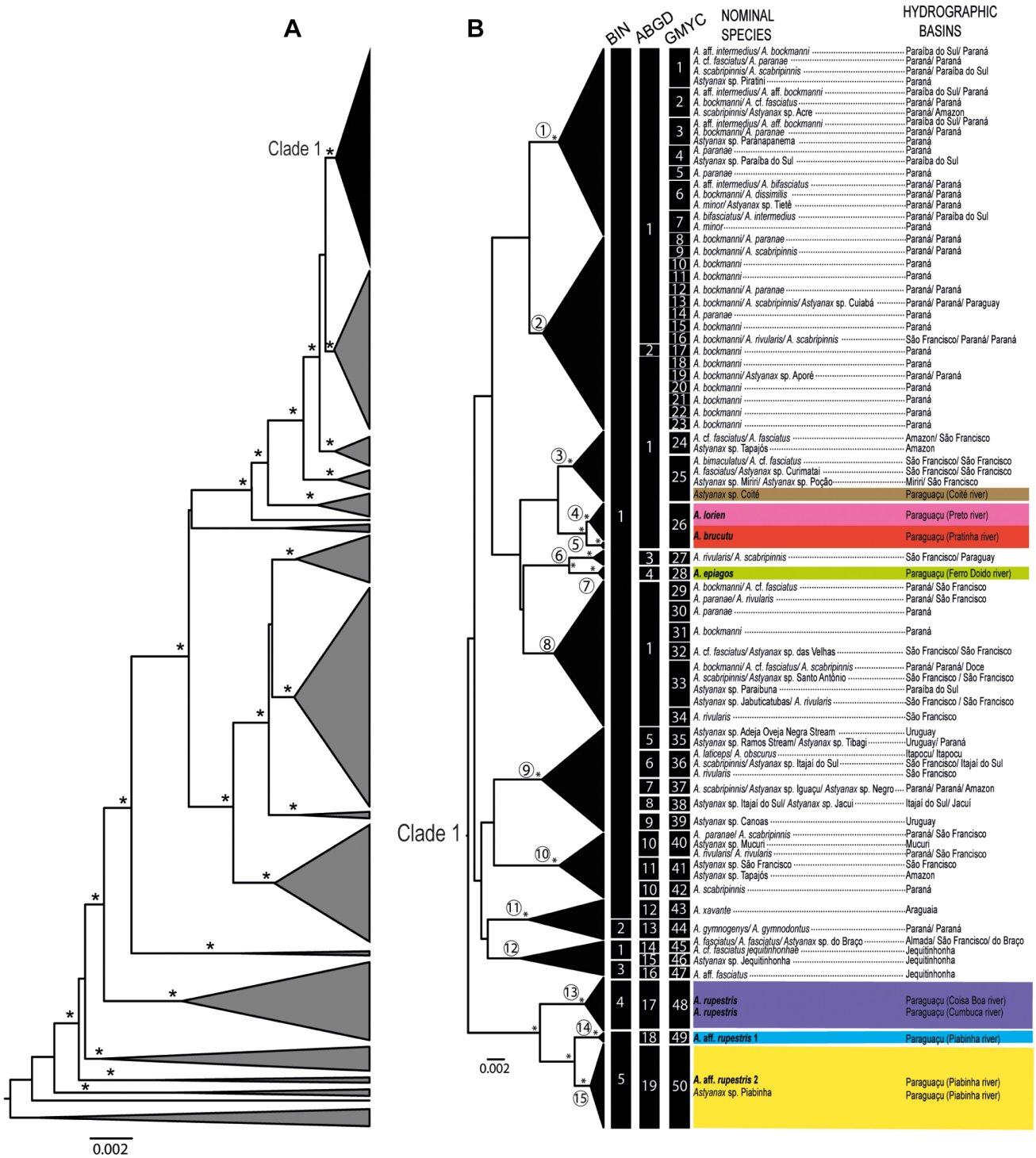

FIGURE 2 | Bayesian tree showing phylogenetic relationships among *Astyanax* species, using 1,792 COI sequences available in the Bold system database, and 75 ones produced in this study for specimens of *Astyanax* endemic to the upper Paraguaçu River basin. **A**. Clusters (black) for the clade 1 formed by *Astyanax* species closely related to the specimens collected in the Paraguaçu River; and clusters (grey) for the remaining analyzed *Astyanax* species. **B**. Clade 1 in details, depicting the delimitation species results using BIN, ABGD, and GMYC approaches. Black rectangles represent the distinct number of MOTUs identified by the three analyses: BIN (MOTU 1-5); ABGD (MOTU 1-19); GMYC (MOTU 1-50). The numbers in the nodes correspond to the main clusters of species (Tab. **S2**). Nodes marked with an asterisk denote posterior probabilities greater than 0.9. Species of *Astyanax* from the upper Paraguaçu River basin are highlighted in colored rectangles. *Astyanax* sp. sequences were download from BOLD system database. Our studied species are in bold letters. The colors of the species name are highlighted in accordance with Fig. 1.

Single locus species delimitation analyses. Our single locus delimitation analyses for the species obtained in Clade 1, with COI sequences, showed different results among the three approaches used herein (BIN, ABGD, GMYC, Fig. 2B). The BIN approach recovered five distinct MOTUs. Focusing on the species from the Paraguaçu River basin, *A. brucutu*, *A. epiagos*, and *A. lorien* were grouped with *Astyanax* sp. Coité and thirteen nominal species in a single MOTU (MOTU 1, BIN AAC5910). *Astyanax rupestris* (MOTU 4, BIN ADI2769) was separated from *A.* aff. *rupestris* (MOTU 5, BIN ACR6356), while *Astyanax* sp. Piabinha was grouped with this latter. The mean divergence within BINs ranged from 0% (MOTU 2, BIN ABZ0055) to 1.7% (MOTU 1, BIN AAC5910), and the pairwise divergence between BINs ranged from 1.8% (MOTU 4, BIN ADI2769 and MOTU 5, BIN ACR6356) to 3.6% (MOTU 4, BIN ADI2769 and MOTU 3, BIN ABZ6219).

The ABGD analysis indicated the presence of 19 distinct MOTUs into Clade 1, with four singletons, *i.e.*, four MOTUs represented only by a single individual. The average genetic distances within and between these MOTUs were 0.18% and 2.5%, respectively. We found *A. brucutu*, *A. lorien*, and *Astyanax* sp. Coité grouped into the MOTU 1 with 12 nominal species. The species *A. epiagos* and *A. rupestris* were separately recovered in the MOTU 4 and 17, respectively. The genetic distance between MOTU 1 and 4 was 1.8%. Differently from the BIN analysis, *A.* aff. *rupestris* was divided in two MOTUs named *A.* aff. *rupestris* 1 (MOTU 18) and *A.* aff. *rupestris* 2 (MOTU 19), with a genetic distance between them equal to 0.7%.

The GMYC results showed a total of 50 MOTUs, of which eight were singletons. The confidence limit for the estimated number of entities ranged from 49 to 60. The null model likelihood (L_0 = 3877.946) was significantly ($p < 0.01$) lower than the GMYC model likelihood (L = 4039.65), indicating that there is probably more than one species in our sample. We observed *A. brucutu* and *A. lorien* grouped in the same MOTU (MOTU 26), while *Astyanax* sp. Coité River was clustered to the species *A. bimaculatus*, *A.* cf. *fasciatus*, and *A. fasciatus* from Miriri and São Francisco basins (MOTU 25). On the other hand, *A. epiagos* (MOTU 18), *A. rupestris* (MOTU 48), *A.* aff. *rupestris* 1 (MOTU 49), and *A.* aff. *rupestris* 2 (MOTU 50) were recovered as independent MOTUs. The average genetic distance values were 0.14% for intra- and 1.8% for inter-MOTUs.

The average genetic distance values calculated between *Astyanax* from the Paraguaçu River basin, defined by the BP&P analysis, were 0.0% (intra-MOTU) and 2.1% (inter-MOTU). The maximum intra-MOTU distance was 0.001% (*A. rupestris*), and the minimum inter-MOTU distance was 0.3% (*A. brucutu* and *A. lorien*). *Astyanax* aff. *rupestris* 1 and *A.* aff. *rupestris* 2 showed 0.7% inter-MOTU distance, while both diverged 1.8% from *A. rupestris* (see Tab. **S3**).

Multilocus species delimitation. After edition and alignment, the dataset for *A. brucutu*, *A. epiagos*, *A. lorien*, *A. rupestris*, and *A.* aff. *rupestris* consisted of 140 S7 sequences with 684 bp, and 75 COI and 73 Cytb sequences, comprising fragments with 614 bp and 1,032 bp, respectively. No stop-codons, deletions or insertions were observed.

The BEAST* analyses used to obtain a guide tree reached apparent convergence, with ESS of at least 300 for all parameters, showing convergence between runs. The BP&P results, using this prior information, separated *A. brucutu*, *A. epiagos*, *A. lorien*, and *A. rupestris*, and, similarly to the ABGD and GMYC results, suggested the existence

of two genetic lineages within *A.* aff. *rupestris* (*A.* aff. *rupestris* 1 and *A.* aff. *rupestris* 2). The speciation probabilities assumed maximum values (1.0) on all nodes. Moreover, the species delimitation results were not affected by different prior settings, and we recovered maximum speciation probability values for all internal nodes in all tested combinations, indicating consistent results among runs (Fig. 3).

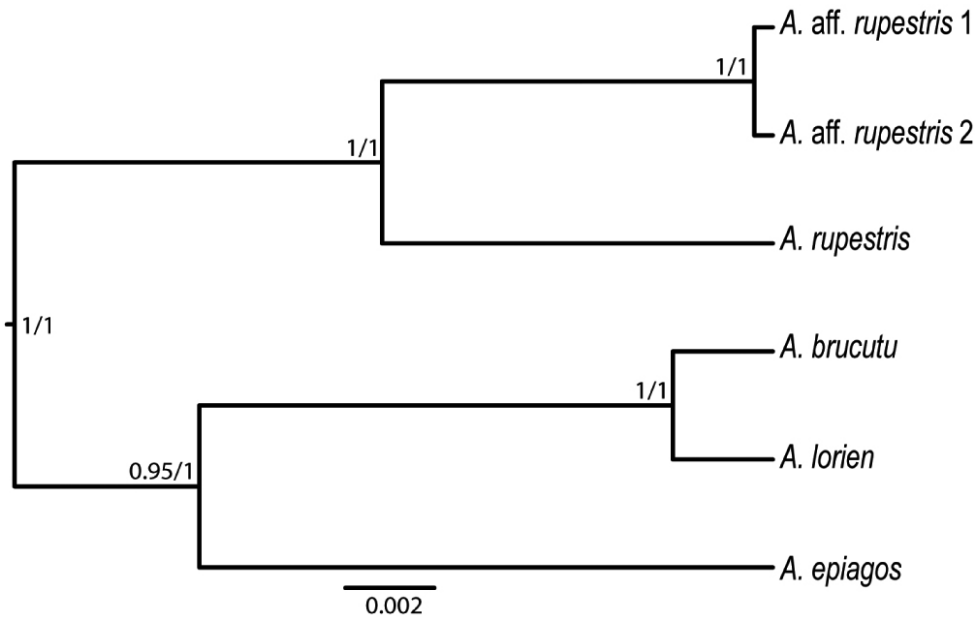

FIGURE 3 | Species tree showing phylogenetic relationships for *Astyanax*'s MOTUs from the upper Paraguaçu River basin. The tree was generated using approximately 2,230 bp obtained for the COI, Cytb, and S7 sequences for the samples indicated in Tab. **S1**. The topology corresponds to the Bayesian tree. The numbers on the branches are bootstrap values for the posterior probability for Bayesian species tree and speciation probability values of BP&P species delimitation. The scale bar indicates nucleotide substitutions per site.

DISCUSSION

Our major phylogenetic analysis recovered all endemic species from the upper Paraguaçu River studied here in a single and large clade (Clade 1). It is noteworthy that although we have included four of the six endemic species, we did not include all *Astyanax* species described for the Paraguaçu River basin (Santos, Caramaschi, 2007, 2011), therefore, Clade 1 must be incomplete. Despite this, our findings suggest that fishes of this basin share an evolutionary history that can result in its high level of endemism (Buckup, 2011; Camelier, Zanata, 2014a; de Pinna *et al*., 2018). Interestingly, we observed a pattern of genetic proximity between species of Clade 1 and species from distinct hydrographic basins, such as the Brazilian crystalline shield and the Atlantic coastal drainages (*i.e*., São Francisco, Paraná, and Paraíba do Sul basins). According to Ribeiro (2006), geological events between upland crystalline drainages and Atlantic tributaries occurred at different times, causing seemingly distant basins to share species or even species complex. The Paraguaçu River has an extensive system of branching headwaters that are adjacent to the eastern streams of the São Francisco basin (Buckup, 2011). In fact, several sister taxa or genetically related species between São Francisco River and NMAF ecoregion, presently separated by the Espinhaço Mountains, have been already reported (*e.g*., Camelier, Zanata, 2014a; Sarmento-Soares *et al*., 2016; Ramirez *et al*., 2017; Anjos *et al*., 2020).

In turn, although the species delimitation methods can be delimiting lineages, but not necessarily species (Carstens *et al*., 2013; Sukumaran, Knowles, 2017), our results are in accordance with the taxa considered valid to the upper Paraguaçu basin (*A. brucutu*, *A. epiagos*, *A. lorien*, *A. rupestris*) and revealed the existence of two genetic lineages within *A.* aff. *rupestris*. However, the number of identified MOTUs was method-dependent, as previously reported in similar studies (*e.g*., Costa-Silva *et al*., 2015; Rossini *et al*., 2016; Machado *et al*., 2018). These discrepancies may likely be due to analytical differences inherent to each method. The BIN and ABGD methods are based on genetic distances. The former is a result of the refined single linkage (RESL), which associates COI sequences with an identifier (BIN) based on a distance value automatically delineated (Ratnasingham, Hebert, 2013), while ABGD requires a priori specification of an intraspecific distance threshold (Puillandre *et al*., 2012). Contrastingly, GMYC uses coalescence approaches, and it requires an ultrametric gene tree in which branches are assigned to one lineage per species or multiple lineages per species (Pons *et al*., 2006). In addition, inconsistencies between the species delimitation methods may be biased by the molecular markers used (Hebert *et al*., 2003) and the limited sample sizes (Carstens *et al*., 2013) common to many investigations. It has been suggested that since distance methods rely heavily on the disparity between intra- and interspecific variation, an incomplete taxonomic sampling could influence the accuracy of the method (Frézal, Leblois, 2008).

The BIN analysis showed more conservative results, grouping the nominal species *A. brucutu*, *A. epiagos*, and *A. lorien*, plus *Astyanax* sp. from Coité River, in a single MOTU; and separating *A. rupestris* from the *A.* aff. *rupestris* and *Astyanax* sp. Piabinha. The BIN method uses 2.2% threshold, splitting species in new BINs when this value is at least twice higher (*e.g*., 4.4%) (Ratnasingham, Hebert, 2013). Although genetic distances equal or higher than 2% are commonly used to separate MOTUs (Hebert *et al*., 2004; Ward, 2009), this threshold can underestimate the number of species when applied to

complex groups such as *Astyanax*. For Neotropical fishes, smaller genetic divergence values have been reported for congeneric species with recent divergence (*e.g.*, Carvalho *et al.*, 2011; Pereira *et al.*, 2011; Ramirez, Galetti, 2015; Machado *et al.*, 2016; Ramirez *et al.*, 2017; Ribolli *et al.*, 2021), and 1% has been acclaimed as the optimal threshold for those belonging to species complexes (Hubert *et al.*, 2008; Pereira *et al.*, 2011). Therefore, the nature of the algorithms used by the BIN method may suffer interference when employed in hyper-diverse groups. For these latter groups, GMYC analyses have been considered more efficient than other methods (Ratnasingham, Hebert, 2013; Costa-Silva *et al.*, 2015; Ribolli *et al.*, 2021), consisting in one of the most accepted approaches for species delimitation based on a single locus analysis (Costa-Silva *et al.*, 2015). In *Astyanax*, this method was already chosen as the most appropriate for species delimitation (Rossini *et al.*, 2016).

Following our single locus analysis and GMYC results, this study showed five MOTUs among the focused species in the Paraguaçu basin, recognizing *A. epiagos* (MOTU 28) and *A. rupestris* (MOTU 48) as distinct species, but joining the two nominal species *A. lorien* and *A. brucutu* in a single clade (MOTU 26). Of note, the multilocus species delimitation approach used here was able to separate the latter species into different MOTUs. Moreover, the description of both species was based on strong diagnostic morphological characters and distinct habitats (Zanata *et al.*, 2017, 2018; Vita *et al.*, 2020). According to these authors, *Astyanax brucutu* presents a unique mandibular morphology similarly found only in specimens of *Creagrutus* Günther, 1864 and *Piabina* Reinhardt, 1867. Furthermore, *A. brucutu* inhabits a geographical region characterized by a distinctive combination of environmental attributes, such as high transparent water, elevated levels of dissolved oxygen, patches of gastropod shells on the bottom and coarse substrate partially covered by aquatic macrophytes, which are not observed elsewhere in the basin or adjacent drainages (Zanata *et al.*, 2017). Additionally, these nominal species could be easily identified by the monophyly criterion and their allopatric distribution.

Although it is parsimonious to consider *A. lorien* and *A. brucutu* as valid species, an alternative hypothesis is to assume that the morphological differences among them are possibly related to local adaptations, since the presence of barriers to gene flow can promote such phenotypic differences in distinct populations or lineages (Zamudio *et al.*, 2016). Rossini *et al.* (2016) argued that *Astyanax* local populations described as new species due only to their restricted geographical distribution or local adaptations could be synonymized in the future. Therefore, further phylogeographic studies incorporating a larger sampling, genomic data, and considering population size and divergence time as relevant parameters should be performed to reassess the genetic relationships between *A. lorien* and *A. brucutu*.

Meantime, low genetic distances among species have been often associated with recent divergences, in which the time to accumulate genetic differences is quite short (Ornelas-García *et al.*, 2008). Previous studies using the COI gene have already detected low genetic distances among species of *Astyanax*, reporting 0.93% between *A.* cf. *fasciatus* and *A. rivularis* (*e.g.*, Carvalho *et al.*, 2011). Low interspecific genetic distance values related to recent divergence have also been described within other fish genera, such as *Parodon* Valenciennes, 1849 (0.4%; Bellafronte *et al.*, 2013), *Zungaro* Bleeker, 1858 (0.4%; Pires *et al.*, 2017), *Megaleporinus* Ramirez, Birindelli & Galetti Jr., 2017 (0.67%; Ramirez *et al.*, 2017), *Leporinus* Agassiz, 1829 (0.7%; Silva-Santos *et al.*, 2018)

Rineloricaria Bleeker, 1862 (0.8%; Costa-Silva *et al*., 2015), *Apareiodon* (0.9%; Bellafronte *et al*., 2013), and *Laemolyta* Cope, 1872 (0.9%; Ramirez, Galetti Jr., 2015). The small body size of specimens of *Astyanax* and the fact of some species are geographically isolated in headwaters may enable the occurrence of vicariance events and speciation by geographic isolation (Castro, 1999). Low genetic divergence between isolated species in distinct tributaries in the same basin may indicate that they had been through a recent vicariance followed by a fast morphological differentiation, without reaching a reciprocal monophyly (Costa-Silva *et al*., 2015). That might explain the low genetic divergence (0.3%) between *A. lorien* and *A. brucutu* herein observed.

The multilocus analysis, separating *A. lorien* from the remaining *Astyanax* species studied, including *A. brucutu* (Fig. 3), reinforces the taxonomic validity of these species. On the other hand, *A. rupestris* and *A.* aff. *rupestris* showed higher values of genetic distances (1.8%; see Tab. **S3**), suggesting that *A.* aff. *rupestris* may be indeed considered distinct from *A. rupestris*. This result is in accordance with the difficulties pointed by Zanata *et al*. (2018) in the description of *A. rupestris*, in which the authors decided not to include the population of the Piabinha River within *A. rupestris*. According to the authors, specimens of *A.* aff. *rupestris* are very similar morphologically to *A. rupestris* but possess variations in some meristic characters that were not observed in the former. The Piabinha population also presents high frequency of specimens with variable lateral-line perforation, four premaxillary teeth in the inner row, and reduction in the number of branched dorsal- and pelvic-fin rays (Zanata *et al*., 2018). *Astyanax* aff. *rupestris* is apparently restrict to the Piabinha River, a Cumbuca's tributary, while *A. rupestris* is known to occurs in a somewhat broader distribution throughout both Cumbuca and Piaba River sub-basins (Zanata *et al*., 2018).

Furthermore, the GMYC approach also pointed *A.* aff. *rupestris* divided in two MOTUs (*A.* aff. *rupestris* 1 and *A.* aff. *rupestris* 2), evidencing hidden genetic diversity and showing MOTUs in sympatry and reciprocal monophyly (see Fig. 3), though with less than 1% of genetic distance between them (0.7%). It appears that some *Astyanax* lineages from the upper Paraguaçu are in a gray zone (*sensu* de Queiroz, 2007), in which speciation is in process, and the boundaries among species are hardly identified (Costa-Silva *et al*., 2015; Anjos *et al*., 2020). In this sense, we agree that further population studies of *A. rupestris*, *A.* aff. *rupestris* 1, and *A.* aff. *rupestris* 2, using methods of integrative taxonomy, including molecular and morphological data, are necessary to clarify the taxonomic status of the *A. rupestris* putative species complex.

Our study was useful in confirming *A. rupestris* from the Piaba and Cumbuca sub-basins as a single molecular unit distinct from *A.* aff. *rupestris* from the Piabinha River. In addition, the data supported the existence of two genetic lineages within the *A.* aff. *rupestris* morphotype. The multilocus analysis was more efficient in identifying species with recent divergence when compared to the single locus analysis using COI sequence only. Altogether, we characterized six distinct MOTUs: *Astyanax epiagos*, *A. brucutu*, *A. lorien*, *A. rupestris*, *A.* aff. *rupestris* 1, and *A.* aff. *rupestris* 2. Regarding the two *Astyanax* sp. previously reported for the Paraguaçu River basin by Rossini *et al*. (2016), the results indicated that *Astyanax* sp. from the Piabinha River and *A.* aff. *rupestris* 2 share the same COI haplotype, and, consequently, belonging to the same taxon (MOTU). On the other hand, *Astyanax* sp. from the Coité tributary needs to be taxonomically assessed, since it clustered to nominal species from São Francisco and Miriri basins, showing no

genetic similarity to the endemic *Astyanax* species from the Paraguaçu River studied here. Overall, these findings contribute to a better understanding of the diversity of this fish group in the upper Paraguaçu River basin, pointing out hidden diversity and reinforcing the relevance of this hydrographic system for the biodiversity ichthyofauna.

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ETHICAL STATEMENT

Permission for the biological sampling was conceived by SISBIO-ICMBio (Sistema de Autorização e Informação em Biodiversidade, Instituto Chico Mendes de Conservação da Biodiversidade, Ministério do Meio Ambiente, Governo Federal, Brazil), under authorization number 13754–1. Access to genetic heritage was registered at SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado, Ministério do Meio Ambiente, Governo Federal, Brazil), under number AAA03B9.

COMPETING INTERESTS

The author declares no competing interests.

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