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Integrative taxonomy reveals disjunct distribution and first record of *Hoplias misionera* (Characiformes: Erythrinidae) in the Amazon River basin: morphological, DNA barcoding and cytogenetic considerations

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The *Hoplias malabaricus* group encompasses six valid species and still is believed to harbors cryptic diversity. In this work, an integrative approach including morphological, DNA barcoding, and cytogenetic considerations was conducted to characterize a population of *H. malabaricus* from the Amazon basin that was recently allocated in the same mitochondrial lineage with *H. misionera*, a species originally described from La Plata basin. The DNA barcoding analysis revealed that the Amazon population nested together with *H. misionera* specimens from the La Plata basin (BIN AAB1732) in the same cluster. The intragroup distance (0.5%) was 12 times lower than the nearest neighbor (6%) distance. The morphometric analysis demonstrated slightly variation between Amazon and La Plata populations, being the former composed by larger specimens. Further morphological data supported the molecular evidence of *H. misionera* inhabiting Amazon basin. The karyotype characterization of *H. misionera* in the Amazon population showed 2n=40 and karyotypic formulae 20m+20sm, that added to C-banding, Ag-NOR and 18S results are suggestive of the similarity to karyomorph C of *H. malabaricus*. This work reveals the first record of *H. misionera* outside of La Plata basin and expands the species distribution for 2500 km northward until the Marajó Island, estuary of Amazonas River.

Keywords: Amazon basin, COI, Cryptic diversity, Karyotype, Trahira.

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O grupo *Hoplias malabaricus* compreende seis espécies válidas e ainda acredita-se que abriga diversidade críptica. Neste trabalho, uma abordagem integrativa incluindo considerações morfológicas, de DNA barcoding e de citogenética foi conduzida para caracterizar uma população de *H. malabaricus* da bacia amazônica que foi recentemente alocada na mesma linhagem mitocondrial de *H. misionera*, uma espécie originalmente descrita para a bacia La Plata. A análise molecular por DNA barcoding revelou que essa população amazônica forma um clado monofilético com espécimes de *H. misionera* provenientes da bacia La Plata (BIN AAB1732). A distância genética intragrupo (0,5%) é 12 vezes menor do que para o vizinho mais próximo (6%). A comparação morfométrica demonstrou pequena variação entre as populações amazônica e La Plata, sendo os primeiros ligeiramente maiores. Entretanto, os dados morfológicos corroboram com evidência molecular e confirmam a ocorrência de *H. misionera* na bacia amazônica. A caracterização cariotípica de *H. misionera* na população amazônica apresentou 2n=40 e fórmula cariotípica 20m+20sm, que aliada aos resultados de banda C, Ag-NOR e 18S sugerem que seja similar ao cariomorfo C de *H. malabaricus*. Esse trabalho revela o primeiro registro de *H. misionera* fora da bacia La Plata e estende a distribuição da espécie por mais de 2500 km ao Norte, até a Ilha do Marajó, estuário do rio Amazonas.

Palavras-chave: Bacia Amazônica, Cariótipo, COI, Diversidade críptica, Traíra.

INTRODUCTION

The wolf fish, locally named as "trahiras" in most part of South America, are classified in the family Erythrinidae, with 19 valid species and 3 genera: *Hoplias* Gill, 1903, *Erythrinus* Scopoli, 1777, and *Hoplerythrinus* Gill, 1896 (Fricke *et al*., 2020). These species have peculiar morphological features, such as, cylindrical body form, rounded caudal fin, absence of adipose fin, 8–15 dorsal-fin rays, 10–11 anal-fin rays, numerous teeth on the palate and 34–47 lateral-line scales (Oyakawa, 2003). This family is restricted to Neotropical region, from Costa Rica to southern Ecuador in the west, and to Argentina in the southeast, being widespread in the South America freshwaters systems (Oyakawa, 2003; Berra, 2007).

Hoplias is the richest genus comprising 14 valid species (Fricke *et al*., 2020). Based on morphological features, the genus is arranged in three groups: *Hoplias aimara* (Valenciennes, 1847), *Hoplias lacerdae* Miranda Ribeiro, 1908 and *Hoplias malabaricus* (Bloch, 1794) (Oyakawa, 1990; Mattox *et al*., 2006; Oyakawa, Mattox, 2009). Recent efforts to try to solve the complex taxonomy of the *Hoplias malabaricus* group have contributed with the re-description (Mattox *et al*., 2014) and description of new species (Azpelicueta *et al*., 2015; Rosso *et al*., 2016, 2018). Currently, this group comprises six species: *H. malabaricus*, *H. microlepis* (Günther, 1864), *H. teres* (Valenciennes, 1847), *H. mbigua* Azpelicueta, Benítez, Aichino & Mendes, 2015, *H. misionera* Rosso, Mabragaña, González-Castro, Delpiani, Avigliano, Schenone & Díaz de Astarloa, 2016, and *H. argentinensis* Rosso, González-Castro, Bogan, Cardoso, Mabragaña, Delpiani & Díaz de Astarloa, 2018. The improvement in taxonomic discrimination intimately agrees with

historical cytogenetic studies demonstrating that *H. malabaricus* is a species complex that hinders cryptic diversity (Bertollo *et al*., 1997, 2000; Born, Bertollo, 2006; Cioffi *et al*., 2009; Blanco *et al*., 2010; Da Rosa *et al*., 2014).

Just recently, molecular results revealed the existence of several fully supported lineages within the once considered to be the continentally distributed *H. malabaricus* (Cardoso *et al*., 2018; Jacobina *et al*., 2018). Many of these lineages were found in the Amazon basin, where Marques *et al*. (2013) earlier demonstrated a conspicuous genetic distinctiveness in a *H. malabaricus* population (Haplogroup Gp2). This population was tentatively assigned to *H. misionera* (Cardoso *et al*., 2018) as it shares the same molecular identity (BIN AAB1732) of the type material of this species. Nevertheless, a taxonomic revision of the Amazon population is lacking. The eventual occurrence of *H. misionera* in the Amazon basin would greatly expand the geographic distribution of this species, since, up to now, its distribution was restricted to the Uruguay, Paraná and Paraguay River Basins in Argentina and southern Brazil (Rosso *et al*., 2016).

Hoplias misionera is distinguished from congeners by the presence of Y-shaped configuration in the medial margin of dentaries, predorsal scales (15–17), total vertebrae count (39–40) and series of the last vertical scales on caudal fin forming a marked curve (Rosso *et al*., 2016). The evaluation of these characters as well as complementary molecular tools would certainly properly define the taxonomic status of the Amazon population postulated to be *H. misionera*. Indeed, modern integrative approaches combining morphological and molecular tools suggest that several divergent lineages may constitute fully independent species in Neotropical Teleosts (Pugedo *et al*., 2016; Rosso *et al*., 2018). Herein, we investigate the taxonomic status of a *Hoplias* population from the Amazon basin by means of an integrative approach including morphological, DNA barcoding and cytogenetic considerations.

MATERIAL AND METHODS

Ethics statement. The Brazilian government System of Authorization and Information in Biodiversity (SISBIO) provided the permits for fish collections (SISBIO 32653–3). The animals were anesthetized and euthanized through immersion in water containing Eugenol solution, following a procedure approved by the Animal Use Ethics Committee (CEUA) of the Universidade Federal do Oeste do Pará (CEUA/UFOPA Nº 09003).

Sampling and study area. We analyzed 23 specimens collected during fieldwork in the lower Amazonas and Trombetas Rivers, in Pará State (Tab. 1). Three specimens from Viçosa Island, in the Marajó archipelago, were purchased in a fish market in the Macapá city, Amapá State.

Morphological analysis. Voucher specimens were fixed in 10% formalin during 72h, rinsed with tap water and preserved in 70% ethanol. Measurements and counts were taken on the left side of the body following Fink, Weitzman (1974), Mattox *et al.* (2006) and Rosso *et al.* (2018) protocols. Counts were obtained by either visual or microscopic inspection. Linear body measurements were taken with a digital caliper to the nearest 0.1 mm. Vertebral counts were obtained using radiographs and included

TABLE 1 | Detailed geographic information of new and former records of *Hoplias misionera* in Amazon Basin. N = number of specimens collected at each location.

Amazon Basin Sector	Municipality	Collecting site	N	Latitude	Longitude	References
Lower Amazon River, Southern bank	Santarém	Maicá Lake	2	-2.4745	-54.5357	Marques *et al*., 2013
Lower Trombetas River, Northern bank	Óbidos	Pororoca-Mamauru streams	8	-1.9368	-55.5206	Marques *et al*., 2013; This study
Lower Amazon River, Northern bank	Oriximiná	Sapucuá Lake	2	-1.7751	-55.8699	Marques *et al*., 2013; This study
Estuarine, Marajó archipelago	Chaves	Viçosa island	3	0.4494	-49.9999	This study
Lower Amazon River, Northern bank	Monte Alegre	Amazon River	5	-2.0105	-54.0713	This study
Lower Amazon River, Northern bank	Alenquer	Amazon River	1	-1.9592	-54.7411	This study
Lower Amazon River, Southern bank	Santarém	Aracampina	1	-2.4383	-54.3233	This study
Lower Amazon River, Northern bank	Alenquer	Centro do Arapiri	1	-2.0821	-54.9944	This study

the four anterior vertebrae of the Weber apparatus. The examined vouchers (n=9) are deposited in the Laboratório de Genética e Biodiversidade, Universidade Federal do Oeste do Pará (UFOPA), Brazil and listed herein in the section material examined. Abbreviation institutions: (UNMDP) Universidad Nacional de Mar del Plata, Argentina.

Material examined (morphological data). *Hoplias misionera*: Brazil: Lower Amazonas River basin, UFOPA AMTRA126–19 - AMTRA128–19; UFOPA AMTRA 130–19 - AMTRA131–19, 5, 214–238 mm SL. Sapucuá Lake: UFOPA AMTRA037–11, 1, female, 188 mm SL. Marajó archipelago, Viçosa Island: UFOPA AMTRA122–19, AMTRA123–19, AMTRA125–19, 3, 228–262 mm SL.

Molecular analysis. Before fixation, a small muscle tissue sample from each specimen was collected and further preserved in absolute ethanol. Total genomic DNA was extracted following an adapted salting-out protocol (Aljanabi, Martinez, 1997; Vitorino *et al*., 2015). DNA barcoding sequences (COI mtDNA) were amplified by Polymerase Chain Reaction (PCR) using standard primers FishF1 and Fish R1 (Ward *et al*., 2005). Details of PCR profiles and sequencing reactions are given in Guimarães *et al.* (2018). In order to explore genetic divergences with already known species, we supplemented our COI data set with sequences downloaded from public repository Barcode of Life Database (www.boldsystems.org) (Ratnasingham, Hebert, 2007) of the following species: *Hoplias misionera* (n=48) (Marques *et al*., 2013; Rosso *et al*., 2016; Cardoso *et al*., 2018), *H. malabaricus* (n=10) (Marques *et al*., 2013; Cardoso *et al*., 2018), *H. microlepis* (n=7) (BOLD Systems), *H. argentinensis* (n=10) (Cardoso *et al*., 2018), *H. mbigua* (n=5) (Cardoso *et al*., 2018), and *H. lacerdae* (n=2) (Cardoso *et al*., 2018). Detailed information on DNA barcoding sequences and specimen origin are listed in **S1**.

The sequences were aligned using the ClustalW Algorithm (Thompson *et al*., 1994) implemented in the software BioEdit (Hall, 1999). Three species delimitation approaches were adopted to recognize Operational Taxonomic Units (OTUs): 1) Generalized Mixed Yule Coalescent – GMYC (Pons *et al*., 2006; Fujisawa, Barraclough 2013); 2) Automatic Barcode Gap Discovery – ABGD (Puillandre *et al*., 2012), and 3) Barcode Index Number (BIN) (Ratnasingham, Hebert, 2013).

For the GMYC method, the repeated haplotypes were removed and an ultrametric tree based on bayesian inference was constructed in BEAST v1.8.0 (Drummond, Rambaut, 2007) with the following settings: GTR evolution model (Gamma distribution), molecular clock lognormal relaxed and Yule process speciation. The bayesian reconstruction was ran with 100 million MCMC iterations, sampled each 1000 iterations with a burn-in of 10%. The convergence and stability were checked with the software Tracer v.1.7.1 and retained for Effective Sample Sizes (ESS) > 200 (Drummond, Rambaut, 2007). The resulting trees were combined with TreeAnotator v1.8.0 (Drummond, Rambaut, 2007). To test the branching events for speciation we used the packages Splits (Species Limits by Threshold Statistics) and Ape (Analyses of Phylogenetics and Evolution), with the single threshold model implemented in R 3.4.0 statistical software (R Core Team, 2014).

We used the method Automatic Barcode Gap Discovery (ABGD) to explore the existence of barcoding gap among the *Hoplias* taxa analyzed. This analysis was processed in the online platform (bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) and setting for K80 model, intraspecific divergence (min. 0.001 and max. 0.1) and barcoding gap as default (X=1.5). The Barcode Index Number (BIN) is implemented in the BOLD System workbench (www.boldsystems.org) that uses the algorithm RESL (Refined Single Linkage Analysis) to find OTUs of DNA barcodes from entry data and the BOLD archived library (Ratnasingham, Hebert, 2013).

For cluster visualization, a Neighbor-joining (NJ) tree based on Kimura-2-parameters (K2P) evolution model (Kimura, 1980) processed with the software MEGA X (Kumar *et al*., 2018) and edited with FigTree v.1.2.2 (http://tree.bio.ed.ac.uk/software/figtree/) was built. We estimated K2P genetic distances using MEGA X (Kumar *et al*., 2018). All the new DNA barcoding sequences were uploaded to the BOLD Systems database linked to the Project AMTRA: "Amazonian Trahiras".

Cytogenetic analysis. We analyzed the karyotype of ten specimens (AMTRA003-11, AMTRA022-11, AMTRA023-11, AMTRA024-11, AMTRA025-11, AMTRA028-11, AMTRA029-11, AMTRA030-11, AMTRA031-11, AMTRA037-11). Chromosome preparations were obtained from kidney cells after 24h of yeast mitosis stimulation (Lee, Elder, 1980) and exposed to 0.025% colchicine (0.01ml/g body mass) following Bertollo *et al.* (1978). The metaphases were examined through 5% Giemsa conventional staining. C-banding followed Sumner (1972). We detected Nucleolar Organizing regions by silver staining (Ag-NOR) following Howell, Black (1980). In situ hybridization (FISH) was applied to mapping ribosomal genes DNAr 18S. The probes were done by PCR using the primers Forward: 18Sf (5' CCG CTT TGG TGA CTC TTG AT 3') and Reverse: 18Sr (5' CCG AGG ACC TCA CTA AAC CA 3') (Martins, Vicari, 2012). The FISH experiments followed procedures described in Pinkel *et al*. (1986) with minor adaptations as described in da Fonseca *et al*. (2018). The chromosomes in the karyotypes were visually classified as metacentrics (m) and submetacentrics (sm) based on arm ratio according to Levan *et al.* (1964) and arranged following Cioffi *et al*. (2009), Santos *et al*. (2009). In order to facilitate comparisons and karyomorph discrimination we analyzed the pattern in size reduction of the first four largest pairs, a criterion previously adopted to distinguish karyomorphs with same diploid number (Bertollo *et al*., 1997). In this comparative analysis we drawn partial idiograms using chromosome figs. from literature for *H. malabaricus* karyomorphs C and F (Bertollo *et al*., 1997), with assistance of the software Drawid V0.26 (Kirov *et al*., 2017).

RESULTS

Morphological analysis (identification). The external morphology and ethanol-preserved coloration are shown in Fig. 1. Medial margins of contralateral dentaries converged to midline in a characteristic Y-shaped (n= 4) or V-shaped (n= 5) (Figs. 2A,B) configuration. Basihial and basibranchials bones bearing tooth plates. A single premaxillary tooth row. First two premaxillary teeth large and caniniform, then four or five very small teeth followed by other two large canines. Maxilla with 35–44 teeth, first five increasing progressively in size. Dentary external series comprised of 4 small teeth followed by two larger canines, then other series of 4–6 small teeth and 8–10 teeth arranged in a repetitive series of one large and one-two small conic teeth. Accessory ectopterygoids not fragmented, anteriorly expanded and bearing 12–14 conical teeth along their ventrolateral margins. Total dorsal-fin rays 14–15 (ii–12 n = 2; ii–13 n = 7). Total anal-fin rays 10–11 (i–9 n= 2; ii–9 n = 7). Total pectoral-fin rays 13 (i–12 n = 9). Tip of pectoral fin separated from pelvic-fin origin by 3–5 scales. Total pelvic-fin rays 8 (i–7 n = 9). Tip of pelvic fin separated from vertical through anus by 2–3 scales. Total caudal-fin rays 17 (i–15–i n = 9). Predorsal scales (15–17) arranged in an irregular series. Last vertical series of scales on caudal peduncle forming a curve (Fig. 3A). Lateral line complete with one or two anterior scales without pores followed by 39–41 perforated scales. Longitudinal series of scales between dorsal-fin origin and lateral line 5–5.5; between lateral line and pelvic-fin origin 4–5. Longitudinal series of scales around caudal peduncle, invariable 20. First epibranchial with 9–12 plate-like denticulated gill rakers. One laminar gill raker on cartilage. First ceratobranchial with 4–6 more elongated rakers and 11–16 plate-like denticulated gill rakers. Laterosensory canal along ventral surface of dentary with four pores. A single laterosensory canal along infraorbitals with invariable 11 pores, with infraorbital 5 lacking pores. Laterosensory system of dorsal surface of head with 11–12 pores. Nasal bone: two pores, frontal bone: four-five pores, pterotic bone: two pores. One pore between parietal bones, on posterior end of symphysis. Supraopercle and extra-scapular bones with following combination of pores: 1:1 and 0:2. Total vertebrae 38–40 (n=3).

FIGURE 1 | *Hoplias misionera*, UFOPA AMTRA131-19, 237 mm SL, Amazonas River, Alenquer, Pará, Brazil. Lateral view. Scale bar = 1 cm. Photo by L.R.R. Rodrigues.

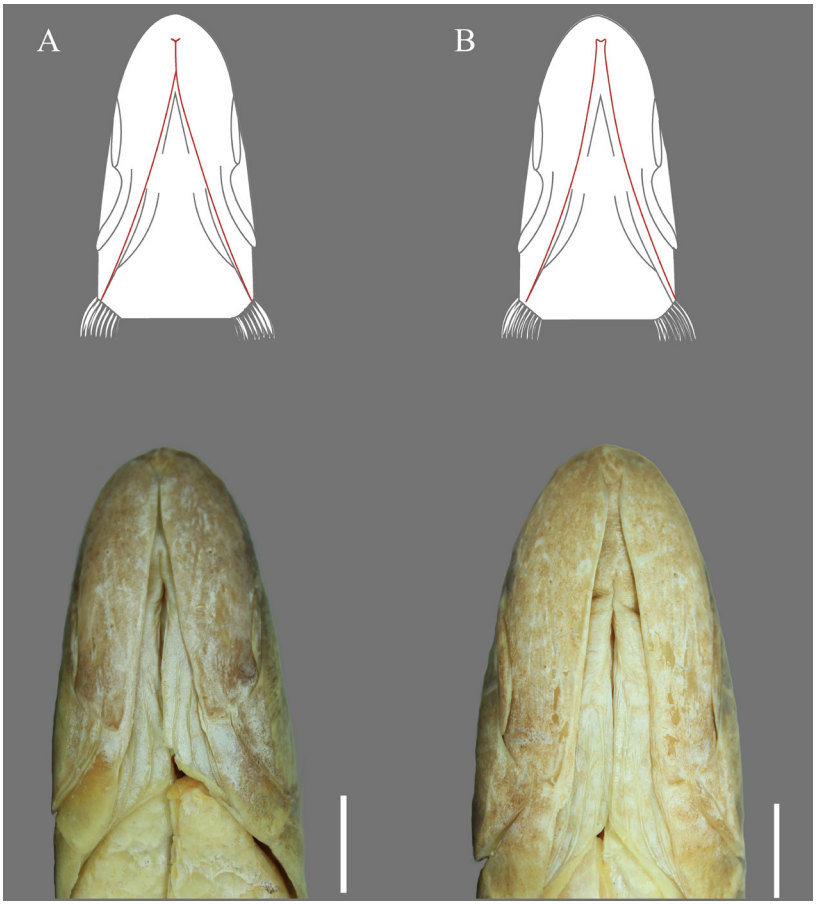

FIGURE 2 | Configuration of the medial margins of the dentary in *Hoplias misionera*. **A.** Y-shaped, UFOPA AMTRA126-19, 214 mm SL. **B.** V-shaped, UFOPA AMTRA127-19, 232 mm SL. Scale bars = 1 cm. Photos by L. R. R. Rodrigues. Illustration by T. M. A. Lima.

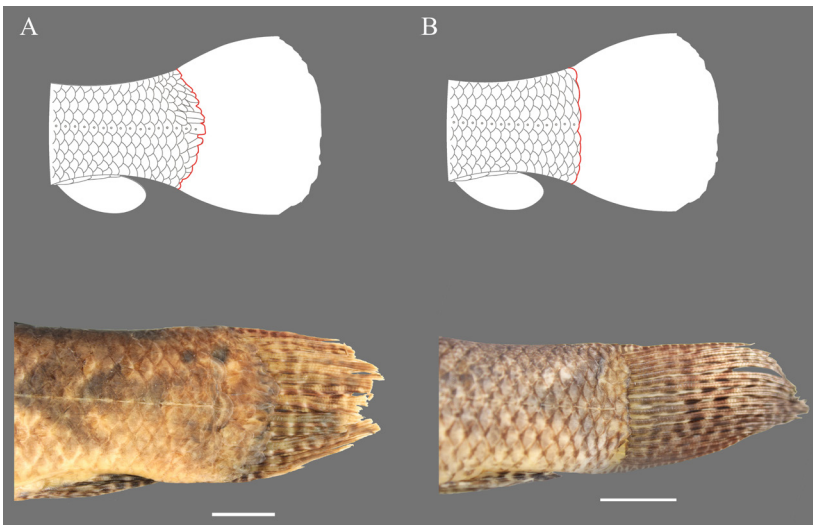

FIGURE 3 | Last vertical series of scales on the base of the caudal-fin rays. Comparison between *Hoplias misionera* (**A**), UFOPA AMTRA131-19, 237 mm SL and *Hoplias* cf. *malabaricus* (**B**), UFOPA AMTRA110, 201 mm SL. Scale bars = 1 cm. Photos by L. R. R. Rodrigues. Illustration by T. M. A. Lima.

Morphometric data of specimens of *H. misionera* from the Amazon River Basin are summarized in Tab. 2. The population of the Amazon basin was composed by specimens of 188 to 262 mm of standard length. A shallower body depth (18.1–20.3% *vs.* 20.6–25.5%), shorter head length (28.0–30.9% *vs.* 30.6–34.6%), smaller predorsal distance (42.4–46.2% *vs.* 46.9–51.8%) and larger snout length (24.0–28.5% *vs.* 20.5–24.7%) characterize this population when compared with *H. misionera* from La Plata River Basin.

Molecular analysis. A data set with 95 DNA partial COI sequences (652 bp) of six species of *Hoplias* was assembled and revealed a base composition of 17% G, 27.9% C, 23.8% A and 31.3% T. The sequences did not show indels nor stop codons. All the species delimiting tools were congruent to confirm the presence of *H. misionera* in the Amazon basin. The *H. misionera* cluster was either delimited in a single cluster (ABGD, BIN) or split in two clusters (GMYC). A tree inference based on distances (NJ) revealed clusters that were congruent with species delimitation (Fig. 4). All the specimens sampled to this work nested within the *H. misionera* clade. Pairwise genetic distances indicated deep divergences (4.7–15.4%) between species. In contrast, *H. misionera* populations from Amazon basin and La Plata Basin diverged by just 0.6% (Tab. 3).

TABLE 2 | Morphometric data of *Hoplias misionera* from the Amazon basin; values 1-14 are percentages of the standard length and values 15-22 are percentages of head length. N = number of specimens; SD = standard deviation.

		N	Mean	Minimum	Maximum	SD
	Standard length (mm)	9	230.4	188	262	-
1.	Body depth	6	19.2	18.1	20.3	0.6
2.	Head length	9	29.2	28.0	30.9	1.0
3.	Pectoral-fin length	9	17.3	15.0	19.0	1.1
4.	Pelvic-fin length	9	18.0	16.1	20.0	1.2
5.	Anal-fin length	9	17.1	15.0	18.9	1.1
6.	Dorsal-fin length	9	32.5	30.4	35.9	1.8
7.	Dorsal-fin base length	9	18.9	17.1	20.9	1.0
8.	Anal-fin base length	9	8.4	7.2	9.4	0.7
9.	Prepectoral distance	9	28.8	25.6	31.0	1.6
10.	Prepelvic distance	9	52.4	48.5	55.0	2.1
11.	Predorsal distance	9	44.1	42.4	46.2	1.2
12.	Preanal distance	9	78.4	72.9	83.5	3.5
13.	Caudal peduncle depth	9	13.2	12.3	14.5	0.7
14.	Caudal peduncle length	9	12.8	11.9	13.5	0.5
15.	Head depth	8	49.4	44.9	55.4	3.1
16.	Snout length	9	25.7	24.0	28.5	1.4
17.	Snout width	9	27.7	22.8	29.7	1.9
18.	Snout depth	9	19.7	17.4	22.6	1.7
19.	Pre-nasal distance	9	15.8	13.7	18.7	1.4
20.	Orbital diameter	9	16.2	13.8	18.0	1.2
21.	Interorbital width	9	30.8	25.8	35.6	2.5
22.	Upper jaw length	9	55.3	48.3	61.3	3.6

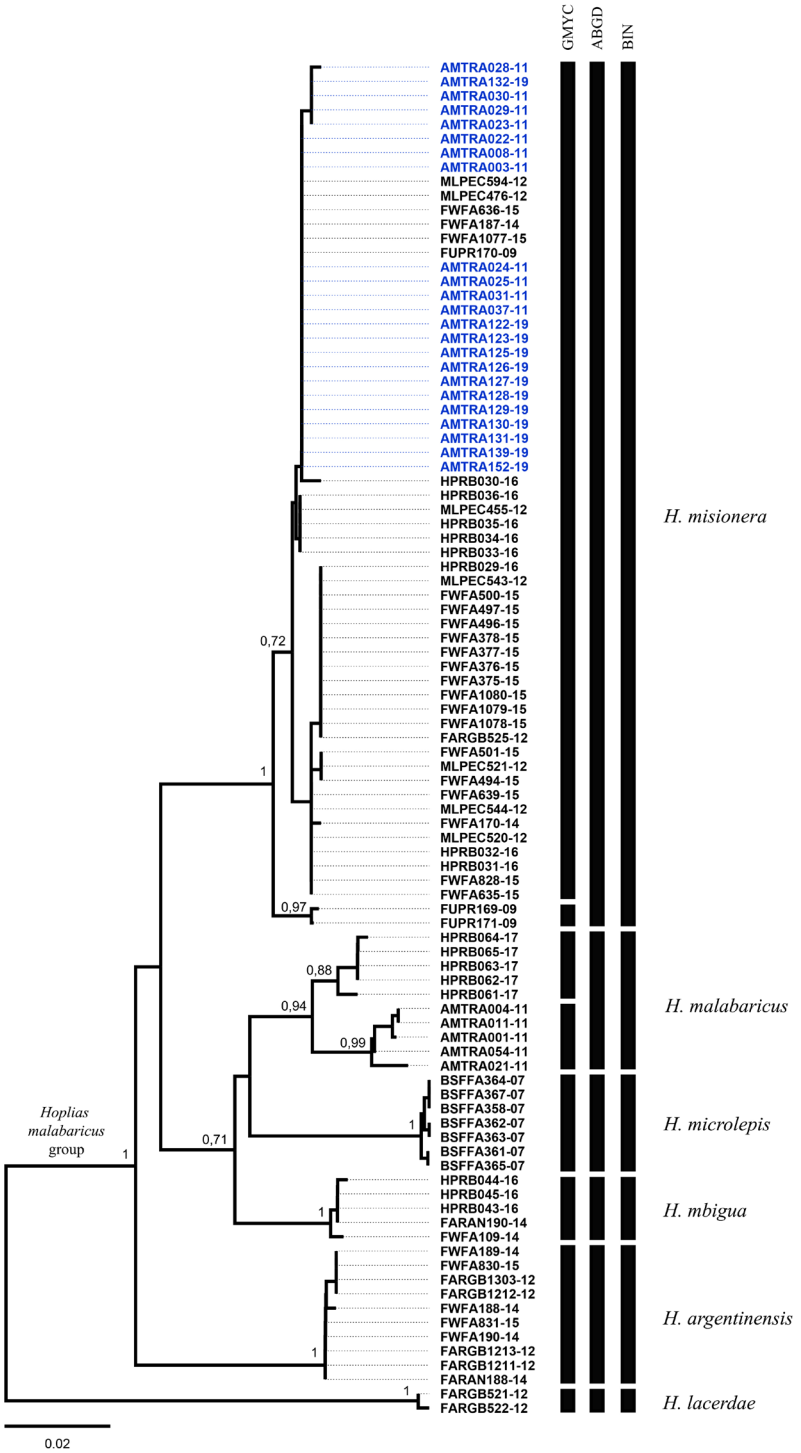

FIGURE 4 | Neighbor joining (NJ) tree of *Hoplias* inferred from partial COI (Cytochrome c Oxidase Subunit I gene) sequences using the Kimura 2-parameter model. The lateral bar indicates the partitions of species delimitation performed by the GMYC, ABGD and BIN analysis. The clade *Hoplias misionera* nested individuals from the La Plata and Amazon basins (blue tips).

Cytogenetic analysis. The karyotype of *H. misionera* from Amazon basin presented 2n=40 chromosomes, where 20 pairs were metacentric and 20 submetacentric (Fig. 5A). C-banding showed heterochromatic regions in the centromeres of all chromosomes and variable amount in the distal region in most of the metacentric pairs (4–10) and few submetacentric pairs (17–20). A conspicuous heterochromatic block was observed in the pericentromeric region of pair 13 (Fig. 5B). The Nucleolar organizing regions

TABLE 3 | Mean genetic distances (Kimura 2-parameter model) between *Hoplias* clusters. Bold values indicate the mean intraspecific distances.

Species/BIN(Population)	*H. misionera*		ABZ3047	AAD3629	AAZ3734	ACO5223	ABW2258
	Amazon	La Plata					
H. misionera AAB1732 (Amazon)	**0.001**						
H. misionera AAB1732 (La Plata)	0.006	**0.005**					
H. malabaricus ABZ3047	0.069	0.071	**0.016**				
H. microlepis AAD3629	0.080	0.079	0.058	**0.002**			
H. argentinensis AAZ3734	0.071	0.061	0.075	0.069	**0.001**		
H. mbigua ACO5223	0.060	0.065	0.047	0.055	0.081	**0.002**	
H. lacerdae ABW2258	0.137	0.136	0.149	0.154	0.140	0.153	**0.002**

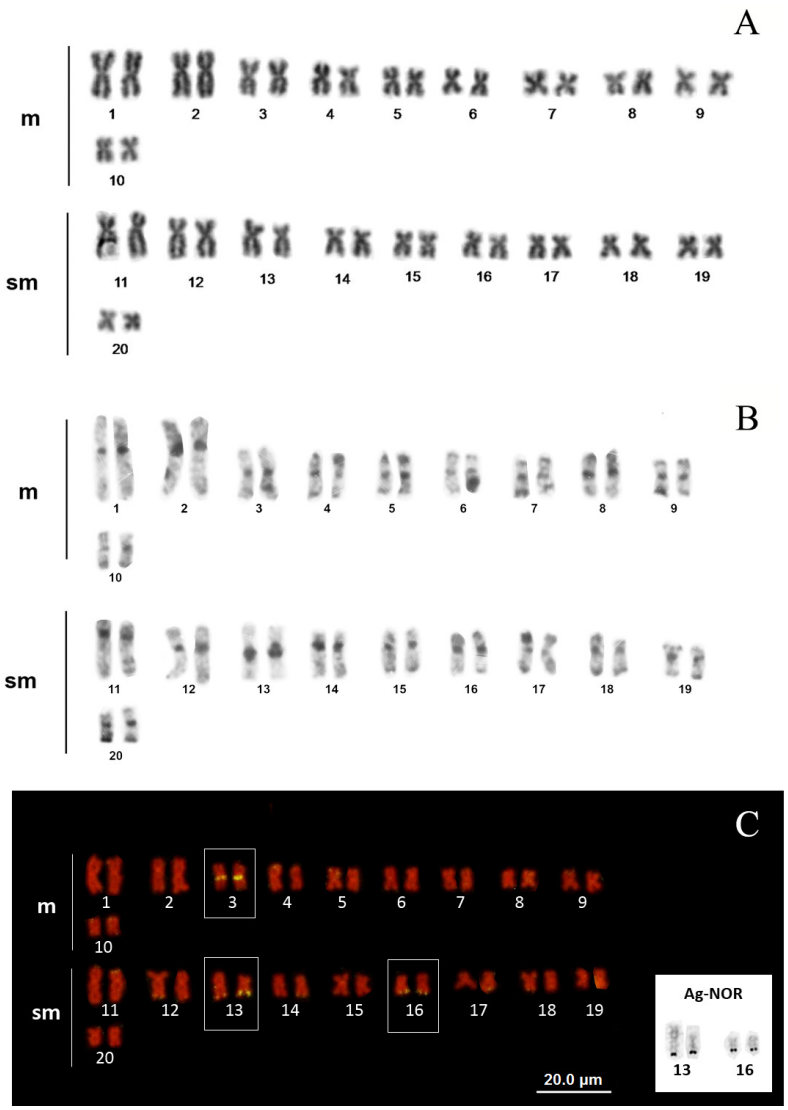

FIGURE 5 | Karyotype of *Hoplias misionera* from Amazon basin (2n=40 chromosomes). Conventional Giemsa stained (**A**), C-banded (**B**) and mapping of 18S rDNA FISH probes (green signals) (**C**). The Ag-NOR bearing chromosomes are showed in the box.

(Ag-NORs) were detected in the telomeric region of two submetacentric pairs. These Ag-NOR positions were coincident with the 18S rDNA hybridization marks in the pairs 13 and 16; an additional fluorescent mark was visualized in the centromeric region of the metacentric pair 3 (Fig. 5C). The size and heterochromatic band comparison between the largest chromosome pairs (1–2, 11–12) are showed in Fig. 6.

Geographic distribution. The easternmost collecting point in the Amazon basin, Viçosa Island, was situated in the Marajó Archipelago, a large island that lies at the mouth of the Amazon River. An updated distribution map of *H. misionera* is provided in the Fig. 7.

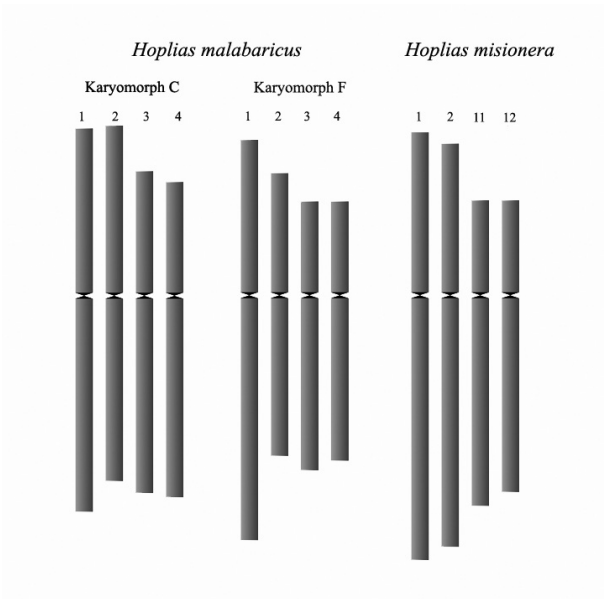

FIGURE 6 | Partial idiogram of the four largest chromosome pairs of *Hoplias malabaricus* (karyomorphs C and F) and *H. misionera* showing marked size reduction from the first to second metacentric pair only in the karyomorph F.

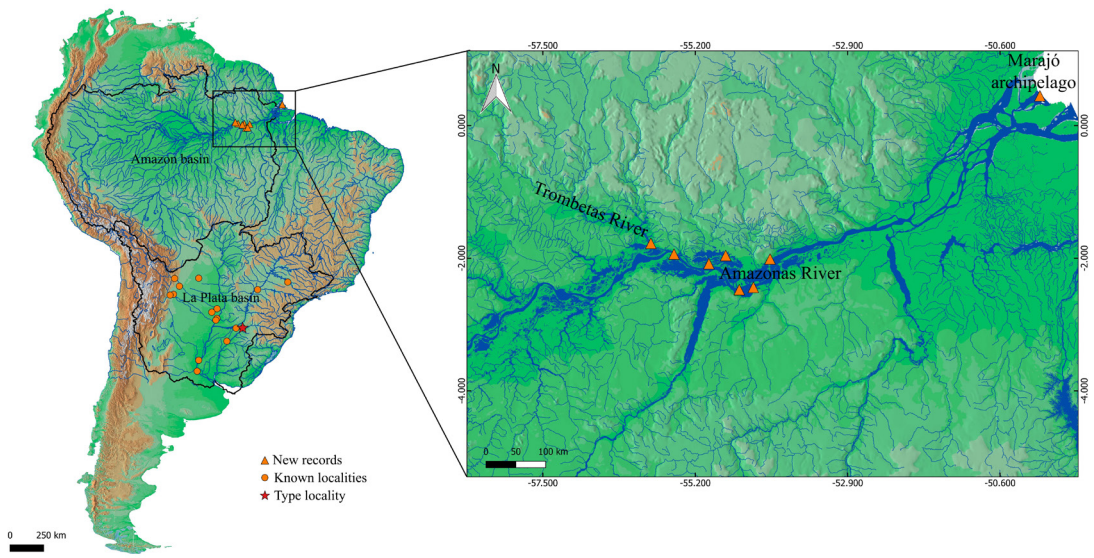

FIGURE 7 | Updated distribution map of *Hoplias misionera* showing former known localities in Argentina and southern Brazil (Rosso *et al.*, 2016) and the new records from Amazon basin (triangles). Star = type locality.

DISCUSSION

Our results combined morphology, DNA and cytogenetics to characterize a population of *H. misionera* from the Amazon basin and represent the first record of this species outside the La Plata River basin. The set of available diagnostic characters examined fits the diagnosis of *H. misionera* (Rosso *et al*., 2016), with remarks on some characters. Overall, when compared with type specimens from La Plata River basin, the population of *H. misionera* from the Amazon basin can be characterized by having a slightly higher number of total anal-fin rays (10–11 *vs.* 10) and scales separating pelvic fin from anus (2–3 *vs.* 2). They also presented lower number of teeth (8–10 *vs.* 10–16) in the posterior repetitive series of the dentary and scales in the lateral line (39–41 *vs.* 40–43). A slightly wider range was observed in counts of gill rakers in epibranchial (9–12 *vs.* 10–11) and ceratobranchial (15–22 *vs.* 17–21) bones.

Morphometric analysis revealed some trends of variation between Amazon and La Plata populations of *H. misionera*. The population of the Amazon basin was composed by larger specimens (188–262 mm *vs.* 39.22–174 mm of standard length) and showed different values in body depth, head length, predorsal distance and snout length.

Morphological differences between natural populations of geographically isolated fish that inhabit different hydrographic systems have been frequently reported (*e.g*., Neves, Monteiro, 2003; Shibatta, Hoffmann, 2005; Silva *et al*., 2009). In particular, these studies propose that this external morphological distinction could be, in part, a response to selective pressure in different environmental conditions. *Hoplias misionera* is a species that inhabits different environments, such as rivers, streams, lakes and dams, where these morphometric differences may be the result of different evolutionary patterns due to environmental conditions, emphasizing the singularity of each basin. Some of the observed variable morphometric characters could also be the result of ontogenetic variation, since there was a complete non-overlapping range of standard length between both populations. The entire range of lateral-line scales (39–43) combining Amazon and La Plata populations was observed by Ota *et al*. (2018) in the upper Paraná River basin. Reia *et al*. (2020) further expanded the lower limit to 38 when revising four specimens in the Sucurí River basin, upper Paraná River.

Some specimens of *H. misionera* from Amazon basin displayed a Y-shaped arrangement on the medial margins of dentaries, a feature firstly proposed to distinguish *H. misionera* from all remainder species of *Hoplias* (Rosso *et al*., 2016). However, we also observed the V-shaped configuration in some specimens, as it was also observed lately for other populations of *H. misionera* from the lower Paraná and Paraguay river basins (Rosso, unpublished data). Despite the variable state of this character, the remaining morphological and meristic characters analyzed in the specimens collected in the Amazon River basin were largely congruent with those provided by Rosso *et al.* (2016).

The molecular evidence also confirms that samples analyzed represent *H. misionera*. The speciation process within *Hoplias* has been linked to deep divergence in COI sequences. For instance, *H. misionera* and the recently described *H. argentinensis* diverged in 5.6 and 9.0% from the nearest neighbor (Rosso *et al*., 2016, 2018) respectively. In the Amazon basin, only one species of *Hoplias malabaricus* group (*H. malabaricus*) has been reported (see Oyakawa, 2003). Our results showed that *Hoplias misionera* of the Amazon basin presented 6.9% of genetic distance to this species. Generally, a 2% divergence

threshold has been widely used to discriminate Neotropical fish species (Pereira *et al*., 2013), but there is also evidence that the interspecific genetic distance value of 2%, may not be useful in many Neotropical fish species, as occurs within the genera *Astyanax* Baird & Girard, 1854 (Rossini *et al*., 2016; Terán *et al*., 2020), *Hypostomus* Lacepède, 1803 (Queiroz *et al*., 2020), *Schizodon* Agassiz, 1829 (Ramirez *et al*., 2020), among others. Our molecular analysis revealed a low divergence between the two populations of *H. misionera* (Amazon *vs*. La Plata) diverging by just 0.5%. Indeed, inferences using COI gene showed that several taxonomically recognized species in the genus *Hoplias* form well defined mtDNA lineages (Cardoso *et al*., 2018). These results strongly suggest that barcode methodology should be considered as an additional diagnostic tool for confirmation of future new records for the genus *Hoplias*.

Hoplias misionera from the Amazonas River showed karyotype 2n=40 and its macrostructure resembles to the karyomorphs C and F of its congener *H. malabaricus* (Bertollo *et al*., 1997, 2000; Cioffi *et al*., 2009; Santos *et al*., 2009). Besides the conservative diploid number both karyomorphs are clearly distinguished based on the relative chromosomal size between the first four largest pairs (Bertollo *et al*., 1997) and some minor differences in the amount of constitutive heterochromatin that is slightly increased in the karyomorph C (see Cioffi, Bertollo, 2010; Santos *et al*., 2009, 2016). These attributes are assumed to validate both karyomorphs as distinct entities. Indeed, Bertollo *et al*. (1997) observed sympatry of karyomorphs C and F in the Tocantins River population without evidence of hybridization.

Our specimens showed karyotypic formulae (20m+20sm) similar to that observed in *H. malabaricus* karyomorph F from São Francisco river (Santos *et al*., 2009) and karyomorph C from Amazon basin (Marques *et al*., 2013; Santos *et al*., 2016). However, it differs from karyomorph C population from Bento Gomes River, a tributary of Paraguay River basin (Cioffi *et al*., 2009; Cioffi, Bertollo, 2010). In addition, *H. misionera* from Amazon basin shared identical pattern of Ag-NOR bearing chromosomes with *H. malabaricus* cytotype F and C from Amazon basin, but diverges from the Paraguay River basin population (see Cioffi *et al*., 2009; Cioffi, Bertollo, 2010).

It is noteworthy that karyomorph C is widespread throughout South America (Bertollo *et al*., 2000) and shows variation in some cytogenetic markers, such as karyotypic formulae, Ag-NOR and 18S FISH marks (Cioffi *et al*., 2009; Cioffi, Bertollo, 2010; Santos *et al*., 2016; Guimarães *et al*., 2017). Variation of karyotypic formulae is frequently explained by the occurrence of chromosomal rearrangements but sometimes it could be an artifact resulted from misinterpretation of chromosome morphology in poor metaphases plates. Given the good quality of *Hoplias* chromosome preparations is plausible that populations from distinct hydrographic basins, showing karyomorph C variants, can diverge by chromosomal rearrangements such as pericentric inversions, which is a good explanation for the transformation of 20m+20sm to 14m+26sm such as observed between *H. malabaricus* (karyomorph C) from Amazon and Paraguay river basins. It has been frequently demonstrated that *H. malabaricus* display multiple and variable Ag-NORs sites among distinct populations (Bertollo, 1996; Born, Bertollo, 2000; Vicari *et al*., 2005; Santos *et al*., 2016). *Hoplias misionera* conserved this cytogenomic feature (multiple Ag-NORs) and because this species shared a similar Ag-NOR pattern with karyomorphs C and F, is reasonable to conclude that based on this trait we cannot resolve its karyotype classification.

The relative size of the first four largest pairs has been considered a reliable trait to separate both karyomorphs C and F (Bertollo *et al*., 1997). The comparative analysis with the karyotype of *H. misionera* failed to detect the marked size reduction from the first to second chromosome pair, which is the main cytogenetic signature of the karyomorph F. In contrast, we observed a gradual size reduction congruent with the karyomorph C. Distinct populations of *H. malabaricus* karyomorph C share these same characters and additionally show high amounts of heterochromatin (Bertollo *et al*., 1997; Santos *et al*., 2016; Cioffi, Bertollo, 2010), a feature that was also clearly observed in our specimens of *H. misionera*. Additionally, the karyomorph C is characterized by a nascent XX/XY sex system that leads to heterochromatin accumulation in the centromere of pair 11 (Cioffi, Bertollo, 2010), homologue to pair 14 (Santos *et al*., 2016) and that we postulate homologue to the *H. misionera* pair 13. In contrast, Santos *et al*. (2009) also recognized a probable XX/XY sex system in the cytotype F but in this case, the chromosomal pair involved is the largest metacentric pair 1.

Based on the cytogenetic markers analyzed herein the karyotype of *H. misionera* from Amazonas River is closer related to the karyomorph C of its congener *H. malabaricus*. This karyomorph has been recorded in *H. malabaricus* populations from eastern and central portions of Amazon basin (Bertollo *et al*., 2000; Santos *et al*., 2016) and distributes southward to Paraná and Paraguay Basins reaching the northeast Argentina in the region of Misiones Province (Lopes, Fennochio, 1994) where is the type locality of *H. misionera* (Rosso *et al*., 2016). Therefore, there is a possibility that the karyomorph C remains conserved throughout the species distribution range. However, we recommend treating this assumption cautiously because karyotypic macrostructure can be a homoplastic character and could lead to mistaken inferences. Indeed, our results do not support the hypothesis that *H. misionera* populations from Argentina must be characterized as cytotype A, as proposed by Jacobina *et al*. (2018) from a geographic distribution interpretation. The sympatry of karyomorphs A and C in the northeast Argentina has been already reported (Lopes, Fennochio, 1994). All these aspects highlight the need for conducting cytogenetic studies only on well-defined taxonomic species, if we wish to improve our knowledge about the relationships between taxonomic and karyotype diversity. Further investigations are also needed to find out other aspects of the cytogenomic patterns of *H. misionera* populations from the Amazon and La Plata basins.

The geographic range of *H. misionera* is widely expanded northerly from the original localities included in the species description. New occurrences reported for the Amazon Basin are situated 2700 km northwards from the northernmost location previously known for *H. misionera* and 3180 km from the type locality of the species (Rosso *et al*., 2016). The disjunct distribution of *H. misionera* in the Amazon and Paraná-Paraguay systems confirms a biogeographic condition formerly suggested by Cardoso *et al*. (2018) grounded only in molecular data. Fish fauna shared between the Amazon and Paraguay rivers has been explained as the result of biotic dispersal events across wetlands connecting the headwaters of neighboring drainages (see Lundberg *et al*., 1998; Carvalho, Albert, 2011; Ota *et al*., 2014). The Paraguay River Basin has approximately 309 species (Britski *et al*., 2007; Ferreira *et al*., 2017), with about one-third shared with the Amazon basin (see Carvalho, Albert, 2011; Dagosta, De Pinna, 2019). Clearly, given the vastness of the Amazon basin, this region might still harbor large extensions of underexplored river systems hindering species diversity (Jézéquel *et al*., 2020). In this scenario, the occurrence

of other populations of *H. misionera* should not be ruled out. Further studies focusing on biogeographic and integrative scopes may fill these sampling gaps and assess the morphological and genetic trait variation of *H. misionera* populations throughout the entire species distribution range.

Comparative material examined. *Hoplias misionera*: **Argentina**: Holotype: Misiones province, stream tributary of the Acaraguá River: UNMDP 574, 1, 164 mm SL. Paratypes: same locality as holotype: UNMDP 3320, 1, 174 mm SL; UNMDP 3391, 1, 149 mm SL. Formosa province, riacho Saladillo: UNMDP 3321, 1, 142 mm SL; UNMDP 3322, 1, 148 mm SL; riacho Salado: UNMDP 3327, 1, 171 mm SL; UNMDP 3328, 1, 146 mm SL; UNMDP 3329, 1, 134 mm SL; riacho Mbiguá: UNMDP 3371, 1, 154 mm SL; UNMDP 3376, 1, 165 mm SL.

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REFERENCES

- **Aljanabi SM, Martinez I**. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Res. 1997; 25(22):4692–93. https://doi.org/10.1093/nar/25.22.4692
- **Azpelicueta MM, Benítez M, Aichino D, Mendez CMD**. A new species of the genus *Hoplias* (Characiformes, Erythrinidae), a tararira from the lower Paraná River, in Missiones, Argentina. Acta Zool Lilloana. 2015; 59(1–2):71–82.
- **Blanco DR, Lui RL, Bertollo LAC, Diniz D, Filho OM**. Characterization of invasive fish species in a river transposition region: evolutionary chromosome studies in the genus *Hoplias* (Characiformes, Erythrinidae). Rev Fish Biol Fish. 2010; 20(1):1–8. https://doi.org/10.1007/s11160-009-9116-3
- **Berra TM**. Freshwater fish distribution. 2nd. ed. The University of Chicago Press, Chicago; 2007.
- **Bertollo LAC**. The nucleolar organizer regions of Erythrinidae fish. An uncommon situation in the genus *Hoplias*. Cytologia. 1996; 61(1):75–81. https://doi.org/10.1508/cytologia.61.75
- **Bertollo LAC, Born GG, Dergam JA, Fenocchio AS, Moreira-Filho O**. A biodiversity approach in the Neotropical Erythrinidae fish, *Hoplias malabaricus*. Karyotypic survey, geographic distribution of karyomorphs and cytotaxonomic considerations. Chromosome Res. 2000; 8(7):603–13. https://doi.org/10.1023/A:1009233907558

- **Bertollo LAC, Moreira-Filho O, Fontes MS**. Karyotypic diversity and distribution in *Hoplias malabaricus* (Pisces, Erythrinidae): Cytotypes with 2n=40 chromosomes. Brazil J Genet. 1997; 20:237–42.
- **Bertollo LAC, Takahashi KS, Moreira-Filho O**. Cytotaxonomic considerations on *Hoplias lacerdae* (Pisces, Erythrinidae). Rev Bras Genet. 1978; 1(2):103–20.
- **Born GG, Bertollo LAC**. An XX/XY sex chromosome system in a fish species, *Hoplias malabaricus*, with a polymorphic NOR-bearing X chromosome. Chromosome Res. 2000; 8(2):111–18. https://doi.org/10.1023/A:1009238402051
- **Born GG, Bertollo LAC**. A new sympatric region for distinct karyotypic forms of *Hoplias malabaricus* (Pisces, Erythrinidae). Braz J Biol. 2006; 66(1):205–10. http://dx.doi.org/10.1590/S1519-69842006000200004
- **Britski HA, Silimon KZS, Lopes BS**. Peixes do Pantanal: manual de identificação. 2nd. ed. Brasília, DF: Embrapa; 2007.
- **Cardoso YP, Rosso JJ, Mabragaña E, González-Castro M, Delpiani M, Avigliano E *et al.*** A continental-wide molecular approach unraveling mtDNA diversity and geographic distribution of the Neotropical genus *Hoplias*. PLoS ONE. 2018; 13(8):e0202024. https://doi.org/10.1371/journal.pone.0202024
- **Carvalho TP, Albert JS**. The Amazon-Paraguay divide. In: Albert JS, Reis RE, editors. Historical biogeography of neotropical freshwater fishes. London: University of California Press; 2011. p.193–202.
- **Cioffi MB, Martins C, Centofante L, Jacobina U, Bertollo LAC**. Chromosomal variability among allopatric populations of Erythrinidae fish *Hoplias malabaricus*: Mapping of three classes of repetitive DNAs. Cytogenet Genome Res. 2009; 125(2):132–41. https://doi.org/10.1159/000227838
- **Cioffi MB, Bertollo LAC**. Initial steps in XY chromosome differentiation in *Hoplias malabaricus* and the origin of an X_1X_2Y sex chromosome system in this fish group. Heredity. 2010; 105(6):554–61. https://doi.org/10.1038/hdy.2010.18
- **Dagosta FCP, De Pinna M**. The fishes of the Amazon: distribution and biogeographical patterns, with a comprehensive list of species. Bull Am Mus Nat Hist. 2019; 2019(431):1–163. https://doi.org/10.1206/0003-0090.431.1.1
- **Drummond AJ, Rambaut A**. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol. 2007; 7(1):214. https://doi.org/10.1186/1471-2148-7-214
- **Ferreira FS, Duarte GSV, Severo-Neto F, Froehlich O, Súarez YR**. Survey of fish species from plateau streams of the Miranda river basin in the Upper Paraguay river region, Brazil. Biota Neotrop, 2017; 17(3):e20170344. http://dx.doi.org/10.1590/1676-0611-BN-2017-0344
- **Fink WL, Weitzman SH**. The so called cheirodontin fishes of Central America with descriptions of two new species (Pisces: Characidae). Smithson Contrib Zool. 1974; 172:1–46. https://doi.org/10.5479/si.00810282.172
- **Fonseca IC, Maciel LAM, Ribeiro FRV, Rodrigues LRR**. Karyotypic variation in the long-whiskered catfish *Pimelodus blochii* Valenciennes, 1840 (Siluriformes, Pimelodidae) from the lower Tapajós, Amazonas and Trombetas Rivers. Comp Cytogenet. 2018; 12(3):285–98. https://doi.org/10.3897/CompCytogen.v12i3.22590
- **Fricke R, Eschmeyer WN, Van der Laan R, editors**. Eschmeyer's catalog of fishes: Genera, species, references [Internet]. San Francisco: California Academy of Science; 2020. Available from: http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp
- **Fujisawa T, Barraclough TG**. Delimiting species using single-locus data and the generalized mixed Yule coalescent approach: A revised method and evaluation on simulated data sets. Syst Biol. 2013; 62(5):707–24. https://doi.org/10.1093/sysbio/syt033
- **Guimarães EMC, Carvalho NDM, Schneider CH, Feldberg E, Gross MC**. Karyotypic comparison of *Hoplias malabaricus* (Bloch, 1794) (Characiformes, Erythrinidae) in Central Amazon. Zebrafish. 2017; 14(1):80–89. https://doi.org/10.1089/zeb.2016.1283

- **Guimarães KLA, Sousa MPA, Ribeiro FRV, Porto JIR, Rodrigues LRR**. DNA barcoding of fish fauna from low order streams of Tapajós river basin. PLoS ONE. 2018; 13(12):e0209430. https://doi.org/10.1371/journal.pone.0209430
- **Hall TA**. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999; 41:95–98.
- **Howell WM, Black DA**. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: A 1-step method. Experientia. 1980; 36(8):1014–15. https://doi.org/10.1007/BF01953855
- **Jacobina UP, Lima SMQ, Maia DG, Souza G, Batalha-Filho H, Torres RA**. DNA barcode sheds light on systematics and evolution of neotropical freshwater trahiras. Genetica. 2018; 146(6):505–15. https://doi.org/10.1007/s10709-018-0043-x
- **Jézéquel C, Tedesco PA, Bigorne R, Maldonado-Ocampo JA, Ortega H, Hidalgo M *et al***. A database of freshwater fish species of the Amazon Basin. Sci Data. 2020; 7(1):1–96. https://doi.org/10.1038/s41597-020-0436-4
- **Kimura M**. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980; 16(2):111–20. https://doi.org/10.1007/BF01731581
- **Kirov I, Khrustaleva L, Van Laere K, Soloviev A, Meeus S, Romanov D *et al***. DRAWID: User-friendly java software for chromosome measurements and idiogram drawing. Comp Cytogenet. 2017; 11(4):747–57. https://doi.org/10.3897/compcytogen.v11i4.20830
- **Kumar S, Stecher G, Li M, Knyaz C, Tamura K**. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol. 2018; 35(6):1547–49. https://doi.org/10.1093/molbev/msy096
- **Lee MR, Elder FFB**. Yeast stimulation of bone marrow mitosis for cytogenetic investigations. Cytogenet Genome Res. 1980; 26:36–40. https://doi.org/10.1159/000131419
- **Levan A, Fredga K, Sandberg AA**. Nomenclature for centromeric position on chromosomes. Hereditas. 1964; 52(2):201–20. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x
- **Lopes PA, Fenocchio AS**. Confirmation of two different cytotypes for the neotropical fish *Hoplias malabaricus* Gill 1903 (Characiformes). Cytobios. 1994; 80:217–21.
- **Lundberg JG, Marshall LG, Guerrero J, Horton B, Malabarba MCSL, Wesselingh F**. The stage for Neotropical fish diversification: a history of tropical South American rivers. In: Malabarba LR, Reis RE, Vari RP, Lucena ZMS, Lucena CAS, editors. Phylogeny and classification of Neotropical fishes. Porto Alegre: Edipucrs. 1998; p.13–48.
- **Marques DF, Santos FA, Silva SS, Sampaio I, Rodrigues LRR**. Cytogenetic and DNA barcoding reveals high divergence within the trahira, *Hoplias malabaricus* (Characiformes: Erythrinidae) from the lower Amazon River. Neotrop Ichthyol. 2013; 11(2):459–66. https://doi.org/10.1590/S1679-62252013000200015
- **Martins C, Vicari MR**. Hibridização *in situ* em cromossomos de peixes. In: Guerra M, coordenador. Citogenética Molecular: Protocolos comentados. Ribeirão Preto: SBG; 2012. p.59–87.
- **Mattox GMT, Toledo-Piza M, Oyakawa OT**. Taxonomic study of *Hoplias aimara* (Valenciennes, 1846) and *Hoplias macrophthalmus* (Pellegrin, 1907) (Ostariophysi, Characiformes, Erythrinidae). Copeia. 2006; 2006(3):516–28. https://doi.org/10.1643/0045-8511(2006)2006[516:TSOHAV]2.0.CO;2
- **Mattox GMT, Bifi AG, Oyakawa OT**. Taxonomic study of *Hoplias microlepis* (Gunther, 1864), a trans- Andean species of trahiras (Ostariophysi: Characiformes: Erythrinidae). Neotrop Ichthyol. 2014; 12(2): 343–52. https://doi.org/10.1590/1982-0224-20130174
- **Neves FM, Monteiro LR**. Body shape and size divergence among populations of *Poecilia vivipara* in coastal lagoons of south-eastern Brazil. J Fish Biol. 2003; 63(4):928–41. https://doi.org/10.1046/j.1095-8649.2003.00199.x
- **Ota RP, Lima FCT, Pavanelli CS**. A new species of *Hemigrammus* Gill, 1858 (Characiformes: Characidae) from the Rio Madeira and Rio Paraguai basins, with a redescription of *H. lunatus*. Neotrop Ichthyol. 2014; 12(2):265–79. https://doi.org/10.1590/1982-0224-20130176

- **Ota RR, Deprá GC, Graça WJ, Pavanelli CS**. Peixes da planície de inundação do alto rio Paraná e áreas adjacentes: Revised, annotated and updated. Neotrop Ichthyol. 2018; 16(2): e170094. https://doi.org/10.1590/1982-0224-20170094
- **Oyakawa OT**. Revisão sistemática das espécies do gênero *Hoplias* (grupo *lacerdae*) da Amazônia brasileira e região leste do Brasil (Teleostei: Erythrinidae). [MSc.Thesis]. São Paulo: Universidade de São Paulo; 1990.
- **Oyakawa OT**. Family Erythrinidae (Trahiras). In: Reis RE, Kullander SO, Ferraris CJ, Jr., organizers. Check list of the freshwater fishes of South and Central America. Porto Alegre: Edipucrs; 2003. p.238–40.
- **Oyakawa OT, Mattox GMT**. Revision of the Neotropical trahiras of the *Hoplias lacerdae* species group (Ostariophysi: Characiformes: Erythrinidae) with descriptions of two new species. Neotrop Ichthyol. 2009; 7(2):117–40. https://doi.org/10.1590/S1679-62252009000200001
- **Pereira LHG, Hanner R, Foresti F, Oliveira C**. Can DNA barcoding accurately discriminate megadiverse Neotropical freshwater fish fauna? BMC Genet. 2013; 14(1):20. https://doi.org/10.1186/1471-2156-14-20
- **Pinkel D, Straume T, Gray J**. Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. PNAS. 1986; 83(9):2934–38. https://doi.org/10.1073/pnas.83.9.2934
- **Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S *et al***. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. Syst Biol. 2006; 55(4):595–609. https://doi.org/10.1080/10635150600852011
- **Puillandre N, Lambert A, Brouillet S, Achaz G**. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Mol Ecol. 2012; 21(8):1864–77. https://doi.org/10.1111/j.1365-294X.2011.05239.x
- **Pugedo ML, Andrade Neto FR, Pessali TC, Birindelli JLO, Carvalho DC**. Integrative taxonomy supports new candidate fish species in a poorly studied neotropical region: The Jequitinhonha river basin. Genetica. 2016; 144(3):341–49. https://doi.org/10.1007/s10709-016-9903-4
- **Queiroz LJ, Cardoso Y, Jacot-des-Combes C, Bahechar IA, Lucena CA, Py-Daniel LR *et al***. Evolutionary units delimitation and continental multilocus phylogeny of the hyperdiverse catfish genus *Hypostomus*. Mol Phylogenet Evol. 2020; 145:106711. https://doi.org/10.1016/j.ympev.2019.106711
- **R Core Team**. R: A Language and environment for statistical computing. R Foundation for Statistical Computing. Vienna: Austria; 2014. Available from: http://www.R-project.org
- **Ramirez JL, Santos CA, Machado CB, Oliveira AK, Garavello JC, Britski HA *et al***. Molecular phylogeny and species delimitation of the genus *Schizodon* (Characiformes, Anostomidae). Mol Phylogenet Evol. 2020; 153:106959. https://doi.org/10.1016/j.ympev.2020.106959
- **Ratnasingham S, Hebert PDN**. BOLD: The Barcode Of Life Data system (www.barcodinglife.org). Mol Ecol Notes. 2007; 7(3):355–64. https://doi.org/10.1111/j.1471-8286.2007.01678.x
- **Ratnasingham S, Hebert PDN**. A DNA-based registry for all animal species: The Barcode Index Number (BIN) system. PLoS ONE. 2013; 8(7):e66213. https://doi.org/10.1371/journal.pone.0066213
- **Reia L, Costa e Silva GS, Garcia-Ayala JR, Vicensotto AMPF, Benine RC**. Ichthyofauna of the ribeirão Sucuri, a tributary of the rio Tietê, upper rio Paraná basin, southeastern Brazil. Check List. 2020; 16(3):711–28. https://doi.org/10.15560/16.3.711
- **Da Rosa R, Vicari MR, Dias AL, Giuliano-Caetano L**. New Insights into the biogeographic and karyotypic evolution of *Hoplias malabaricus*. Zebrafish. 2014; 11(3):198–206. https://doi.org/10.1089/zeb.2013.0953
- **Rossini BC, Oliveira CAM, Melo FAG, Bertaco VA, Díaz de Astarloa JM, Rosso JJ *et al***. Highlighting *Astyanax* species diversity through DNA barcoding. PloS One. 2016; 11(12):e0167203. https://doi.org/10.1371/journal.pone.0167203
- **Rosso JJ, Mabragaña E, González-Castro M, Delpiani MS, Avigliano E, Schenone N *et al***. A new species of the *Hoplias malabaricus* species complex (Characiformes: Erythrinidae) from the La Plata River basin. Cybium. 2016; 40(3):199–208.

- **Rosso JJ, González-Castro M, Bogan S, Cardoso YP, Mabragaña E, Delpiani M *et al***. Integrative taxonomy reveals a new species of the *Hoplias malabaricus* species complex (Teleostei: Erythrinidae). Ichthyol Explor Freshw. 2018; 28:235–52. https://doi.org/10.23788/IEF-1076
- **Santos U, Völcker CM, Belei FA, Cioffi MB, Bertollo LAC, Paiva SR *et al***. Molecular and karyotypic phylogeography in the Neotropical *Hoplias malabaricus* (Erythrinidae) fish in eastern Brazil. J Fish Biol. 2009; 75(9):2326–43. https://doi.org/10.1111/j.1095-8649.2009.02489.x
- **Santos FA, Marques DF, Terencio ML, Feldberg E, Rodrigues LRR**. Cytogenetic variation of repetitive DNA elements in *Hoplias malabaricus* (Characiformes - Erythrinidae) from white, black and clear water rivers of the Amazon basin. Genet Mol Biol. 2016; 39(1):40–48. https://doi.org/10.1590/1678-4685-gmb-2015-0099
- **Silva EL, Centofante L, Miyazawa CS**. Análise morfométrica em *Thoracocharax stellatus* (Kner, 1858) (Characiformes, Gasteropelecidae) proveniente de diferentes bacias hidrográficas Sul-americanas. Biota Neotrop. 2009; 9(2):71–76. https://doi.org/10.1590/S1676-06032009000200006
- **Shibatta OA, Hoffmann AC**. Variação geográfica em *Corydoras paleatus* (Jenyns) (Siluriformes, Callichthyidae) do sul do Brasil. Rev Bras Zool. 2005; 22(2):366–71. https://doi.org/10.1590/S0101-81752005000200010
- **Sumner AT**. A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res. 1972; 75:304–06.
- **Terán GE, Benitez MF, Mirande JM**. Opening the Trojan horse: Phylogeny of *Astyanax*, two new genera and resurrection of *Psalidodon* (Teleostei: Characidae). Zool J Linnean Soc. 2020; 1–18. https://doi.org/10.1093/zoolinnean/zlaa019
- **Thompson JD, Higgins DG, Gibson TJ**. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994; 22(22):4673–80. https://doi.org/10.1093/nar/22.22.4673
- **Vicari MR, Artoni RF, Bertollo LAC**. Comparative cytogenetics of *Hoplias malabaricus* (Pisces, Erythrinidae). A population analysis in adjacent hydrographic basins. Genet Mol Biol. 2005; 28(1):103–10. https://doi.org/10.1590/S1415-47572005000100018
- **Vitorino CA, Oliveira RCC, Margarido VP, Venere PC**. Genetic diversity of *Arapaima gigas* (Schinz, 1822) (Osteoglossiformes: Arapaimidae) in the Araguaia-Tocantins basin estimated by ISSR marker. Neotrop Ichthyol. 2015; 13(3):557–68. https://doi.org/10.1590/1982-0224-20150037
- **Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN**. DNA barcoding Australia's fish species. Philos Trans R Soc B. 2005; 359(1462):1847–57. https://doi.org/10.1098/rstb.2005.1716

AUTHOR'S CONTRIBUTION

Karen L. A. Guimarães: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing-original draft, Writing-review and editing.

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Mendelsohn F. B. Souza: Methodology, Software, Validation, Visualization, Writing-review and editing.

Juan M. Díaz de Astarloa: Investigation, Resources, Supervision, Validation, Visualization, Writing-review and editing.

Luís R. R. Rodrigues: Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing-review and editing.

ETHICAL STATEMENT

The Brazilian government System of Authorization and Information in Biodiversity (SISBIO) provided the permits for fish collections (SISBIO 32653–3). The animals were anesthetized and euthanized through immersion in water containing Eugenol solution, following a procedure approved by the Animal Use Ethics Committee (CEUA) of the Universidade Federal do Oeste do Pará (CEUA/UFOPA Nº 09003).

COMPETING INTERESTS

The authors declare no competing interests.

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