Neotropical Ichthyology

Original article

https://doi.org/10.1590/1982-0224-2023-0117

Allozyme and cytogenetic analysis in two species of *Hypostomus* (Siluriformes: Loricariidae) from the Paraguai River basin, Brazil: occurrence of B microchromosome and intrapopulation heterochromatic polymorphism in *H. boulengeri*

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Hypostomus is distributed by Central and South America basins, with diverse species with taxonomic conflicts. This way, the integration of auxiliary techniques contributes to understanding the systematics and phylogeny of the group. Thus, this study aimed to investigate the *Hypostomus cochliodon* and *H. boulengeri* from the Onça stream (Paraguai River basin) by allozyme and cytogenetic techniques. *Hypostomus boulengeri* showed a diploid number of 68 chromosomes (14m+14sm+18st+22a), multiple NOR revealed by Ag-NOR and 18S rDNA FISH, a polymorphism of heterochromatin in acrocentrics and the presence of B microchromosome. *Hypostomus cochliodon* showed a diploid number of 64 chromosomes (16m+26sm+14st+8a); despite the single NOR, some individuals showed NOR in both telomeres detected by Ag-NOR and 18S rDNA FISH. Isozyme identified two diagnostic loci (Idh-A and Gdh-A)

Submitted October 24, 2023
Accepted May 21, 2024
by Claudio Oliveira
Epub July 22, 2024

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Online version ISSN 1982-0224
Print version ISSN 1679-6225

Neotrop. Ichthyol.
vol. 22, no. 2, Maringá 2024

between the two species and multiple loci with unique alleles in *H. boulengeri.* The genetic variability indicated by the mean heterozygosity (He) was 0.2461 and 0.0309 in *H. boulengeri* and *H. cochliodon*, respectively. Thus, this study reports the first cytogenetic data for *H. boulengeri* and the first isozymatic data for *H. boulengeri* and *H. cochliodon.* The two species presented evident cytogenetic and isoenzymatic differences with the obtaining of exclusive genetic markers providing support for future evolutionary studies in the group.

Keywords: Ribosomal DNA, C-Banding, Diagnostic Loci, Isoenzymes.

Hypostomus está distribuído por bacias da América Central e do Sul, com grande diversidade de espécies com conflitos taxonômicos. Desta forma, a integração de técnicas auxiliares contribui para a compreensão da sistemática e filogenia do grupo. Assim, este estudo teve como objetivo investigar *Hypostomus cochliodon* e *H. boulengeri* do riacho Onça (bacia do rio Paraguai) por meio de técnicas aloenzimáticas e citogenéticas. As análises citogenéticas em *H. boulengeri* mostraram número cromossômico igual a 2n = 68 (14m+14sm+18st+22a), sistema de NOR múltiplo revelado por Ag-NOR e 18S-FISH, um polimorfismo de heterocromatina em acrocêntricos e a presença de microcromossomos Bs. *Hypostomus cochliodon* mostrou um número diploide de 64 cromossomos (16m+26sm+14st+8a); apesar do sistema de NOR simples, alguns indivíduos apresentaram NOR em ambos os telômeros detectados por Ag-NOR e 18S-FISH. Uma isozima identificou dois loci diagnósticos (Idh-A and Gdh-A) entre as duas espécies e múltiplos loci com alelos únicos em *H. boulengeri*. A variabilidade genética indicada pela heterozigosidade média (He) foi de 0,2461 e 0,0309 em *H. boulengeri* e *H. cochliodon*, respectivamente. Assim, este estudo relata os primeiros dados citogenéticos para *H. boulengeri* e os primeiros dados isoenzimáticos para *H. boulengeri* e *H. cochliodon*. As duas espécies apresentaram evidentes diferenças citogenéticas e isoenzimáticas com a obtenção de marcadores genéticos exclusivos fornecendo suporte para futuros estudos evolutivos no grupo.

Palavras-chave: DNA Ribossomal, Banda C, Loco diagnóstico, Isoenzimas.

INTRODUCTION

Loricariidae is the most prominent family among the Siluriformes, distributed throughout Central and South America, and it is composed of fish popularly known as catfish. Among the subfamilies, Hypostominae has 500 species and 45 valid genera (Fricke *et al*., 2023), that despite the monophyly, present phylogenetic conflicts, mainly in genus *Hypostomus* Lacepède, 1803 which presents a large number of species with significant morphological variation (Zawadzki *et al*., 2001, 2008a; Armbruster, 2003, 2004; Reis *et al*., 2006; Ferraris, 2007; Cramer *et al*., 2011; Lujan *et al*., 2015; Roxo *et*

al., 2019). Thus, the integration of phylogenetic techniques can elucidate taxonomic uncertainties; for example, integrative taxonomy associates molecular, cytogenetic, and morphological methods, which together contribute to aspects related to genetic variability and cryptic diversity of the genus (Pugedo *et al*., 2016; Dias, Zawadzki, 2018; Azevedo *et al*., 2021).

Studies using the allozyme technique to identify species of *Hypostomus* were carried out mainly in specimens present in the Paraná River basin, contributed to the evaluation of the genetic variability of populations, identification, and distinction of species, in addition to the inference of phylogenetic relationships and systematic approach of this genus that represents a complex subject (Zawadzki *et al*., 1999, 2002, 2004, 2008a; Paiva *et al*., 2005; Ito *et al*., 2009).

On the other hand, cytogenetic studies in *Hypostomus* show wide variation in chromosome number, ranging from 2n = 64 in *H. faveolus* Zawadzki, Birindelli & Lima, 2008, *H. cochliodon* Kner, 1854, *H. soniae* Hollanda Carvalho & Weber, 2005 (Bueno *et al*., 2013; Oliveira *et al*., 2019) to 2n = 84 in *H. perdido* Zawadzki, Tencatt & Froehlich, 2014 (Cereali *et al*., 2008; Zawadzki *et al*., 2014). In addition, interspecific and intraspecific karyotypic variations have been reported in different populations. This diversity has been attributed to chromosomal rearrangements throughout the karyotypic evolution of *Hypostomus* (Artoni, Bertollo, 2001; Bueno *et al*., 2012; Ferreira *et al*., 2019). In addition, inferences about the taxonomy and phylogeny have been made from the physical mapping of some regions of the chromosomes, such as the nucleolus organizer region (NOR), where the single NOR is considered a pleisiomorphic character, while multiple NOR located in the terminal region is the most commonly found character and deemed apomorphic character in the genus (Artoni, Bertollo, 1996, 2001; Alves *et al*., 2006; Rubert *et al*., 2016; Lorscheider *et al*., 2018). Moreover, constitutive heterochromatin co-localized with NOR sites may be involved in the dispersion of extra copies of rDNA genes along the genome in *Hypostomus,* where unequal mating events and amplification of heterochromatin would explain the occurrence of multiple NORs. Additionally, transposable elements have also been suggested as agents of dispersion of copies of these genes throughout the genome of the group (Bueno *et al*., 2014; Rubert *et al*., 2016; Lorscheider *et al*., 2018).

In addition, variations in the distribution of constitutive heterochromatin across the karyotype have made it possible to discuss the dispersion mechanisms of heterochromatin in different populations of *Hypostomus* (Artoni, Bertollo, 1999; Bitencourt *et al*., 2011; Traldi *et al*., 2012; Baumgärtner *et al*., 2014). Conspicuous blocks of heterochromatin located on the long arm of acrocentric chromosomes have been commonly found in the group, and even reports of polymorphism involving these chromosomes have been detected intra- and interpopulationally (Artoni, Bertollo, 1999, 2001; Rubert *et al*., 2011; Bitencourt *et al*., 2012). The hypothesis that the amplification of heterochromatic segments could have caused the dispersion of heterochromatin along the karyotype, in addition to other elements such as transposable elements and chromosomal rearrangements, was also suggested (Bitencourt *et al*., 2012; Traldi *et al*., 2012, 2019; Baumgärtner *et al*., 2014; Oliveira *et al*., 2015; Ferreira *et al*., 2019).

In this article, we aimed to expand on the cytogenetic and isozymatic data of *H. cochliodon* and *H. boulengeri* (Eigenmann & Kennedy, 1903) collected in a tributary of the Paraguai River. Here are the first cytogenetic data for *H. boulengeri* and the first isoenzymatic data for both species.

MATERIAL AND METHODS

Study area and sampling. The individuals of *Hypostomus boulengeri* and *H. cochliodon* (Figs. 1A, B) were collected in the Onça stream, a tributary of the Taquari River, upper Paraguai River basin, Mato Grosso do Sul (Coxim-MS; 18°30'S 54°40'W and 18°32'S 51°25'W). Specimens were anesthetized and sacrificed by immersion in eugenol, fixed in 10% formalin solution, and later preserved in 70% ethanol (Griffiths, 2000). Specimens of both species were deposited in the ichthyological collection of the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupélia) of the Universidade Estadual de Maringá (NUP 9821, *H. boulengeri*; NUP 9822, *H. cochliodon*).

Cytogenetic analysis. Ten individuals of *H. boulengeri* (three males; five females; two unidentified) and sixteen of *H. cochliodon* (seven males; eight females; one unidentified) were analyzed. Mitotic chromosomes were obtained from kidney cells, according to the technique described by Bertollo *et al*. (1978). The Nucleolar Organizer Regions (NOR) were detected by the silver nitrate staining (Howell, Black, 1980) and Fluorescent *in situ* Hybridization (FISH) technique, using 18S rDNA probes obtained

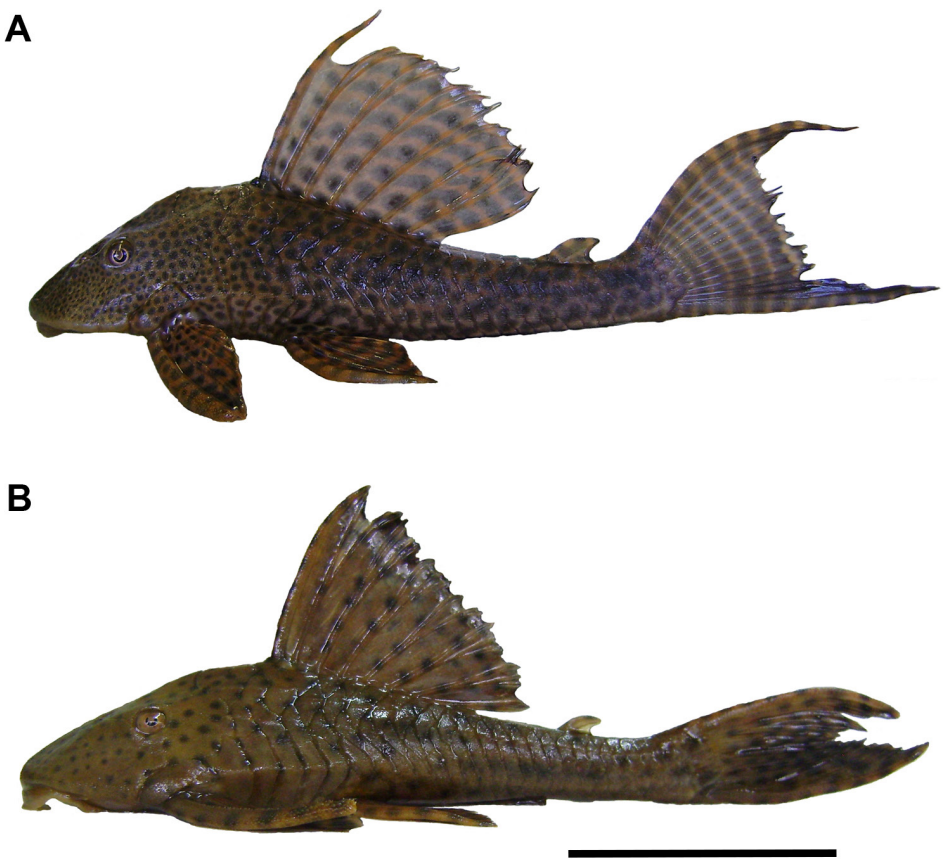

FIGURE 1 | Specimens of *Hypostomus boulengeri* (**A**) and *H. cochliodon* (**B**) from the Onça stream, upper Paraguai River basin. Scale bar = 100 mm.

from 18S rDNA fragments of *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka, Galetti, 2004), following the methodology described by Pinkel *et al*. (1986). The C-banding technique determined the heterochromatin distribution (Sumner, 1972) and stained with propidium iodide (Lui *et al*., 2012). The arms ratio, as proposed by Levan *et al*. (1964), established chromosome morphology and classified it as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a).

Allozyme analysis. Muscle, liver, and heart samples were collected from both species and preserved at low temperatures (-20°C). Starch gels (15%) were prepared using three different buffer systems in pH 7.4 (Murphy *et al*., 1996), each one specific for the other enzymatic systems and tissues (Tab. 1). Tissue samples were homogenized with 0.02 M Tris-HCl buffer, pH 7.5, and centrifuged at 25.000 rpm for 30 min at a low temperature. The protein extract was applied to the gel, subjected to continuous horizontal electrophoresis, and subsequently incubated in specific histochemical solutions (Murphy *et al*., 1996). The enzymatic systems were analyzed (Tab. 1), and the genetic interpretation of the zymograms was based on the quaternary structure of the enzymes, according to Ward *et al*. (1992). Data were analyzed using Popgene 1.31 (Yeh *et al*., 1999). Loci and alleles were named according to Simonsen (2012), and data was analyzed using Popgen 1:32 software (Yeh *et al*., 1997). Genetic variability was determined by calculating heterozygosity (expected and observed) according to Nei (1978). The identity (I) and the genetic distance (D) were calculated with the values of the allele frequencies. We employed the dendrogram (grouping method by the algorithm UPGMA- Unweighted Pair Group Method with Arithmetic Means) of the populations, assuming Hardy-Weinberg equilibrium.

TABLE 1 | Allozymes analyzed in species *Hypostomus boulengeri* and *H. cochliodon*: enzyme name, enzyme commission (EC) number, tissues, and buffers. EC n - Enzyme Commission Number; L - liver; M - muscle; H - heart; TBE - Tris-borate-EDTA; TC - Tris-citrate; TEM - Tris -EDTA-maleate.

Enzyme (abbreviation)	nº EC	Tissues	Buffers
Alcohol dehydrogenase (ADH)	1.1.1.1	L	TBE
Aspartate aminotransferase (AAT)	2.6.1.1	L	TEM
Acid Phosphatase (ACP)	3.1.3.2	L	TC
Glucose-3-phosphate dehydrogenase (G3PDH)	1.1.1.8	L	TC
Glucose-6-phosphate isomerase (GPI)	5.3.1.9	M, H	TC
Glucose dehydrogenase (GCDH)	1.1.1.118	L	TEM
Isocitrate dehydrogenase (IDH)	1.1.1.42	L, M, H	TC
Malate dehydrogenase (MDH)	1.1.1.37	L, M, H	TC
Superoxide dismutase (SOD)	1.15.1.1	L	TBE

RESULTS

Cytogenetic data. Individuals of *Hypostomus boulengeri* presented a diploid number of 2n = 68 distributed in 14m+22sm+10st+22a and a fundamental number (FN) equal to 114 (Fig. 2A). In addition to the basic karyotype, all male and female individuals presented a variation from zero to one B microchromosomes in the somatic cells without homology with the other chromosomes (Fig. 2A, in the box). These elements are smaller than any chromosome of the standard A complement and presented 6% of frequency in the cells analyzed (Tab. 2). *Hypostomus cochliodon* showed 2n = 64 with the karyotypic formula of 16m+22sm+18st+8a and FN = 120 (Fig. 3A). There were no karyotypic differences between males and females in both species.

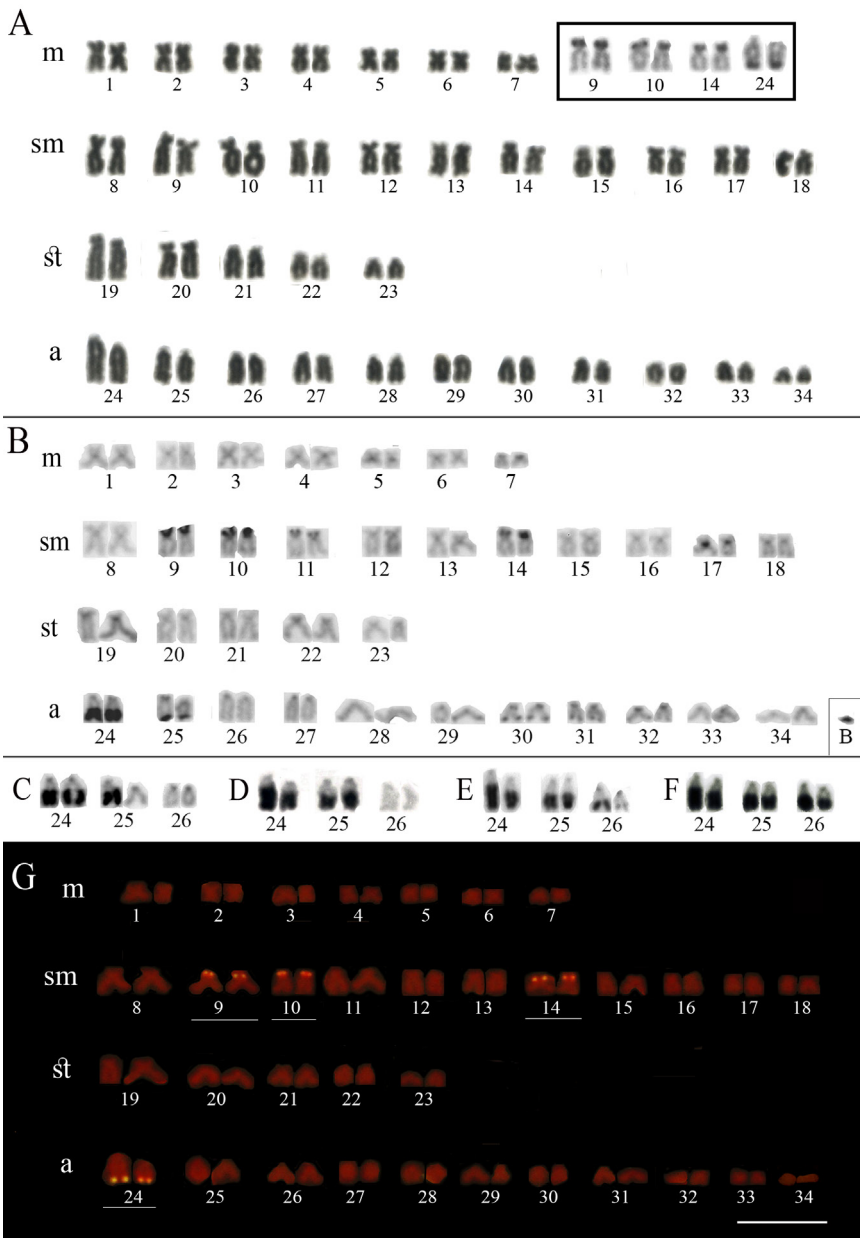

FIGURE 2 | Karyotypes of *Hypostomus boulengeri* subjected to **A.** Giemsa, **B.** C-banding and **G.** FISH with 18S rDNA probe (yellow). Polymorphism of the heterochromatin in pairs 24, 25, and 26 are shown in **C**, **D**, **E**, and **F**. The Ag-NOR-bearing chromosomes are boxed beside the karyotype stained with Giemsa. The B microchromosomes are boxed beside the karyotypes stained with Giemsa and C-banding. Scale bar = 10 µm.

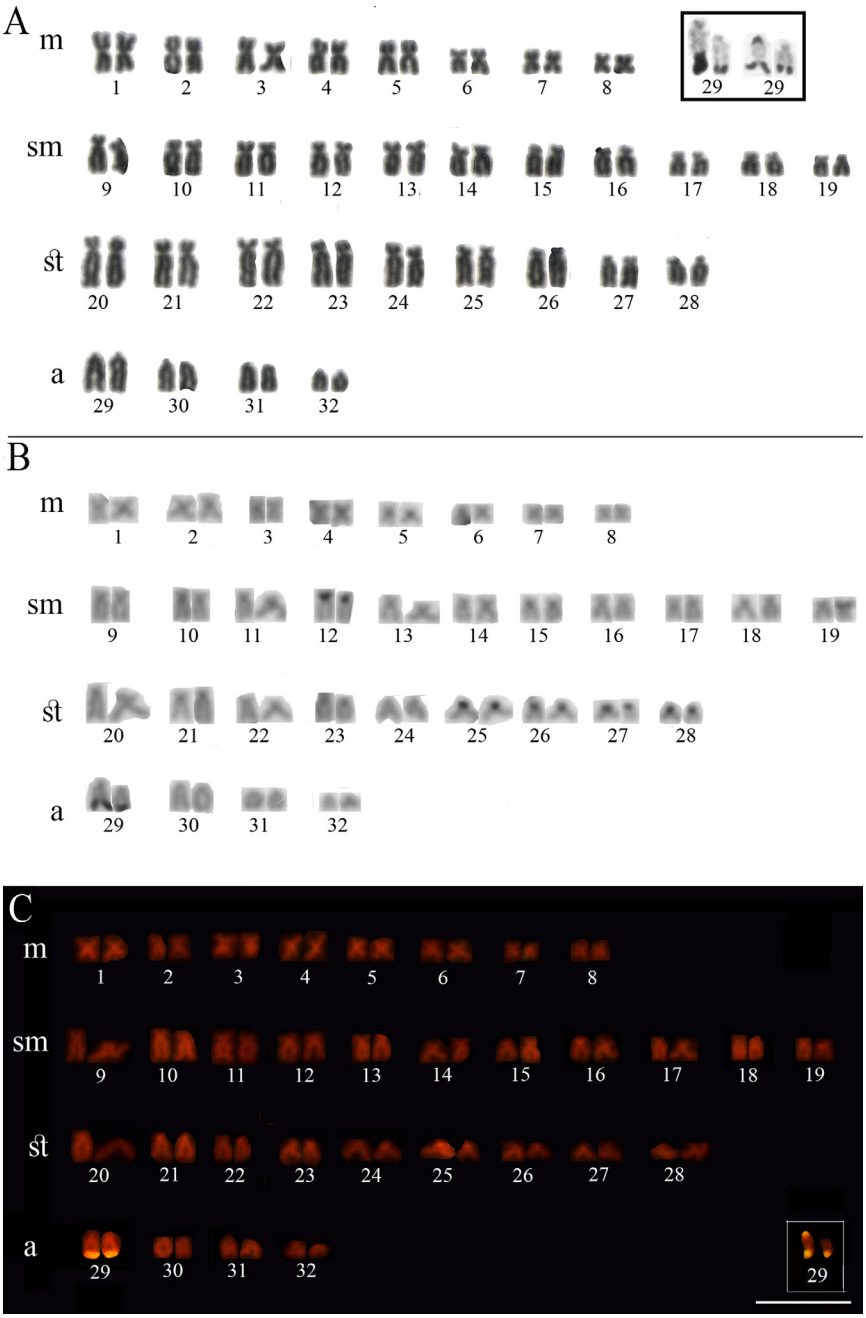

FIGURE 3 | Karyotypes of *Hypostomus cochliodon* subjected to **A.** Giemsa, **B.** C-banding, and **C.** FISH with 18S rDNA probe (yellow). The Ag-NOR-bearing chromosomes are boxed beside the karyotype stained with Giemsa. Note one of the homologs of pair 29 with marking in both telomeres after stained Ag-NOR (Box in **A**), C-banding (Box in **B**), and FISH with 18S rDNA probe (Box in **C**). Scale bar = 10 µm.

Analysis of the nucleolus organizer region performed with Ag-NOR and 18S rDNA FISH techniques in *H. boulengeri* showed a multiple NOR located on the short arm of three pairs of submetacentric chromosomes (pairs: 9, 10, and 14; Fig. 2A, in the box) and in the telomere region on the long arms of pair 24 (Fig. 2A, in the box). In *H. cochliodon*, the single NOR was detected on the long arm of the first pair of acrocentric chromosomes at the telomeric position (pair 29; Figs. 3A, C, in the boxes). However, in some individuals of this species, additional staining was detected in the short arm in one of the homologs of the NOR organizing pair (NORs in both telomeres, Figs. 3A, C, in the boxes).

TABLE 2 | Frequency of B microchromosomes in *Hypostomus boulengeri*.

Individual	Cells analyzed	Cells with B microchromosomes
1	17	2 (11.76%)
2	4	0 (0%)
3	30	2 (6.7%)
4	35	1 (2.86%)
5	19	0 (0%)
6	62	4 (6.45%)
7	23	3 (13.04%)
8	3	0 (0%)
9	4	0 (0%)
10	3	0 (0%)
Total (Frequency)	**200**	**12 (6.0%)**

In *H. boulengeri*, the C-banding revealed pericentromeric heterochromatin blocks in most metacentric, submetacentric, and subtelocentric chromosomes and the short arm of pairs 9, 10, and 14 coinciding with the NOR regions (Fig. 2B). Furthermore, a conspicuous heterochromatic segment can also be observed in the long arm of some acrocentric chromosomes in both sexes (pairs: 24, 25, and 26), and the number of chromosomes containing this type of heterochromatin varied among individuals in the population (two to six chromosomes; Figs. 2C–F), characterizing a numerical polymorphism. Additionally, some individuals have heterochromatic B microchromosome (Fig. 2B, in the box). Heterochromatin in *H. cochliodon* was evidenced mainly in the pericentromeric regions of metacentric and submetacentric chromosomes and blocks on the short arm of the submetacentric and subtelocentric chromosomes (Fig. 3B). Further, the NOR region was C-banding positive (Fig. 3B, in the box).

Allozyme data. Nine enzymatic systems allowed the analysis of 15 loci of the species *H. boulengeri* and *H. cochliodon*, presenting 30 alleles; among these were diagnoses (Idh-A and Gcdh-A; Tab. 3). In *H. boulengeri,* several exclusive alleles were detected with variable frequencies at the loci: Aat-A-*a* and *c*, Adh-A-*b* and *c*, Gpi-B-*a* and *d*, G3pdh-A-*b* and *c*, G3pdh-B-*b* and Idh-B-*a*. Regarding the genetic variability of the two populations, values of 0.2461 and 0.0309 were found for the average expected heterozygosity (H_e) (Tab. 3) for *H. boulengeri* and *H. cochliodon*, respectively.

TABLE 3 | Allelic frequencies were obtained from the polymorphic loci of the species analyzed in this study. Number of *Hypostomus boulengeri* analyzed - n (*H. b.*), number of *H. cochliodon* analyzed - n (*H. c.*), Loci - polymorphic loci, percentage of polymorphic loci (P%), number of alleles per locus (K), average heterozygosity obtained (Ho) and expected (He). In parentheses are the respective standard deviations.

Loci	Allelic	*Hypostomus boulengeri*	n (*H. b.*)	*Hypostomus cochliodon*	n (*H. c.*)
Aat-A	*a*	0.0526	19	—	24
	b	0.5789		1.0000	
	c	0.3684		—	
Aat-B	*a*	1.0000	20	1.0000	22
Acp-A	*a*	1.0000	20	1.0000	24
Adh-A	*a*	0.3000	20	1.0000	24
	b	0.6500		—	
	c	0.0500		—	
Gcdh-A	*a*	1.0000	20	—	24
	b	—		1.0000	
Gpi-A	*a*	1.0000	20	1.0000	24
Gpi-B	*a*	0.2250	20	—	24
	b	0.4000		0.6522	
	c	0.2000		0.3478	
	d	0.1750		—	
G3pdh-A	*a*	0.5750	20	1.0000	24
	b	0.3250		—	
	c	0.1000		—	
G3pdh-B	*a*	0.2000	20	1.0000	24
	b	0.8000		—	
Idh-A	*a*	—	20	1.0000	24
	b	0.2000		—	
	c	0.6500		—	
	d	0.1500		—	
Idh-B	*a*	0.6000	20	—	22
	b	0.4000		1.0000	
Mdh-A	*a*	1.0000	20	1.0000	24
Mdh-B	*a*	1.0000	20	1.0000	24
Mdh-C	*a*	1.0000	20	1.0000	24
Sod-A	*a*	1.0000	20	1.0000	24
P		7		1	
P%		46.67		6.67	
K		1.8667		1.0667	
H_o		0.0981 (0.1702)		0.0000 (0.0000)	
H_e		0.2461 (0.2836)		0.0309 (0.1171)	

DISCUSSION

Cytogenetics analysis. *Hypostomus cochliodon* from the Onça stream showed a diploid number (2n = 64) similar to other cytogenetically characterized populations. However, the karyotype formula, FN, nucleolar organizer pair's location, and constitutive heterochromatin distribution detected in the present study differed (Tab. 4). Although this species belongs to the *H. cochliodon* group (Ambruster, 2003), considered a monophyletic clade with 20 valid species distributed throughout South America (Tencatt *et al*., 2014), cytogenetic studies are scarce. Bueno *et al*. (2013) related cytogenetic data of *Hypostomus* species with their respective geographic distributions along the watershed, considering that *H. cochliodon* is one of the species with the highest diploid number widely distributed in the North basin (Paraguai and Amazonia), despite the great diversity of species spread across these basins, cytogenetic data on karyotypic variety are also scarce for the genus. The present study extends the cytogenetic data of *Hypostomus* belonging to the Paraguai basin; in addition, *H. boulengeri* presented 68 chromosomes. Therefore, it corroborates recent studies that demonstrate that in the southern basins, the species of *Hypostomus* contain a high number of chromosomes (Bueno *et al*., 2013; Becker *et al*., 2014; Rubert *et al*., 2016; Ferreira *et al*., 2019). Thus, it is necessary to increase such data to understand the karyotypic evolution of the group; in addition, together with morphological and molecular studies, they can help to understand the systematics and phylogeny of this species (Becker *et al*., 2014; Rubert *et al*., 2016; Ferreira *et al*., 2019).

Furthermore, the present study shows the first cytogenetic description of *H. boulengeri* by detecting a constitutive heterochromatin polymorphism involving acrocentric chromosomes that presented conspicuous heterochromatin blocks (Figs. 2C–F). This type of heterochromatin pattern in acrocentrics was also found in other species of *Hypostomus* (Artoni, Bertollo, 1999; Kavalco *et al*., 2004; Baumgärtner *et al*., 2014; Oliveira *et al*., 2015; Ferreira *et al*., 2019). In addition, in some populations, polymorphisms related to this type of heterochromatin distribution pattern in acrocentric chromosomes were also observed, suggesting that the amplification of heterochromatic regions originated the intra and interpopulational variations in the genus (Traldi *et al*., 2012; Baumgärtner *et al*., 2014; Oliveira *et al*., 2015; Ferreira *et al*., 2019). In *H. regani* (Ihering, 1905), also

TABLE 4 | Comparison among cytogenetic studies in the species *Hypostomus cochliodon*. FN: Fundamental number; m: metacentric; sm: submetacentric; st: subtelocentric; a: acrocentric.

Species/ Sampling site	Diploid Number	Karyotype	FN	NOR System/ NOR pair	Heterochromatin distribution	References
H. cochliodon/ Iguaçu River	64	12m+16sm+16st+20a	108	Simple/ 28	-	Bueno *et al*. (2013)
H. aff. *cochliodon*/ Esparramo stream	64	18m+20sm+26st/a	102	Multiple/ 22, 26	large heterochromatic blocks in pairs: 20, 21 e 22	Becker *et al*. (2014)
H. aff. *cochliodon*/ Pitaluga stream	64	18m+20sm+26st/a	102	Multiple/ 22, 26	large heterochromatic blocks in pairs: 20, 21 e 22	Becker *et al*. (2014)
H. cochliodon/ Piraputanga River	64	16m 20sm 28st-a	100	Multiple	-	Rubert *et al*. (2016)
H. cochliodon/ Onça stream	64	16m+22sm+18st+ 8a	120	Simple/ 29	absence of large heterochromatic blocks in pairs	Present study

collected from the Onça stream, a chromosomal heteromorphism was detected by the C-banding technique, which allowed the distinction of two karyotypes, suggesting that the origin of this heteromorphism occurred from the amplification of heterochromatin that allowed the difference of two karyotypes (Ferreira *et al*., 2019). In *H. strigaticeps* (Regan, 1908), from the upper Paraná River basin, heterochromatin amplification supposedly caused the interpopulation polymorphism, considered that the unequal crossing over processes and the proximity of homologous segments in the interphase nucleus would probably facilitate unequal exchanges and dispersion of heterochromatin and that such events could be involved in the amplification process of this region by the genome (Baumgärtner *et al*., 2014).

In addition, the association of transposable elements (TEs) to heterochromatin would promote its reorganization due to the ability of TEs to disperse throughout the genome, thus contributing to chromosomal evolution (Baumgärtner *et al*., 2014). Rex1 transposable elements (TEs) were associated with heterochromatin in *H. ancistroides* (Ihering, 1911), and *H. nigromaculatus* (Schubart, 1964). Transposable elements (TEs) Rex1 were found to be associated with heterochromatin in *H. ancistroides* and *H. nigromaculatus*. Accumulation of TEs in some chromosomes of *Hypostomus* species indicates the involvement of these elements with the organization of constitutive heterochromatin (Pansonato-Alves *et al*., 2013; Traldi *et al*., 2019). Thus, the heterochromatin polymorphism involving acrocentric chromosomes detected in *H. boulengeri* in the present study that could occurred by amplifying the constitutive heterochromatin, unequal crossing-over and/or transposable elements associated with heterochromatin, which plays an essential role in the karyotypic evolution of *Hypostomus*.

Regarding the B microchromosome detected in *H. boulengeri* in the present study, this type of chromosome is uncommon in *Hypostomus*, with B chromosomes being observed only in *Hypostomus* sp. from Xingu-3 (Milhomem *et al*., 2010) and *Hypostomus* sp. 3 (Cereali *et al*., 2008). In both studies described previously, the frequency of this chromosome in the populations analyzed was not mentioned. In five individuals of *H. boulengeri*, these chromosomes were present in 6% of the cells analyzed (Tab. 2), showing an inter and intra-individual variabilities of these elements, suggesting mitotic instability, probably due to their non-Mendelian behavior that may be related to chromosomal non-disjunction during meiosis, leading to uneven segregation of genetic material between germ cells (Rosa *et al*., 2014).

Furthermore, the B microchromosome observed in *H. boulengeri* were completely heterochromatic, while in *Hypostomus* sp. from Xingu-3 (Milhomem *et al*., 2010) and *Hypostomus* sp. 3 (Cereali *et al*., 2008), these B microchromosomes were neither heterochromatic. This indicates that these B microchromosomes can have a different DNA composition, mainly concerning repetitive sequences. In other species of the family Loricariidae, B chromosomes are rarely found, having been reported only in *Hisonotus leucofrenatus* (Miranda Ribeiro, 1908) (Andreata *et al*., 1993), *Loricaria* sp. and *Proloricaria prolixa* (Isbrücker & Nijssen, 1978) (Scavone, Júlio-Jr, 1994), *Neoplecostomus paranensis* Langeani, 1990 (Alves *et al*., 1999), *Rineloricaria pentamaculata* Langeani & de Araujo, 1994 (Porto *et al*., 2010) and *Harttia longipinna* Langeani, Oyakawa & Montoya-Burgos, 2001 (Blanco *et al*., 2012).

Regarding the nucleolar organizer region, although most individuals of *H. cochliodon* presented a number and location of the NOR, considered conserved in *Hypostomus* (Artoni, Bertollo, 1996, 2001; Kavalco *et al*., 2005; Alves *et al*., 2006; Cereali *et al*., 2008; Milhomem *et al*., 2010; Bitencourt *et al*., 2011, Martinez *et al*., 2011; Rubert *et al*., 2011; Lorscheider *et al*., 2018), some individuals showed an additional NOR site (NORs in both telomeres) in one of the homologs of the NOR organizer pair. *Hypostomus* with NORs in both telomeres has been reported in *H. cochliodon*, *H. hermanni* (Ihering, 1905), *H. albopunctatus* (Regan, 1980), and *H.* aff. *paulinus* (Ihering, 1905) (Rubert *et al*., 2016). In some fish species such as in the genus *Psalidodon* Eigenmann, 1911 (Mantovani *et al*., 2005; Fernandes, Martins-Santos, 2006; Fernandes *et al*., 2009; Abelini *et al*., 2014), *Hoplias malabaricus* (Bloch, 1794) (Cioffi *et al*., 2009; Blanco *et al*., 2010), *Pyrrhulina* cf. *australis* (Oliveira *et al*., 1991) and *Poecilia latipunctata* Meek, 1904 (Galetti, Rash, 1993), NOR in both telomeres has been reported.

For the variation in the distribution of 18S rDNA sites in the Loricariidae, it has been suggested that the dispersion of such sites throughout the genome could jointly or/and separately have contributed to the karyotypic evolution of the group (Porto *et al*., 2011, 2014a,b; Rubert *et al*., 2016). Rubert *et al*. (2016) observed interspecific variation in four species of *Hypostomus* (*H. cochliodon*, *H. hermanni*, *H. albopunctatus*, and *H.* aff. *paulinus*); it was proposed that the association between heterochromatin and rDNA sites contributes to the occurrence of unequal crossing-over generating new rDNA loci. In addition, the proximity between telomeres in the interphase nucleus would facilitate the translocation of some copies of the rDNA genes located in telomeric regions, resulting in the translocation/transfer of genetic material among the chromosomes (Schweizer, Loidl, 1987; Fernandes, Martins-Santos, 2006; Cioffi *et al*., 2010; Porto *et al*., 2014a; Rubert *et al*., 2016). The transposable elements associated with rDNA copies may also contribute to the dissemination of these genes due to their ability to disperse throughout the genome in fish (Silva *et al*., 2011; Piscor *et al*., 2013; Bueno *et al*., 2014; Rubert *et al*., 2016).

We suggest that in *H. cochliodon*, the NOR in both telomeres is a derived character. The NOR sites on the long arm probably occurred duplication or amplification and were later inserted in a new region of the same chromosome (a short arm of pair 29). Thus, the NOR in both telomeres in *H. cochliodon* and the multiple NOR in *H. boulengeri* corroborate the data for other species, characterized as apomorphies in *Hypostomus* (Lorscheider *et al*., 2018). Rubert *et al*. (2016) suggest that intrinsic genus factors led to different karyoevolutionary mechanisms and would explain the NOR variability, the chromosomal behavior, and the dispersion of specific rearrangements that occurred differently in each population.

Allozyme analysis. Concerning the isozyme analysis, the two diagnostic loci and the exclusive alleles for *H. boulengeri* showed a distinction between the two species. Allozyme studies among populations of this group have made it possible to identify genetic differences that contribute to the differentiation between them. Renesto *et al*. (2007) identified diagnostic loci for species *H. boulengeri* and *H. cochliodon* from the upper Paraguai River basin (sAat-2, Idh-2 and Mdhp-B) that differed from those found in the present work (Idh-A and Gdh-A). Furthermore, the sAat-2 locus separated *H. boulengeri* and *H. cochliodon* from seven other species (*H. latifrons* Weber, 1986, *H. regani*,

Hypostomus sp. 1, *Hypostomus* sp. 2, *Hypostomus* sp. 3, *H.* cf. *latirostris*, and *Pterygoplichthys ambrosettii* (Holmberg, 1893) from the Manso River (Manso Reservoir) and the Cuiabá River. The Idh-2 locus reported by Renesto *et al.* (2007) is equivalent to the Idh-B locus of the present study, while the Mdhp (malic enzyme) was not analyzed.

The average expected heterozygosity (He) of *H. boulengeri* (He = 0.2461) was the highest ever verified among the species of this genus studied by isozyme analysis. The highest He values previously found was 0.199 for *H. hermanni* from the Ivaí River (upper Paraná River basin; Paiva, 2006). For *H. boulengeri* in the present work, the value of He represents more than four times the expected average heterozygosity value for fish (He = 0.051), obtained by Ward *et al.* (1992). However, Renesto *et al.* (2007) verified in *H. boulengeri* from the upper Paraguai River basin that He equals 0.078. Distinct values were found between populations of *H. margaritifer* (Regan, 1908) from the Itaipu reservoir in Paraná (He = 0.104) and from the Corumbá Reservoir in Goiás (He = 0.061) (Zawadzki *et al.*, 2002). *Hypostomus cochliodon* revealed a He value of 0.0309, similar to that found for the population of this same species collected in the Itaipu reservoir (0.039) (Zawadzki *et al.*, 2005), however with a lower He value (0.070) described for *H. cochliodon* of the upper Paraguai River (Renesto *et al.*, 2007).

A study of three populations of *H. regani* from the Corumbá, Itaipu, and Manso reservoirs showed that they differed in terms of heterozygosity values, 0.0527, 0.0712, and 0.0317, respectively (Zawadzki *et al.*, 2008b). Thus, heterozygosity is a measure of genetic variability, which can be similar or variable between populations of the same species of *Hypostomus*. Several biotic and abiotic factors are proposed in the literature. They may be involved in the process of genetic differentiation of this group, such as natural inbreeding barriers that impede gene flow, differences in temperature, water velocity, food resources, and reproductive strategies (Zawadzki *et al.*, 1999, 2002, 2005, 2008b; Paiva, 2006; Ito *et al.*, 2009).

There are few studies on the biology of Neotropical fish, especially those from the Paraguai River basin. Thus, there is difficulty in correlating multivariate biological factors with greater or lesser heterozygosity in these fish. Although it is impossible to confirm the causes of this high genetic variability, it is known that it is crucial because, as expressed by Vida (1994), "the future of maintaining species diversity lies in the genetic diversity of species. Generally, the greater genetic diversity maintained, the greater adaptability and the probability of species survival in a changing environment".

The present study presents cytogenetic and isozymatic data of *H. cochliodon* and *H. boulengeri* collected in a tributary of the Paraguai River offered the first cytogenetic data for *H. boulengeri* and the first isozymatic data for both species, with the detection of two diagnostic loci, exclusive alleles and high genetic variability for *H. boulengeri*, in addition, the two species presented evident cytogenetic and isoenzymatic differences with the obtaining of exclusive genetic markers providing support for future evolutionary studies in the group.

ACKNOWLEDGMENTS

We authors thank Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupélia) for logistic support. This study was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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ETHICAL STATEMENT

Fishes were collected under permits from the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio nº 72521/2019). The procedures followed the 'Ethical Principles in Animal Research' guidelines adopted by the National Council of Control of Animal Experimentation (CONCEA).

COMPETING INTERESTS

The author declares no competing interests.

HOW TO CITE THIS ARTICLE

- **Paiva S, Porto FE, Codognotto FJ, Fernandes CA, Rossi MMV, Borin-Carvalho LA, Portela-Castro ALB, Zawadzki CH, Renesto E, Martins-Santos IC.** Allozyme and cytogenetic analysis in two species of *Hypostomus* (Siluriformes: Loricariidae) from the Paraguay River basin, Brazil: occurrence of B microchromosomes and intrapopulation heterochromatic polymorphism in *H. boulengeri*. Neotrop Ichthyol. 2024; 22(2):e230117. https://doi.org/10.1590/1982-0224-2023-0117

Neotropical Ichthyology

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Official Journal of the
Sociedade Brasileira de Ictiologia
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