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A N I N T E R N A T I O N A L J O U R N A L

Comparative Cytogenetics Among Three Sympatric *Hypostomus* Species (Siluriformes: Loricariidae): An Evolutionary Analysis in a High Endemic Region

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ABSTRACT

The karyotypes of three armored catfish species (Loricariidae) from the Iguaçu river, southern of the Brazil, were compared using different techniques: C-banding, Ag-NOR and fluorescence in situ hybridization (FISH), which used 5S and 18S rDNAs and total C_ot-1 fraction as probes. Hypostomus commersoni and Hypostomus derbyi presented 2n = 68 chromosomes, with karyotype formulae 12m+12sm+14st+30a and 12m+12sm+10st+34a, respectively; whereas Hypostomus myersi presented 2n = 74 chromosomes and 12m+16sm+12st+34a. The chromosomal localization of the Ag-NORs, 5S and 18S rDNAs differed in number of sites and chromosomal localization among the studied species. The total C_ot-1 probe permitted the visualization of the repetitive DNA fraction in karyotypes of each species. Cross-hybridizations using total C_ot-1 probe revealed that these species have repetitive DNAs in common. However, this does not occur in H. commersoni in relation to the other species. The apparent karyotype similarity suggests a close relationship between the sympatric H. commersoni and H. derbyi species, but the small differences detected in the examined chromosomal markers indicate evolutionary divergence due to gene flow restriction among them. Hence, the present findings indicate different composition of repetitive sequences among studied species, which permit to infer its role in chromosomal differentiation of Hypostomus.

Keywords: Armored catfish; FISH; Iguaçu river; karyotype evolution.

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INTRODUCTION

The armored catfishes are characterized by a body covered by bony plates and a ventral suckermouth [1]. Loricariidae is the largest family of the Siluriformes, accounting 974 valid species [2], and has phylogenetic studies that shown constant systematic reallocations in its subfamilies and genus [1,3-6]. The genus *Hypostomus* Lacépède, 1803 contains the greatest number of species in the Hypostominae subfamily (29 genera in total) [1,7-9], occurring in various freshwaters ecosystems throughout tropical and subtropical regions in South America [1].

Hypostomus possesses apomorphic morphological and cytogenetic characters among loricariids [10], such as broad diploid number (2n) ranging (64 to 84 chromosomes) and, usually, multiple sites of rDNAs [11-14], while some species have chromosomal characteristics considered to be plesiomorphic in the family, like sinteny in ribosomal genes families [10]. In *Hypostomus*, karyotype formulae, heterochromatin distribution, and number and localization of rDNAs sites are considered important evolutionary chromosome markers [10,15-18]. Chromosome variability at the intra- and interspecific level are detected in comparative cytogenetic studies [16-18]. In addition, recent cytogenetic data showed that evolutionary breakpoint regions clustered in repetitive DNA regions promoted genomic reshuffle and chromosome evolution in Loricariidae species [19-25].

Iguaçu River is an important tributary of Paraná River basin in the south region of Brazil and presents high endemism of its ichthyofauna due to the isolation caused by the formation of the Iguaçu waterfalls 22 Mya ago [26]. Five species of *Hypostomus* are described in the Iguaçu River: *Hypostomus albopunctatus* (Regan, 1908), *Hypostomus commersoni* Valenciennes, 1836, *Hypostomus derbyi* (Haseman, 1911), *Hypostomus myersi* (Gosline, 1947) and *Hypostomus nigropunctatus* Garavello, Britski & Zawadzki, 2012, being *H. derbyi* and *H. nigropunctatus* restricted to this basin [27]. The aim of the present study was to perform cytogenetical analysis in three *Hypostomus* species (*H. commersoni*, *H. derbyi*, and *H. myersi*) collected in in the middle Iguaçu River region (União da Vitória, Paraná State, Brazil – the type-localization of *H. derbyi* and *H. myersi* description), in order to establish chromosomal characterization among these sympatric and syntopic armored catfishes.

MATERIALS AND METHODS

Specimens of *H. commersoni* (4 females and 4 males), *H. derbyi* (3 females and 6 males), and *H. myersi* (8 females and 6 males) were collected in the Iguaçu River (Fig. 1), Paraná River Basin, Brazil (26°15'1.11" S and 51°6'10.67" W). The specimens were identified and desposited in the ichthyologic collection of the *Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura* (Nupélia; acronym = NUP) of *Universidade Estadual de Maringá*, Brazil (*H. commersoni* NUP 13581; *H. derbyi* NUP 13582; *H. myersi* NUP 13579). The procedures were performed according to the Ethics Committee in Animal Experimentation (Process CEUA 29/2016) of the *Universidade Estadual de Ponta Grossa*.

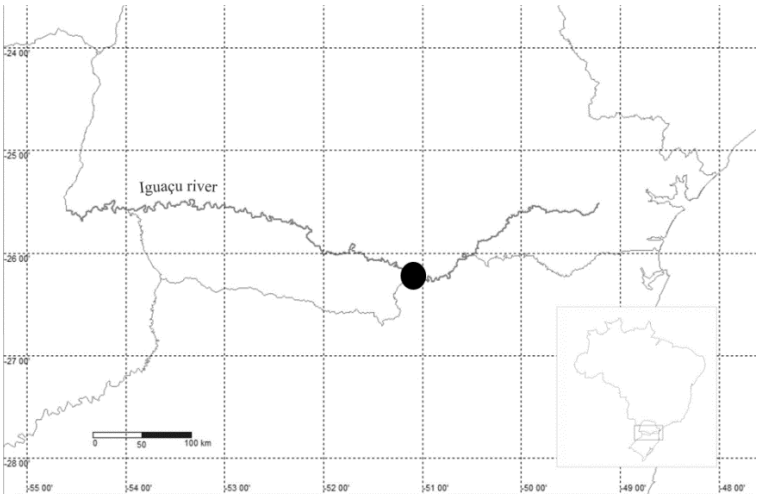

Figure. 1. Map of Brazil, highlighting the state of Paraná and the Iguaçu river drainage. The circle refers to sampled collection region at União da Vitória.

Chromosome preparations were obtained by the air-drying method [28], and then subjected to conventional Giemsa staining, C-banding for identification of heterochromatin regions [29-30] and silver nitrate impregnation ($AgNO_3$) for detection of nucleolar organizer regions (NORs) [31]. Genomic DNA was obtained from liver using cetyltrimethylammonium bromide (CTAB) method [32]. The DNA fraction containing moderately and highly repetitive DNAs was obtained by the C_ot-1 DNA reassociation kinetic technique [33-34], with modifications [35]. Fragments obtained by C_ot-1 ranged from 100 to 400 bp and were used as probe in cross-fluorescence *in situ* hybridization (cross-FISH) among the *Hypostomus* species. In addition, FISH experiments were performed using 18S [36] and 5S rDNAs [37] as probes, obtained from each of the three species studied. The probes were labeled by nick translation, using biotin-16-dUTP or digoxigenin-11-dUTP, following the manufacturer protocol (Roche Applied Science, Mannheim, Germany).

The general protocol for FISH procedure [38] was followed under stringency condition ~ 80 % (2.5 ng/μL probe, 50% formamide, 2x SSC – sodium saline citrate -, 10% dextran sulfate, for 18 h at 42 °C). Post-hybridization washes were performed in high stringency (50 % formamide at 42 °C for 20 min, 0.1x SSC at 60 °C for 15 min, and 4x SSC 0.05 % Tween at room temperature for 10 min). Signal detection was performed using antibodies Streptavidin Alexa Fluor 488 (Molecular Probes, Carlsbad, CA, USA) and anti-digoxigenin rhodamine (Roche Applied Science). Chromosomes were counterstained with 4´,6-diamidino-2-phenylindole (DAPI 0.2 μg/mL) in mounting medium Vectashield (Vector, Burlingame, CA, USA) and analyzed using an epifluorescence microscope. Chromosomes were classified according to Levan et al. [39], like metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) chromosomes. Fundamental number (FN) considered the m, sm and st chromosomes with two arms and acrocentric chromosomes with one arm.

RESULTS

Hypostomus commersoni

Hypostomus commersoni presented 2n = 68 chromosomes (12m+12sm+14st+30a), FN = 106 (Fig. 2A), and multiple Ag-NORs localized at the terminal region of the short arm of chromosome pair 15 and in the long arm of chromosome pair 28, both presenting size heteromorphism between the homologous (Fig 2). C-banding revealed positive heterochromatin predominantly at centromeric regions of most chromosomes, terminal bands in the short arm of the pair 13 and, one of the chromosome pairs 15 and 19, in the long arm of chromosome pairs 26 and 28, and one of the homologous of chromosome pair 4 (Fig. 2B). FISH mapping of 18S rDNA probe showed signals in the terminal regions of the short arm of chromosome pairs 13 and 15, and in terminal region of short arm in one chromosome of the pair 14, and the long arm of the pair 28. In addition, 5S rDNA sites were localized in the interstitial region of the short arm of chromosome pairs 5 and 19 and at the terminal region of the short arm of pair 9, 13 and 15 (Fig. 3A). Therefore, there was a synteny (18S/5S rDNA) at the terminal region in the short arm of chromosome pairs 13 and 15. Metaphases of *H. commersoni* subjected to FISH with *H. commersoni* total C_ot-1 DNA probe showed low repetitive DNA accumulation at the terminal and interstitial regions of some chromosomes (Fig. 4A). *Hypostomus commersoni* metaphases hybridized with *H. derbyi* total C_ot-1 DNA probe presented markers in many chromosomes (Fig. 4B). However, *H. commersoni* chromosomes did not show evident markers using *H. myersi* total C_ot-1 DNA as probe (Fig. 4C).

Hypostomus derbyi

All specimens of *H. derbyi* analyzed presented 2n = 68 chromosomes (12m+12sm+10st+34a), FN = 102 (Fig. 2C) and presented single NOR at the terminal region of the short arm of pair 14 (Fig. 2). C-banding pattern showed the heterochromatin at the pericentromeric region of the short arm of the chromosome pair 2 and, at the terminal region of the short arm of pair 14 (Fig. 2D). 18S rDNA sites were localized in the chromosome pair 14, while the 5S rDNA sites were presented at the pericentromeric region of the short arm of the metacentric pair 2 (Fig. 3B). *Hypostomus derbyi* metaphases were subjected to hybridization with total C_ot-1 probes of *H. commersoni* and *H. derby* total C_ot-1 DNA probe revealing repetitive DNAs at the terminal and interstitial regions of some chromosomes in common (Fig. 4D and E, respectively). In addition, *H. derbyi* metaphases hybridized with *H. myersi* total C_ot-1 probe presented signals predominantly on three chromosome pairs (Fig. 4F).

Hypostomus myersi

Specimens of *H. myersi* showed 2n = 74 chromosomes (12m+16sm+12st+34a) and FN = 114 (Fig. 2E). A single NOR site was detected at the terminal region of the long arm in chromosome pair 32 (acrocentric) and, frequently this site showed a size polymorphism between the homologous regions (Fig. 2). The heterochromatin was localized at centromeric regions of most chromosome pairs. We also detected heterochromatin at terminal regions of the long arm of the pairs 14, 20, 29, 30, 31, 32, 34, 36, and 37. In addition, the short arm of chromosome pair 7 and, and the interstitial region in the long

arm of pairs 20, 21, and 23 had heterochromatic regions. A size heteromorphism was observed in heterochromatin block of the chromosome pair 20 in both, males and females. It was visualized an evident overlapping signal between heterochromatic sites and NORs on chromosome pair 32, that show a size heteromorphism (Fig. 2F). 18S rDNA site was localized in chromosome pair 32, while the 5S rDNA site was visualized in the m pair 2 (Fig. 3C). Metaphases of *H. myersi* were hybridized with *H. commersoni* total C_ot-1 probe and not showed evident signals in the chromosomes (Fig. 4G). In addition, when metaphases of *H. myersi* were subjected to *H. derbyi* and *H. myersi* total C_ot-1 probes, it was observed repetitive DNA accumulations at terminal and interstitial regions of many chromosomes (Fig. 4H and I, respectively).

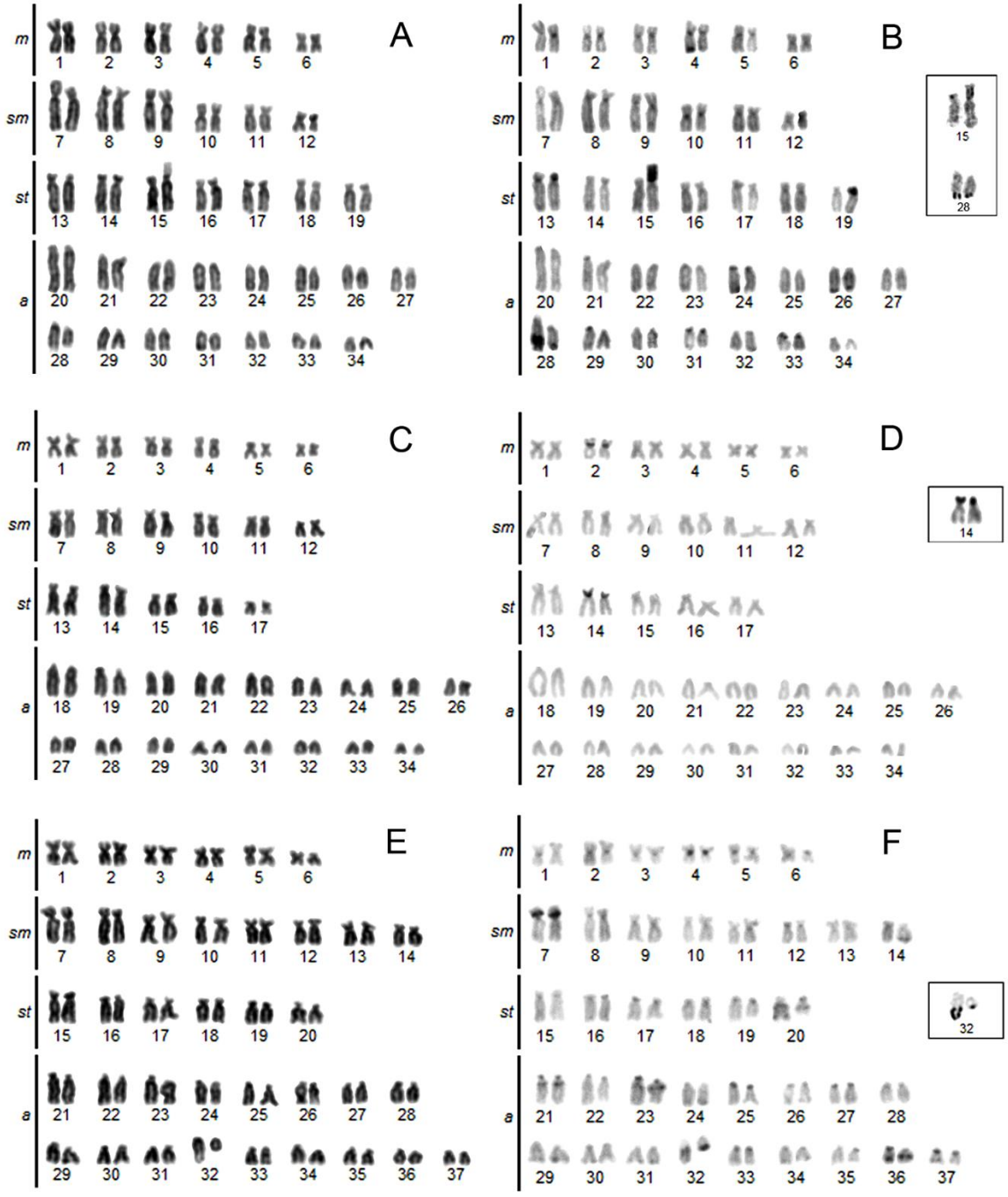

Figure. 2. Karyotypes of *H. commersoni* (**A**, **B**), *H. derbyi* (**C**, **D**) and *H. myersi* (**E**, **F**). submitted to Giemsa-stained (**A**, **C**, **E**) and C-banded chromosomes (**B**, **D**, **F**). Chromosome pairs carrying Ag-NORs are boxed. Bar = 10 µm.

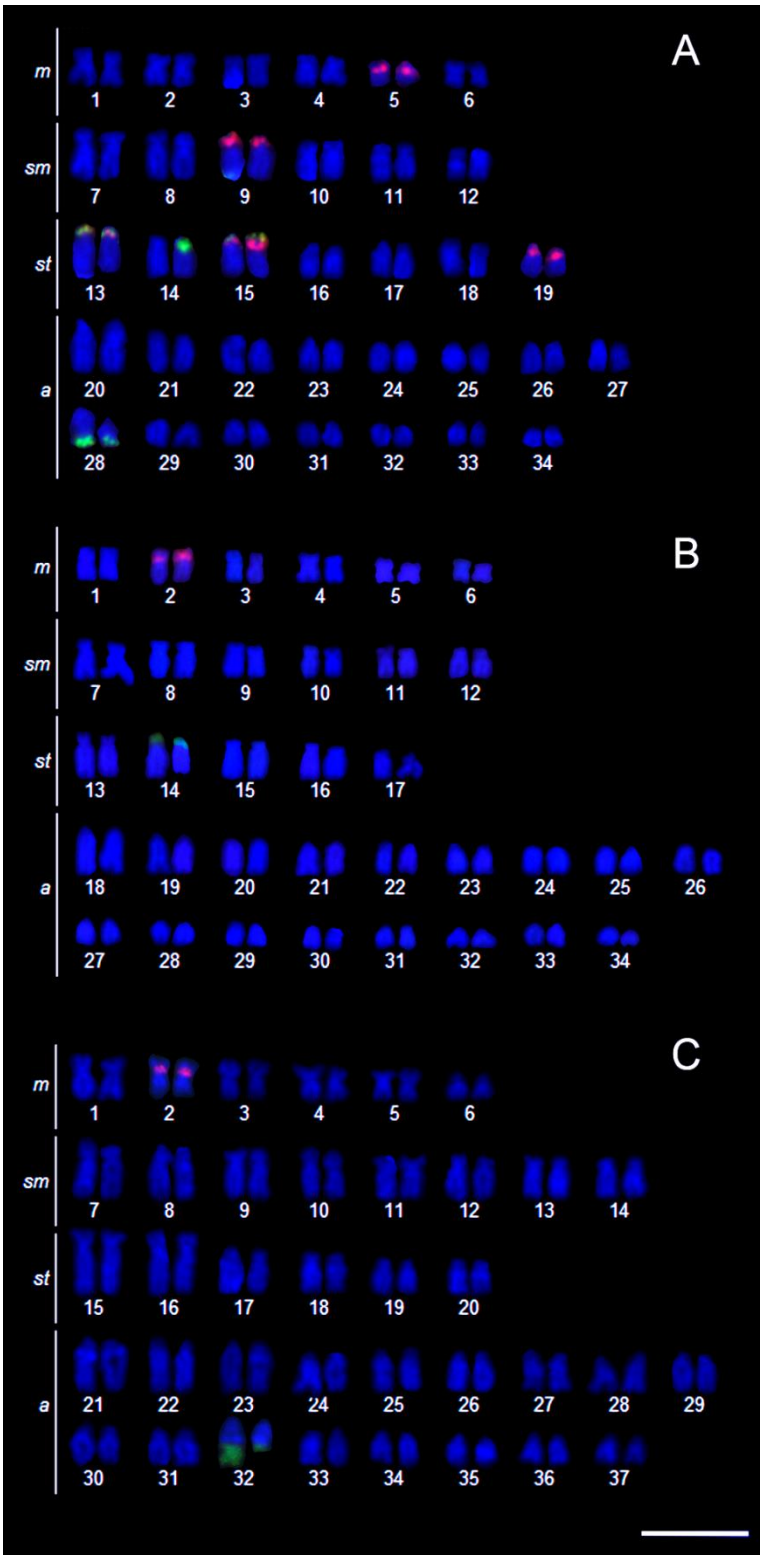

Figure. 3. Double FISH karyotypes with 5S rDNA (red markers) and 18S rDNA (green markers) probes. **(A)** *Hypostomus commersoni*, **(B)** *H. derbyi* and, **(C)** *H. myersi*. Bar = 10 µm.

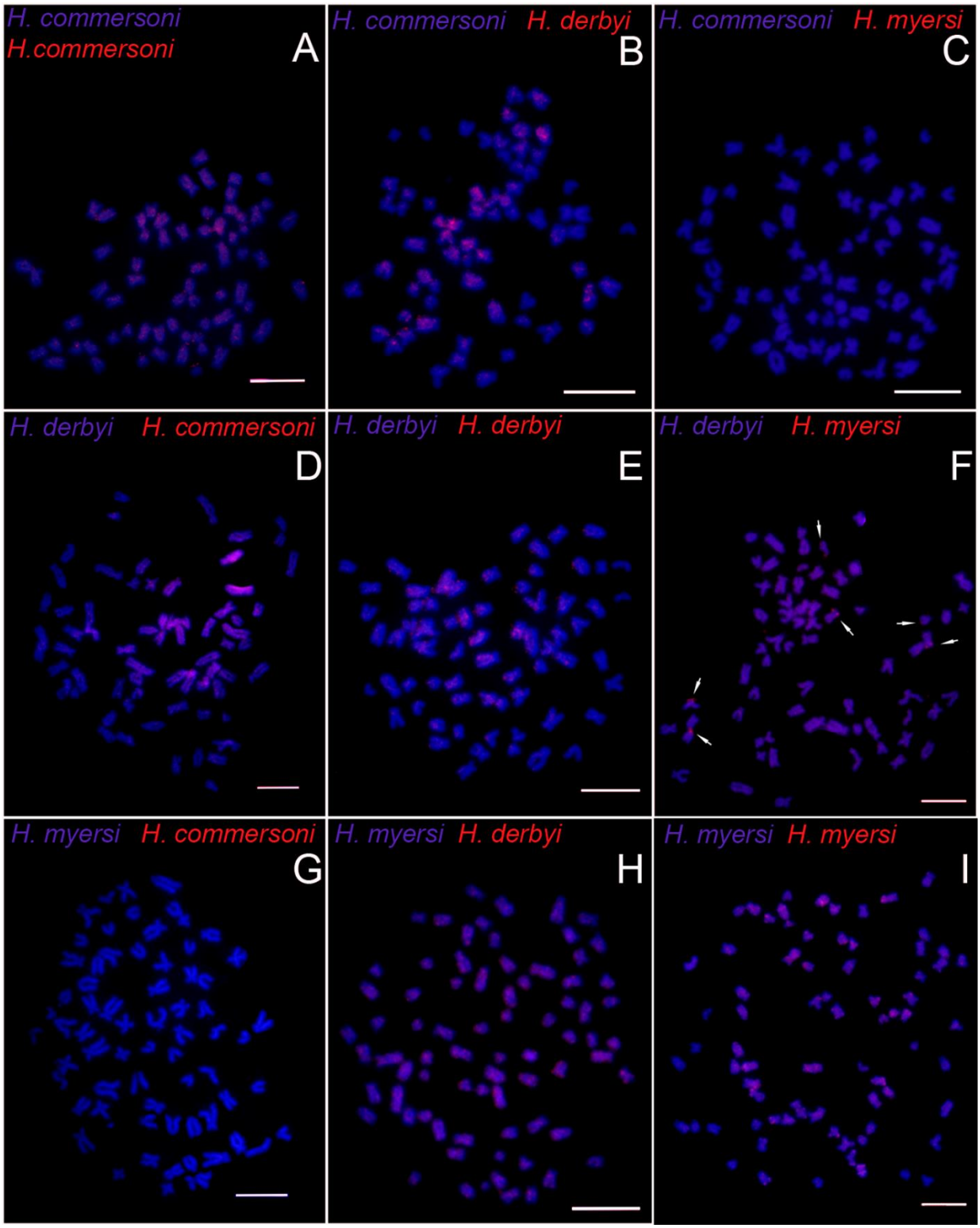

Figure. 4. Metaphases of *H. commersoni* **(A-C)**, *H. derbyi* **(D-F)** and *H. myersi* **(G-I)** after cross-FISH, using *H. commersoni* total C_ot-1 DNA probe **(A, D, G)**, *H. derbyi* total C_ot-1 DNA probe **(B, E, H)** and, *H. myersi* total C_ot-1

DNA probe **(C, F, I)**. Red markers correspond to similar repeat DNA chromosomal regions among the species. Bar = 10 µm.

DISCUSSION

Several cytogenetic studies in *Hypostomus* revealed a great complexity and diversity of information [10-18, 40-46], showing extensive karyotype variation in this group. Cytogenetic analysis evidenced 2n = 54 chromosomes as an ancestral characteristic in Loricariidae, since it was reported in the basal genera and in the sister group Trichomycteridae [47]. Karyotype analyses in *Hypostomus* have allowed well-defined evolutionary trends, as the occurrence of high variation in the 2n, due to chromosomal rearrangements, like centric fissions [12-20,40-46], which could explain the increased 2n present in the *H. commersoni* (2n = 68 chromosomes), *H. derbyi* (2n = 68 chromosomes) and *H. myersi* (2n = 74 chromosomes).

Chromosome variability in *Hypostomus* may be assessed by intraspecific variation of 2n, karyotype formula, heterochromatin localization, and number and localization of NORs sites [15,17,44]. *Hypostomus plecostomus* (Linnaeus, 1758) was described with 2n = 54 chromosomes [48]. However, it was considered a misidentification case, once the recent cytogenetic studies in this species has demonstrated 2n = 68 chromosomes [49]. Thus, in *Hypostomus* species the 2n is ranging from 64 chromosomes in *Hypostomus* sp. Xingu-1, *Hypostomus faveolus* Zawadzki, Birindelli & Lima, 2008 and *Hypostomus cochliodon* Kner, 1854 to 84 chromosomes in *Hypostomus perdido* Zawadzki, Tencatt & Froehlich, 2014, cited as *Hypostomus* sp. 2 Perdido River [11-13, 50]. The chromosomal data in this study showed that 2n = 68 and different karyotype formulae between *H. commersoni* and *H. derbyi* could be explained by pericentric inversions. Cytogenetic analysis in *H. derbyi* from the Iguaçu river in Curitiba, Paraná state, showed a different karyotype formula [51] and can suggest chromosomal diversification and cryptic species for *H. derbyi*. In *Hypostomus*, the number of st/a chromosomes was postulated to be directly proportional to an increase in 2n [13]. Although *H. myersi* presented small numbers of st/a chromosomes and the highest 2n among the *Hypostomus* species analyzed in this work, the pericentric inversions occurred in the chromosomal diversification of the genus, as proposed by Bueno et al.[13].

In situ localization of ribosomal sites are considered important cytotaxonomic and evolutionary markers for understanding the karyotype differentiation in fishes [10, 22-25]. *Hypostomus commersoni*, *H. derbyi* and *H. myersi* differed in localization and number of chromosome pairs bearing 18S rDNA sites. In *H. derbyi* and *H. myersi*, the presence of a single NOR indicated the maintenance of the plesiomorphic condition for Loricariidae [17,47,52-53], while *H. commersoni*, with multiple NORs, showed a apomorphic condition [10,12,15-16,18,40,45]. A heterochromatin/rDNA association was found to be polymorphic in size between the homologous of the pairs 15 and 28 of *H. commersoni* and pair 32 of *H. myersi*, probable due an unequal crossing over and/or amplification of this region, like observed in other *Hypostomus* [12,15,18,41,44] and Loricariidae species [47,52-53].

The heterochromatin/rDNA association visualized in the analyzed species is a common feature in this genus. According to Vicari et al. [54], the association between NORs and heterochromatin permit the dispersion of the rDNA sites throughout the genome, as visualized for *H. commersoni*, *H. derby* and *H. myersi*, which showed heterochromatin/rDNA associated chromosomal sites. In addition, *H. commersoni* and *H. myersi* presented additional heterochromatic blocks at centromeric, pericentromeric, and terminal regions, when compared to *H. derby*. *Hypostomus myersi* showed heterochromatic interstitial blocks in the long arms of some st and acrocentric

chromosomes. The size heteromorphism of the heterochromatin block in pair 20 of the *H. myersi* is due to in cis accumulation of repeat sequences [46]. Interstitial bands have also been observed in *Hypostomus regani* (Ihering, 1905) [18,41], *Hypostomus topavae* (Godoy, 1969) [13], *Hypostomus unae* (Steindachner, 1878) [43] and *Hypostomus wuchereri* (Günther, 1864) [42]. The pattern of heterochromatin bands at interstitial positions, which are equidistant in relation to the centromere and equilocal in non-homolog chromosomes of the same diploid group, may occur due to chromosome organization in nucleic interphase that would facilitate the transposition these segments[55-56].

A syntenic chromosomal region of 18S and 5S rDNAs presenting a size heteromorphism between the homologous chromosomes was observed in *H. commersoni*. This size heteromorphism of 5S and 18S rDNA clusters may be resulted from unequal crossing-over, from the association of repetitive *cis*-sequences caused by mismatches of repeated DNA units [57] or, being mediated by the movement of transposable elements (TEs) associated with rDNA [58]. These mechanisms traditionally promote an increase or decrease of the heterochromatin block [57]. Syntenic 5S and 18S rDNAs observed on chromosome pairs 13 and 15 of *H. commersoni* may be considered a vestige of the ancestral condition of Loricariidae [47] or a recurrent feature in these species due to an intense movement of rDNA sites, especially when associated with TEs [16,58]. In addition to the syntenic rDNA sites, *H. commersoni* presented multiple sites of 18S and 5S rDNAs, considered to be derived in *Hypostomus*, with origin by dispersion of theses sequences throughout the genome, which was confirmed by Traldi et al.[10]

The repetitive fraction obtained by the C_ot-1 reassociation kinetics has been considered an excellent methodology to recover repetitive DNA units, especially TEs, minisatellites, and microsatellites [60-61]. Except the satellite DNA, which is a highly in tandem repetitive sequence and the main heterochromatin marker [62-63], the TEs, microsatellites, and minisatellites are disperse throughout the genomes, accumulating at heterochromatic regions [61,64-65]. The cross-FISH with DNA C_ot-1 probes of *H. commersoni*, *H. derbyi*, and *H. myersi* showed different distribution of repetitive DNAs among the species analyzed. We observed that *H. derbyi* shares sequences of repetitive DNAs with *H. commersoni* and *H. myersi*, whereas *H. commersoni* and *H. myersi* not possesses shared repeat DNA sequences. These data suggest a higher evolutionary dynamic for the repetitive fraction of the genome of the species *H. commersoni* and *H. myersi*, according to the evolutionary model combined with repetitive units of the genome [66]. Following this model, mutated copies of repetitive DNAs could spread throughout the genome by mechanisms as unequal cross-over [59] or hitchhiking with TEs, leading to sequence homogenization [67] in the same genome. However, the effect of these mechanisms, when we compare different genomes, would be the differentiation of repetitive DNA copies, as verified for these species. Chromosome differentiation caused by the movement of repetitive sequences seems to be very important for speciation [58,68-70]. Symonová et al.[58] provided indirect evidence in which rDNA uses the spreading mechanism of retrotransposons, subsequently affecting recombination rates in genomes, leading to rapid genome divergence in close relationship species. Hence, these extensive genome rearrangements in *Hypostomus* could be associated with speciation event induced by retrotransposon genome spreading, leading to numeric and structural chromosome diversification.

Cytogenetic data obtained in the present study for the three sympatric and syntopic species of *Hypostomus* of the middle portion of the Iguaçu River revealed that although *H. commersoni* and *H. derbyi* presented the same diploid number, they diverged in relation to heterochromatic regions and 18S and 5S rDNA sites. *Hypostomus derbyi* and *H. myersi*, although presenting different diploid number and heterochromatin distribution, shared

repetitive DNAs and 18S and 5S rDNA single sites. On the other hand, *H. commersoni* and *H. myersi* were more divergent in all cytogenetic characteristics analyzed.

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