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Chromosomal diversity in *Hypostomus* (Siluriformes, Loricariidae) with emphasis on physical mapping of 18S and 5S rDNA sites

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ABSTRACT. We examined chromosomes of three species of the genus *Hypostomus*, in order to contribute to the understanding of the karyotype evolution of this group. Specimens of *H. ancistroides* and *H. nigromaculatus* displayed differences in karyotype formulas, distribution and location of heterochromatin and nucleolus organizer regions when compared to other populations of the same species. We made the first cytogenetic characterization of *H. tapijara*, an endemic species in the Ribeira de Iguape River. These specimens had 2n = 66 chromosomes, while *H. ancistroides* showed 2n = 68 and *H. nigromaculatus* 2n = 76 chromosomes. Physical mapping of 18S and 5S rDNA sites of the three species showed simple, multiple and syntenic clusters. Synteny of ribosomal sites was found in *H. ancistroides* and *H. tapijara*, and an interspersed pattern between these sites in all chromosomes bearing the synteny was observed. We conclude that the genus *Hypostomus* has a high chromosome complexity that is accompanied by great

morphological variation. It is evident that this group comprises an interesting model for understanding the chromosome evolution of Neotropical ichthyofauna.

Key words: Fish; Hypostominae; Fiber-FISH; Chromosome evolution

INTRODUCTION

In eukaryotes, the ribosomal genes comprise two distinct multigene classes that are composed of tandemly arrayed repeated sequences. The major rDNA (45S) corresponds to the nucleolus organizer region (NOR) and includes the genes encoding 18S, 5.8S and 28S rRNAs, and an intergenic non-transcribed spacer; the minor rDNA is comprised of a gene class encoding 5S rRNA molecules (Long and David, 1980). Due to the apparent molecular conservation of ribosomal sites among closely related groups of organisms, such sequences can be useful tools for cytotaxonomic and evolutionary studies.

In fish, the active 45S rDNA sites in interphase were initially identified by silver-staining (AgNOR). Since then, fluorescence *in situ* hybridization (FISH) has allowed for the localization of all major and minor rDNA sites. High-resolution variations of FISH have been used on many groups of organisms (Guerra, 2004). Among these techniques, hybridization on extended chromatin fiber (fiber-FISH), although not yet widely used in cytogenetic studies of fish (Barros et al., 2011), is a tool with great potential to contribute to various types of studies, as is observed for other classes of organisms (Muñoz-Pajares et al., 2011).

Among fish, Loricariidae is a highly diversified group with wide geographical distribution; it comprises the armored catfishes (Ferraris Jr., 2007). According to Reis et al. (2006), this group is subdivided into six subfamilies: Loricariinae, Hypoptopomatinae, Hypostominae, Neoplecostominae, Lithogeninae, and Delturinae. In Hypostominae, the diploid number (2n) varies from 34 chromosomes in *Ancistrus cuiabae* (Mariotto et al., 2011) to 84 chromosomes in *Hypostomus* sp 2 (Cereali et al., 2008). Most cytogenetic studies show great variability in some chromosome features; however, molecular analyses, such as physical mapping of ribosomal genes, have been done for only a small number of species (Mariotto et al., 2011; Mendes-Neto et al., 2011; Rubert et al., 2011; Ziemniczak et al., 2012; Traldi et al., 2012).

Considering the importance of ribosomal genes in karytoype evolutionary studies and the relatively few studies that involve physical mapping of these genes in Hypostominae, we analyzed the species *H. ancistroides*, *H. nigromaculatus* and *H. tapijara* by classical (conventional staining, C-banding and silver-staining) and molecular cytogenetic methods (FISH of mitotic chromosomes and extended chromatin fiber), in order to contribute to the understanding of the karyotype evolution of the genus *Hypostomus*, especially regarding the physical location of 18S and 5S ribosomal genes.

MATERIAL AND METHODS

We collected specimens of *H. ancistroides* (11 females and 10 males) and *H. nigromaculatus* (13 females and 13 males) from the Lapa Stream, Ipeúna city, São Paulo, Brazil, and specimens of *H. tapijara* (26 females and 14 males) from Ribeira de Iguape River, Registro city, São Paulo, Brazil. The specimens were deposited in the Museum of Zoology

at the University of São Paulo, under voucher numbers MZUSP 110802, MZUSP 110801 and MZUSP 109785, respectively. Cell suspensions were obtained from the anterior portion of the kidney of these specimens, according to the procedures described by Foresti et al. (1993). The slides with extended chromatin fibers were obtained according to the protocols described by Fidlerova et al. (1994) and Muñoz-Pajares et al. (2011), adapted for fish (Barros et al., 2011).

The mitotic chromosomes were stained with a solution of 5% Giemsa and divided into four karyotype groups: metacentrics (m), submetacentrics (sm), subtelocentrics (st), and acrocentrics (a) (Levan et al., 1964). The heterochromatic distribution pattern was determined according to the protocol described by Sumner (1972), with modifications in the coloring step (Lui et al., 2012). The NORs were identified by AgNOR, described by Howell and Black (1980).

The physical mapping of 18S and 5S rDNAs in mitotic chromosomes and extended chromatin fiber was performed according to Pinkel et al. (1986), with probes obtained from *Prochilodus argenteus* (Hatanaka and Galetti, 2004) and *Leporinus elongatus* (Martins and Galetti Jr., 1999), respectively. The 18S rDNA probe was labeled with biotin-16-dUTP and the 5S rDNA probe with digoxigenin-11-dUTP, by nick translation, according to manufacturer instructions (Roche Applied Science). The hybridization procedure was performed under high-stringency conditions - 77% (200 ng of each probe, 50% deionized formamide, 10% dextran sulfate, 2X SSC, pH 7.0-7.2, at 37°C overnight). After hybridization, the slides were washed in 15% formamide/0.2X SSC 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. The last step was performed in two 5-min washes. The signal detection was performed using avidin-FITC (Sigma) against the 18S rDNA and anti-digoxigenin-rhodamine (Roche Applied Science) for the 5S rDNA probes. The chromosomes were counter-stained with a solution of antifading/DAPI (40 µL antifading + 1 µL DAPI - 0.2 mg/mL) and analyzed under an Olympus BX51 epifluorescence microscope with an image capture system (Olympus DP72).

RESULTS

H. ancistroides showed 2n = 68 chromosomes (14m + 16sm + 22st + 16a), fundamental number (FN) = 120 (Figure 1A). AgNOR revealed multiple NORs, in the terminal regions of the short arms of chromosome pairs 8, 13 and 15 (Figure 1B, box). C-banding revealed small amounts of heterochromatin, distributed over terminal and centromeric regions of several chromosomes (Figure 1B). In chromosome pair 15, there was overlapping between heterochromatic sites and the NOR (Figure 1B). *H. nigromaculatus* showed 2n = 76 chromosomes (12m + 22sm + 30st + 12a), with FN = 140 (Figure 1C). AgNOR revealed a single NOR, in the terminal position of the long arm of acrocentric pair 33 (Figure 1D, box). C-banding revealed that the heterochromatin was located in terminal and centromeric portions of a few chromosome pairs (Figure 1D); there was overlapping between heterochromatic sites and NORs (Figure 1D). *H. tapijara* showed 2n = 66 chromosomes (14m + 24sm + 14st + 14a), FN = 118 (Figure 1E). AgNOR revealed multiple NORs, allocated in the terminal portion of the short arm of the metacentric pair 4 and in the long arm of acrocentric pair 29 (Figure 1F, box). In C-banding the most prominent heterochromatic blocks overlapped with AgNORs (Figure 1F).

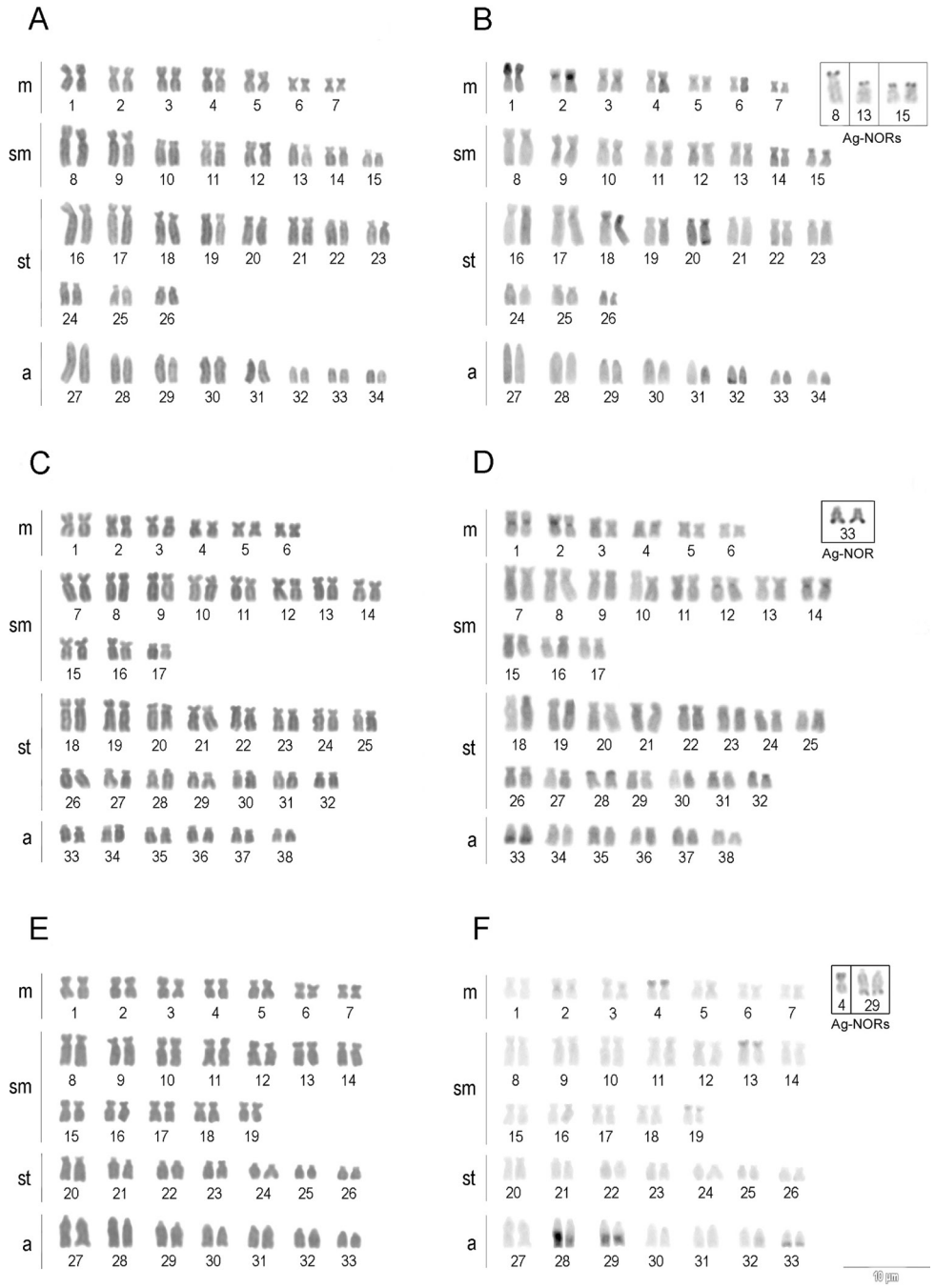

Figure 1. Karyotypes of *Hypostomus ancistroides* (**A**, **B**), *H. nigromaculatus* (**C**, **D**) and *H. tapijara* (**E**, **F**) with conventional Giemsa-staining (**A**, **C**, **E**) and C-banding (**B**, **D**, **F**). m = metacentrics; sm = submetacentrics; st = subtelocentrics; a = acrocentrics.

In *H. ancistroides*, the FISH with 18S rDNA probe confirmed the results obtained by silver staining; however, only one chromosome of pair 15 showed a signal (Figure 2A). There were three chromosome pairs bearing 5S rDNA sites: pair 2, with an interstitial marking on the short arm, and pairs 8 and 13, which presented a syntenic condition with the 18S rDNA site (Figure 2A). In *H. nigromaculatus*, FISH with 18S rDNA probe confirmed the result obtained by silver staining, showing only acrocentric pair 33 bearing this cistron (Figure 2B). There was one chromosome pair bearing 5S rDNA sites: pair 2, with an interstitial marking on the short arm (Figure 2B). In *H. tapijara*, the FISH with 18S rDNA revealed a larger number of sites than was observed with AgNOR, showing sites in the terminal portion of the short arm of chromosome pairs 4 and 10, and in the terminal portion of the long arm of pair 29 (Figure 2C). There were four chromosome pairs bearing 5S rDNA sites: pair 2, with an interstitial marking on the short arm, pair 31 with an interstitial marking on the long arm, and pairs 4 and 10, which presented a syntenic condition with the 18S rDNA site (Figure 2C).

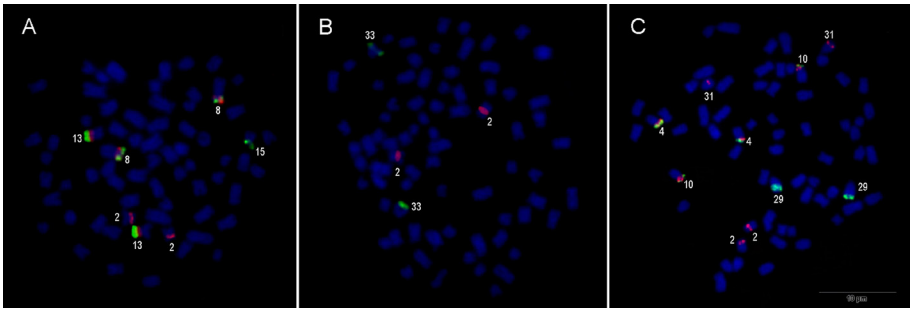

Figure 2. Metaphases of *Hypostomus ancistroides* (**A**), *H. nigromaculatus* (**B**) and *H. tapijara* (**C**) after double-FISH using 18S rDNA (green signal) and 5S rDNA (red signal) probes. The numbers show the chromosome pairs bearing ribosomal sites.

Physical mapping of the ribosomal genes on extended chromatin fiber in *H. ancistroides* showed fibers at one of these sites, which corresponds to chromosomes bearing one type of rDNA and fibers with both rDNA sites, co-localized (pairs 8 and 13) (Figures 3A-F). Physical mapping of the ribosomal genes on extended chromatin fiber in *H. tapijara* showed fibers with one of these sites, which correspond to chromosomes bearing one type of rDNA and fibers with both rDNA sites co-localized (pairs 4 and 10) (Figures 3G-L).

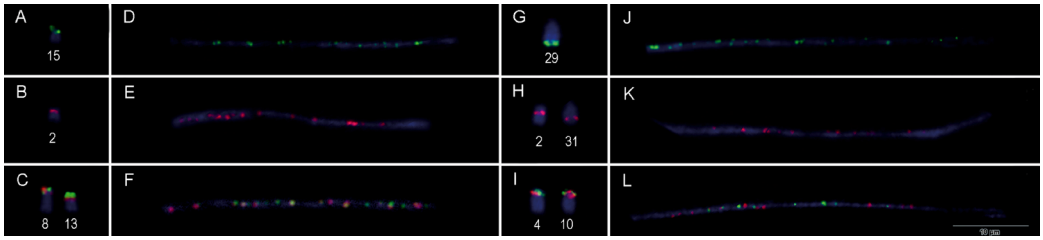

Figure 3. Mitotic chromosomes (**A**, **B**, **C**, **G**, **H**, **I**) and extend chromatin fibers (**D**, **E**, **F**, **J**, **K**, **L**) after double-FISH using 18S rDNA (green signal) and 5S rDNA (red signal) probes of *Hypostomus ancistroides* (**A**, **B**, **C**, **D**, **F**) and *H. tapijara* (**G**, **H**, **I**, **J**, **K**, **L**). The numbers show the chromosome pairs bearing ribosomal sites.

DISCUSSION

Hypostomus is the most prevalent genus of armored catfishes in Brazilian rivers. It is widespread all over South America and occurs in a great variety of freshwater ecosystems (Oyakawa et al., 2005). Among Hypostominae, *Hypostomus* is one of the most widely studied genera by cytogenetic methods; however, the high diversity in this group remains unexplored (Rubert et al., 2011). The characterization we made of *H. tapijara* (endemic species of Ribeira de Iguape basin) comprises the first chromosomal data on this species; a large number of species of *Hypostomus* have not had chromosomal analysis yet.

This genus is known for its wide degree of chromosome diversification (Artoni and Bertollo, 2001; Mendes-Neto et al., 2011; Bueno et al., 2012), with 2n ranging from 54 chromosomes in *H. plecostomus* (Muramoto et al., 1968) to 84 chromosomes in *Hypostomus* sp 2 (Cereali et al., 2008). Nevertheless, *H. plecostomus* could be a case of misidentification and could belong to another Loricariidae genus. In this case, *H. cochliodon* (Armbruster, 2003) with 2n = 62 chromosomes (Cereali et al., 2004) would have the lowest 2n value in *Hypostomus*. Artoni and Bertollo (2001) considered 2n = 54 chromosomes as a putative primitive condition in Loricariidae, since it is reported in the basal genera and in the sister group Trichomycteridae. Thus, rearrangements like centric fissions could have led to the increased diploid number observed in several species of the group. In fact, all so-far-studied species of *Hypostomus* show a 2n higher than 54 chromosomes (Bueno et al., 2012), as in the three species that we studied here (*H. ancistroides* with 2n = 68, *H. nigromaculatus* with 2n = 76, and *H. tapijara* with 2n = 66).

Karyotype variations with conservation of the 2n over distinct populations of the same species are common in *Hypostomus* (Artoni and Bertollo, 1996; Alves et al., 2006; Mendes-Neto et al., 2011; Rubert et al., 2011). We found this situation in *H. ancistroides* and *H. nigromaculatus*, species that are widespread in Brazilian basins. All populations of *H. ancistroides* and *H. nigromaculatus* that have been studied showed 2n = 68 chromosomes (Artoni and Bertollo, 1996; Alves et al., 2006; Rubert et al., 2011) and 76 chromosomes (Rubert et al., 2008), respectively, with differences in karyotype formulae, heterochromatic pattern and NOR location. According to Bueno et al. (2012), in addition to the centric fissions, pericentric and paracentric inversions also have an important role in karyotype diversification of *Hypostomus*, which could explain these population differences. On the other hand, Rosa et al. (2011) indicated that gametic combinations in chromosome number of polymorphic populations of Loricariidae have also led to fundamental number variation.

The ribosomal sites are important cytotaxonomic and evolutionary markers for understanding chromosome diversity in fish. According to Alves et al. (2006), multiple NORs in terminal position are common in species of *Hypostomus*, but the simple NOR condition is also observed in some species of this genus. The variability of this feature in this group is obvious, contributing to its chromosome diversity. In the genus *Hypostomus*, variations in the heterochromatic pattern among different species are commonly observed, as we found here. Heterochromatic segments that overlap with or are adjacent to ribosomal sites are frequent in Neotropical fish, as we observed with *H. ancistroides*, *H. nigromaculatus* and *H. tapijara*, a fact that, probably, enabled the dispersion of NOR sites over the genome (Moreira-Filho et al., 1984; Vicari et al., 2008).

FISH confirmed the variation of the 18S rDNA sites noticed in species of *Hypostomus* and made possible the identification of the numerical and positional diversity of rDNA 5S sites (Kavalco et al., 2004; Mendes-Neto et al., 2011; Rubert et al., 2011; Traldi et al., 2012).

Apparently, the interstitial site of 5S rDNA in a metacentric pair is constant in species of *Hypostomus*, as found in *H. regani* (Mendes-Neto et al., 2011), *H. iheringii* (Traldi et al., 2012), *H. ancistroides*, *H. nigromaculatus*, and *H. tapijara* (this paper). Thus, it is possible that this site represents a primitive condition within this genus. However, variations in the number and location of 5S rDNA sites are observed among species of *Hypostomus*; they could be a result dispersion of this sequence mediated by transposable elements (Da Silva et al., 2011).

In *Hypostomus*, absence of rDNA sites detected by FISH in both homolog chromosomes is recurrent. This condition, that we found for the 18S rDNA site of chromosome pair 15 in *H. ancistroides*, was previously observed in *H. regani* (Mendes-Neto et al., 2011) and *H. iheringii* (Traldi et al., 2012). Possibly, it is due to the limited sensitivity of the technique for detecting very small sites (Schwarzacher and Heslop-Harrison, 2000).

In fish, arrangement of ribosomal sites in distinct chromosomes is the situation that is most often found (Lui et al., 2009). According to Martins and Galetti Jr. (1999), this condition could provide advantages compared to the syntenic arrangement. Gene conversion and unequal crossing mechanisms are likely to occur in 18S and 5S rDNA sites with syntenic arrangements; thus, the location of these genes on different chromosomes avoids possible unfavorable rearrangements (Dover, 1986). However, several groups of Neotropical fish carry the major and minor ribosomal genes in the same chromosome in an adjacent, distant or co-located position (Hatanaka and Galetti Jr., 2004; Moraes-Neto et al., 2011). The synteny between ribosomal genes in metacentric/submetacentric pairs observed in *H. ancistroides* and *H. tapijara* is the first report of this condition in the genus. The analysis of FISH on extended chromatin fibers of these species revealed that these genes are interspersed in the chromosomes bearing synteny. In eukaryotes, the transcription of 18S rDNA is performed by RNA polymerase I, whereas the 5S rDNA sequence is transcribed by RNA polymerase III (Snustad and Simmons, 2006). Given the transcriptional activity of 18S rDNA sites confirmed by AgNOR, and the possible physical restriction between the performances of the two transcription complexes in the same chromosomal segment, the sites of 5S rDNA co-located with the 18S rDNA could be pseudogenes. However, in pair 10 of *H. tapijara*, no sites had AgNOR and thus it is not possible to define the functional condition of ribosomal genes in this chromosome pair. On the other hand, it is possible that these genes are transcribed from different DNA strands; thus, both could be active, as was observed for other contiguous genes in crustaceans (Barzotti et al., 2000).

In Loricariidae, the most widely species analyzed display distinct chromosomes with ribosomal sites (Mariotto et al., 2011; Mendes-Neto et al., 2011; Rosa et al., 2011; Ziemniczak et al., 2012). A syntenic condition of these genes was observed in *H. ancistroides* and *H. tapijara* (this paper), and in species of the subfamilies Neoplecostominae and Hypoptopomatinae (Ziemniczak et al., 2012), Hypostominae - tribe Ancistrini (Mariotto et al., 2011) and Loricariiane (Kavalco et al., 2004). This arrangement is considered as a probable primitive condition of this family because this condition is found in the outgroup Trichomycteridae (Ziemniczak et al., 2012). The synteny of *H. ancistroides* and *H. tapijara* could be a vestige of this primitive condition since they showed a low 2n number, whereas in *H. nigromaculatus* chromosome rearrangements could have promoted a derived condition.

Among the Loricariidae, *Hypostomus* is considered a derived genus. Chromosome studies have identified a great karyotype variation in the group, including derived features, such as high diploid numbers and multiple NORs, as well as primitive characteristics, like synteny between ribosomal genes. Thus, it is evident that there is chromosome complexity in this genus,

which is accompanied by considerable morphological variation, showing that this genus could be an interesting model for studies of chromosome evolution in Neotropical ichthyofauna.

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