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Research Article

Chromosome mapping of repetitive sequences in four Serrasalmidae species (Characiformes)

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Abstract

The Serrasalmidae family is composed of a number of commercially interesting species, mainly in the Amazon region where most of these fishes occur. In the present study, we investigated the genomic organization of the 18S and 5S rDNA and telomeric sequences in mitotic chromosomes of four species from the basal clade of the Serrasalmidae family: *Colossoma macropomum*, *Mylossoma aureum*, *M. duriventre*, and *Piaractus mesopotamicus*, in order to understand the chromosomal evolution in the family. All the species studied had diploid numbers 2n = 54 and exclusively biarmed chromosomes, but variations of the karyotypic formulas were observed. C-banding resulted in similar patterns among the analyzed species, with heterochromatic blocks mainly present in centromeric regions. The 18S rDNA mapping of *C. macropomum* and *P. mesopotamicus* revealed multiple sites of this gene; 5S rDNA sites were detected in two chromosome pairs in all species, although not all of them were homeologs. Hybridization with a telomeric probe revealed signals in the terminal portions of chromosomes in all the species and an interstitial signal was observed in one pair of *C. macropomum*.

Key words: 5S rDNA, telomeric sequences, tambaqui and pacu.

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Introduction

The family Serrasalmidae comprises approximately 80 species distributed among 15 genera, with a number of commercially important species of the genera *Colossoma*, *Mylossoma*, and *Piaractus*, which are valued for fishing or aquaculture in the Amazon region (Araújo-Lima and Goulding, 1998; Oliveira and Araújo-Lima, 1998; Jégu, 2003; Nelson, 2006). The species of this family are popularly known as "pacus", "piranhas" and "tambaqui" (*C. macropomum*), which have high, laterally compressed bodies with abdominal spines and long dorsal fins. Their distribution is exclusively Neotropical and they inhabit environments such as floodplains, seasonally flooded forests, white-waters, and rivers main channels throughout South America, mainly in the Amazon, Paraguay, and Orinoco river basins (Goulding, 1980; Jégu, 2003).

According to Calcagnotto *et al.* (2005), Serrasalmidae is strongly supported as a monophyletic family and, according to the phylogeny based on mitochondrial DNA proposed by Ortí *et al.* (2008), the species of this family can be divided into herbivorous clades represented by "pacus" and "*Myleus*" and a carnivorous clade composed of "piranhas". "Pacus" are considered basal and "piranhas" are thought to be the most derived clade. Supporting this classification, cytogenetic studies have demonstrated variations in the diploid numbers in the family, with 2n = 54 for the "pacus" clade (Nirchio *et al.*, 2003; Nakayama *et al.*, 2012), 2n = 58 for the *Myleus* clade (Porto JIR, 1999, PhD Thesis. INPA/UFAM, Manaus, AM) (García-Parra WJ, 2000, PhD Thesis. INPA/UFAM, Manaus, AM), and 2n = 58 to 64 for the most derived "piranhas" clade (Muramoto *et al.*, 1968; Nakayama *et al.*, 2001). Although the karyotypes of many species of this family have been described (Almeida-Toledo *et al.*, 1987; Cestari and Galetti Jr, 1992a,b; Nakayama *et al.*, 2000, 2001, 2002, 2008, 2012; Centofante *et al.*, 2002; Nirchio *et al.*, 2003; Gaviria *et al.*, 2005) (García-Parra WJ, 2000, PhD Thesis. INPA/UFAM, Manaus, AM), analyses using molecular cytogenetic techniques, mainly in the most basal genera (*Colossoma*, *Mylossoma* and *Piaractus*), are still scarce.

Fluorescence *in situ* hybridization (FISH) allows mapping specific DNA sequences on the chromosomes, with repetitive sequences, such as telomeric, 18S and 5S rDNA, being the most commonly studied (Martins, 2007). Mapping of these sequences has been useful in studying

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questions related to karyotypic evolution, sexual and supernumerary chromosomes origin, and genomic organization in many fish species (Voltolin *et al.*, 2010; Gross *et al.*, 2010; Schneider *et al.*, 2012; Terencio *et al.*, 2012). Within this context, the present work aimed to investigate the genomic organization of 18S rDNA, 5S rDNA and telomeric sequences in species of the basal clade of Serrasalmidae in order to understand the family chromosomal evolution.

Material and Methods

Four species belonging to the Serrasalmidae family were analyzed: *Colossoma macropomum*, *Mylossoma aureum*, *M. duriventre*, and *Piaractus mesopotamicus*. Thirty-eight specimens collected in the central Amazon region or from pisciculture farms (Table 1) were anesthetized with eugenol (a 5 mL stock solution diluted into 12 L of water) and sacrificed to obtain chromosome preparations. Mitotic chromosomes were obtained from kidney cells (Bertollo *et al.*, 1978). Constitutive heterochromatin detection was achieved using the C-banding technique (Sumner, 1972), and the nucleolus organizing regions (NORs) were evidenced by silver nitrate impregnation (Howell and Black, 1980).

Total DNA extraction was performed using muscle tissue samples of *C. macropomum*, *M. aureum* and *M. duriventre* following Sambrook *et al.* (1989), and quantification was performed in agarose gels by comparison with a standard lambda marker. The 18S and 5S rDNA genes were amplified by polymerase chain reaction (PCR), employing the oligonucleotide primers 18Sf (5'-CCG CTT TGG TGA CTC TTG AT-3') and 18Sr (5'-CCG AGGACC TCA CTA AAC CA-3') (Gross *et al.*, 2010), 5Sa (5'-TAC GCC CGA TCT CGT CCG ATC-3'), and 5Sb (5'- CAGGCT GGT ATG GCC GTA AGC-3') (Martins and Galetti Jr., 1999). PCR reactions were performed in a final volume of 25 μL containing genomic DNA (200 ng), 10x buffer with 1.5 mM $MgCl_2$, Taq DNA polymerase (5 U/μL), dNTPs (1 mM), the primer pairs (5 mM), and deionized water. Conditions for the 18S rDNA amplification reaction were: 1 min at 95 °C, 35 cycles of 1 min at 94 °C, 1 min at 56 °C, and 90 s at 72 °C; with a final extension of 5 min at 72 °C. Conditions for the 5S rDNA amplification reaction were: 1 min at 95 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 59 °C and 90 s at 72 °C; with a final extension of 5 min at 72 °C. PCR reactions for the telomeric sequences (TTAGGG)n were performed in a final volume of 25 μL containing 10x buffer with 1.5 mM of $MgCl_2$, dNTPs (1 mM), 0.2 μL (TTAGGG)5 primer, 0.2 μL (CCCTAA)5 primer, and 2 U of Taq DNA polymerase (Ijdo *et al.*, 1991). The first part of the amplification process was conducted under low stringency (4 min at 94 °C; followed by 12 cycles of 1 min at 94 °C, 45 s at 52 °C and 90 s at 72 °C), followed by 35 cycles at high stringency conditions (1 min at 94 °C, 90 s at 60 °C and 90 s at 72 °C).

Telomeric sequences and 18S rDNA products were labeled with digoxigenin-11-dUTP (Dig Nick Translation mix; Roche), whereas the 5S rDNA products were labeled with biotin-14-dATP (Biotin Nick Translation mix; Roche), according to the manufacturer's instructions. Avidin-FITC (Sigma-Aldrich), biotinylated anti-avidin (Sigma-Aldrich), and anti-digoxigenin-rhodamine (Roche) were used to immunodetect the probes. Intra- and interspecific hybridizations were performed following the protocols described by Pinkel *et al.* (1986) for a 77% stringency (2.5 ng/μL of 18S rDNA, 5S rDNA, or telomeric probe, 50% formamide, 10% dextran sulfate, and 2x SSC at 37°C for 18 h). Chromosomes were counterstained with DAPI (2 mg/ml) in VectaShield (Vector) mounting medium.

After FISH, the metaphases were analyzed under an Olympus BX51 epifluorescence microscope. Images were captured using a coupled Olympus DP71 digital camera and the Image-Pro MC 6.3 software. Metaphases were processed with Adobe Photoshop CS3 and the chromosomes were measured with Image J and classified according to the nomenclature proposed by Levan *et al.* (1964).

Results

Karyotypic descriptions

The four species analyzed had diploid numbers of 2n = 54 chromosomes and fundamental numbers FN = 108 - although their karyotypic formulas differed. A subtelocentric pair was present in *M. duriventre*. None of the species had heteromorphic sex chromosomes. The karyotypic formulas were: 26m+28sm in *Colossoma*

Table 1 - Data of the Serrasalmidae specimens analyzed.

Species	Collection localities	Numbers of specimens
Colossoma macropomum	Lago Catalão (confluence of the Negro and Solimões rivers- 3º11'59" S and 59º53'59" W)	3 (2 females and 1 male)
	Pisciculture - Fazenda Santo Antônio (AM)	2 (undetermined sex)
Mylossoma aureum	Lago Catalão (confluence of the Negro and Solimões rivers - 3º11'59" S and 59º53'59" W)	4 (1 female and 3 males)
Mylossoma duriventre	Lago Catalão (confluence of the Negro and Solimões rivers - 3º11'59" S and 59º53'59" W)	13 (5 females and 8 males)
Piaractus mesopotamicus	Pisciculture - Centro de Educação Tecnológica em Aquacultura (Monte Aprazível, SP)	16 (undetermined sex)

macropomum (Figure 1a); 24m+30sm in *Piaractus mesopotamicus* (Figure 1b); 40m+14sm in *Mylossoma aureum* (Figure 1c), and 28m+12sm+14st in *M. duriventre* (Figure 1d).

The constitutive heterochromatin of *C. macropomum*, *M. duriventre*, and *P. mesopotamicus* was concentrated in the centromeric regions of several chromosomes (Figure 1e, f, h). In *M. aureum*, besides the heterochromatic blocks in the centromeric regions, pairs 2, 6, 12, 19 and 23 had heterochromatin at interstitial positions of the long arms and pair 21 on its short arm (Figure 1g).

Nucleolus organizing regions (NOR) were located on the terminal portions of the long arms of metacentric pairs 6 and 12 of *C. macropomum* (Figure 1a), in terminal regions of chromosome 4 in *P. mesopotamicus* (Figure1b) and in the short arms of one homologue of pair 12 in species of *Mylossoma* (Figure 1c, d).

Repetitive sequences mapping

In situ hybridization experiments with 18S sequences as probes were performed numerous times in *Mylossoma* species but no labeling of ribosomal sites could be detected. This result may reflect methodological problems or may be

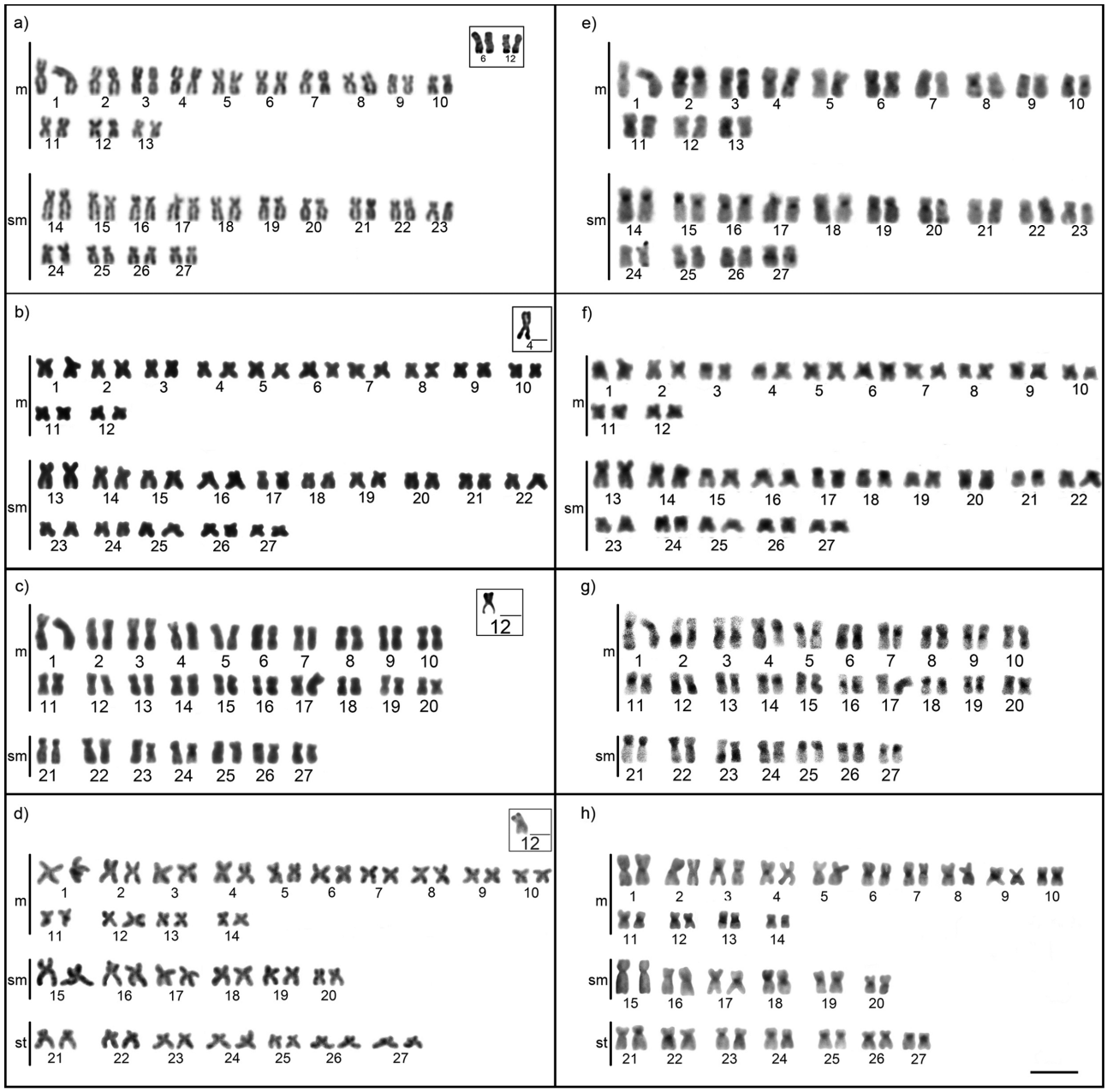

Figure 1 - Conventionally stained and C- banding karyotypes of: *C. macropomum* (a and e), *P. mesopotamicus* (b and f), *M. aureum* (c and g), and *M. duriventre* (d and h). In the insets, AgNORs-bearing chromosomes. Bar = 10 μm.

due to the small size of these sites which would prevent their visualization. The 18S rDNA sequences were visualized in the terminal regions of the long arms of metacentric pairs 6, 10 and 12 of *C. macropomum* (Figure 2a), with pairs 6 and 12 coinciding with the Ag-NOR. In *P. mesopotamicus*, signals were observed in the terminal regions of the long arms of metacentric pairs 4 and 6 (Figure 2b). 5S rDNA sequences mapped to euchromatic and heterochromatic regions of two chromosome pairs in each of the four species (Figure 3e, f, g, h). Labeling was observed in an interstitial position of pair 11 and in the terminal region of the short arm of pair 13 in *C. macropomum* and *P. mesopotamicus* (Figure 3a, b). In both species, the 5S rDNA sites of pair 11 co-localized with heterochromatic blocks and the 5S rDNA sites of pair 13 were adjacent to heterochromatic regions (Figure 3e, f). The 5S rDNA sites were present in pairs 4 and 12 of *Mylossoma aureum* (Figure 3c, g) and in pairs 4 and 8 of *M. duriventre* (Figure 3d). The interstitial signals in the long arms coincided with the heterochromatic blocks in both species.

FISH with telomeric probes revealed signals at the terminal portions of both arms of all chromosomes in the four species (Figure 4a, b, c, d). An interstitial telomeric site (ITS) was observed in one metacentric pair of *C. macropomum* (Figure 4a) and coincided with a positive C-banding region.

Discussion

Cytogenetic descriptions

According to the phylogeny based on mtDNA data proposed by Ortí *et al.* (2008), the Serrasalmidae family may be divided into three main clades: "pacus", "*Myleus"*, and "piranhas". All of the species analyzed herein belong to

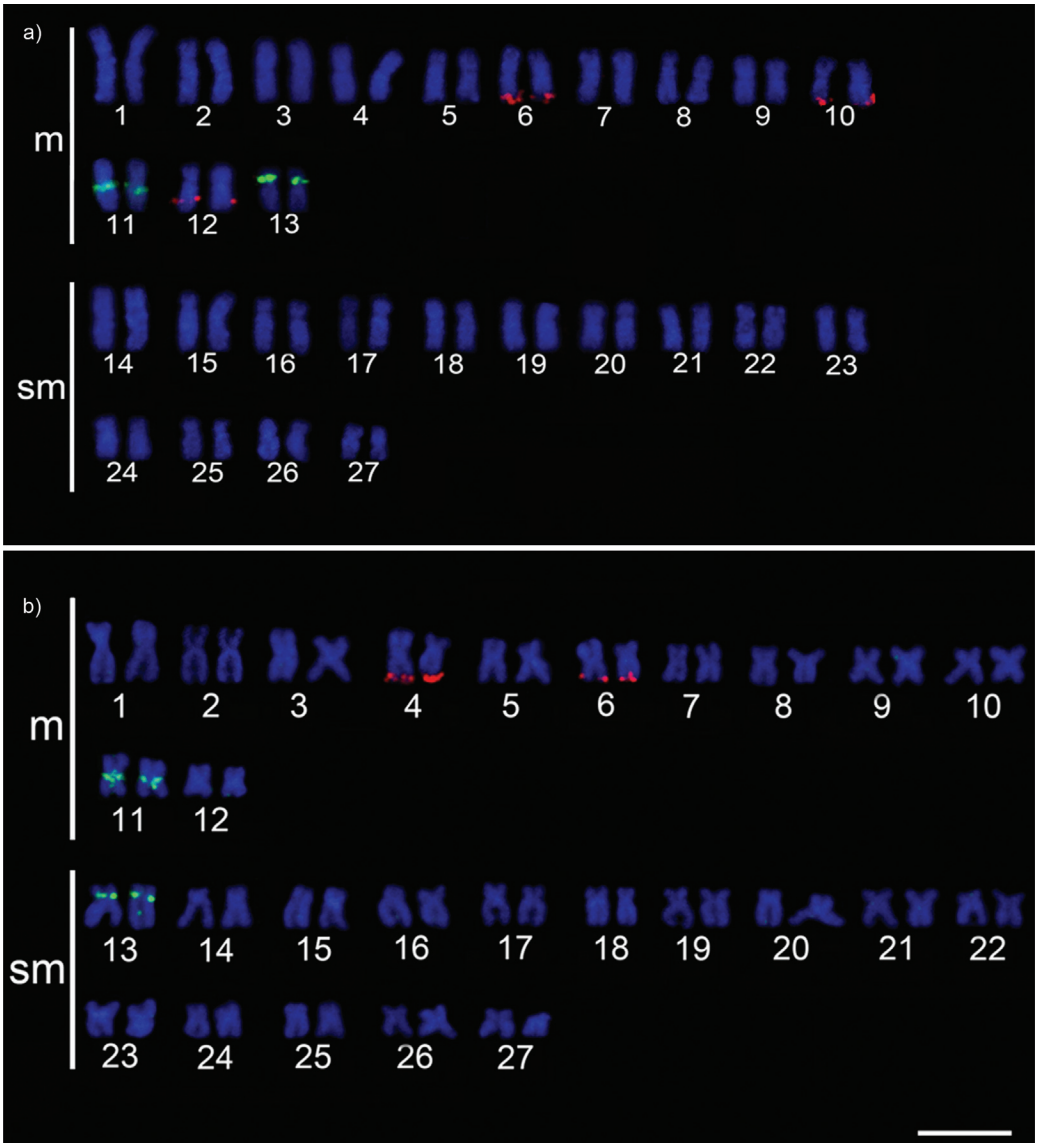

Figure 2 - 18S rDNA sites (red) and 5S rDNA sites (green) in the karyotypes of: (a) *C. macropomum* and (b) *P. mesopotamicus*. Bar = 10 μm.

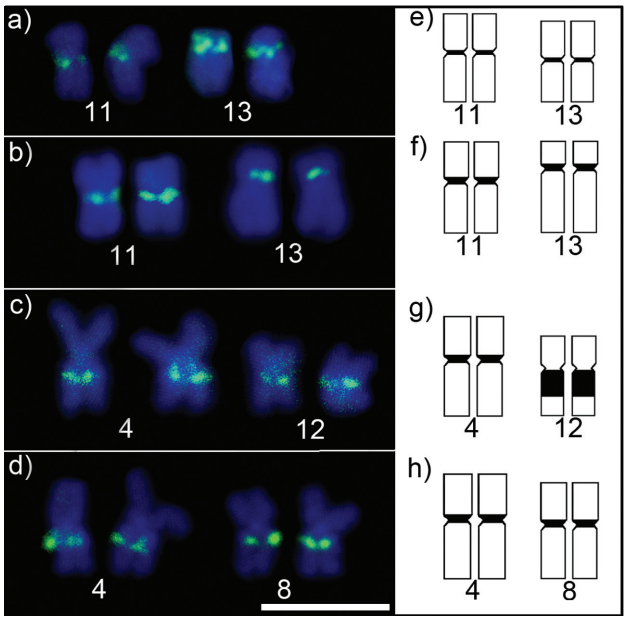

Figure 3 - Co-localization of some 5S rDNA sites with heterochromatic blocks. On the left column, chromosomes after FISH with 5S rDNA sequences; on the right column, heterochromatic blocks in the same chromosomes: (a, e) *C. macropomum*, (b, f) *P. mesopotamicus*, (c,g) *M. aureum*, and (d, h) *M. duriventre*. Bar = 10 µm.

the "pacus" clade, which comprises the *Piaractus*, *Colossoma*, and *Mylossoma* genera, considered basal among Serrasalmidae (Ortí *et al.*, 1996). The evolutionary relationships among Serrasalmidae are not well defined and have been the focus of several studies. Molecular data have been used to supplement morphological studies that confirm the monophyly of this fish group from species to family and/or subfamily levels (Calcagnotto *et al.*, 2005; Freeman *et al.*, 2007).

Chromosomal data separate the species into three groups that corroborate Ortí *et al.* (1996) phylogenetic proposal for Serrasalmidae - with the "pacus" group, considered the most basal, having 2n = 54 biarmed chromosomes, the "*Myleus*" group showing 2n = 58 (Porto JIR, 1999, PhD Thesis. INPA/UFAM, Manaus, AM) (García-Parra WJ, 2000, PhD Thesis. INPA/UFAM, Manaus, AM), and the "piranhas" clade, considered the most derived, presenting diploid numbers that vary from 58 to 64 and the presence of acrocentric pairs (Muramoto *et al.*, 1968; Nakayama *et al.*, 2002, 2008, 2012). This scenario suggests an evolutionary trend towards increasing chromosome numbers mainly due to chromosomal fissions.

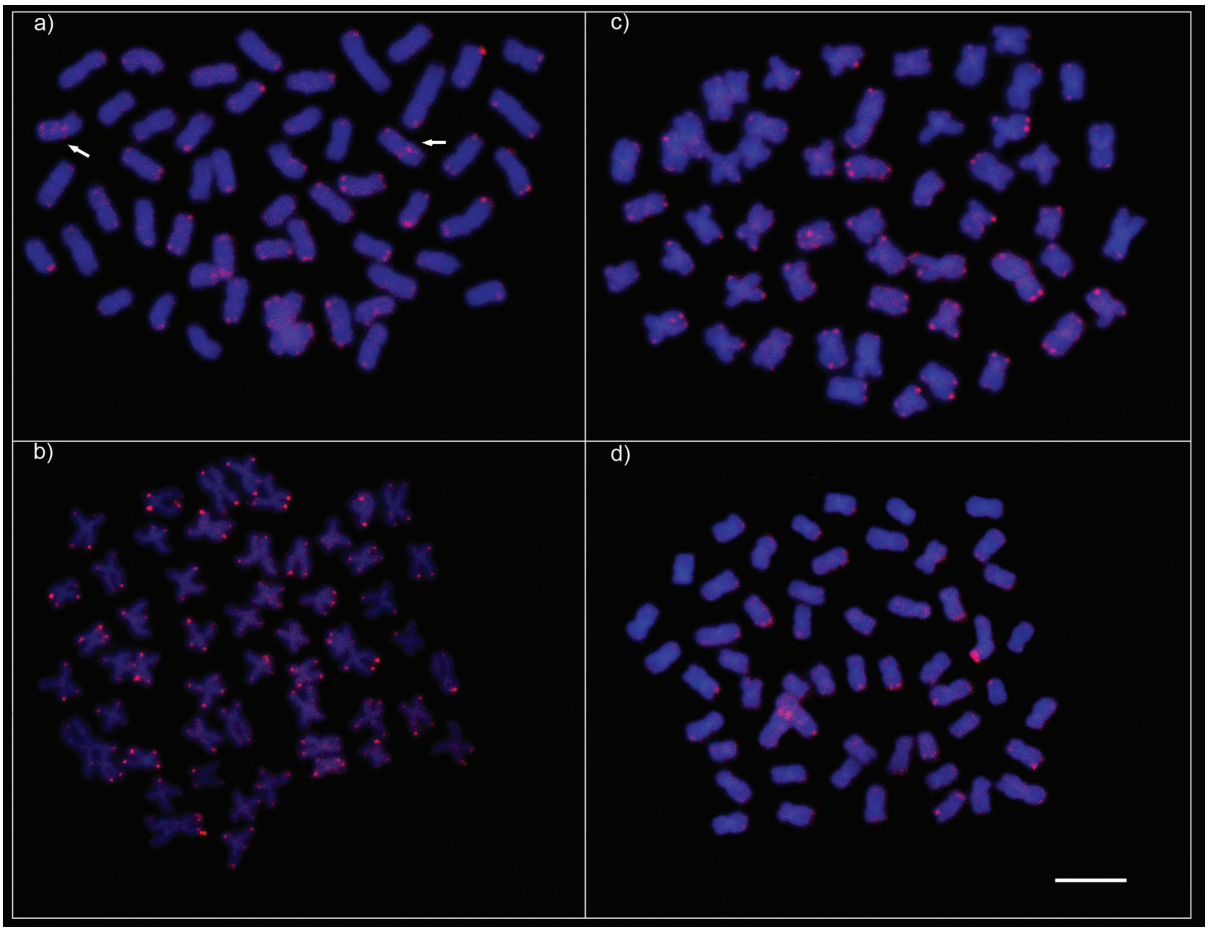

Figure 4 - Metaphases after FISH with telomeric probes (red): (a) *C. macropomum*, (b) *P. mesopotamicus* (c) *M. aureum* and (d) *M. duriventre.*

All the species analyzed here shared the same diploid number, although their karyotypic formulas differed, indicating the occurrence of non-Robertsonian rearrangements. This kind of rearrangements have been reported for a number of other fish species (Garcia and Moreira-Filho, 2005; Mazzuchelli *et al.*, 2007; Nakayama *et al.*, 2008, 2012; Schneider *et al.*, 2012). Besides having 2n = 54 as its exclusive diploid number, the "pacus" clade is the only one whose species consistently present only biarmed chromosomes (metacentric, submetacentric, and subtelocentric).

The karyotypes of *Colossoma macropomum* and *P. mesopotamicus* were different from those previously described. The karyotypic formula of our specimens of *C. macropomum* differed from that described by Nirchio *et al.* (2003); *P. mesopotamicus* presented the same karyotypic formula described when this species was still classified as *Colossoma mitrei* (Almeida-Toledo *et al.*, 1987), but differences were detected in the distribution of heterochromatic blocks, with small quantities of constitutive heterochromatin observed in the present work. These differences may be due to different factors, including environmental influences. The great diversity of habitats may expose organisms to different selection pressures that may activate or inactivate certain genes through epigenetic mechanisms. The appearance of distinct heterochromatic regions in different populations could well reflect adaptive processes that are part of the necessary metabolic fine-tuning for species survival. According to Richards *et al.* (2010), epigenetic changes induced by hybridization or environmental stress may be passed on for many generations and contribute for the adaptation of these fish to new environments.

Repetitive sequences mapping

Fluorescence *in situ* hybridization with 18S rDNA sequences showed multiple signals in two species, as previously reported for other Serrasalmidae (Nakayama *et al.*, 2008, 2012). The 18S rDNA distribution in three chromosome pairs of *C. macropomum* corroborated earlier findings for that same species (Nirchio *et al.*, 2003; Nakayama *et al.*, 2012), in contrast to the Ag-NOR staining that evidenced only two pairs. The pattern observed in *P. mesopotamicus* (two labeled pairs) differed, however, from that described in other species of the same genera (*P. brachypomus*) which presented a single 18S rDNA-bearing chromosome pair (Nirchio *et al.*, 2003). The smaller number of NOR evidenced by silver nitrate could be explained by the fact that this agent only interacts with proteins in recently active regions, whereas FISH detects all rDNA sites despite their recent activity.

If the presence of single NOR are considered a plesiomorphic characteristic, as in other fish groups (*e.g.*, Feldberg *et al.*, 2003 - family Cichlidae), a similar situation may well exist in the Serrasalmidae family, with the basal species *P. brachypomus* (Orti *et al.*, 2008) bearing 18S rDNA sequences in a single chromosome pair (Nirchio *et al.*, 2003), followed by other species of the same genus with two 18S rDNA-bearing pairs and by *C. macropomum* with these sequences present in three pairs. The "piranhas" clade has five to eight chromosome pairs bearing 18S rDNA sites (Nakayama *et al.*, 2012).

FISH with 5S rDNA sequences resulted in two chromosome pairs labeled in the four species. The pairs bearing the 5S rDNA sites (pairs 11 and 13) in *C. macropomum* and *P. mesopotamicus* appeared to be homeologs, as their sizes and morphologies were very similar (Figure 3a, b). Although the *Mylossoma* species also showed 5S rDNA sites in two chromosome pairs, these did not seem to be intra- or intergeneric homeologs, as the morphology and interstitial labeling location in one of the pairs differed from those observed in the other species (Figure 3c, d). Additionally, the 5S rDNA localization in *C. macropomum* differed from that previously reported for this same species, in which the single 5S rDNA site coincided with a heterochromatic region (Nakayama *et al.*, 2012). This variation in the number of chromosome pairs with multiple 5S rDNA sites in *C. macropomum* contradicts Nakayama *et al.* (2012) observation that the pattern of a single 5S rDNA-bearing site found in *Serrasalmus* was a characteristic shared by the entire family. Additionally, the synteny of the 5S and 18S rDNA sites previously described in *C. macropomum* was not observed in the present work, perhaps due to the fact that their chromosomes morphological similarities led to errors in their identification and the5S and 18S rDNA probes were not hybridized simultaneously.

These observations also do not corroborate the hypothesis that the localization of interstitial sites on a single chromosome pair is characteristic of basal species (Martins and Galetti Jr., 1999). In the Serrasalmidae family, for example, the opposite pattern was observed, with the species of the basal genera (*Colossoma*, *Mylossoma*, and *Piaractus*) having two 5S rDNA-bearing chromosome pairs and the most derived species (of the genus *Serrasalmus*) showing a single chromosome with 5S rDNA sequences (Nakayama *et al.*, 2012). The location of the 5S rDNA sites in heterochromatic regions was the only similarity observed in relation to other serrasalmids.

Telomeric sequences were present in the terminal regions of all the chromosome arms in all the analyzed species, confirming the hypothesis that the observed variations between the karyotype were due to inversions. Interstitial telomeric sequences (ITSs) in one chromosome pair were only observed in *C. macropomum* and did not seem to result from fusion, as the diploid number was maintained, neither to be due to inversions. These ITS coincided with heterochromatic regions, which could explain their presence. Other repetitive DNA sequences present in constitutive heterochromatin regions have been associated with telomeric sequences (Schneider *et al.*, 2012). Additionally, ITS observed in centromeric positions (as in *C.*

macropomum) may be related to satellite DNAs transferred from other regions by processes such as unequal crossing-over, transpositions, or duplications in the heterochromatin (Schneider *et al.*, 2012).

Based on the data presented herein, we conclude that the basal clade of Serrasalmidae, composed of *C. macropomum*, *M. aureum*, *M. duriventre*, and *P. mesopotamicus* has karyotypic features consistent with the previously published data for these species, including their 2n = 54. This is compatible with the phylogeny of the group. The data presented herein demonstrate the need of additional molecular studies within this clade to aid the identification of the distribution patterns of the repetitive sequences that are effectively determining the evolutionary trend within this group.

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