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Research Article

Cytogenetic analysis and chromosomal characteristics of the polymorphic 18S rDNA in the fish *Prochilodus lineatus* (Characiformes, Prochilodontidae)

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Abstract

We used differential staining techniques (BSG, GTG, $AgNO_3$, DAPI and CMA_3 banding) and fluorescent in situ hybridization (FISH) with 5S and 18S probes to investigated the karyotypic and cytogenetic chracteristics of *Prochilodus lineatus* specimens from a population in Vila Velha state park (Parque Estadual de Vila Velha, Ponta Grossa, Paraná state, southern Brazil). The specimens studied showed the same karyotype as that found in other *P. lineatus* populations, *i.e.* 2n = 54 biarmed chromosomes (40m + 14 sm) and c-positive heterochromatin preferentially located pericentromerically in all chromosomes. The presence of partial or totally heterochromatic supernumerary chromosomes with numeric intra-individual variation was confirmed in the analyzed population. The nucleolar organizing regions (NORs) were interstitially situated on the long arm of chromosome pair 4 directly beneath the centromere. The differential banding techniques and FISH revealed NOR size polymorphism due to structural events such as breaks and duplication of the larger rDNA site cluster. We also observed syntenic localization of the 5S ribosomal genes in the distal segment of the 45S cluster.

Key words: fish cytogenetics, *Prochilodus,* karyotype, minor and major rDNA, NOR polymorphism.

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Introduction

The karyotypes of representatives of the genus *Prochilodus* (Prochilodontidae) are characterized by the presence of an evolutionarily conserved karyotype of 2n = 54 biarmed chromosomes (Pauls and Bertollo, 1990). However, a few species and/or populations present intra- and interpopulational karyotype variation related to supernumerary microchromosomes, as seen in *Prochilodus lineatus* (Pauls and Bertollo, 1983; 1990; Oliveira *et al*., 1997; Dias *et al*., 1998; Maistro *et al*., 2000; Cavallaro *et al*., 2000; Jesus *et al*., 2003).

As in the majority of Neotropical fish karyotypes, the *P. lineatus* karyotype contains only one chromosome pair with nucleolar organizing regions (NORs) (Pauls and Bertollo, 1990). The *in situ* location of ribosomal genes indicates synteny for the 18S and 5S rDNA sites of *P. lineatus* and *Prochilodus argenteus* as well as polymorphism in the number of 18S genes (Jesus and Moreira-Filho, 2003; Hatanaka and Galetti Jr., 2004). To investigate the level of conservation of these characters between different *P. lineatus* populations we analyzed the 18S and 5S rDNA sites of a specific *P. lineatus* population, giving special attention to polymorphism analysis of the 18S sites as revealed by different chromosome banding methods.

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Material and Methods

The population (n = 30) consisted of 19 female and 11 male *Prochilodus lineatus* (Prochilodontidae) specimens from a Brazilian site at Lagoa Dourada (25°14'09" S, 50°00'17" W) in the Upper Tibagi River basin in Vila Velha state park (Parque Estadual de Vila Velha, Ponta Grossa, Paraná state, southern Brazil). Analyzed specimens were deposited in the Zoology Museum, Londrina State University Paraná, Brazil as voucher number 1737.

Chromosome preparations were obtained from anterior kidney cells using *in vivo* colchicine treatment (Bertollo *et al*., 1978). Constitutive heterochromatin was detected by C-banding (Sumner, 1972) and NORs by silver

nitrate staining (Ag-NORs) as described by Howell and Black (1980). Counterstain-enhanced fluorochrome staining with GC-specific chromomycin A_3 and AT specific 4',6-diamidino-2-phenylindole (DAPI) was according to Schweizer (1976) and longitudinal GTG chromosomal bands were visualized after treatment with trypsin (Gold *et al*., 1990).

Fluorescent *in situ* hybridization (FISH) was used to map the major and minor rDNA sites in the chromosomes using 18S rDNA (approximately 1,800 bp) obtained by PCR from the nuclear DNA of *Prochilodus argenteus* (Hatanaka and Galetti Jr., 2004), using the NS1 5'-GTAGT CATATGCTTGTCTC-3' and NS8 5'-TCCGCAGGTTC ACCTACGGA-3' primers (White *et al.*, 1990) and a 5S rDNA probe from *Leporinus elongatus* (Martins and Galetti Jr., 1999). The probes were labeled with 14-dATP biotin by nick translation according to the manufacturer's instructions (Bionick Labelling System, Invitrogen). The metaphase chromosomes were treated according to the procedure described by Pinkel *et al*. (1986) and analyzed using an Olympus BX50 epifluorescence microscope. The chromosome figures were captured using CoolSNAP-pro software (Media Cybernetics).

For karyotyping the chromosomes were arranged into two groups as metacentric (m) or submetacentric (sm) based on their arm ratios (Levan *et al*., 1964) and arranged in order of decreasing size.

Results and Discussion

The diploid chromosome number of *P. lineatus* is 2n = 54, corresponding to 40 metacentric and 14 submetacentric chromosomes (Figure 1). This is a basal and conserved condition in *Prochilodus*, also evidenced in a few other phylogenetically close families such as Parodontidae, Anostomidae and Curimatidae (Galetti Jr. *et al*., 1994). However, this standard diploid number may be extended to up to 2n = 57 chromosomes due to the presence of B microchromosomes that may vary in number intra- or inter-individually. Various studies (Pauls and Bertollo, 1990; Oliveira *et al.*, 1997; Cavalaro *et al.,* 2000*;* Jesus *et al*., 2003) have shown that *P. lineatus* is a useful Neotropical fish model for studies concerning the origin, behavior and evolution of B chromosomes, which although highly diversified in regards to morphology and distribution of satellite DNAs are always heterochromatic and small in relation to the chromosomes of the standard complement (Artoni *et al.*, 2006).

Besides being present on B chromosomes, constitutive heterochromatin also occurs at the pericentromeric region of all the other chromosomes of the complement and at the NORs of the 4th metacentric pair (Figure 1b), which appear GC-rich when stained with Chromomycin A_3 (Figures 2a, b and 3a, c, d, e, f). The correlation of NOR sites with GC-rich sites is relatively common among fish, although staining with GC-specific fluorochromes is not considered

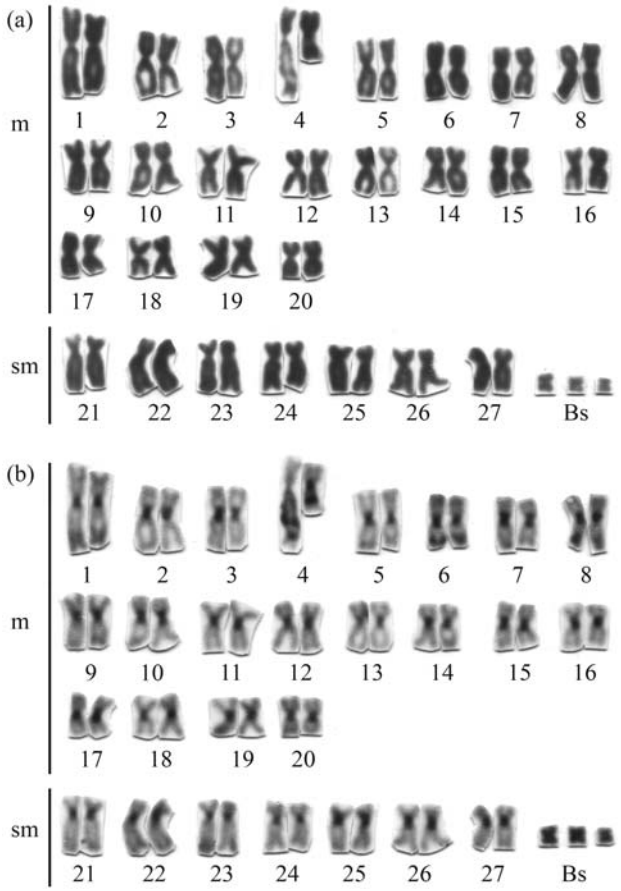

Figure 1 - Karyotypes of *Prochilodus lineatus* from Dourada Lagoon. (a) standard karyotype with conventional Giemsa staining and (b) karyotype showing C-bands. Bar represents 5 μm.

as a method for the direct determination of ribosomal genes but of GC-rich heterochromatins associated with a gene cluster (Artoni *et al*., 1999). Pendás *et al*. (1993) used *in situ* hybridization with 18S rDNA probes to show that the ribosomal genes of Atlantic salmon (*Salmo salar*) were interspersed throughout the heterochromatic chromosomal regions and that this resulted in an apparent coincidence between ribosomal genes and heterochromatic regions when the chromosomes subjected to silver nitrate staining and C-banding, these observations being supported by our results with *P. lineatus* (Figure 3a, e).

The *P. lineatus* NOR site, located directly beneath the centromere in the interstitial region of the long arm of chromosome pair 4, frequently showed a size polymorphism in one of the homologues (Figures 2a, b, d, and 3). Size polymorphisms of the NORs are relatively common in Neotropical fishes (Foresti *et al.*, 1981; Brum *et al.*, 1998; Vicari *et al*., 2005) and are sometimes also associated with sex chromosomes (Galetti Jr., 1998; Molina *et al*., 1998; Born and Bertollo, 2000, Artoni and Bertollo, 2002; Vicari *et al*., 2003). Interestingly, a lethal effect in the rainbow trout (*Oncorhynchus mykis*) has been found to be related to a NORs size polymorphism in homozygotes, with evidence

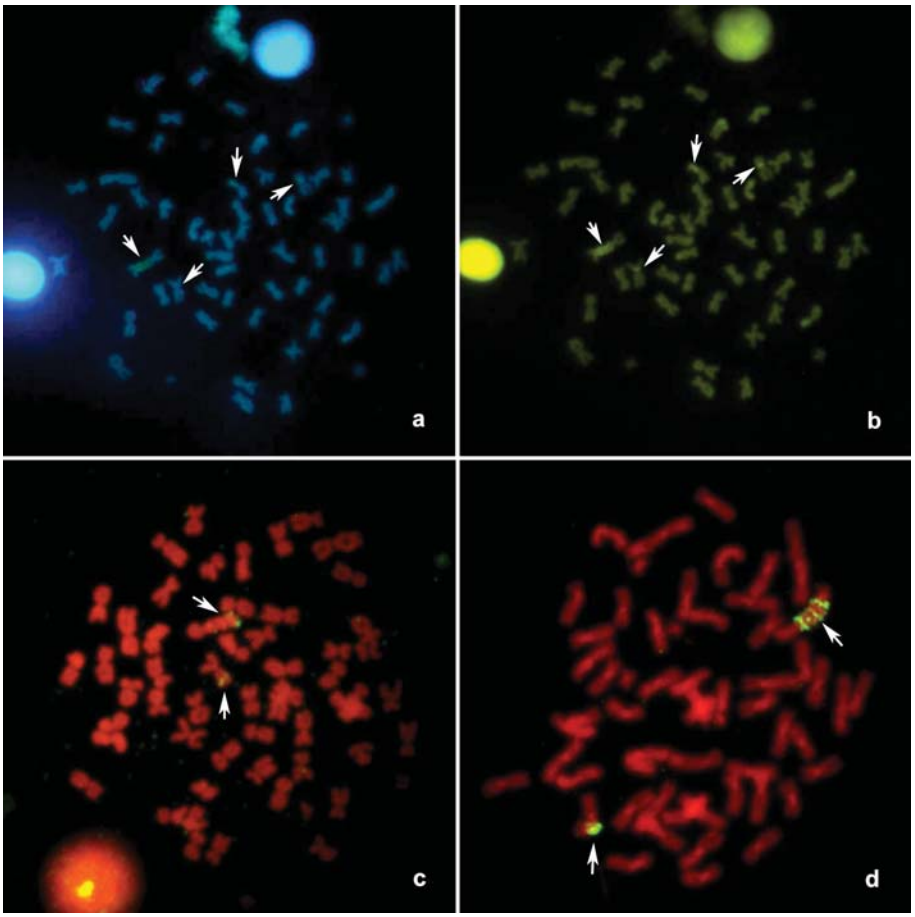

Figure 2 - Metaphases of *Prochilodus lineatus* showing (a) negative DAPI regions, (b) positive chromomycin A_3 regions, (c) rDNA 5S sites and (d) rDNA 18S sites. The arrows indicate the sites.

also showing that heterozygotes present a higher adaptive value (Porto-Foresti *et al.*, 2004). In piscines, the involvement of heterochromatin in the accumulation of rDNA *loci* through unequal crossing-over or sister chromatid exchanges involving repeated sequences and adjacent loci has been proposed as the main mechanism for these rDNA polymorphisms (Pendás *et al*. 1993). However, structural chromosomal alterations such as duplications, deletions and dissimilar crossing-over are all mechanisms which may produce structural polymorphisms in NORs (Gold, 1990). Our *P. lineatus* data permitted a more detailed analysis of NOR polymorphisms, our FISH analysis clearly showing that NOR polymorphisms in this species was due to up to three tandemly repeated 18S rDNA clusters (Figure 3f). Accordingly, the GTG banding pattern showed that this region was formed from three G-negative band segments separated by small G-positive band segments (Figure 3b), indicating that breaks in these segments and localized duplications were responsible for the observed polymorphism.

Pauls and Bertollo (1990) used silver nitrate (Ag) staining to analyze the NORs of members of the genus *Prochilodus* and found AgNORs in a secondary constriction located interstitially on the long arm of a large metacentric pair and also described a third metacentric chromosome sporadically bearing active NOR on the telomeric region in *Prochilodus marggravii* (now *P. argenteus*) and *Prochilodus affinis* (now *P. costatus*). In some *P. lineatus* specimens from the Mogi Guaçu river (São Paulo State, Brazil) Jesus and Moreira-Filho (2003) detected not only a large metacentric pair but also a variable number of ribosomal sites (one or two additional lower inactive sites) suggesting inter-individual numerical polymorphism of the 18S rDNA regions of the this fish. Hatanaka and Galetti Jr.

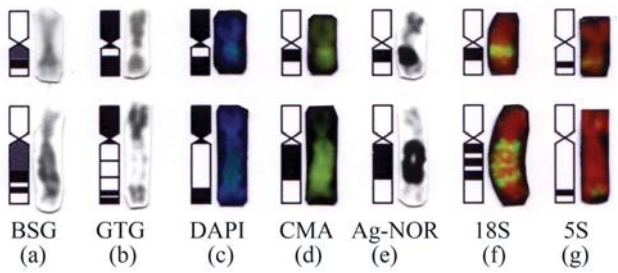

Figure 3 - Idiogram of *Prochilodus lineatus* showing the NOR polymorphism on the 4th chromosome pair and syntenic 5S site: (a) C-banding, (b) GTG-banding, (c) DAPI staining, (d) Chromomycin A_3 staining, (e) Ag-NOR sites, (f) 18S rDNA FISH and (g) 5S rDNA FISH.

(2004) observed a number of additional rDNA clusters in *P. argenteus* but, on the other hand, Maistro *et al*. (2000) reported that in *P. lineatus* the major rDNA region seems to be a large metacentric pair, a finding supported in our present study.

Our data also shows that the 5S ribosomal site is syntenic with the 18S rDNA site, being located subterminally on the long arm of chromosome pair 4m, adjacent and distal to the 18S site (Figures 2c and 3g). The mappings of the 5S rDNA sites in fish have demonstrated a higher frequency for a single chromosome locus, which may correspond to the ancestral condition in this group (Martins and Galetti Jr., 1999). Nevertheless, the occurrence of multiple 5S rDNA sites has also been observed in a few species, such as *Astyanax scabripinnis* (Ferro *et al*., 2001) and *Hoplerythinus unitaeniatus* (Diniz and Bertollo, 2003). On the other hand, the *P. argenteus* population of the São Francisco River (Minas Gerais State, Brazil), occasionally presented a third hybridization signal corresponding to an additional 5S rDNA site (Hatanaka and Galetti Jr, 2004). Despite being adjacent and distal to the 18S rDNA, the 5S rDNA was not involved in polymorphism of chromosome pair 4 (Figure 3g). Syntenic organization of 18S and 5S rDNAs has also been described in *P. lineatus* from the Mogi-Guaçu River and in *P. argenteus* from the São Francisco River (Jesus and Moreira-Filho, 2003; Hatanaka and Galetti Jr, 2004, respectively). In conclusion, the observations regarding the localization and variability of the number of ribosomal genes and the number of sites in *P. lineatus* show that these may be variable among different populations. The presence of a single 18S rDNA-bearing chromosome pair must be a plesiomorphic condition in relation to the other *P. lineatus* populations and other species of this genus that present multiple NORs. Similarly, synteny between the two ribosomal families may indicate plesiomorphy in the genus *Prochilodus*.

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