



## Karyotypic conservatism in five species of *Prochilodus* (Characiformes, Prochilodontidae) disclosed by cytogenetic markers

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### Abstract

The family Prochilodontidae is considered a group with well conserved chromosomes characterized by their number, morphology and banding patterns. Thence, our study aimed at accomplishing a cytogenetic analysis with conventional methods (Giemsa staining, silver staining of the nucleolus organizer regions-AgNOR, and C-banding) and fluorescence in situ hybridization (FISH) with 18S and 5S ribosomal DNA probes in five species of the *Prochilodus* genus (*Prochilodus argenteus*, *Prochilodus brevis*, *Prochilodus costatus*, *Prochilodus lineatus* and *Prochilodus nigricans*) collected from different Brazilian hydrographic basins. The results revealed conservatism in chromosome number, morphology, AgNORs 18S and 5S rDNAs location and constitutive heterochromatin distribution patterns. The minor differences observed in this work, such as an Ag-NOR on a *P. argenteus* chromosome and a distinct C-banding pattern in *P. lineatus*, are not sufficient to question the conservatism described for this group. Future work using repetitive DNA sequences as probes for FISH will be interesting to further test the cytogenetic conservatism in *Prochilodus*.

**Keywords:** AgNOR, C-banding, hydrographic basins, conserved karyotype, FISH.

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### Introduction

Fishes of the family Prochilodontidae are significant components of the fauna of Neotropical rivers and are considered one of the most important elements of commercial and subsistence freshwater fisheries in South American environments, except in Chile, where they are not found (Lowe-McConnell, 1975; Goulding, 1981; Vari, 1983; Flecker, 1996).

Cytogenetic data on *Prochilodus* species revealed a conserved karyotype with  $2n = 54$  chromosomes and a fundamental number  $FN = 108$  (Pauls and Bertollo, 1983, 1990), suggesting that the family Prochilodontidae exhibits a predominantly conserved chromosomal evolution (Pauls and Bertollo, 1990). However, a few populations and/or species, such as *P. brevis*, *P. lineatus*, *P. mariae* and *P. nigricans*, showed karyotypic variation due to the presence

of supernumerary chromosomes (Pauls and Bertollo 1983, 1990; Oliveira *et al.*, 1997, 2003; Dias *et al.*, 1998; Venere *et al.*, 1999; Maistro *et al.*, 2000; Cavallaro *et al.*, 2000; Jesus and Moreira-Filho, 2003; Artoni *et al.*, 2006; Voltolin *et al.*, 2009).

Conventional cytogenetic markers, such as Ag-NORs, evidenced a single chromosome pair bearing NORs in some species of *Prochilodus* (Pauls and Bertollo, 1990; Venere *et al.*, 1999; Oliveira *et al.*, 2003; Jesus and Moreira-Filho, 2003; Vicari *et al.*, 2006; Voltolin *et al.*, 2009).

Data on the localization of the 5S and 18S ribosomal genes by FISH in the genomes of species of Prochilodontidae are still scarce (Jesus and Moreira-Filho, 2003; Hatanaka and Galetti Jr, 2004; Vicari *et al.*, 2006; Grass *et al.*, 2007; Terêncio *et al.*, 2012). FISH with the 5S and 18S ribosomal genes showed that they are syntenic in *P. lineatus* and *P. argenteus*, and evidenced a polymorphism in the number of 18S rRNA genes (Jesus and Moreira-Filho, 2003; Hatanaka and Galetti Jr, 2004; Voltolin *et al.*, 2009).

C-banding analyses carried out in *Prochilodontidae* representatives showed that the constitutive heterochromatin is frequently restricted to centromeric blocks in all chromosomes of the standard (A) complement (Pauls and Bertollo, 1990; Venere *et al.*, 1999; Cavallaro *et al.*, 2000; Oliveira *et al.*, 2003; Jesus and Moreira-Filho 2003; Artoni *et al.*, 2006; Voltolin *et al.*, 2009). Additionally, the supernumerary chromosomes were usually entirely heterochromatic in these species (Pauls and Bertollo 1990; Maistro *et al.*, 2000; Cavallaro *et al.*, 2000; Jesus and Moreira-Filho, 2003; Artoni *et al.*, 2006; Voltolin *et al.*, 2009).

Therefore, the objective of the current study was to conduct a comparative analysis with conventional and molecular cytogenetic markers in five species of the genus *Prochilodus* collected in different Brazilian hydrographic basins to look for chromosome differences that may have accumulated in these populations over the years allowing their cytogenetic differentiation.

## Materials and Methods

We analyzed 20 samples of *P. lineatus* from the Mogi-Guaçu river, Pirassununga, (São Paulo); 17 individuals of *P. nigricans* from the Tocantins Araguaia basin (Tocantins); 15 individuals of *P. costatus* acquired from the Aquicultura Tropical pisciculture, Propiá (Sergipe); six samples of *P. argenteus* from the São Francisco basin; and five individuals of *P. brevis*, acquired from the Departamento de Nacional de Obras Contra a Seca (DNOCS) dam, in Natal (Rio Grande do Norte).

Mitotic chromosomes were obtained from anterior kidney fragments (Foresti *et al.*, 1981) and through lymphocyte culture (Fenocchio and Bertollo, 1988) with some adjustments. The karyotypes were arranged according to Levan *et al.* (1964).

Active NORs were identified after silver nitrate staining (Howell and Black, 1980) and the constitutive heterochromatin was detected after C-banding (Sumner, 1972).

FISH was carried out according to Pinkel *et al.* (1986) using 5S rDNA probes obtained by PCR from *Prochilodus* genomic DNA using the primers A (5'-TACGCCGATCTCG TCCGATC-3') and B (5'-CAGGCTGGTATGGCCGTAAGC-3') (Pendás *et al.*, 1994). The 18S rDNA probe was obtained by PCR using the NS1 (5'-GTAGTCATATGCTTGCTC-3') and NS8 (5'-TCCGCAGGTTACCTACGGA-3') primers (White *et al.*, 1990).

The 5S probe was labeled with biotin-dUTP and the 18S probe was labeled with digoxigenin-dUTP (Roche) by PCR, according to the manufacturer's instructions.

The 5S and 18S rDNA probes were denatured in 70% formamide:2xSSC for 5 min. The hybridization occurred at 37 °C overnight in a moist chamber (0.3 µg of denatured probe, 50% formamide, 10 mg/mL of dextran sulfate; 2xSSC, 5 mg/mL of salmon sperm DNA).

The 5S and 18S probes were immunodetected with avidin-FITC and anti-digoxigenin-rhodamine, respectively, and the preparations were counterstained with DAPI (4-6-diamidino-2-phenylindole) and examined under an epifluorescence photomicroscope (BX 61, Olympus) equipped with an Olympus DP70 cooled digital camera. Photomicrographs were taken using the Pro MC 6.0 software.

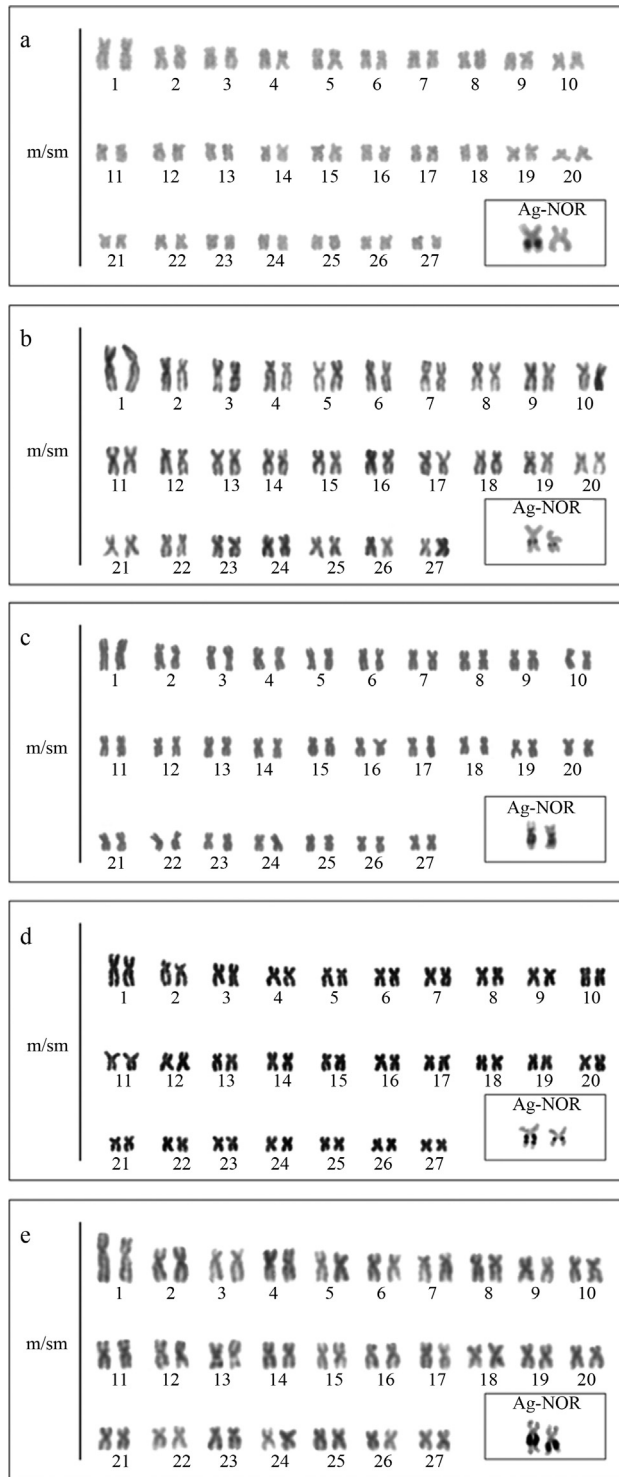
## Results

All specimens of *Prochilodus* (*P. argenteus*, *P. brevis*, *P. costatus*, *P. lineatus* and *P. nigricans*) collected in the different Brazilian hydrographic basins presented a karyotype with  $2n = 54$ ,  $FN = 108$  and metacentric and submetacentric chromosomes (Figure 1a-e). All specimens of *P. lineatus* had supernumerary chromosomes (Figure 2a), whereas one *P. nigricans* specimen had a single B chromosome that showed intraindividual variation, with 23 cells out of 30 exhibiting the extra chromosome (Figure 2b).

After Ag-NOR staining, only one homolog of a submetacentric pair presented a NOR in *P. brevis*, *P. costatus*, *P. lineatus* and *P. nigricans* (Figure 1b-e, highlighted). In *P. argenteus*, the NOR was observed only on one homologue of the second largest submetacentric pair. This Ag-NORs pattern was found in approximately 40 metaphases of all *P. argenteus* specimens.

FISH with the 5S and 18S rDNA probes was performed to confirm if there was a karyotypic difference in the 18S gene location in *P. argenteus*. Synteny between these two genes was observed in all samples tested, including *P. argenteus*, in which the 18S rDNA labeled both homologues of the second submetacentric pair, which was not observed after Ag-NOR (Figure 3a, b, c, d and e). Furthermore, the location of these genes was identical in all the species, *i.e.*, the 5S gene was located near the terminal region of the long arm of the submetacentric chromosome pair and the 18S gene, in a pericentromeric position, syntenic with the 5S gene. Clusters of these ribosomal sequences were not detected in any of the species studied, nor in the B chromosomes present in the genome of *P. lineatus* and *P. nigricans*.

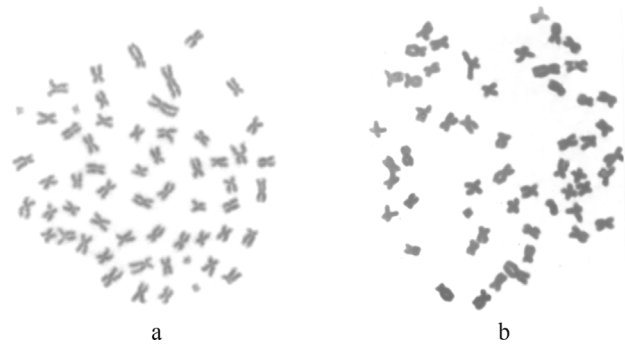
C-banding was performed to identify the distribution of constitutive heterochromatin in all the species. The results allowed us to differentiate among the specimens of *P. lineatus* and those of the other four *Prochilodus* species. Conspicuous heterochromatic blocks present only in the centromeric regions of the standard A chromosome set were observed in *P. lineatus* (Figure 4d). In *P. argenteus*, *P. brevis*, *P. costatus* and *P. nigricans*, besides the presence of heterochromatic regions in the centromere, a large heterochromatic block on the long arm of a submetacentric chromosome pair was also observed (Fig. 4b-e). The supernumerary chromosomes of *P. lineatus* and *P. nigricans* were totally heterochromatic (Figure 4d, e).



**Figure 1** - Karyotypes of the five *Prochilodus* species analyzed in this work: (a) *P. argenteus*; (b) *P. brevis*; (c) *P. costatus*; (d) *P. lineatus* and (e) *P. nigricans*. In the boxes, the AgNOR-bearing pair.

**Discussion**

Pioneer studies in *Prochilodus* cytogenetics conducted by Pauls and Bertollo (1983, 1990) evidenced a con-



**Figure 2** - Metaphases of (a) *P. lineatus*, with four supernumerary chromosomes (arrows). Supernumerary chromosomes occurred in all individuals of this species; (b) metaphase of *P. nigricans* with only one supernumerary chromosome (arrow).

spicuous homogeneity in karyotypes. Several studies have shown that specimens of *Prochilodus* presented  $2n = 54$ ,  $FN = 108$  and biarmed chromosomes (Pauls and Bertollo, 1983, 1990; Oliveira *et al.*, 1997; Cavallaro *et al.*, 2000, Jesus and Moreira-Filho, 2003; Voltolin *et al.*, 2009). Our results are consistent with these data (Figure 1a-e).

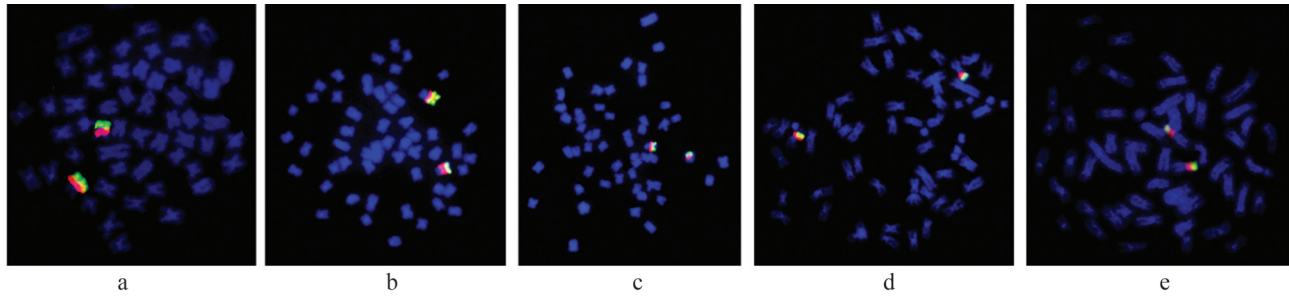
The presence of supernumerary microchromosomes in some species of *Prochilodus* enabled us to study aspects concerning their origin, evolution, structure and maintenance. First described in *P. lineatus* by Pauls and Bertollo (1983), up to two B microchromosomes were also identified in *P. brevis* (= *P. cearensis*) by these same authors (Pauls and Bertollo (1990). Venere *et al.* (1999) described the occurrence of one or two B chromosomes in *P. nigricans* and Oliveira *et al.* (2003) identified up to three supernumerary chromosomes in some individuals of *P. mariae* from the Orinoco river basin in Venezuela.

The specimens of *P. brevis*, *P. costatus*, *P. lineatus* and *P. nigricans* studied herein had only one Ag-NOR situated on the long arm of the second largest submetacentric pair (inbox in Figure 1b-e), as already described for these species (Pauls and Bertollo, 1983.1990; Venere *et al.*, 1999; Maistro *et al.*, 2000; Jesus and Moreira-Filho, 2003; Hatanaka and Galetti Jr, 2004; Vicari *et al.*, 2006; Artoni *et al.*, 2006; Voltolin *et al.*, 2009).

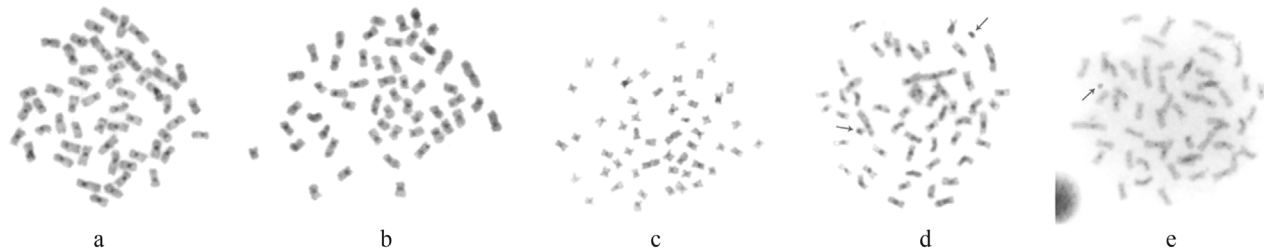
In *P. argenteus*, a single AgNOR was observed on the long arm of one homologue of a submetacentric chromosome (inbox in Figure 1a). This result is inconsistent with the literature data for this species, in which the single AgNOR was observed on the second largest submetacentric pair (Hatanaka and Galetti Jr, 2004).

Silver nitrate does not directly bind to rDNA, but to the proteins associated with the nucleolar structure, restricting the identification to the NORs that had been active in the preceding interphase (Miller *et al.*, 1976). This is the most reasonable hypothesis to explain the single AgNOR in *P. argenteus*.

The position of the ribosomal genes was further investigated with FISH with the 18S and 5S rDNA probes.



**Figure 3** - Fluorescence *In situ* Hybridization (FISH) with 5S (green) and 18S (red) rDNA probes in: (a) *P. argenteus*; (b) *P. brevis*; (c) *P. costatus*; (d) *P. lineatus* with a supernumerary chromosome, and (e) *P. nigricans* with a supernumerary chromosome.



**Figure 4** - C-banded cells of: (a) *P. argenteus*; (b) *P. brevis*; (c) *P. costatus*; (d) *P. lineatus* and (e) *P. nigricans*. In *P. lineatus*, constitutive heterochromatin was only present in the centromeric regions of all A chromosomes. All supernumerary chromosomes were heterochromatic (arrows).

These probes were syntenic in agreement with previous reports (Jesus and Moreira-Filho, 2003; Hatanaka and Galetti Jr, 2004; Vicari *et al.*, 2006). No additional 18S clusters were found in this species, as previously reported (Maistro *et al.*, 2000; Vicari *et al.*, 2006) and neither additional 5S clusters, as already described by Jesus and Moreira-Filho (2003) and by Vicari *et al.* (2006) in specimens of *P. lineatus* from the Mogi Guaçu river and Dourada lagoon, respectively.

The syntenic organization of the 5S and 18S ribosomal genes is a rare event among vertebrates. In addition to *P. lineatus* (Jesus and Moreira-Filho, 2003; Vicari *et al.*, 2006; Voltolin *et al.*, 2009), this synteny was also observed in *Salmo salar* (Pendás *et al.*, 1994), *Oncorhynchus mykiss* (Móran *et al.*, 1996), *Astyanax* (Almeida-Toledo *et al.*, 2002), in amphibians (Lucchini *et al.*, 1993) and, more recently, in *Pimelodus britskii* (Moraes-Neto *et al.*, 2011). On the other hand, these loci have been mapped on different chromosomes in many fish species (Martínez *et al.*, 1996; Morán *et al.*, 1996; Born and Bertollo, 2000; Ferro *et al.*, 2001; Vicente *et al.*, 2001; Wasko *et al.*, 2001; Noleto *et al.*, 2007), representing the most frequent condition in vertebrates (Lucchini *et al.*, 1993; Drouin and Muniz De Sá, 1995; Suzuki *et al.*, 1996).

Neither the 5S nor the 18S ribosomal genes have been found in the B microchromosomes of *P. lineatus* (Jesus and Moreira-Filho, 2003) and of *P. nigricans*.

C-banding has proven very useful in cytogenetic studies of fish, permitting the identification of constitutive heterochromatin regions. Differences in the amount or distribution of heterochromatin identified by C-banding are

considered important for some fish groups and the distribution of C-bands may characterize genera, species and populations (Montovani *et al.*, 2000).

Several C-banding studies have been performed in Prochilodontidae, especially in *Prochilodus lineatus*. Maistro *et al.* (2000) reported centromeric and subtelomeric constitutive heterochromatin in the A complement of curimatás collected in the Mogi-Guaçu river, Pirassununga, São Paulo. However, telomeric C-bands were absent in the chromosome preparations analyzed herein, but the heterochromatic nature of the supernumerary chromosomes was corroborated (Jesus and Moreira-Filho, 2003; Artoni *et al.*, 2006; Voltolin *et al.*, 2009).

Specimens of *Prochilodus* are characterized by the presence of conspicuous centromeric heterochromatic blocks in the A complement and in all supernumerary chromosomes (Jesus and Moreira-Filho, 2003; Artoni *et al.*, 2006; Voltolin *et al.*, 2009).

In this study, we observed different patterns of heterochromatin distribution in the genomes of some species of *Prochilodus*. In *P. lineatus*, constitutive heterochromatin was observed only in the pericentromeric regions of all chromosomes of the standard (A) complement (Figure 4d) and all B chromosomes were heterochromatic. In *P. argenteus*, *P. brevis*, *P. costatus* and *P. nigricans*, in addition to the centromeric region of all A chromosomes, we also observed a large heterochromatic block on a submetacentric chromosome pair (Figure 4a, b, c, e). In addition to the centromeric C-bands in specimens of *P. lineatus* from the Mogi Guaçu river, Jesus and Moreira-Filho (2003) also evidenced heterochromatic blocks close to the telo-

meric region of a submetacentric pair. Despite these small differences in the constitutive heterochromatin distribution in *Prochilodus*, we can still consider them a cytogenetically conserved group.

Oliveira *et al.* (2003) confirmed the conservative nature of the chromosome number and morphology in Prochilodontidae and reinforced the idea that small structural chromosome rearrangements may be the main cause of karyotypic diversification in this group. In that study, the authors observed that in *Prochilodus mariae* from the Orinoco river basin, Venezuela, in addition to the occurrence of constitutive heterochromatin in all centromeres of the autosomes, a heterochromatic block was present in a submetacentric pair, as observed herein in *P. argenteus*, *P. brevis*, *P. costatus* and *P. nigricans*. Oliveira *et al.* (2003) also identified differing C-banding patterns between *Semaprochilodus kneri* and *Semaprochilodus laticeps*. In *S. kneri*, constitutive heterochromatin was present in the centromeric regions of all A chromosomes and a conspicuous heterochromatic block occurred on the long arm of pair 24. In *S. laticeps*, heterochromatin was only found in the pericentromeric regions, as we also observed in *P. lineatus*.

The presence of heterochromatin only in centromeric regions of the standard A chromosome set, as described herein in *P. lineatus*, differs from the data published by Maistro *et al.* (2000) and Jesus and Moreira-Filho (2003) for *P. lineatus* from the Mogi Guaçu River, the same location of our collections. These authors described the presence of heterochromatin in the centromeric region of all autosomes and in the telomeric regions of some pairs of the A complement.

Nevertheless, the four species analyzed (*P. argenteus*, *P. brevis*, *P. nigricans* and *P. costatus*) exhibited the same heterochromatin distribution pattern, in full agreement with literature data (Pauls and Bertollo, 1993; Venere *et al.*, 1999; Hatanaka and Galetti Jr, 2004).

Pauls and Bertollo (1983, 1990) stated that the family Prochilodontidae, especially the *Prochilodus* genus, presented a conserved karyotype, resulting from a conservative chromosome evolution. The data obtained herein are in agreement with this proposed conservatism. The small cytogenetic variations found herein, such as the constitutive heterochromatin distribution and the position of the NOR among the studied *Prochilodus*, are not sufficient to contradict the strong conservatism proposed for the species of this genus by various authors of numerous cytogenetic studies.

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