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Cytogenetic polymorphism in *Prochilodus lineatus* (Pisces: Characiformes) from the middle Paraná River, Santa Fe City, Argentina

D.E. Gras[1], M.S. Brassesco[2], R. Markariani[3], H.A. Roncati[4], E.T. Sakamoto-Hojo[5], A.S. Fenocchio[6], M.C. Pastori[7]

[1, 2, 5]Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes 3900 CEP 14040-900, Ribeirão Preto, São Paulo, Brasil. [3]Departamento de Ciencias Naturales, Facultad de Humanidades y Ciencias, Universidad Nacional del Litoral, Ciudad Universitaria, Paraje El Pozo s/n, CP S3000ZAA, Santa Fe, Argentina. [4, 6, 7]Laboratorio de Citogenética General, Facultad de Ciencias Exactas, Químicas y Naturales, Universidad Nacional de Misiones, Félix de Azara 1552 CP N3300LQH, Posadas, Misiones, Argentina.

E-mails: [1]diagras@rge.fmrp.usp.br, [1]diagras@hotmail.com, [2]marsol@rge.fmrp.usp.br, [3]markari@fbcb.unl.edu.ar, [4]citog@fceqyn.unam.edu.ar, [5]etshojo@usp.br, [6]afenocch@fceqyn.unam.edu.ar, [7]nelida@fceqyn.unam.edu.ar

Abstract. The species of the family Prochilodontidae have a stable karyotype, made of 54 biarmed chromosomes, and one pair bearing the nucleolus organizer regions (NORs). Despite its considerable economic importance in fluvial basins, no cytogenetic data are available for the Argentinian populations of the species *Prochilodus lineatus* (Valenciennes, 1847). Forty five *P. lineatus* specimens from the middle Paraná River (Santa Fe City, Argentina) were analyzed cytogenetically. Chromosome preparations were obtained by kidney cell suspension analyzed directly or after short-term culture, and processed for Giemsa staining, C- and NOR-banding. Fluorescence *In Situ* hybridization with a 28S rDNA probe was also performed. All specimens exhibited the expected familiar karyotypic features, with 2n = 54 and fundamental number of chromosomes (FN) = 108, and C-banding pattern. Silver staining and FISH detected the NORs on a single chromosome pair. In spite of this invariable condition, positional differences on the NORs-bearing chromosomes were observed. Seventy percent of the specimens showed interstitial NORs, while the remaining 30% presented telomeric positive bands.

Key words: chromosome banding, FISH, pisces cytogenetics, cytogenetic polymorphism, *Prochilodus lineatus*.

Introduction

The prochilontids are widely distributed throughout South America; their occurrence has been recorded in almost all hydrographic basins (Mago-Leccia, 1972). They are one of the most important components of commercial and subsistence fishery of freshwater environments.

The genus *Prochilodus* (Agassiz, 1829) comprises 24 species, commonly referred as "*sábalo*" or "*curimbatá*". In most cases they are the main

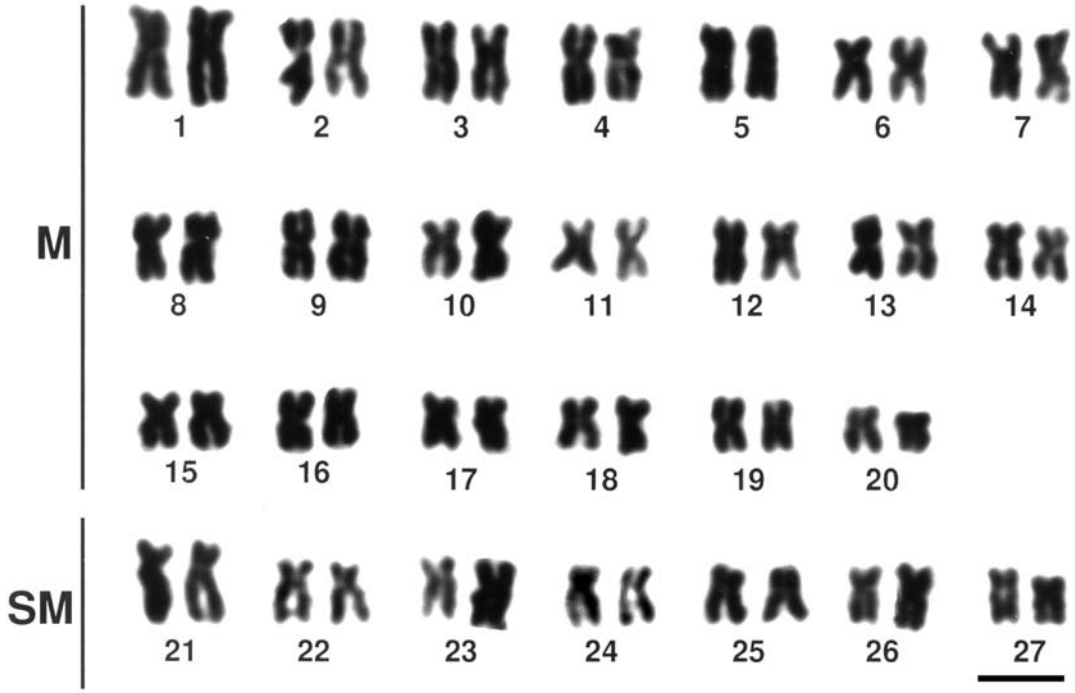

Fig. 1. *Prochilodus lineatus*, karyotype showing 40 metacentric (M) and 14 submetacentric (SM) chromosomes. Bar=5 µm.

type of detritivorous fish and can account more than 50% of the ichthyofauna (Vari, 1983), thus playing a fundamental ecological role in the streams.

Cytogenetic studies of Neotropical fishes have revealed wide chromosome diversity. Among the Characiforms, two strong trends are apparent: karyologically homogeneous groups, versus taxa with high cytogenetic variability (Bertollo et al., 1986). The family Prochilodontidae falls into the first group and its members rarely diverge from the standard karyotype. All the species so far studied have a diploid number 54. The chromosomes are all biarmed, with a fundamental number (FN) = 108. A single NORs-bearing chromosome pair is present in the karyotype (Rebelo-Porto et al.,1992; Brum, Galetti, 1997).

According to Pauls, Bertollo (1990), *Prochilodus* species, including *P. lineatus* (Valenciennes, 1847) (= *P. scrofa* Steindachner, 1881), are characterized by the presence of 40 metacentric and 14 submetacentric chromosomes, with pericentromeric constitutive heterochromatin and nucleolar organizer regions located on an interstitial secondary constriction in the long arm of chromosome pair number 2. In numerous cases, the karyotype stability is altered by the presence of a variable number (0-7) of B-chromosomes, as described for many Brazilian populations (Pauls, Bertollo, 1983, 1990; Dias et al., 1998; Maistro et al., 2000).

Despite the available data, there is no karyotype information about *P. lineatus* in Argentine fluvial basins, even though it represents half of the total fish biomass with approximately 500 Kg/ha within the Paraná River (Oldani, 1990). Thus, considering its importance for the region's economy, we aimed to aquire basic chromosomal data

on individuals from the Paraná River (Argentina), taking into account some cytotaxonomic and evolutionary considerations.

Material and methods

Forty five specimens (17 males, 14 females and 14 immatures) of *Prochilodus lineatus* from the middle Paraná River (Santa Fe City, Santa Fe, Argentina) were cytogenetically studied. Three sampling sites were included: Laguna Setúbal (31º35'S/60º37'W), Alto Verde (31º39'S/60º 42'W) and Canal de Derivación (31º38'S/ 60º40'W). The specimens were checked by taxonomists A.S. Fenocchio and H.A. Roncati who provided the correct species identification.

Mitotic chromosome preparations were obtained from anterior kidney cell suspensions, by direct (Bertollo et al.,1978) and short-term culture methods (Fenocchio et al., 1991), and they were analyzed by conventional and differential staining. Nucleolar organizer regions were identified by silver nitrate staining described by Howell, Black (1980), and constitutive heterochromatin was revealed according to Sumner (1972).

Flourescence *In Situ* Hybridization (FISH) was performed according to Pinkel et al. (1986). The bee 28S rDNA probe (GenBank acc. no. AJ302936) was obtained by PCR using the following primers: Forward 5'-TGCTACTA-CCACCAAGATCT-3' and Reverse 5'ACGAC-CTCACCTATTCTCA-3'.

The amplified fragments were purified using the Wizard SV Gel and PCR Clean-up System Kit (Promega Corporation, Madison, WI, USA) and the sequence identity was verified by cloning into the pGEM-T vector (Promega), and sequencing in an ABI 377 automatic sequencer (Applied Biosystems). Afterwards, the probes were labelled with biotin-16-dUTP (Roche, Germany) by another round of PCR (Boei et al., 1996). The labelled DNA probes were re-suspended in hybridization buffer (50% formamide, 2xSSC, 9% dextran sulfate, 40mM phosphate buffer) in the presence of competitor DNA, Cot-1 (Boehringer Ingelhein, Germany). The DNA probes were denatured at 70º C for 5 minutes, chilled on ice, and incubated for competition for 1 h at 37º C. Before the *In Situ* hydridization *per se*, the chromosome preparations were pretreated with RNAse (Boehringer Ingelhein, Germany) and pepsin (Sigma Chemical Co., St. Louis, USA) for 1 h and 15 minutes at 37º C, respectively. Denaturation of the chromosomes was achieved by adding 70% deionized formamide, 2xSSC, and 10mM phosphate (pH 7) to the slides, which were afterwards heated during 4 minutes in a 80ºC oven, and then dehydrated in ethanol series. The preannealed probes were mixed and placed on the slides and kept overnight at 37º C in order to allow the hybridization to the target DNA.

After hybridization the slides were washed in 50% formamide/2xSSC, pH 7, at 42º C, followed by washes in 0.1xSSC at 60º C and 4xSSC/ 0.05% Tween 20 for 5 minutes at room temperature. The slides were incubated with 5% natural non-fat dry milk for 15 minutes followed by washes in 4xSSC/0.05% Tween 20. Then, they were alternatively incubated with fluorescein isothiocyanate conjugated with avidin D (Sigma Chemical Co., St. Louis, USA), 3 times for 30 minutes each, and anti-avidin D antibodies (2 x 30 minute each), with 4xSSC/0.05% Tween 20 washes between incubations. Finally, the preparations were dehydrated, dried, and counterstained with propidium iodide (Sigma Chemical Co., St. Louis, USA) plus the antifade Vectashield (Vector Laboratories, Burlingame CA 94010, USA).

Results

The *P. lineatus* specimens from the Argentinean middle Paraná showed karyotypes composed of 54 metacentric and submetacentric chromosomes and a fundamental number equal to 108, with no apparent differences between males and

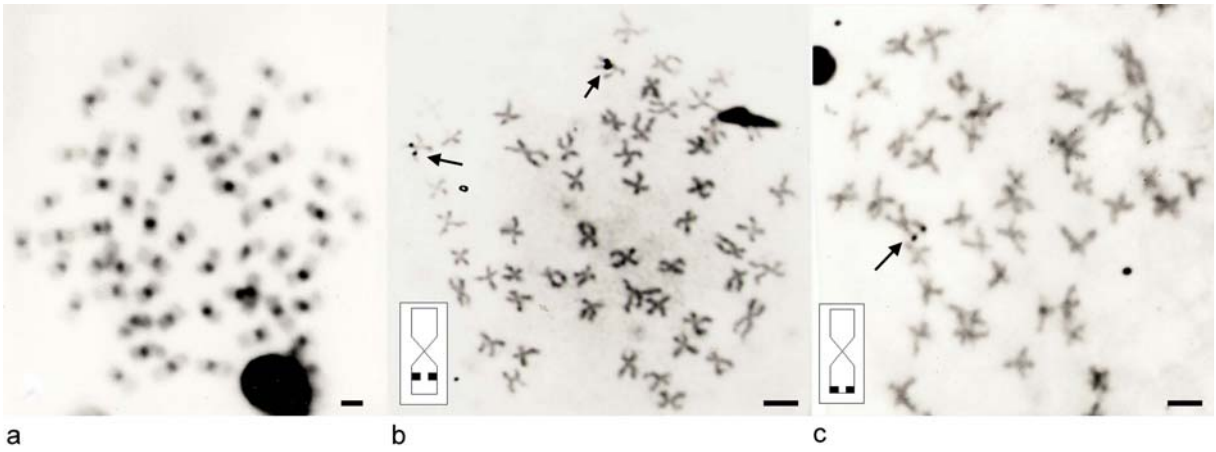

Fig. 2, a-c. *Prochilodus lineatus*, metaphases. **a** - constitutive heterochromatin distribution (C-banding). **b, c** - Ag-staining showing interstitial and telomeric NORs, respectively. Bar=5 µm.

females (Fig. 1). Supernumerary chromosomes were not observed in the 885 cells analyzed.

C-banding showed large heterochromatic pericentromeric segments and pale bands in the telomeric regions of some chromosomes (Fig. 2, a). The silver staining revealed the constant presence of only one NORs-bearing chromosome pair. In spite of this invariable condition, positional differences of the NORs were observed. Seventy percent of the specimens showed interstitial subtelomeric NORs (Fig. 2, b), while the remaining 30% demonstrated telomeric Ag-NOR-positive bands (Fig. 2, c). FISH with 28S rDNA specific probes indicated that the silver-positive regions correspond to the location of ribosomal genes and confirmed the positional variability of the NORs (Fig. 3).

Discussion

The members of the family Prochilodontidae show a wide geographic distribution and good aptitude to spread throughout the hydrographic system, constituting large populations that make seasonal migrations associated with reproductive and feeding habits (Vari, 1983). Cytogenetic studies have shown that prochilodontids have constant diploid numbers, with a conservative chromosome macrostructure and morphology (Bertollo et al., 1986).

The cytogenetic data obtained in the present study are in agreement with the previous results for Brazilian populations. The karyotypic macrostructure found in all *P. lineatus* specimens from the Santa Fe City region confirms the analysis by Pauls, Bertollo (1990), and a complement of 2n = 54 with 40 metacentrics and 14 submetacentric chromosomes. This typology of karyotype is shared by the Curimatidae, Parodontidae and Anastomidae, which, together with the Prochilodontidae, form a monophyletic group (Vari, 1983), suggesting that this chromosome number was probably present in a common ancestor (Venere, Galetti, 1989).

However, despite this conservatism, the speciation process within this family has been accompanied by microstructural rearrangements, as evidenced by species-specific patterns of heterochromatic and NOR bands, which are important cytotaxonomic markers.

Former studies on C-banding showed that most *Prochilodus* species have similar patterns of heterochromatin distribution, usually pericentromeric. The karyotype of our *P. lineatus* population displayed a large number of C-positive bands,

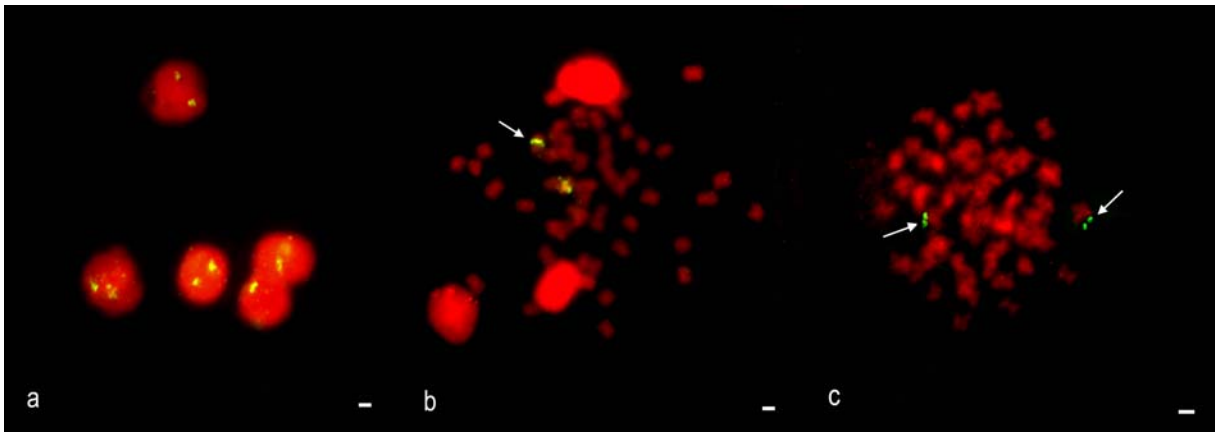

Fig. 3, a-c. Chromosomes of *Prochilodus lineatus* after Fluorescence *In Situ* Hybridization with a 28S rDNA probe. **a** - two fluorescent signals per nucleus indicating a single pair of NORs-bearing chromosomes. **b, c** - NOR positional polymorphism, with interstitial and telomeric bands, respectively. Bar=5 µm.

mainly in the centromeric and pericentromeric regions of almost all chromosomes; in some cases we observed the presence of pale constitutive hetochromatin in telomeric regions. This distribution is comparable with the previously described for *P. cearensis* (Steindachner, 1911), *P. argenteus* (Spix, Agassiz, 1829), *P. nigricans* (Spix, Agassiz, 1829), *P. affinis* (Reinhardt, 1874), *P. marggravii* (Walbaum, 1792) (Pauls, Bertollo, 1990), *P. mariae* (Eigenmann, 1922) (Oliveira et al., 2003), and for *P. lineatus* (Valenciennes, 1847) (Maistro et al., 2000). However, the pattern of C-bands of the last showed that the long arm of chromosome pair number 5 was entirely heterochromatic, which was not observed in the Argentinean population.

In addition, a single NORs-bearing chromosome pair (generally, number 2), seems to be the most widespread condition within the Prochilodontidae (Pauls, Bertollo, 1990). In our population, silver impregnation showed the conspicuous presence of NORs, localized within the long arm of a big metacentric pair (which, based on its size, might also be the second), not associated with C-positive heterochromatin. However, in contrast to the previous studies, where the NORs are localized in an interstitial band, our analyses revealed an unambiguous positional polymorphism. While the majority of individuals showed interstitial bands, the other 30% showed terminal NORs.

A single additional terminal Ag-NOR site has already been reported in the genus *Prochilodus*. *P. margravii* and *P. afinis* showed a not yet identified chromosome that occasionally displays an active NOR on its telomeric region (Pauls, Bertollo, 1990). A third sporadic metacentric chromosome bearing active NORs has also been described in *P. argentus* (Hatanaka, Galetti, 2004). Since silver nitrate staining allows the detection of only NOR sites that were active in the precedent mitotic interphase, pointing to the possibility of extra rDNA clusters, we decided to examine the observed location variability by means of FISH, that identifies rDNA irrespectively of its transcriptional activity. Our FISH experiments with a 28S probe confirmed the presence of only one NOR-bearing chromosome pair and allowed us to explain the variation in NOR location (interstitial and terminal) by structural chromosome changes (probably an inversion event) rather than by differences in transcriptional activity of the rDNA loci.

In some cases, a transcriptional difference,

evidenced by polymorphic NOR size, has been described for several *Prochilodus* species (Oliveira et al., 2003), which is a common event among fish (Almeida-Toledo, Foresti, 1985) and has been explained by illegitimate recombination and/or transposition events. Contrarily, our analysis did not show such size variation and despite the sympatric presence of both NOR localizations, we were unable to identify heterozygous individuals.

Another remarkable feature of species belonging to the genus *Prochilododus* is the extensive presence of small supernumerary chromosomes. In general, the B-chromosomes appear in numbers that vary from 0 to 7 (Dias et al., 1998), are heterochromatic (Pauls, Bertollo, 1983) and mitotically unstable, showing interinvidual and interpopulational variations (Oliveira et al., 2003). However, none of the 45 individuals collected at the Santa Fe region showed supernumerary chromosomes, contrasting with all the previous data for Brazilian populations.

It is well known that natural geographic barriers and hydroelectric plants create obstacles for the dispersion of migrant freshwater species, affecting directly their survival and reproduction. The Brazilian and Argentinean populations, within the Paraná River basin, are separated by several barriers, such as the Itaipú and Yacyretá dams, pointing to the possibility of impaired migration followed by genetic variation and allelic frequency changes. Although these barriers originated recently, they probably play important role in the isolation of these populations and subsequent cytogenetic changes, evidenced by observed NOR positional polymorphism, C-band pattern and absence of B-chomosomes in the specimens collected in Argentinean waters.

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