



Cytogenetic characterization of a population of *Bryconamericus aff. iheringii* (Characidae, Tetragonopterinae)

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Abstract

A cytogenetic analysis of thirteen specimens of *Bryconamericus aff. iheringii* revealed a diploid number of 52 chromosomes and a karyotype of 8M+22SM+10ST+12A with a fundamental number (FN) of 92. The nucleolus organizer regions (NORs) were studied by means of AgNO₃ and CMA₃ staining as well as by FISH using an 18S rDNA probe. NORs were found to be located at the terminal position on the short arm of the submetacentric chromosome pair. C-banding showed strong telomeric and centromeric staining in the majority of the chromosomes, and a similar pattern was observed after treatment with *AluI* restriction enzyme

Key words: AgNOR, *Alu I*, *Bryconamericus*, C-banding, CMA₃, rDNA 18S.

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Introduction

The Characidae family is the largest group of fresh water fish in South America, containing about 170 genera and 885 species (Nelson, 1994). The large number of species that comprise the Characidae family can be grouped into different sub-families. The Tetragonopterinae sub-family in Brazil contains the most species and consists of approximately 50 genera and 400 to 500 species. Most of these fish are small in size and live in various environments. They are mostly omnivorous and also serve as food for other fish (Britski *et al.*, 1988).

The *Bryconamericus* genus is one of the least diversified in the Tetragonopterinae subfamily, consisting of 30 to 40 species that are very similar and distributed among different regions of South and Central America (Géry, 1997).

Although there have been few cytogenetic studies on this group of fish, most of the species examined have been found to have a diploid number of 52 chromosomes (Portela *et al.*, 1988; Wasko and Galetti, 1999). The variable position of nucleolus organizer regions (NORs) has been used to characterize *Bryconamericus aff. iheringii* as a group that has multiple NORs (Wasko *et al.*, 1996; Wasko and Galetti, 1999; Portela-Castro, 1999).

The objective of this study was the cytogenetic characterization of a population of *Bryconamericus aff. iheringii*, using different treatments (AgNOR, fluorochrome chromomycin A₃ and fluorescent *in situ* hybridization)

to detect the NORs and to locate heterochromatin by C banding and treatment with *AluI* restriction enzyme.

Material and Methods

Thirteen specimens of *Bryconamericus aff. iheringii* (8 males, 4 females and 1 undetermined sex) were analyzed collected from the Água da Floresta river, part of the Tibagi river basin (Paraná, Brazil), from August 1999 to February 2001. Mitotic chromosome preparations were obtained from kidney cells, according Bertollo *et al.* (1978). The nucleolus organizer regions (NORs) were analysed by the Howell and Black technique (1980), and the treatment with fluorochrome chromomycin A₃ followed the Schmid technique (1980). C-banding was obtained using the method described by Sumner (1972), and the standard reactions by restriction endonuclease *AluI* were performed using the method described by Mezzanotte *et al.* (1983), with some modifications as proposed by Maistro (1996). Chromosome morphology was determined on the basis of arm ratio as proposed by Levan *et al.* (1964). Chromosomes classified as metacentric (M), submetacentric (SM) and subtelocentric (ST) were considered as biarmed. Acrocentric (A) chromosomes were considered as uniarmed.

The 18S rDNA segment containing 1700 bp of the fish *Oreochromis niloticus* was used for *in situ* hybridization and labeled with biotin-14-dATP by nick translation (Gibco cat n. 18247-015), according to the manufacturer's instructions. The hybridization technique, post-hybridization washes and visualization were carried out in a

manner slightly modified from that reported by Heslop-Harrison *et al.* (1991) and Cuadrado and Jouve (1994). The slides were incubated with RNase A (100 µg/mL in 2xSSC) in a moist chamber at 37 °C, afterwards washed with 2xSSC and 4% paraformaldehyde at room temperature and dehydrated in ethanol series. The hybridization mixture was prepared with 50% formamide, 20xSSC, 50% sulphate dextrane, 10% SDS, 1 mg/mL Bovine Calf Thymus DNA and approximately 100 ng probe. 30 µL of mixture was dropped per slide. The probe was predenatured at 70 °C for 10 min and further put in ice for 5 min. The chromosomes and probe were denatured at 90 °C for 10 min, 48 °C for 10 min, 38 °C for 10 min, using a thermal cycler (PTC 100 MJ Research) and further hybridized overnight at 37 °C in a moist chamber. Post-hybridization washes were carried out at 42 °C in a shaker (2xSSC, 20% formamide/0.1xSSC, 0.1xSSC, 2xSSC, 4xSSC / 0.2% Tween 20) for 5 min each. Hybridization was detected with avidin-FITC conjugate. The chromosomes were counterstained with 1 µL (50 µg/mL) propidium iodide, and slides were mounted in 25 µL Vectashield antifade H1000 (Vector). Chromosomal images were obtained with Fuji color film, ISO 100.

Results and Discussion

The *Bryconamericus aff. iheringii* samples studied had a diploid number of 52 chromosomes distributed as 8M+22SM+10ST+12A with a fundamental number (FN) of 92, for both males and females (Figure 1). The same diploid number had also been found in the River Keller *Bryconamericus aff. iheringii* population from the River Ivaí basin (PR) (Portela-Castro, 1999). However, three different cytotypes were detected in the latter population:

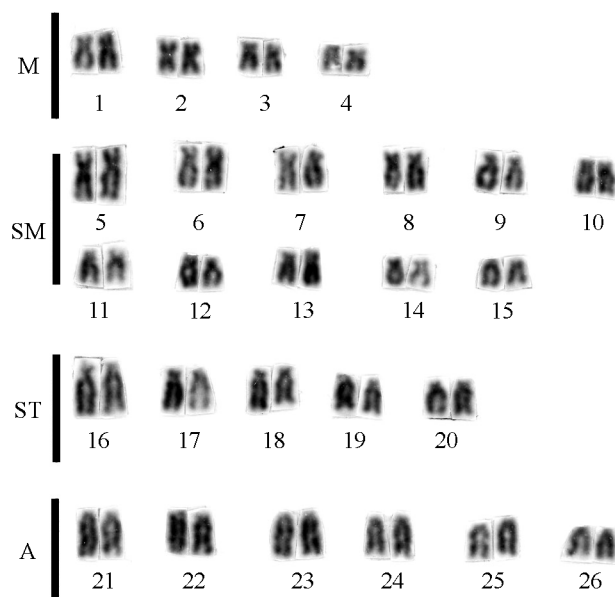


Figure 1 - Giemsa-stained karyotype of *Bryconamericus aff. iheringii*.

cytotype 1 with 12M+18SM+8ST+14A, cytotype 2 with 10M+22SM+8ST+12A and cytotype 3 with 8M+28SM+6ST+10A. Portela-Castro (1999) suggested that these variations may have been the result of pericentric inversion and that perhaps these cytotypes corresponded to different species of the *Bryconamericus* genus. The same author also observed $2n = 52$ in two other *Bryconamericus* species from the River Ivaí basin (PR) with different karyotypes: *Bryconamericus* spp. 1 with 4M+14SM+10ST+24A and *Bryconamericus* spp. 2 with 8M+20SM+8ST+16A.

Portela *et al.* (1988) found $2n = 52$ in *Bryconamericus stramineus* distributed as 26M-SM+26ST-A. Wasko and Galetti Jr. (1998) studied five *Bryconamericus* species from three Brazilian river basins, which all had $2n = 52$ chromosomes, except *Bryconamericus* spp. E from the Avoadeiras Stream (MT) with $2n = 54$. The chromosome formulae found for these species were also different, and the fundamental numbers varied from 88 to 102.

Although the diploid number of 52 chromosomes is characteristic of *Bryconamericus*, as previously described, the karyotypes for the different species in this genus vary, suggesting that chromosome rearrangements may be involved in the karyotypic evolution of these groups of fish. The karyotypic structure of the River Agua da Floresta population (8M+22SM+10ST+12A) is very similar to the *Bryconamericus aff. iheringii* cytotype 2 from the River Keller, River Ivaí basin (PR), differing only in the number of metacentric and subtelocentric chromosomes, which

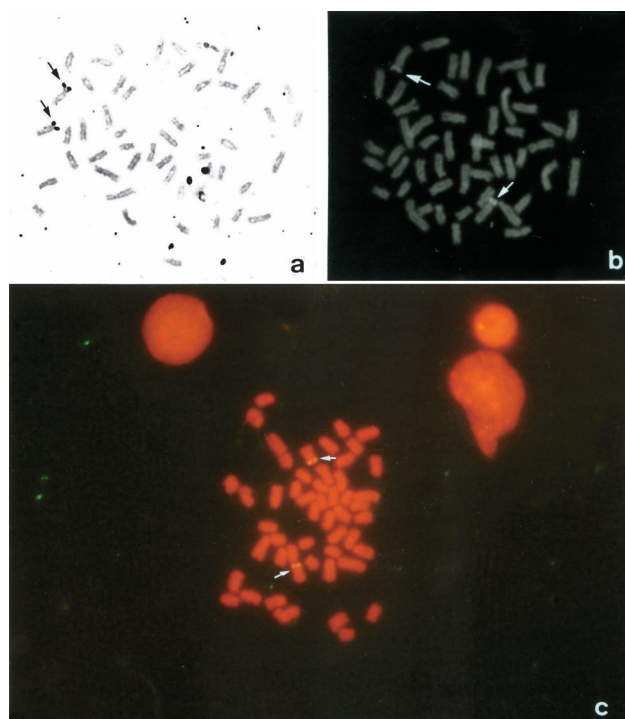


Figure 2 - Differentially stained metaphases of *Bryconamericus aff. iheringii*. (a) AgNOR staining, (b) CMA₃ staining, (c) Fluorescence in situ hybridization with biotin-labeled 18S rDNA probe. The arrows show the nucleolar chromosomal pair.

also suggests that there may have been chromosome rearrangements, probably pericentric inversions, responsible for these differences between the two populations.

NORs were identified in the telomeric region on the short arm of a pair of large-sized submetacentric chromosomes in *Bryconamericus* aff. *iheringii* from the River Água da Floresta (PR) (Figure 2a). All studies carried out to date on the *Bryconamericus* genus have shown multiple NORs for this group. Wasko and Galetti Jr. (1999) observed nine phenotypes, based on the number and localization of NORs in four *Bryconamericus* species examined, two populations from the River Piracicaba (SP), one population from the Três Bocas Stream (PR) and another from the Avoadeiras Stream (MT). Portela-Castro (1999) studied three species from the River Ivaí basin (PR) and also observed a variation of 2 to 4 nucleolar chromosomes; in spite of this inter- and intraindividual variability, the presence of one pair of large submetacentric chromosomes was relatively constant in two species, *Bryconamericus* spp. 2 and *Bryconamericus* aff. *iheringii* (Cytotypes 1 and 2).

Thus, the *Bryconamericus* aff. *iheringii* population investigated in this study is the first of the *Bryconamericus* genus found to have simple NORs. According to Hsu *et al.* (1975), species with a single pair of NORs could be considered ancestors of related species whose NORs are distributed among several chromosomes. Therefore, regarding this trait, the *Bryconamericus* populations studied up to now could be considered more recent compared to the *Bryconamericus* aff. *iheringii* population of the River Água da Floresta. In addition, the presence of a pair of large submetacentric chromosomes, as observed in some species from the River Ivaí basin, including *Bryconamericus* aff. *iheringii*, would support this hypothesis.

Other genera of the Tetragonopterinae sub-family such as *Moenkhausia* (Portela, Galetti and Bertollo, 1988), *Tetragonopterus* and *Gymnocorymbus* (Alberdi and Fenocchio, 1997) also show simple NORs. However, the multiplicity of NORs is fairly frequent in other genera of this subfamily, mainly *Astyanax* (Morelli *et al.* 1983; Mizoguchi and Martins-Santos, 1998; Maistro *et al.*, 2000a, among others).

Treatment with the fluorochrome chromomycin A₃ was also used to show NORs because these regions are associated with GC-rich DNA families in some fish species (Pendás *et al.*, 1993). *B.* aff. *iheringii* chromosomes studied with this fluorochrome showed agreement between the AgNORs and CMA₃ sites detected (Figure 2b), indicating that the NORs in this species are rich in GC. Portela-Castro (1999) also used CMA₃ fluorochrome in different *Bryconamericus* species and observed sites corresponding to AgNOR sites. Wasko (1996) observed in all the species studied that most of the chromosomes labeled with mitramicine also had AgNORs, except for *Bryconamericus* spp. E.

Fluorescent *in situ* hybridization was used with an 18S rDNA probe to indicate the location of the ribosomal genes with greater precision. In the metaphases analyzed, the signals appeared on the short arm of a pair of submetacentric chromosomes (Figure 3a), corresponding to results with silver nitrate and CMA₃. This is the first study on the *Bryconamericus* genus where FISH was used to identify the ribosome cistrons. Marco-Ferro *et al.* (2001) used 18S and 5S rDNA probes on the Tetragonopterinae sub-family to characterize four *Astyanax scabripinnis* populations. A probable differential activation of the NORs among the populations was observed with the 18S probe and AgNORs, and the 5S probe showed 8 sites in all the populations. This technique has also been used recently in other groups of fish to better characterize NORs, for example in *Pirinampus pirinampu* (Swarça *et al.*, 1999) and *Zungaro zungaro* (Swarça *et al.* 2001b) of the Pimelodidae family, where the 18s rDNA probe showed heteromorphisms the size of NORs between homologous chromosomes.

C banding showed a heterochromatic block in the centromeric and telomeric regions in most of the *B.* aff. *iheringii* chromosomes. The 16, 17, 18, 19 and 20 pairs, all subtelocentric, displayed stronger staining in the telomeric regions (Figure 3A), and can be considered heterochromatic markers in this species. The distribution of heterochromatin is variable in the *Bryconamericus* genus. Wasko and Galetti Jr. (1998) observed heterochromatic blocks in the interstitial, telomeric and centromeric regions in five *Bryconamericus* spp. populations, and one of them had at least four entirely heterochromatic arms from different chromosomes. In *Bryconamericus* aff. *iheringii*, cytotypes 1 and 2, and *Bryconamericus* spp. 2 from the River Ivaí basin (PR) investigated by Portela-Castro (1999), the heterochromatic blocks are restricted to the centromeric regions of practically all the chromosomes and also on the short arms of pairs corresponding to those with NOR staining.

Treatment with restriction enzymes has been used to help in chromosome classification for better karyotype determination and also in the study of chromosome polymorphisms. The *AluI* restriction enzyme recognizes and cleaves specific DNA (AG/CT) sequences. The heterochromatic regions resistant to the action of the *AluI* represent regions that have a low frequency of DNA sequences recognized by this enzyme. The treatment with *AluI* did not digest the heterochromatic regions of the subtelocentric marker pairs 16, 17, 18, 19 and 20 (Figure 3B) in *Bryconamericus* aff. *iheringii* from the River Água da Floresta (PR), indicating that these regions do not possess blocks with sequences rich in AG/CT. However, in the centromeric regions of the 1, 2, 3 and 4 pairs, all metacentric, which are C+ banded, were cleaved by *AluI*, showing that the heterochromatin of these regions had a different composition from that found in the telomeric re-

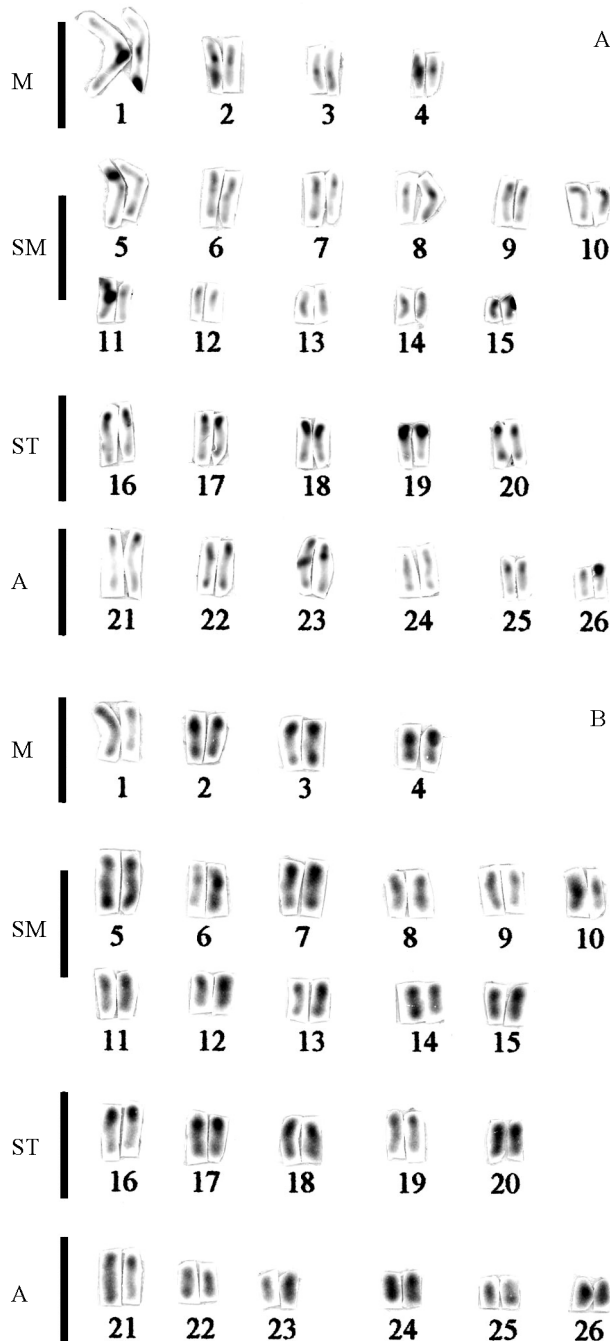


Figure 3 - C-banded karyotype (A) and restriction enzyme *AluI*-treated karyotype (B) of *Bryconamericus* aff. *iheringii*.

gions of the 16, 17, 18, 19 and 20 pairs. Therefore, the treatment with *AluI* enzyme associated with C banding allowed the identification of different types of heterochromatin in the chromosome structure of this *Bryconamericus* aff. *iheringii* population.

This is the first study to examine the effect of a restriction enzyme, namely *AluI*, on the cytogenetic study of *Bryconamericus*, and it would be interesting to use this treatment in other species of this genus, for better character-

ization of the heterochromatic regions of the chromosomes in these fish.

Souza *et al.*, (1996) observed different types of heterochromatin in *Astyanax scabripinnis*, with the use of different chromosome banding techniques such as C banding, CMA₃ fluorochrome, mitramicine and DAPI. *AluI* has also produced a linear differentiation in other groups of fish, similar to that with C banding (Abuín *et al.*, 1994; Bouza, *et al.*, 1994; Maistro *et al.*, 2000b; Swarça *et al.*, 2001a, among others).

The data provided here permit a fairly specific characterization of *Bryconamericus* aff. *iheringii* from the River Água da Floresta (PR). This population was shown by different types of chromosome banding to be more conservative compared to the other species studied by other authors, since variation in this population in regard to karyotype or even in the NOR number and localization, which are so common in other *Bryconamericus* species, was not observed.

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