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Research Article

Chromosome divergence and NOR polymorphism in *Bryconamericus* aff. *iheringii* (Teleostei, Characidae) in the hydrographic systems of the Paranapanema and Ivaí Rivers, Paraná, Brazil

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Abstract

Cytogenetic studies were carried out in three populations of *Bryconamericus* aff. *iheringii* from two hydrographic systems of the Paranapanema and Ivaí Rivers, separated by a watershed, both belonging to the upper Paraná River basin. Specimens had a constant diploid number 2n = 52 chromosomes. However, three karyotype formulae were identified in the three populations: *B.* aff. *iheringii* from the Maringá stream had 12M+18SM+8ST+14A (FN = 90); specimens from Keller River showed 8M+28SM+6ST+10A (FN = 94) and specimens from the Tatupeba stream had 8M+20SM+8ST+16A (FN = 88). Nucleolar organizer regions (NORs) were identified by silver nitrate staining and fluorescent *in situ* hybridization (FISH) with an 18S rDNA probe. Specimens from Tatupeba stream had a simple NOR system located in a terminal position of the short arm of a pair of large submetacentric chromosomes. Ag-NOR and FISH methodologies revealed multiple NORs in specimens of the Maringá stream and Keller River. Differences in chromosome structure and in NOR patterns in the three populations of *B.* aff. *iheringii* revealed fixed evolutionary chromosome divergence. Aspects related to karyotypic variations and to geographic isolation of these populations are discussed.

Key words: Bryconamericus, Characid fish, chromosome divergence, fluorescent *in situ* hybridization, NOR polymorphism.

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Introduction

An important characteristic of nucleolar organizer regions (NORs) in fish is its inter- and intra-species polymorphism. NOR characterization can be a cytogenetic marker of for cytotaxonomic studies and can even aid in constructing phylogenetic hypotheses (cytosystematics) for several fish groups (Amemyia and Gold, 1988; Galetti Jr, 1998; Almeida-Toledo, 2000). Some fish groups present a simple NOR system characterized by ribosomal cistrons on only one chromosome pair, whereas others have a multiple NOR system composed of cistrons dispersed over several chromosomes (Galetti Jr., 1998).

Small characids are characterized by extensive heterogeneity with regard to NOR patterns. A simple NOR system was identified in the species *Gymnocorymbus ternetzi* (Alberdi and Fenocchio, 1997), *Tetragonopterus argenteus* (Alberdi and Fenocchio, 1997), *Moenkhausia intermedia* (Portela *et al.*, 1988; Portela-Castro and Julio Júnior, 2002) and *Moenkhausia costae* (Portela *et al.*, 1988). In others characids, occurrence of a multiple NOR system is common, as has been observed in the genus *Astyanax*, chiefly in the *Astyanax scabripinnis* complex (*e.g.*, Maistro *et al.*, 1998; Mizoguchi and Martins-Santos, 1998; Mantovani *et al.*, 2000; Marco-Ferro *et al.*, 2001; Souza *et al.*, 2001; Kavalco and Moreira-Filho, 2003; Mantovani *et al.*, 2005). Both conditions have been reported in some cases: specimens of *Moenkhausia sanctaefilomenae* from the Tietê River (SP, Brazil) analyzed by Foresti *et al.* (1989) had a multiple NOR system, whereas *Moenkhausia sanctaefilomenae* from the Paraná River (PR, Brazil) analyzed by Portela-Castro and Júlio Jr. (2002) exhibited a simple NOR pattern. Differentiation in the localization of 18S and 5S ribosomal sites was detected between two *Hyphessobrycon anisitsi* populations which presented similar karyotype in number and formulae (Centofante *et al.*, 2003).

Bryconamericus is one of the 88 genera listed as "Incertae sedis" group of the Characidae family with 51 valid species (Lima *et al.*, 2003). Actual diversity in the ge-

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nus *Bryconamericus* is unknown and its systematics is unresolved. The genus comprises small-sized species, not more than 10 cm in length, that are distributed throughout several continental aquatic ecosystems in South and Central America (Vari and Siebert, 1990).

Chromosome analyses of the genus *Bryconamericus* are rare and the diploid number of the species studied up to the moment is restricted to 2n = 52 (Portela *et al.*, 1988; Wasko *et al.*, 1996; Wasko and Galetti Jr., 1998; Paintner-Marques *et al.*, 2002a, 2003), although great diversity in chromosome structure has been revealed.

In the current study three populations of *Bryconamericus* aff. *iheringii* from two hydrographic systems of the Paranapanema and Ivaí River basins, separated by a watershed were studied. Karyotypes were analyzed with emphasis on the identification of NORs by silver nitrate staining ($AgNO_3$) and fluorescent *in situ* hybridization (FISH) with 18S rDNA probes.

Material and Methods

Cytogenetic studies were carried out in three populations of *Bryconamericus* aff. *iheringii* from two hydrographic system of the upper Paraná River basin in the state of Paraná, Brazil (Figure 1): the Maringá stream belongs to the Paranapanema River basin; Keller River and the Tatupeba stream belong to the Ivaí River basin. Amongst the 54 specimens analyzed, 21 (8 males and 13 females) were collected from the Tatupeba stream; 16 specimens (5 males and 11 females) were collected from Maringá stream and 17 specimens (10 males and 7 females) were collected from Keller River. Mitotic metaphases were obtained from kidney cells by air-drying, as described by Bertollo *et al.* (1978). NORs were identified by silver nitrate ($AgNO_3$) following the Howell and Black (1980) method, and by fluorescent *in situ* hybridization (FISH) method with 18S rDNA probes. Two types of probes were used to detect 18S rDNA segments in FISH analysis: (1) genomic DNA of *Astyanax scabripinnis* amplified by PCR using the primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS8 (5'-TCCGCAGGTTCACCTACGGA-3'), recommended by White *et al.* (1990); (2) amplified and cloned fragments of *Oreochromis niloticus* (kindly provided by Dr. Cesar Martins of the Universidade Estadual Paulista, Botucatu, SP, Brazil). The probes were labeled with biotin 14-dATP by nick translation (Bio Nick Labeling System- Gibco, BRL). The FISH protocol followed the methods of Heslop-Harrison *et al.* (1991) and Cuadrado and Jouve (1994).

Results

Bryconamericus aff. *iheringii* specimens had a constant diploid number of 2n = 52 chromosomes, however, three karyotype formulae were identified. No chromosome differences were found between the sexes.

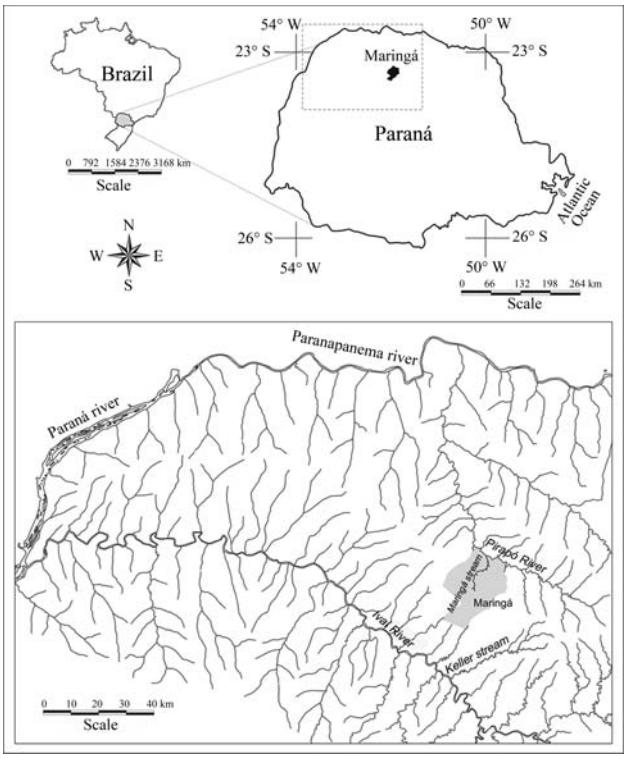

Figure 1 - Hydrographic map showing the draining basins of the Paranapanema and Ivaí Rivers, with collection sites of *Bryconamericus* aff. *iheringii*.

Bryconamericus aff. *iheringii* population of the Maringá stream (Cytotype I)

This karyotype is composed of 12M+18SM+8ST+14A with fundamental number (FN) of 90, (Figure 2a). Silver nitrate-stained metaphases (Ag-NOR) showed terminal labeling on the short arm of two to four chromosomes, with inter- and intra-individual variation in signal numbers (Figure 3a). By FISH we detected six fluorescent signals of which four corresponded to Ag-NOR chromosomes (Figure 3b). Four fluorescent signals on the telomeres of the short arm of the submetacentric pairs 7 and 10 showed greater intensity, whereas a low intensity fluorescent spot was detected on the telomere of the short arm of submetacentric pair 14. A size heteromorphism occurred on pair 7.

Population of *Bryconamericus* aff. *iheringii* from Keller River (Cytotype II)

This karyotype is composed of 8M+28SM+6ST+10A and FN = 94, (Figure 2b). Silver nitrate staining revealed terminal labels in the short arm of two to four chromosomes (Figure 3c), with inter- and intra-individual numerical variations. Ag-NORs sites were eventually detected in the telomere of the long arm of one of the homologs of the large subtelocentric pair. FISH revealed ten ribosomal sites that included Ag-NOR markings (Figure 3d). Intense fluorescent signals were detected in the short

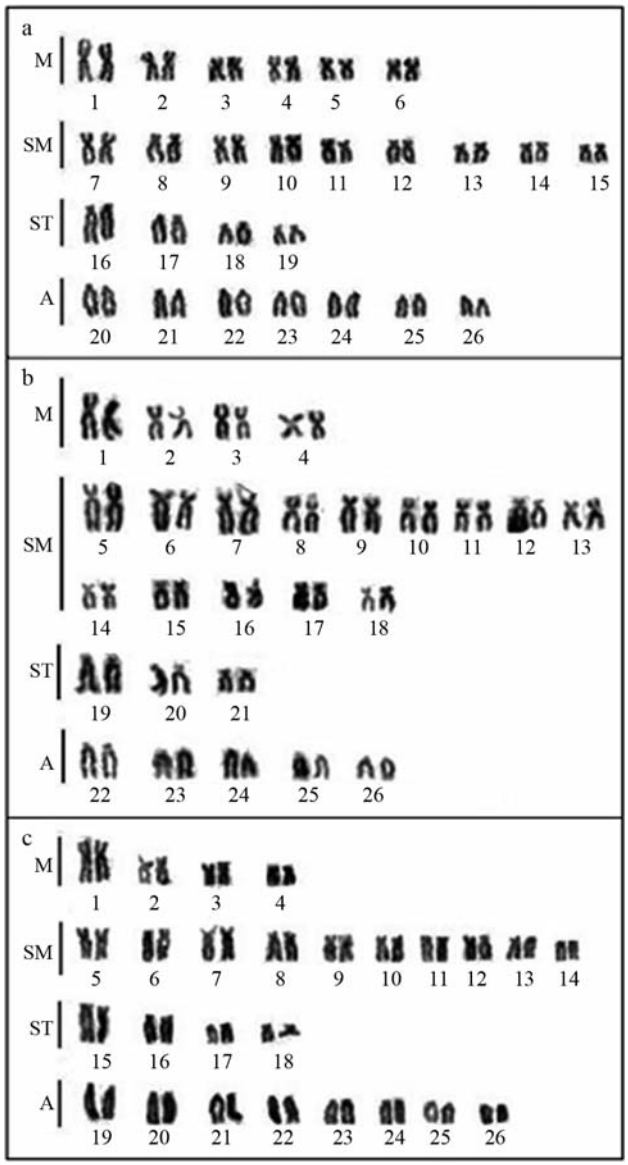

Figure 2 - Giemsa-stained karyotypes of *Bryconamericus* aff *iheringii* (a) cytotype I, population from Maringá stream; (b) cytotype II, population from Keller River; (c) cytotype III, population from Tatupeba stream.

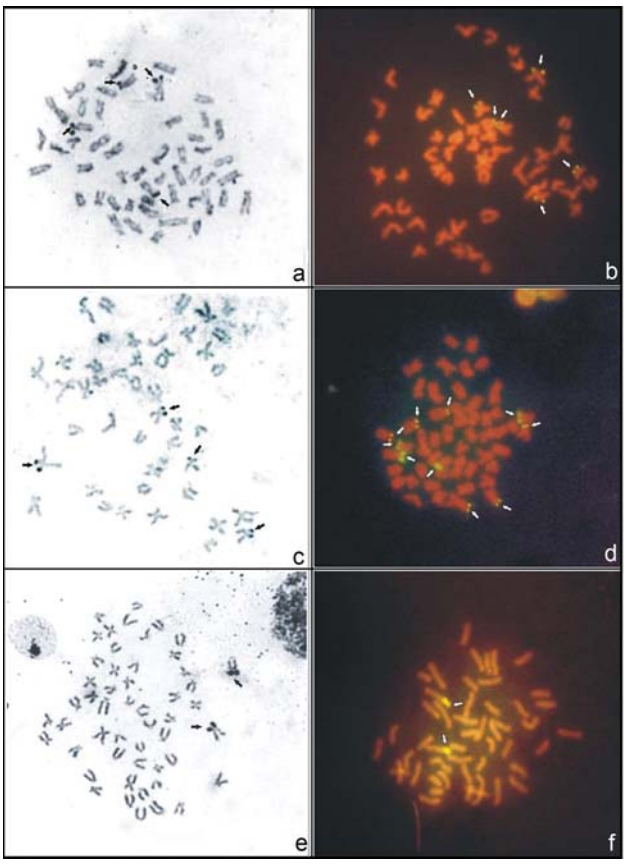

Figure 3 - Metaphases of *Bryconamericus* aff *iheringii* specimens showing Ag-NOR – bearing chromosomes (a, c and e) and fluorescent *in situ* hybridization (FISH) using a 18S rDNA probe (b, d and f). The arrows indicate: (a) four Ag-NOR chromosomes and (b) six 18S rDNA sites in the population from Maringá stream; (c) four Ag-NOR chromosomes and (d) ten 18S rDNA sites in the population from Keller River; (e) two Ag-NOR chromosomes and (f) two 18S rDNA sites in the population from Tatubepa stream.

arm of submetacentric telomeres (pairs 7 and 8). In addition to the above chromosomes, smaller signs were visible in six more chromosomes which included the Ag-NOR region in a subtelocentric chromosome. Size heteromorphism was detected in chromosome pair 7.

Population of *Bryconamericus* aff. *iheringii* from Tatupeba stream (Cytotype III)

The karyotype structure of these specimens consists of 8M+20SM+8ST+16A, FN = 88 (Figure 2c). Silver nitrate-stained metaphases showed only one pair of NOR-bearing submetacentric chromosomes (n. 7) with labeling on the short arm, coinciding with a secondary constriction (Figure 3e). Fluorescent signals confirmed the Ag-NOR pair by FISH. This chromosome pair also had a NOR-size heteromorphism (Figure 3f).

Discussion

Although the diploid number of 2n = 52 chromosomes is the most frequent one in the genus *Bryconamericus*, karyotype formulae are variable even at the intra-specific level. Structural chromosome diversity is corroborated by results of the current analysis. Paintner-Marques *et al.* (2003) reported 2n = 52 chromosomes in *B.* aff. *iheringii* specimens from the Água da Floresta River (Tibagi River basin, Paraná) distributed as 8M+22SM+10ST+12A (FN = 92). The three karyotype formulae obtained in the present study and the karyotype reported by Paintner-Marques *et al.* (2003) suggest the occurrence of fixed extensive evolutionary chromosome diversification in this species. The fundamental number in this genus ranges from 84 to 102 (Portela *et al.*, 1988; Wasko *et al.*, 1996; Wasko and Galetti Jr., 1998; Paintner-Marques *et al.*, 2002a, 2003). Differences in karyotype structure have been

evidenced mainly as divergence in acrocentric chromosomes number. Variation in the fundamental number (FN) may be the result of chromosome rearrangements of the pericentric inversion type which are considered to be the main mechanism of karyotype evolution in this fish group (Wasko and Galetti Jr., 1998).

Chromosome banding in *Bryconamericus* species has contributed towards a better understanding of the structural chromosome diversity of the group. *Bryconamericus* showed extensive variability of NORs (Wasko and Galetti Jr., 1999; Paintner-Marques *et al.*, 2002b), and each species could be characterized by a specific C-banding pattern (Wasko and Galletti Jr., 1998).

A multiple NOR system is the most frequent condition in the genus *Bryconamericus*. Although the two populations of *B.* aff. *iheringii* from the Maringá stream and Keller River have multiple ribosomal sites, they differ in the number of NOR-bearing chromosomes, as revealed by the FISH technique. Chromosome rearrangements, such as transposition and/or translocations resulting in dispersion of ribosomal genes, seem to occur in several fish species (Galetti Jr *et al.*, 1995; Castro *et al.*, 1996; Mantovani *et al.*, 2000). These mechanisms may explain NOR variation in each isolated population of *B.* aff. *iheringii*. Silver nitrate-stained chromosomes (Ag-NOR) in populations of *B*. aff. *iheringii* with multiple NORs (Maringá stream and Keller River) are less than the number of FISH-identified ribosomal sites. This variation shows that not all ribosomal sites (Ag-NOR) are active. A similar condition has been reported in *Bryconamericus* aff. *exodon*, where 2 to 5 Ag-NOR sites and eight 18S rDNA sites were detected (Paintner-Marques *et al.*, 2002b). The FISH approach using an 18S rDNA probe was extremely important for the distinction between cytotypes I and II with regard to numbers of structural NORs. Additionally, both methodologies revealed a simple NOR pattern in the Tatupeba stream *B*. aff. *iheringii* population. Although a single nucleolar pair is an uncommon condition in the genus, results are in accordance with the simple NOR system reported in *B*. aff. *iheringii* of the Tibagi River basin analyzed by Paintner-Marques *et al.* (2003).

The three populations analyzed have a common NOR phenotype, or rather a pair of large submetacentric chromosomes is always labeled. This submetacentric pair in the three studied populations also showed a more intense fluorescent signal, suggesting that it contains a larger number of rDNA gene copies. These chromosomes may be considered as the main nucleolus organizer and probably contain a cytogenetic marker preserved in this species. Wasko and Galetti Jr. (1999) detected up to 9 Ag-NOR phenotypes in four *Bryconamericus* species and registered the occurrence of a NOR phenotype (NOR in the short arm of a medium acrocentric chromosome) in three species, indicating that this pair may be an ancient NOR feature among these fishes.

The karyotype divergence and NOR polymorphism detected between the populations of *B.* aff. *iheringii* suggests that their geographic isolation could favor the fixation of chromosomal rearrangements that probably occurred during karyotype evolution of the genus *Bryconamericus.* Differential selective pressures in each environment may have been decisive for karyotype differentiation and may have produced the detected chromosome diversification. The above hypothesis is based on the biological characteristics of small characids, which is a group that comprises species with high levels of endemism and fast speciation rates (Böhlke *et al.*, 1978).

According to Silva (2004), *Bryconamericus* is a polyphyletic genus with many groups of species. Based mainly on the position and shape of maxillary teeth, this author recognized three groups of *Bryconamericus* species in South America; the groups *exodon*, *microcephalus* and *iheringii*. The latter includes all species found in the southern region of South America. The occurrence of different *B.* aff. *iheringii* cytotypes requires a detailed taxonomic evaluation of this species, and cytogenetic data can be important tools in their identification.

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