B chromosome and NORs polymorphism in *Callichthys callichthys* (Linnaeus, 1758) (Siluriformes: Callichthyidae) from upper Paraná River, Brazil

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**Introduction**

B chromosomes are extra chromosomes from the normal chromosomal set that follow their own evolutionary pathway (Camacho et al., 2000). Many studies concerning B chromosomes seek to clarify the molecular organization, ways of transmission (Camacho et al., 2000; Jesus et al., 2003; Jones & Houben, 2003), origin and evolution of these chromosomes (Camacho et al., 2000; Mestriner et al., 2000; Jesus et al., 2003; Poletto et al., 2010); however it is not always possible to precisely determine their origin (Jamilena et al., 1994; Camacho et al., 2000).

In their differentiation process, B chromosomes would develop biologically meaningful but not essential functions (Mião et al., 1991; Plowman & Bougourd, 1994), being considered selfish chromosomes, genomic parasites or accessories (Jones & Houben, 2003; Poletto et al., 2010). Some authors suggest a correlation between their presence and environmental factors (Néo et al., 2000), or the possibility that these chromosomes convert into a reservoir of genetic
variability, showing an evolutionary role (Rejón et al., 1987).

These additional chromosomes were found in different organisms, like in insects (Amos & Dover, 1981), plants (Jones & Houben, 2003), fungi (Mião et al., 1991), amphibians (Sharbel et al., 1998; Green, 2004), birds (Pigozzi & Solari, 1998) and fishes. In fishes, they have been already described in Characiformes (Mizoguchi & Martins-Santos, 1997; Voltolin et al., 2010), Labriformes (cited as Perciformes, Roncatti et al., 2007; Poletto et al., 2010), Siluriformes (Oliveira et al., 1993; Shimabukuro-Dias et al., 2005; Blanco et al., 2012), Gymnotiformes (Mendes et al., 2012) and Tetraodontiformes (Alves et al., 2008).

Cytogenetic studies in the Callichthyinae subfamily show the presence of B chromosomes only in Callichthys Linnaeus, 1758. These extra chromosomes were observed in different populations of C. callichthys, varying in size, quantity and morphology (Oliveira et al., 1993; Shimabukuro-Dias et al., 2005). In the present study, chromosomal structure of one population of C. callichthys was studied focusing the distribution of ribosomal sites and the biology of supernumerary chromosomes in this species, highlighting the occurrence of a morphologically ill-defined B chromosome and a hypothesis about its origin.

Fig. 1. Callichthys callichthys karyotypes stained by Giemsa: (a) with an acrocentric B chromosome and the third NORs bearing chromosome (interstitial) and (b) with the ill-defined acrocentric B chromosome and the third NORs bearing chromosome (terminal). The bar represents 5µm.
Material and Methods

Cytogenetic studies were carried out on *Callichthys callichthys* (6 males and 1 female) sampled from the Paraná River (Guaíra, Paraná State, Brazil). Voucher specimens were deposited in the Coleção Ictiológica do Núcleo em Pesquisas em Limnologia, Ictiologia e Aquicultura, Universidade Estadual de Maringá (NUP 6095 - *C. callichthys*). Metaphasic cells were obtained from the kidney (Bertollo et al., 1978; Foresti et al., 1993). Fish were anesthetized and sacrificed with clove oil according to Griffiths (2000). Heterochromatin was revealed through C banding (Sumner, 1972) and nucleolar organizer regions (NORs) were revealed through silver nitrate impregnation (Howel & Black, 1980). Staining with the specific base fluorochromes Chromomycin A₂ (CMA₂) and 4′,6-diamidino-2-phenylindole (DAPI) was performed following the procedure described by Schweizer (1980). Chromosomes types were classified in metacentric (*m*), submetacentric (*sm*), subtelocentric (*st*) or acrocentric (*a*) based on the arm relationship criteria proposed by Levan et al. (1964).

The localization of the 5S and 18S rDNA sites in the chromosomes was performed using the fluorescence *in situ* hybridization (FISH) method (Pinkel et al., 1986 with modifications, Margarido & Moreira-Filho, 2008), with probes obtained from the fish species *Leporinus elongatus* (Martins & Galetti Jr., 1999) and *Prochilodus argenteus* (Hatanaka & Galetti Jr., 2004), respectively. The probes were labelled through nick translation, with digoxigenin-11-dUTP (5S rDNA) and biotin-16-dUTP (18S rDNA) (Roche). Detection and amplification of the hybridization signal were made using avidin-FITC and anti-avidin biotin (Sigma) for probes labelled with biotin, and anti-digoxigenin rhodamine (Roche) for probes labelled with digoxigenin. Slides were counterstained with DAPI (50 μg/mL) and analyzed in epifluorescence microscope (Olympus BX61). The images were captured using the software DP controller (Media Cybernetics).

Results

The analysis of mitotic cells revealed a diploid number of 56 chromosomes (26 *m-sm* + 30 *st-a*) for both sexes, with the sporadic occurrence of an acrocentric B chromosome, which appears in three males e one female individuals (from 2.3% to 7.14% of metaphasic cells frequencies; 100 metaphase plates/individual analyzed) (Fig. 1a). Also, one male individual had a B chromosome, like a morphologically ill-defined acrocentric one, present in all analyzed metaphases (Fig. 1b).

C-banding revealed centromeric heterochromatin, with pericentromeric and telomeric markings on some chromosomes and coincident markings with the intercalary region of the ribosomal sites in all specimens analyzed. Also, both the B chromosomes were completely heterochromatic (Figs. 2a, b). The intercalary region of the NORs was positive for CMA₂ and negative for DAPI (Figs. 3a, b, c, d); indicating that the intercalary region of the 45S rDNA has a GC rich composition. The ill-defined acrocentric B chromosome was not differentially stained by base-specific fluorochromes (Fig. 3c).

The NORs (Ag-staining and 18S rDNA) were simple, on the short arm of the chromosome pair 25, although two individuals presented an extra marking, located on the short arm of one of
chromosome from the m-sm pair 4. This additional marking was interstitial in one individual and terminal on the other (Figs. 1a, b, box and Fig. 4b, c). The individual with the third NOR on terminal position had the ill-defined acrocentric B chromosome.

The physical mapping of ribosomal genes through FISH showed 5S rDNA sites on four m-sm chromosome pairs. However, two individuals showed differences in the number of rDNA sites, one with seven and another with nine chromosomes bearing 5S rDNA. Both individuals also had a third chromosome bearing 18S rDNA. Three conditions were observed for the chromosomal pair 4: standard, 5S rDNA in the terminal position of the short arm of both chromosomes (Fig. 4a); variant 1, 5S rDNA in the terminal position of the short arm, syntenic to interstitial 18S rDNA in only one homologous of the pair (Fig. 4b); variant 2, 5S rDNA in the interstitial position of the short arm syntenic to 18S rDNA in terminal position on only one chromosome, with the occurrence of the ill-defined acrocentric B chromosome (Fig. 4c).

Fig. 3. Callichthys callichthys metaphases spreads with the third NOR bearing chromosome. The arrowheads indicate the NORs, marked in the interstitial position stained by (a) CMA3 and confirmed by (b) DAPI; and in terminal position stained by (c) CMA3 and confirmed by (d) DAPI. The bar represents 5µm.
cause a fast heterochromatinization of extra elements, constituting the basis for differentiation of B chromosomes that isolates them from the rest of the genome (Camacho et al., 2000) or just keeps the gene dosage (Amos & Dover, 1981). This segment possibly had its gene organization modified through rearranges like proposed by Jamilena et al. (1994). The presence of this B chromosome in all observed cells suggests that a neo-centromere might have been originated, capable of anchoring proteins from the kinetochore like observed by Depinet et al. (1997), differing from other reports of the occurrence of B chromosomes in some populations of C. callichthys that presented intra-individual variation (Oliveira et al., 1993; Sanchez & Fenocchio, 1996), apart from a report of a B chromosome present in all cells of one individual (Shimabukuro-Dias et al., 2005).

On the present study, the presence of intercalary heterochromatin on the NORs might have facilitated the dispersion and origin of the B chromosome. The presence of specific repetitive DNAs in B chromosomes has been described for many fish species (Mestriner et al., 2000; Jesus et al., 2003). However, the composition of the ill-defined acrocentric B chromosome was not similar to its origin (GC-rich) (Fig. 3c), suggesting different evolutionary mechanisms for B chromosomes (Camacho et al., 2000).

Discussion

B chromosomes have been described for approximately 5% of all Neotropical fishes (Oliveira et al., 2009) showing predominance in some groups. Callichthyidae has 199 species (Froese & Pauly, 2013), with B chromosomes described for Corydoras (Oliveira et al., 1988) and Callichthys (Oliveira et al., 1993; Sanchez & Fenocchio, 1996; Shimabukuro-Dias et al., 2005), varying in number, size and morphology.

A hypothesis was raised about the origin of the third NOR bearing chromosome and the B chromosome. The process would have begun with the amplification of the ribosomal genes on the main acrocentric chromosomes pair (25). It is possible that an event of dispersion to a third chromosome happened, as proposed by Schweizer and Loidl (1987), with the transposition of terminal segments from acrocentric chromosomes to non-homologous, followed by homogenization. This dispersion carried terminal 5S rDNA to chromosome 4 (m-sm), originating the variant 1, with 5S rDNA in the terminal position of the short arm, syntenic to interstitial 18S rDNA in only one chromosome from the pair (Fig. 4b). Possibly, a paracentric inversion happened afterwards, originating variant 2, with 5S rDNA in the interstitial position of the short arm, syntenic to 18S rDNA in terminal position. In this process, a small segment would have been lost by a fission following the paracentric inversion, corresponding to the additional chromosome (Fig. 4c). Although 18S rDNA sites have already been observed on B chromosomes (Polleto et al., 2010), the B chromosome observed in C. callichthys does not have ribosomal cistrons, which was verified through silver nitrate impregnation and confirmed through 18S rDNA-FISH.

The ill-defined acrocentric B chromosome is completely heterochromatic (Fig. 5). It is possible that the ribosomal cistrons were eliminated, as seen by Jamilena et al. (1994) in Crepis capillaris. This suggests the presence of cell mechanisms which
Terminal NORs were described in many populations of *C. callichthys* (Oliveira et al., 1993; Sanchez & Fenocchio, 1996; Shimabukuro-Dias et al., 2005). On the present study, the analyzed population shows the same pattern, except for two individuals. These individuals showed the presence of a third NOR bearing chromosome, similar to a situation described for one individual by Porto & Feldberg (1993). The presence of an additional chromosome bearing NORs is a recurring condition for *C. callichthys*. It is possible that these regions have undergone homogenization like the model proposed by Schweizer & Loidl (1987); however, this issue demands further studies. Further studies performing in situ hybridization with probes obtained from the ill-defined acrocentric B chromosome are necessary to confirm the proposed hypothesis for the origin of this chromosome in *C. callichthys*.

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