

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

Jociléia Thums Konerat<sup>1</sup>, Vanessa Bueno<sup>1</sup>, Lucas Baumgartner<sup>2</sup>,  
Isabel Cristina Martins-Santos<sup>2</sup> and Vladimir Pavan Margarido<sup>1</sup>

B chromosomes are extra chromosomes from the normal chromosomal set, found in different organisms, highlighting their presence on the group of fishes. *Callichthys callichthys* from the upper Paraná River has a diploid number of 56 chromosomes (26 *m-sm* + 30 *st-a*) for both sexes, with the presence of a sporadically acrocentric B chromosome. Moreover, one individual presented a diploid number of 57 chromosomes, with the presence of a morphologically ill-defined acrocentric B chromosome in all analyzed cells. The physical mapping of 5S and 18S rDNA shows multiple 5S rDNA sites and only one pair of chromosomes with 18S sites in *C. callichthys*, except for two individuals. These two individuals presented a third chromosome bearing NORs (Ag-staining and 18S rDNA) where 5S and 18S rDNA genes are syntenic, differing only in position. The dispersion of the 18S rDNA genes from the main *st-a* chromosome pair 25 to one of the chromosomes from the *m-sm* pair 4 would have originated two variant individuals, one of which with the ill-defined acrocentric B chromosome. Mechanisms to justify the suggested hypothesis about this B chromosome origin are discussed in the present study.

Cromossomos B são cromossomos extras ao conjunto cromossômico normal, encontrado em diferentes organismos, com destaque para sua presença no grupo de peixes. *Callichthys callichthys* do alto rio Paraná tem um número diploide de 56 cromossomos (26 *m-sm* + 30 *st-a*) para ambos os sexos, com a presença esporádica de um cromossomo B acrocêntrico. Além do mais, um indivíduo apresentou número diploide de 57 cromossomos, com a presença de um cromossomo B acrocêntrico morfológicamente mal definido em todas as células analisadas. O mapeamento físico do DNAr 5S e 18S mostrou múltiplos sítios de DNAr 5S e apenas um par de cromossomos com sítio para o DNAr 18S em *C. callichthys*, com exceção para dois indivíduos. Estes dois indivíduos apresentaram um terceiro cromossomo portador das RONS (Ag-RONS e 18S rDNA), onde os genes DNAr 5S e 18S são sintênicos, diferindo apenas na posição. A dispersão dos genes DNAr 18S do par de cromossomos principal *st-a* 25 para um dos cromossomos do par *m-sm* 4 teria originado dois indivíduos variantes, um dos quais com cromossomo B acrocêntrico mal definido. Mecanismos para justificar a hipótese sugerida sobre a origem deste cromossomo B são discutidos no presente estudo.

**Key words:** 18S rDNA, 5S rDNA, Chromosomal rearrangements, FISH, Supernumerary chromosome.

## **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

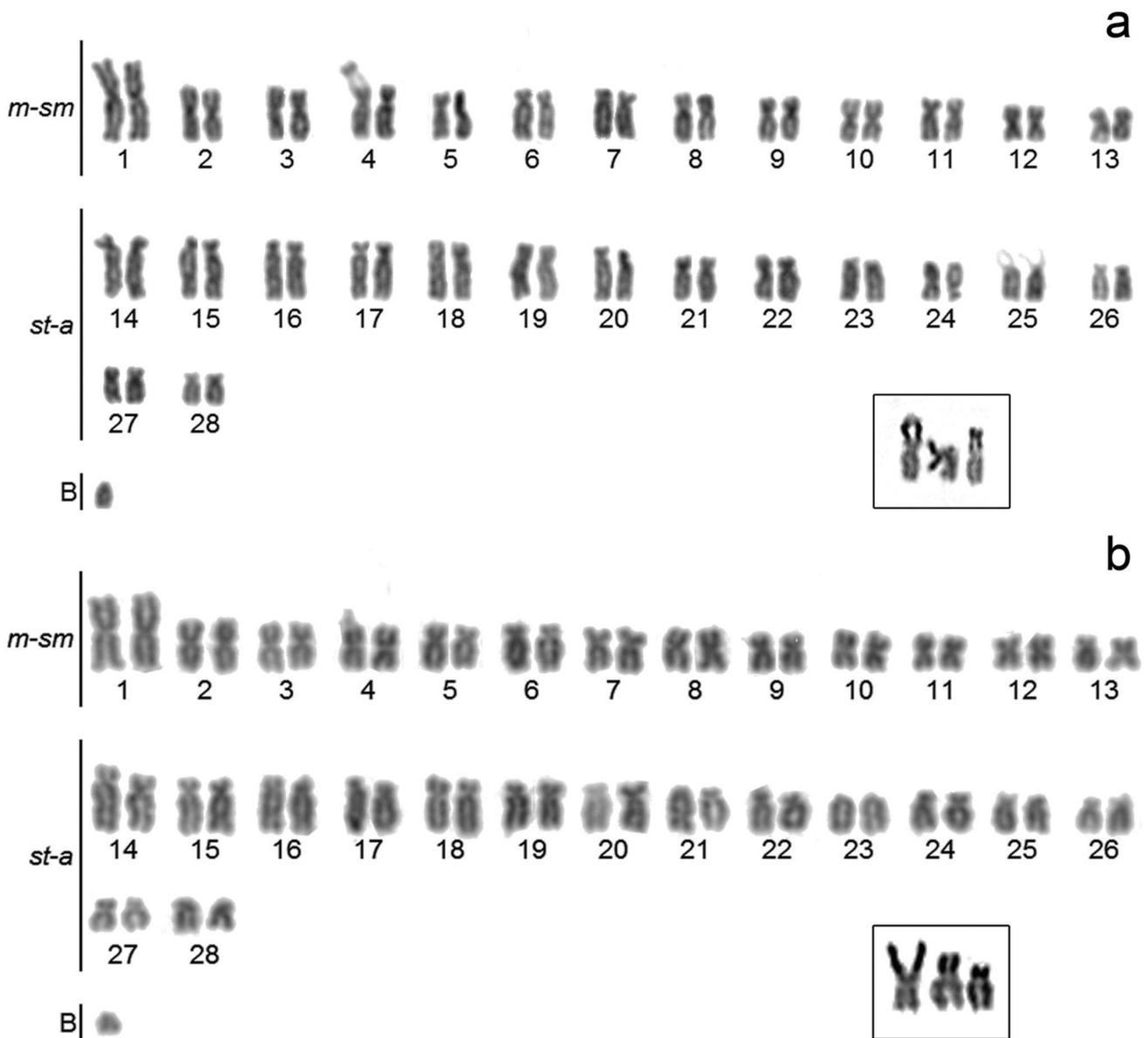
<sup>1</sup>Centro de Ciências Biológicas e da Saúde, Universidade Estadual do Oeste do Paraná. Rua Universitária 2069, 85814-110 Cascavel, PR, Brazil. vladimir.margarido@unioeste.br

<sup>2</sup>Departamento de Biologia Celular e Genética, Universidade Estadual de Maringá. Avenida Colombo, 5790, 87020-900 Maringá, PR, Brazil.

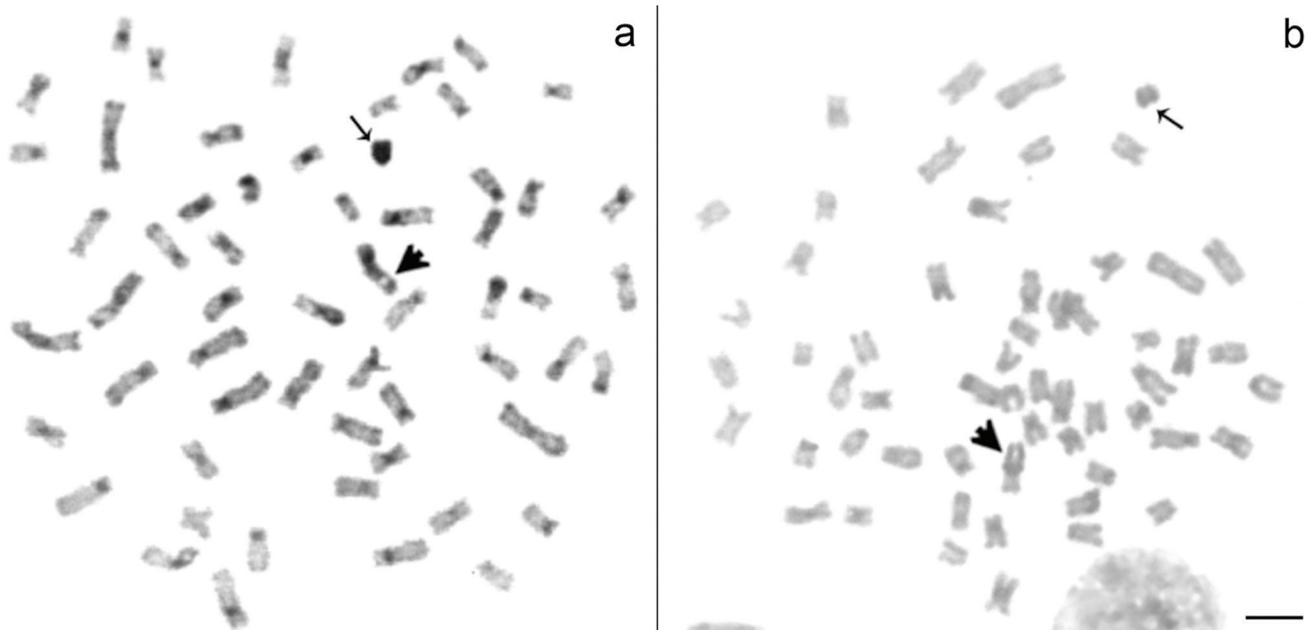
variability, showing an evolutionary role (Rejón *et al.*, 1987).

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 5 μm.



**Fig. 2.** C-banded metaphases of *Callichthys callichthys*: the arrowhead indicates the third NORs bearing chromosome and the arrow indicates the (a) acrocentric B chromosome and the (b) ill-defined acrocentric B chromosome. The bar represents 5 $\mu$ m.

### Material and Methods

Cytogenetic studies were carried out on *Callichthys callichthys* (6 males and 1 female) sampled from the Paran river (Guara, Paran State, Brazil). Voucher specimens were deposited in the Coleo Ictiolgica do Ncleo em Pesquisas em Limnologia, Ictiologia e Aquicultura, Universidade Estadual de Maring (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 A<sub>3</sub> (CMA<sub>3</sub>) 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  $\mu$ g/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).

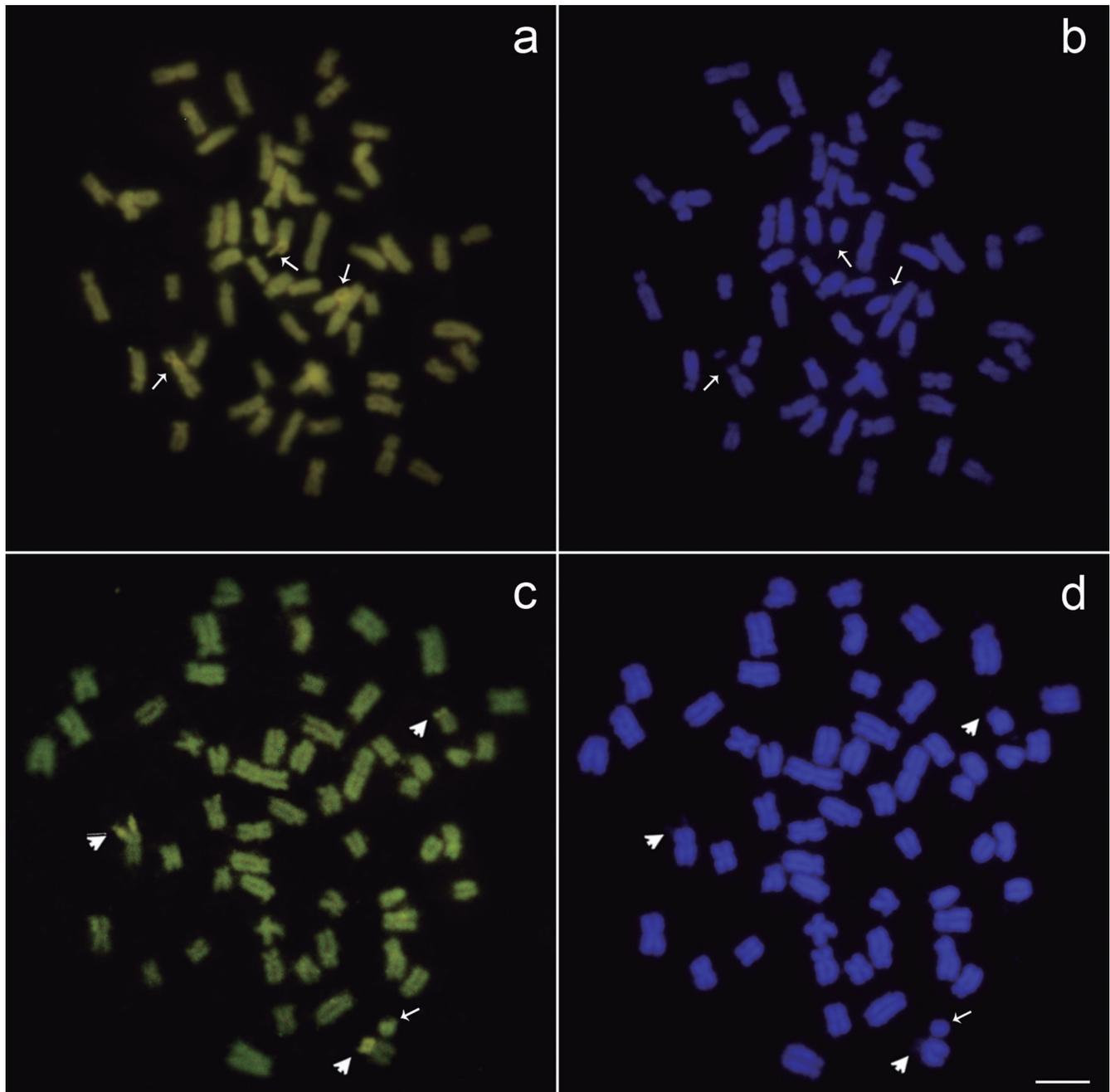
C-banding revealed centromeric heterochromatin, with pericentromeric and telomeric markings on some chromosomes and coincident markings with the intercalary region of the ribosomal sites in all specimens analyzed. Also, both the B chromosomes were completely heterochromatic (Figs. 2a, b). The intercalary region of the NORs was positive for CMA<sub>3</sub> 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

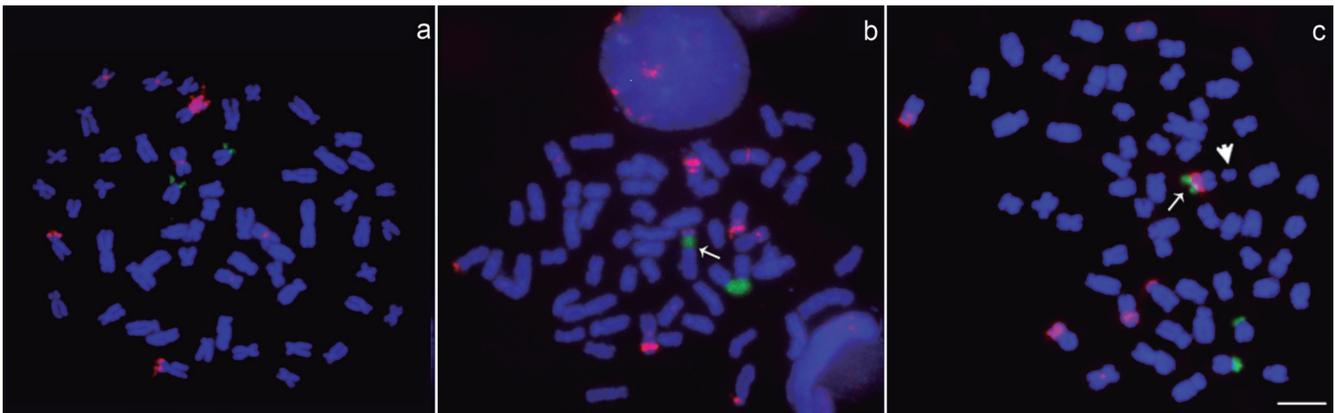
chromosome from the *m-sm* pair 4. This additional marking was interstitial in one individual and terminal on the other (Figs. 1a, b, box and Fig. 4b, c). The individual with the third NOR on terminal position had the ill-defined acrocentric B chromosome.

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) CMA<sub>3</sub> and confirmed by (b) DAPI; and in terminal position stained by (c) CMA<sub>3</sub> and confirmed by (d) DAPI. The bar represents 5µm.



**Fig. 4.** Double FISH metaphase spreads of *Callichthys callichthys* with 5S (red) and 18S (green) rDNA probes. Standard metaphase (a) with 56 chromosomes and only the pair 25 bearing NORs. The arrows indicate the *m-sm* chromosome 4 with (b) 18S rDNA in interstitial position and terminal 5S rDNA and (c) 18S rDNA in terminal position. The arrowhead shows the ill-defined acrocentric B chromosome. The bar represents 5  $\mu$ m.

### Discussion

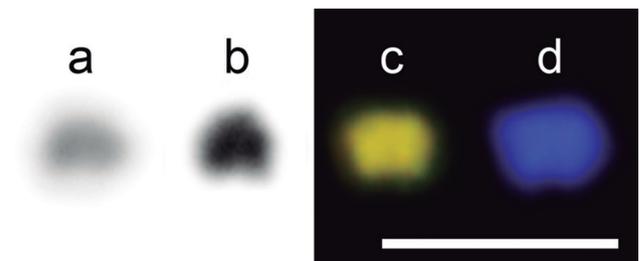
B chromosomes have been described for approximately 5% of all Neotropical fishes (Oliveira *et al.*, 2009) showing predominance in some groups. Callichthyidae has 199 species (Froese & Pauly, 2013), with B chromosomes described for *Corydoras* (Oliveira *et al.*, 1988) and *Callichthys* (Oliveira *et 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

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).



**Fig. 5.** The ill-defined acrocentric B chromosome: (a) Giemsa stained; (b) C-banded; stained by (c) CMA<sub>3</sub> and by (d) DAPI. The bar represents 5  $\mu$ m.

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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