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Short Note

Characterization of the Constitutive Heterochromatin of *Astyanax* sp. D (Characiformes: Characidae) from the Upper Iguaçu River (PR)

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Chromosomal characterization of constitutive heterochromatin was performed in *Astyanax* sp. D, a species found in the Iguaçu river, using C-banding, fluorescent *in situ* hybridization (FISH) of the repetitive DNA family As51, localization of the 18S rDNA sites and base-specific fluorochromes. The sites of the satellite DNA coincide with large heterochromatic blocks present in this species, namely CMA_3^- and $DAPI^-$. The many 18S rDNA sites are CMA_3^+. Similarity between the large heterochromatic blocks is suggested, as well as the identification of two types of heterochromatin, one related to the 18S rDNA domains and the other to the As51 regions.

Key words: Fluorescent *in situ* hybridization, satellite DNA, chromosome, As51, neotropical region.

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Satellite DNAs are sequences frequently found in the genome of eukaryotes in which they can represent from 1% to more than 70% of the genetic material (LI & GRAUR 1991), being characterized by highly repetitive and tandemly-arranged monomers. The isolation, sequencing and subsequent *in situ* localization of satellite DNA in chromosomes constitutes a precise way of characterizing the heterochromatic regions of the genome (HAAF *et al.* 1993; REED & PHILLIPS 1995; GARRIDO-RAMOS *et al.* 1994; OLIVEIRA & WRIGHT 1998; JESUS *et al*. 2003). In the genus *Astyanax*, MESTRINER *et al*. (2000) identified a satellite DNA of 51 bp composed of monomers (59% AT) in *A. scabripinnis* (Jenyns 1942), named As51, located in the distal regions of a few acrocentric chromosomes, in the nucleolar organizing regions, as well as in the supernumerary chromosomes present in this species.

Astyanax sp. D is an endemic characid species of the Iguaçu river (Brazil) and, even though it has not yet been formally described, it is considered a valid taxonomical entity (SEVERI & CORDEIRO 1994; AGOSTINHO & GOMES 1997; KANTEK *et al*. 2003; VITULE & ABILHOA 2003; INGENITO *et al*. 2004; KANTEK *et al*. 2007), with SAMPAIO (1988) and KANTEK *et al*. (2007) considering *Astyanax* sp. D and *Astyanax scabripinnis* very close taxa.

The objective of the present study was to characterize the composition of the heterochromatic regions of *Astyanax* sp. D from the Iguaçu River through the use of base-specific fluorochromes and fluorescent *in situ* hybridization (FISH) with As51 satellite DNA and 18S rDNA probes.

Material and Methods

Astyanax sp. D specimens (4 males and 27 females – MHNCI 9095 – Museu de História Natural do Capão da Imbuia) were collected in the Bicudo River, an affluent of the Upper Iguaçu River, in the municipal district of Balsa Nova (Paraná State, Brazil) (UTM 22J0635133/7171020). The mitotic metaphases were obtained through short duration

cell culture (FENOCCHIO *et al*. 1991). Chromosome classification was established according to LEVAN *et al.* (1964). The detection of the constitutive heterochromatin was performed following the methodology of SUMNER (1972). Chromosome staining with chromomycin A_3 (CMA_3) and 4',6-diamidino-2-phenylindole (DAPI) fluorochromes were used on the same slide, following SCHWEIZER (1980). The localization of both the As51 satellite DNA and the 18S rDNA sites in the chromosomes was performed through fluorescent *in situ* hybridization (FISH) according to PINKEL *et al.* (1986), with probes obtained from the fish *Astyanax scabripinnis* (MESTRINER *et al.* 2000) and *Prochilodus argenteus* Spix and Agassiz, 1829 (HATANAKA & GALETTI Jr 1994), respectively. The slides bearing the chromosomes were incubated with 90 μl of 0.4% RNAse solution in 2 x SSC at 37°C for 1 h, covered by coverslips and washed three times with 2 x SSC and once with 1 x PBS at room temperature for 5 min. The slides were then dehydrated in cold ethanol (50% and 100%) for 5 min each and air dried. Chromosome denaturation was performed with 70% formamide in 2 x SSC at 70°C for 5 min. and dehydrated as described above. The probes were marked with 14-dATP-biotin by nick translation according to the manufacturer's instructions (Bionick Labelling System – Invitrogen). The marked probes were denatured in hybridization solution (50% formamide, 10% dextran sulphate in 2 x SSC) at 100°C for 10 min. The slides with denatured chromosomes were incubated overnight with 50 μl of the hybridization solution at 37°C. After the hybridization, the slides were washed with 1 x SSC at 37°C, with 1 x SSC at room temperature for 5 min for 5 min each and with washing solution (0.5% Tween in 4 x SSC) for 15 min (twice). The slides were incubated in an NFDM solution (skim milk powder) for 15 min at room temperature and rinsed twice with washing solution for five min each. Afterwards, they were incubated with flouorescein-avidin (FITC-avidin) in an NFDM solution in a dark humid chamber at room temperature for 30 min (detection procedure) and washed two times with washing solution for 5 min each. The signal was amplified by incubation with 90 μl of anti-avidin solution (biotin conjugated) in NFDM for 30 min in a dark humid chamber at room temperature. The slides were then rinsed three times with washing solution for 5 min each, followed by another detection procedure. The amplification step was repeated. After that, the slides were dehydrated in cold ethanol (50% and 100%) for 5 min each and air dried. The chromosomes were counterstained with DAPI (0.2 mg/ml) and analyzed in an Olympus BX50 epifluorescence microscope. The software Image-Pro Plus (Media Cybernetics) was used for image capture.

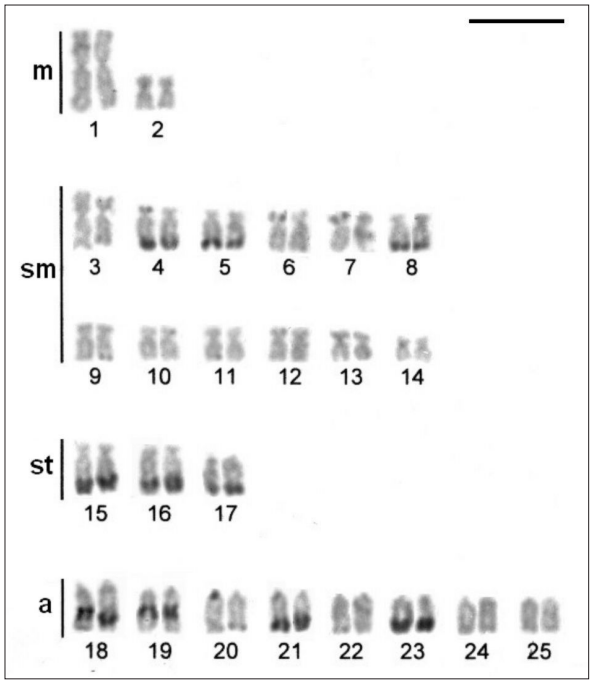

Fig. 1. *Astyanax* sp. D karyotype with C-banding. Bar = 5 μm. m – metacentric, sm – submetacentric, st – subtelocentric, a – acrocentric.

Results

The heterochromatin of *Astyanax* sp. D is mainly located in telomeric and interstitial regions of acrocentric and subtelocentric chromosomes (Fig. 1). It was possible to observe the existence of congruence between the bands obtained by the As51 probe (Fig. 2b) and the more conspicuous heterochromatic blocks, while the smaller C^+-band sites were not evidenced by hybridization. Therefore, when comparing the number of C^+ blocks (Fig. 1) with the signals produced by the As51 probe (Fig. 2b), the existence of a larger number of heterochromatic sites was perceived. Besides being As51 DNA sites, the more conspicuous C^+-bands are also $DAPI^-$ (Fig. 2c) and CMA_3^- (Fig. 2d). Approximately twenty-one 18S rDNA sites were distributed throughout many chromosomes (Fig. 2a), all of which were CMA_3^+ (Fig. 2c) with variation regarding the number of sites between different individuals (data not shown). The karyotypes shown in Figure 2 are from different specimens.

Discussion

Both the karyotypic structure and the heterochromatic polymorphism of *Astyanax* sp. D from the Upper Iguaçu River were studied by KANTEK *et al*. (2007) and KANTEK *et al*. (2008). Four populations have been studied by these authors and the C-banding patterns were similar in all populations.

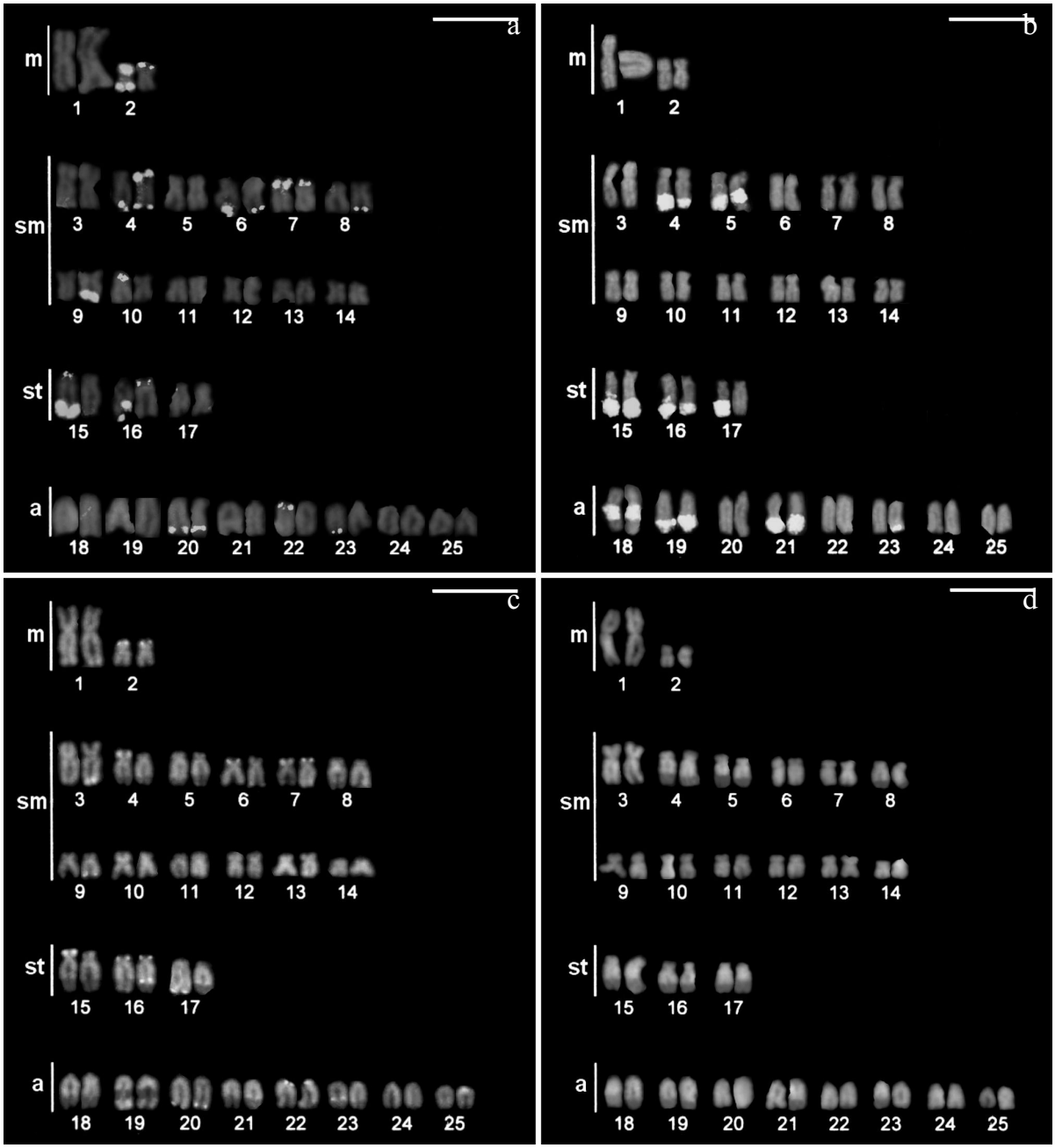

Fig. 2. *Astyanax* sp. D karyotypes. (a) Chromosomal distribution of the 18S rDNA sites and (b) of the As51 satellite DNA; staining by (c) CMA_3 and (d) DAPI. Bars = 5 μm.

However, within each population, inter-individual variation concerning the number and localization of heterochromatic bands was observed. Some of this variation was quantified in each population (KANTEK *et al*. 2008), and the results indicate that the samples were similar when studying the variable frequencies (one of these samples was used in the present article for FISH analyses and base-specific fluorochrome staining). In many species of *Astyanax*, intra-populational variation in the localization and amount of C-bands was observed (MANTOVANI *et al.* 2000; PAZZA *et al*. 2006).

The results for *Astyanax* sp. D obtained in this study show that all large heterochromatic blocks (Fig. 1), present mainly in the acrocentric and subtelocentric chromosomes, possess the same constitution. In fact, besides being As51 positive (Fig. 2b), all of these regions are also negatively stained by CMA_3 (Fig. 2c) and DAPI (Fig. 2d). In previous studies, KANTEK *et al*. (2007) had already pointed out the possibility of these chromosomal domains being similar, based on the characteristics presented after cleavage with restriction enzyme *AluI*.

In the genus *Astyanax*, as well as in many fish species, the association between heterochromatin

(CMA_3^+) and Ag-NOR (18S rDNA sites) is common (AMEMIYA & GOLD 1986). Nevertheless, if the the number of CMA_3^+ and 18S rDNA sites are compared (Figs. 2c and 2a, respectively), a predominance of the latter is evident, probably due to the inter-individual polymorphism observed in this species, considering that the Figures presented correspond to different specimens of *Astyanax* sp. D. Other *Astyanax* species, such as *A. fasciatus* (PAZZA *et al.* 2006) and *A. scabripinnis* (MANTOVANI *et al.* 2005) also possess intra-populational variation regarding the number and localization of 18S rDNA sites.

The numerical difference between the As51 blocks (Fig. 2b) and the C+-bands (Fig. 1) may be explained through the fact that the 18S sites were also heterochromatic. Two distinct types of heterochromatin occur in the chromosomes of *Astyanax* sp. D, one related to the As51 domains (CMA_3^-) and the other to the 18S rDNA regions (CMA_3^+). However, it is likely that not all of the 18S rDNA sites were evidenced through C-banding, especially considering that many sites are much reduced in size (Fig. 2c), thus hindering the detection of heterochromatin in these regions.

Based on positive or negative staining using the base-specific fluorochromes mithramycin A (MM) plus the non-fluorescent counterstaining distamycin A (DA) and DAPI with and without DA counterstaining, MANTOVANI *et al.* (2004) concluded that the As51 blocks from two populations of *Astyanax scabripinnis* possess a different structural organization. These observations demonstrate that C-banded segments containing As51 satellites may differ structurally. In the present study, different fluorochrome combinations were used, hampering the comparison between the present results and those of MANTOVANI *et al.* (2004). KANTEK *et al*. (2007) observed that large heterochromatic blocks in another population of *Astyanax* sp. D are CMA_3 positive. However, the fluorochrome was used without counterstaining, making a comparison difficult between these studies.

MESTRINER *et al*. (2000) observed that As51 satellite DNA is composed of 59% AT bases. Thus, the occurrence of negative CMA_3 in chromosomal domains corresponding to the As51 hybridization sites in *Astyanax* sp. D is expected, since CMA_3 is a GC-specific dye. Contrarily, it was not expected for these regions to also be negatively stained by DAPI, which despite being capable of linkage to GC and AT base pairs, its fluorescence is only significantly potentialized by AT-rich regions (LIN *et al*. 1977). Thus, it is possible that the AT base composition (59%) of the As51 repetitive DNA is insufficient to produce DAPI-positive bands. COMINGS and DRETS (1976) suggest that the lack of fluorescence in heterochromatic regions may be due to the effect of chromosomal proteins on the access of the fluorochromes to satellite DNA. SAITOH and LAEMMLI (1994) argue that the structural organization of the heterochromatic sequences may also be a factor in whether or not fluorescence occurs. Therefore, different factors may be involved in the DAPI- result observed in the As51 repetitive DNA sites.

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