



Evidence of the differentiated structural arrangement of constitutive heterochromatin between two populations of *Astyanax scabripinnis* (Pisces, Characidae)

Monique Mantovani, Luciano Douglas dos Santos Abel, Carlos Alberto Mestriner and Orlando Moreira-Filho

Universidade Federal de São Carlos, Departamento de Genética e Evolução, São Carlos, SP, Brazil.

Abstract

The composition of heterochromatin classes along the chromosomes of specimens from two populations of the fish *Astyanax scabripinnis* was examined using fluorescence banding with GC- and AT-DNA specific fluorochromes and fluorescence *in situ* hybridization (FISH) with an AT-rich satellite DNA (As51) probe. For the pericentromeric heterochromatin blocks neither GC/AT-DNA specific fluorochromes nor the FISH technique produce any response with chromosomes from either of the populations. On the other hand, the telomeric distal heterochromatin blocks of both populations fluoresced when the FISH technique was applied but showed distinct responses after GC-specific fluorochrome treatments, leading us to propose different structural arrangements of the FISH-positive heterochromatins. Such differences in chromosome banding patterns together with other karyotypic differences suggest differentiation of these populations at taxonomic level.

Key words: satellite DNA, chromosome structure, base-specific fluorochromes, heterochromatin heterogeneity, fluorescence *in situ* hybridization

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Introduction

The constitutive heterochromatin of fishes is usually studied by C-banding to characterize distinct patterns of heterochromatin distribution in karyotypes. Comparative analyses of these patterns have led to a greater understanding of the genetic and evolutionary relationships within and between different groups and have contributed significantly to cytotaxonomic studies (Garcia *et al.*, 1987; Galetti Jr. *et al.*, 1991; Almeida-Toledo *et al.*, 1996). However, because the composition of constitutive heterochromatin may vary and C-banding does not reveal the content of nitrogenous bases, other techniques are required (see Verma, 1988; Sumner, 1990). Base-specific fluorochromes were initially used to identify adenine/thymine (AT)- or guanine/cytosine (GC)-rich heterochromatin portions (Schweizer, 1976; Schmid, 1980). This led to substantial advances in the analysis of the constitutive heterochromatin of fishes, allowing for the identification of different heterochromatin classes (Mayr *et al.*, 1988; Caputo *et al.*, 1997; Molina *et al.*, 1998; Artoni and Bertollo, 1999;

Artoni *et al.*, 1999; Margarido and Galetti Jr., 2000; Sola and Gornung, 2001).

Nevertheless, it has been shown that longitudinal Q- and R-bands result from differential folding paths of sequences associated with the chromosome scaffold (SARs), leading to the assumption that positive or negative fluorochrome responses are dependent not only on the base composition of the DNA but also on the arrangement of the bases in the chromosomal structure (Saitoh and Laemmli, 1994). Hence, it is through isolation, sequencing and *in situ* location of satellite DNA that chromosome heterochromatic regions can really be characterized (Haaf *et al.*, 1993; Reed and Phillips, 1995; Garrido-Ramos *et al.*, 1994; 1995; 1998; Oliveira and Wright, 1998; Mestriner *et al.*, 2000; Phillips and Reed, 2000).

Owing to its high morphologic and karyotypic diversity, *Astyanax scabripinnis* has been considered a complex of species (Moreira-Filho and Bertollo, 1991). Constitutive heterochromatin has been involved in the chromosomal differentiation of several *A. scabripinnis* populations (Moreira-Filho and Bertollo, 1991; Maistro *et al.*, 1998; Mizoguchi and Martins-Santos, 1998; Maistro *et al.*, 2000; Mantovani *et al.*, 2000) and fluorescent staining has already revealed constitutive heterochromatin heterogeneity in one population (Souza *et al.*, 1996).

In one *A. scabripinnis* population a 51 bp (59% AT) satellite DNA called As51 has been cloned and sequenced and its chromosomal position found to be in the non-centromeric heterochromatin, *i.e.* the distal blocks of acrocentric chromosomes, the nucleolus organizer regions (NORs) and the supernumerary chromosome (Mestriner *et al.*, 2000).

The purpose of our work was to use the fluorescence *in situ* hybridization (FISH) technique to locate this repetitive DNA in the chromosomes of other *A. scabripinnis* populations and to carry out base-specific fluorochrome analyses which would allow us to evaluate the factors involved in fluorochrome staining based on homology between As51 and the heterochromatic regions. In addition, such data may shed light on the heterogeneity of heterochromatin and on the cytotaxonomic relationships within this species complex.

Materials and Methods

Specimens

We collected specimens of *Astyanax scabripinnis* (Pisces, Characiformes, Characidae) from two different Brazilian populations, seven females and three males from the Marrecas stream near the city of Londrina and four females and four males from the Centenário stream near the town of Maringá, both these streams being within the Paranapanema river basin in the state of Paraná.

Chromosome preparations and C-banding

Mitotic chromosomes were obtained from anterior kidney tissue using the cell suspension technique described by Bertollo *et al.* (1978). The constitutive heterochromatin was visualized by the C-banding technique of Sumner (1972) and the chromosomes were classified according to the criteria of Levan *et al.* (1964).

Base-specific fluorochrome staining

The GC-specific fluorochrome mithramycin A (MM) plus the non-fluorescent counterstain distamycin A (DA) were used according to the method of Schmid (1980) and the AT-specific fluorescent compound 4',6-diamidino-2-phenylindole (DAPI) was used with and without DA counterstaining (Schweizer *et al.*, 1978). The DA/MM preparations were stored in the dark for at least 15 days before being exposed to ultraviolet (UV) light through a 450-490 nm filter, while the DAPI and DA/DAPI slides were kept in the dark for 24 h before exposure to UV light through a 360-390 nm filter. All the preparations were examined using an Olympus BX50 fluorescence microscope and the metaphase photographs taken using Kodak TMAX 100 ISO film.

Fluorescence *in situ* hybridization (FISH)

As the probe we used the As51 satellite DNA sequence inserted in a pGEM4 plasmid (Mestriner *et al.*, 2000) labeled with dATP-biotin by nick translation using the BioNick™ Labeling System (Gibco BRL) according to the manufacturer's instructions.

The FISH technique was carried out according to the method of Pinkel *et al.* (1986), with some modifications. The chromosomes were denatured in 70% (v/v) formamide in 2x saline sodium citrate (2xSSC) solution at 70 °C for 5 min followed by a further 10 min denaturation at 100 °C after which 50 µL of hybridization solution (containing 50% formamide in 2xSSC, 10% (w/v) dextran sulfate, 200 ng/µL of human placenta DNA and about 125 ng of probe) was applied to each slide under a coverslip. Hybridization was performed for 15 h at 37 °C in a moist chamber containing 60% (v/v) formamide, after which the slides were rinsed with 50% formamide in 2xSSC at 42 °C for 20 min followed by 0.1xSSC at 60 °C for 15 min. Detection of the hybridized probe was carried out using three cycles of avidin-fluorescein isothiocyanate (avidin-FITC) and biotinylated anti-avidin (Sigma). The slides were mounted in 25 µL of Vectashield antifade (Vector) and the chromosomes stained with 1 µL of a 50 µg/mL solution of propidium iodide, the metaphase chromosomes being examined using an Olympus BX50 fluorescence microscope and photographed on Kodak Gold Ultra 400 ISO film.

Results

The chromosome complement of the *A. scabripinnis* specimens from the Marrecas population was $2n = 48$ while those from the Centenário population was $2n = 50$ (Figure 1).

Both populations displayed constitutive heterochromatin in the centromeric region of most chromosomes and heterochromatic blocks (most of them large) in the telomeric regions of the long arms, principally in the subtelocentric and acrocentric chromosomes (Figures 2a and 3a). A numerical and quantitative inter-individual polymorphism was observed in the distribution of the distal heterochromatin (terminal heterochromatic blocks) in some chromosomes of both populations (data not shown).

FISH using the As51 satellite DNA probe showed the homology of this repetitive DNA family with the constitutive heterochromatin revealed by C-banding, except for the pericentromeric heterochromatic blocks (Figures 1, 2a and 3a). This technique also confirmed the polymorphism of the distal heterochromatin (*e.g.* pair 25, Figure 1b).

The distal heterochromatic blocks of both populations were revealed to be DA/DAPI negative (Figures 2c and 3c) but provide no response when only DAPI was employed (Figures 2 and 3). This chromosomal domain was DA/MM negative in the $2n = 48$ Marrecas population

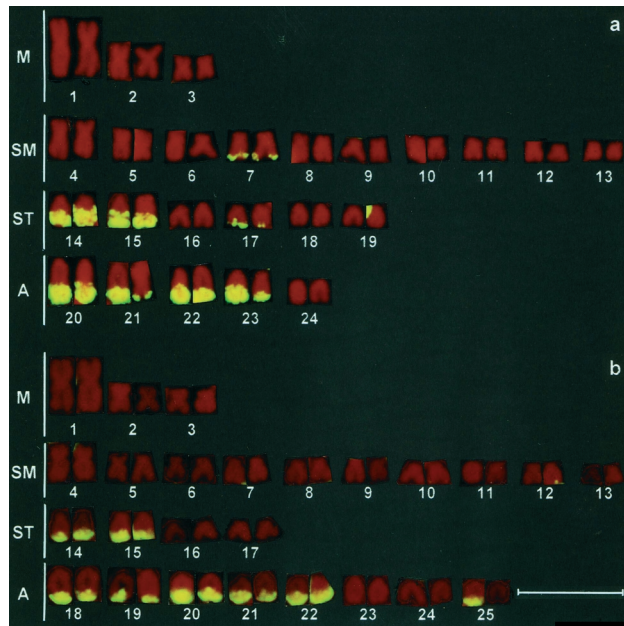


Figure 1 - Karyotypes of *Astyanax scabripinnis* produced using fluorescence in situ hybridization (FISH) and the As51 satellite DNA as probe. The distribution of this repetitive DNA family is restricted to the distal region, mainly of the subtelocentric and acrocentric chromosomes. (a) $2n = 48$ specimen from the Marrecas population (Londrina, Paraná), (b) $2n = 50$ specimen from the Centenário population (Maringá, PR). M = metacentric, SM = submetacentric, ST = subtelocentric and A = acrocentric. Bar = 5 μm .

(Figure 2b) and DA/MM positive in the $2n = 50$ Centenário population (Figure 3b).

The pericentromeric heterochromatins of the two populations did not respond to the GC and AT fluorochrome ligand treatments (Figures 2b-d and 3b-d).

Discussion

The karyotypes and fundamental numbers of *A. scabripinnis* from the Marrecas and Centenário stream populations agree with the results of Moreira-Filho and Bertollo (1991) and Mantovani *et al.* (2000). The distribution and polymorphism of the constitutive heterochromatin observed here were described and extensively discussed by Mantovani *et al.* (2000).

A comparison between the C-banding pattern and our FISH results enabled us to distinguish at least two groups of constitutive heterochromatin: i) pericentromeric heterochromatin, which showed no homology with the As51 repetitive DNA family and ii) a set of distal heterochromatins which were homologous with the As51 sequence.

These data provide evidence of the particular homogeneity of each heterochromatin chromosomal domain (distal and pericentromeric) and of the heterogeneity between these two domains. Both the homogeneity and the heterogeneity are related to the equilocal distribution of heterochromatic regions between members of the complement (John, 1988). According to the “bouquet” polariza-

tion model, the physical contact of equilocal heterochromatic DNA of homologues and non-homologues is provided by the spatial disposition of chromosomes in the nucleus during the initial stages of meiosis when molecular processes favor the homogenization of satellite DNA sequences (Schweizer and Loidl, 1987).

According to Mantovani *et al.* (2000), both the populations analyzed by us show equilocal heterochromatin distribution, and it may be that the above results derive from this distribution.

A similar lack of response of the pericentromeric heterochromatic domain after base-specific fluorochrome staining as that observed in the two populations studied by us has also been found in the chromosomes of other fish species (Sola *et al.*, 1992; Rossi *et al.*, 1996; Sola *et al.*, 2000) and of other *A. scabripinnis* populations (Souza and Moreira-Filho, 1995; Souza *et al.*, 1996). Considering the common pattern displayed by the *A. scabripinnis* populations studied to date, it may be that pericentromeric heterochromatins contain sequences with preserved compositions (seen in several species of the family Sparidae, see Garrido-Ramos *et al.*, 1995) and preserved structures, since pericentromeric heterochromatin plays a fundamental role in the centromeric structure (Haaf *et al.*, 1992).

In contrast, the distal heterochromatic domains of both populations were DA/DAPI negative and showed no response to DAPI staining, which seems at variance with the presence of the AT-rich As51 satellite DNA in the distal heterochromatins. However, the presence of DA/DAPI negative bands may agree with the base-pair composition of this heterochromatic domain because distamycin has high affinity with and is specific to AT-rich domains, forming a very stable complex with DNA and causing structural changes in the double strand (Zimmer *et al.*, 1971) which may reduce or block the accessibility of DAPI to the chromosomal DNA, as has been previously described for another *A. scabripinnis* population (Souza *et al.*, 1996).

Although DAPI is known to bind to both GC and AT base pairs its fluorescence is significantly enhanced by AT-rich regions (Lin *et al.*, 1977) so that the AT base content of the As51 satellite DNA (59%) may be insufficient to produce DAPI-positive fluorescence, as has already been described for daunomycin, an antibiotic that only fluoresces when bound to highly AT-rich (65%) DNAs (Comings and Drets, 1976; Comings, 1978; Johnston *et al.*, 1978). Moreover, when discussing other fluorescent compounds, Comings and Drets (1976) pointed out that lack of fluorescence in heterochromatic regions may be due to the effect of chromosomal proteins on access of the fluorochromes to satellite DNA, while Saitoh and Laemmli (1994) stated that the structural organization of the chromosomal heterochromatic sequences may also be a factor in whether or not fluorescence occurs.

As regards the GC-specific fluorochrome staining, the two *A. scabripinnis* populations showed distinct fluo-

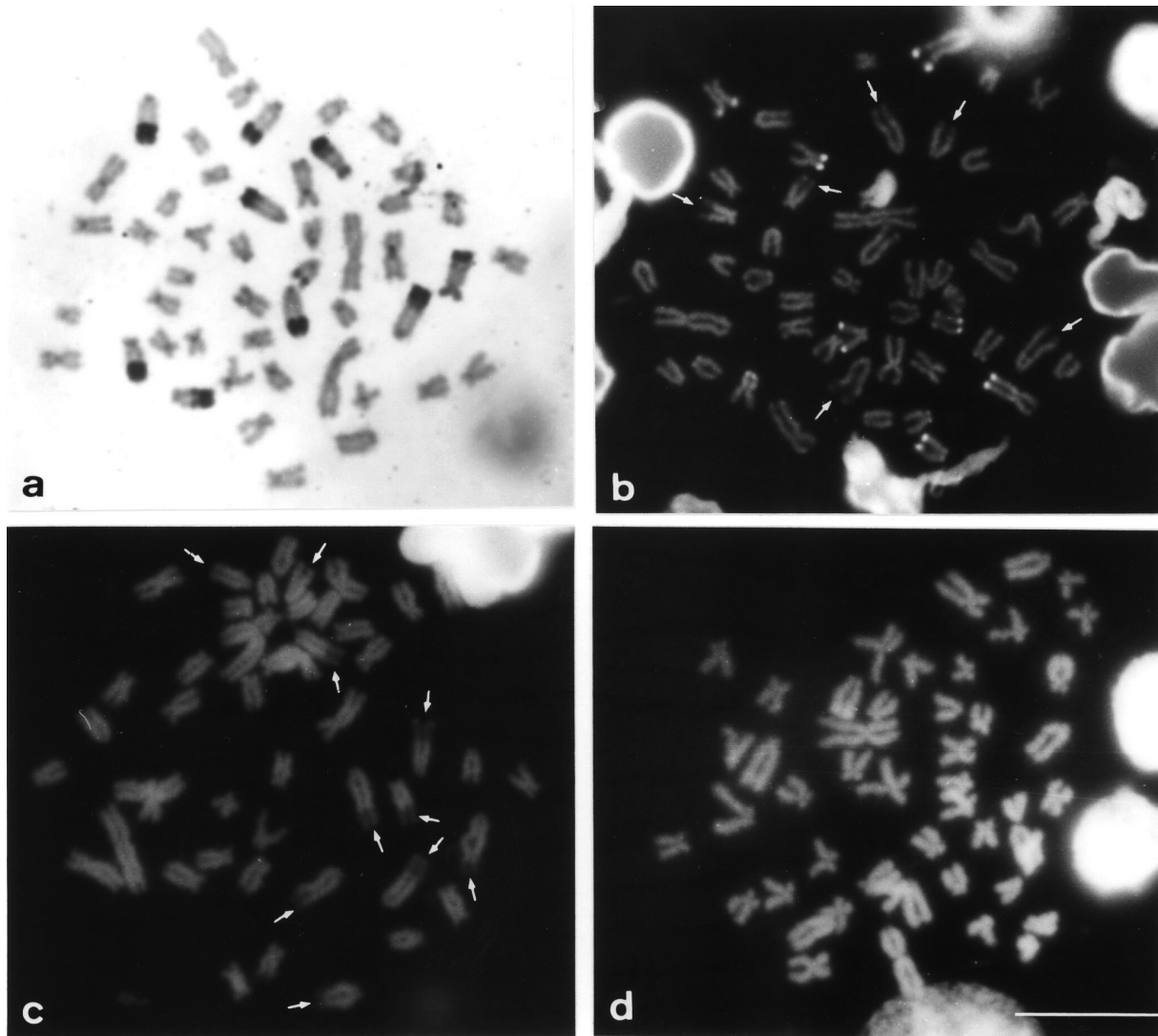


Figure 2 - Metaphase chromosomes of *Astyanax scabripinnis* from the Marrecas population showing distinct responses to different base-specific fluorochromes. (a) Incomplete C-banded metaphase chromosome showing constitutive heterochromatin (dark regions). The telomeric heterochromatic blocks are indicated by arrows as DA/MM negative in (b) and DA/DAPI negative in (c), while pericentromeric heterochromatin did not respond to these treatments. (d) DAPI treatment showing homogeneous staining of the chromosomes. The DA/MM-positive staining in (b) corresponds to the nucleolus organizer regions. Bar = 5 μ m.

rescent patterns for distal heterochromatins. In the $2n = 48$ Marrecas population the heterochromatin was DA/MM negative, in agreement with the base pair composition of this domain but, in contrast, the distal heterochromatin of the $2n = 50$ Centenário population was DA/MM positive, which might suggest a higher proportion of GC bases were it not for its homology with the As51 satellite DNA.

Even though the NORs of fishes frequently fluoresce after GC-specific fluorochrome staining (Schmid and Guttenbach, 1988) these regions have been detected associated to the distal heterochromatic blocks in only one chromosome of the Centenário population karyotype (Mantovani *et al.*, 2000), indicating that the DA/MM-positive pattern is resultant from characteristics of the heterochromatin itself.

Since the distal heterochromatic domains of the Marrecas and Centenário populations show homology with the same sequence, as revealed by the FISH-As51 method, the dissimilar responses described above cannot be attributed to base pair composition and our data may be evidence of differentiated structural arrangements of the distal constitutive heterochromatins in the populations studied.

The DA/MM-positive staining of the distal heterochromatin of the $2n = 50$ Centenário population does not exclude the possibility of the presence of a second, GC-rich, satellite DNA family besides the As51 family in the same heterochromatic domain. The GC clusters of this putative family might be exposed on the chromosomes of the Centenário population, while the $2n = 48$ Marrecas population may possess inverse structural organization that ex-

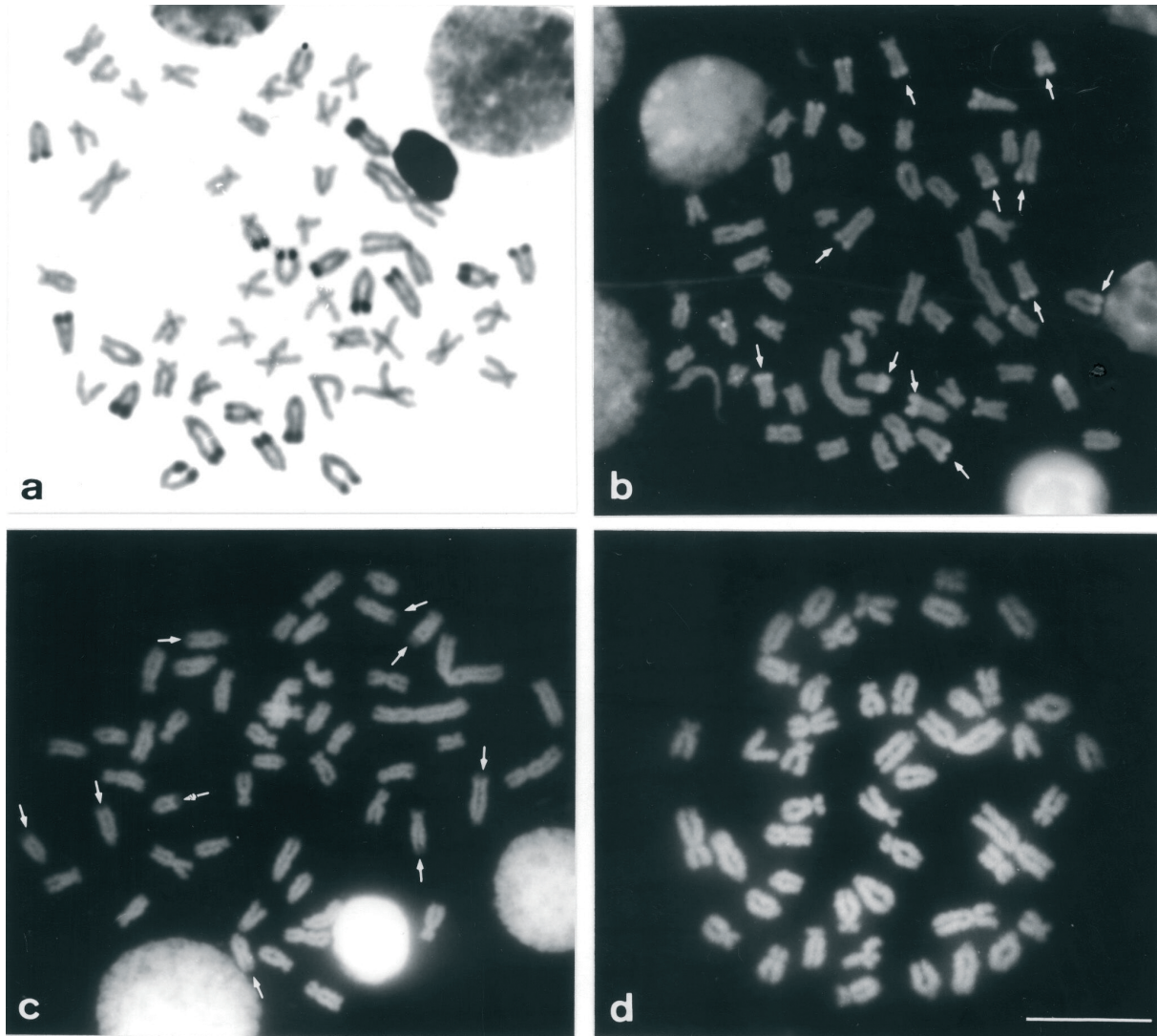


Figure 3 - Metaphase chromosomes of *Astyanax scabripinnis* from the Centenário population showing distinct responses to different base-specific fluorochromes. (a) C-banding showing constitutive heterochromatin (dark regions). The telomeric heterochromatic blocks are indicated by arrows as DA/MM positive in (b) and DA/DAPI negative in (c), while pericentromeric heterochromatin did not respond to these treatments. In (d) the chromosomes are uniformly stained with DAPI. The DA/MM-positive areas, not indicated by arrows in (b), are nucleolus organizer regions. Bar = 5 μ m.

poses the As51 sequences. These assumptions support the presumed influence of the non-random arrangement of nucleotide sequences based on the fluorescent patterns obtained with specific fluorochromes (Johnston *et al.*, 1978; Saitoh and Laemmli, 1994), and again support the proposition that the distal heterochromatins of the Centenário and Marrecas populations are structurally dissimilar. The presence of a GC-rich satellite sequence in the distal heterochromatin of the Centenário population could be checked using the method of Rab *et al.* (1996), which employs differential denaturation based on the fact that GC-rich clusters have higher thermal stability.

The DA/MM negative pattern of the distal heterochromatic domain of the 2n = 48 Marrecas population has also been observed for a 2n = 48 population of the

scabripinnis complex from another river basin (I. L. Souza, personal communication) and therefore appears to be an exclusive characteristic of 2n = 48 populations studied to date. This indicates that the same structural conformation of heterochromatin is shared by these cytotypes, reinforcing the idea that 2n = 48 *A. scabripinnis* populations from different river basins are more closely related to each other than to the 2n = 50 populations from the same river basin (Oliveira *et al.*, 1998; Mantovani *et al.*, 2000).

In addition to highlighting the karyotypic diversity of the *scabripinnis* complex, this work also reveals that a combination of techniques can be valuable in the qualitative investigation of constitutive heterochromatin in fish, since the results allow one to conclude that other factors besides the base composition of DNA may influence the pattern of fluorescence obtained on fluorochrome staining.

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