ZOOLOGIA 29 (2): 159–166, April, 2012
doi: 10.1590/S1984-46702012000200008

# A comparative structural cytogenetic study in three allopatric populations of *Astyanax scabripinnis* (Teleostei: Characidae)

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ABSTRACT. Individual samples from five populations of the characid fish *Astyanax scabripinnis* (Jenyns, 1842) from the Tietê and Paranapanema river basins (Brazil) were studied. All individuals analyzed presented 50 chromosomes but three different karyotypic forms were observed. Additionally, some individuals of one karyomorph presented a macro supernumerary chromosome. No chromosomal differentiation was observed between males and females in any sample analyzed. C-banding revealed two distinct distribution patterns in these populations in which strong terminally located heterochromatic blocks were detected in the long arms of some chromosomes of the specimens from Cascatinha, Cintra, and Funari streams, whereas populations of the Capão Bonito stream and the Barbosa waterfall revealed less evident heterochromatic blocks on the chromosomes. The comparative study of the 18S rDNA localization by fluorescent *in situ* hybridization, and detection of active nucleolus organizing regions by silver stain (Ag-NORs) showed differences in rDNA content and expression of this gene site among the individuals analyzed. Chromosome mapping of the 5S rDNA revealed the presence of four sites located in two distinct chromosomal pairs, with no apparent differences among karyomorphs. Based on the biogeographical distribution and specific biological characteristics of the species, the data focus on the chromosome differentiation mechanisms involved in the speciation process acting on the populations of the *Astyanax scabripinnis* species complex.

KEY WORDS. Chromosome markers; karyoevolution; karyomorphs; nucleolus organizer region.

Characiformes is the most important group of fish in number of individuals and species in the Neotropics (Artoni *et al*. 2000). It constitutes a morphologically and an ecologically diverse group with complex biogeography and evolutionary history (Ortí & Meyer 1997). *Astyanax scabripinnis* (Jenyns 1842) a species of this group, is commonly found in the headwaters of small rivers and streams of the Upper Paraná River Basin (Britski 1972, Garutti & Britski 2000, Veregue & Orsi 2003). Considered as a complex of species by Moreira-Filho & Bertollo (1991), *A. scabripinnis* shows great morphological diversity (Souza *et al*. 1996), mainly in terms of body length, body height and snout length (Moreira-Filho & Bertollo 1991). Cytogenetic data on this species also reveal a variable karyotype with diploid numbers ranging from 2n = 46 to 2n = 50 chromosomes (Moreira-Filho & Bertollo 1991, Souza & Moreira-Filho 1995, Maistro *et al*. 1998, among others). Karyological analyses of specimens of *A. scabripinnis* have also revealed the occurrence of supernumerary chromosomes, with different types of B chromosomes identified in different populations based on their morphology and constitutive heterochromatin distribution patterns (Salvador & Moreira-Filho 1992, Mizoguchi & Martins-Santos 1997, Néo *et al*. 2000).

In the present work, samples of *A.* scabripinnis from five Brazilian localities were analyzed in order to determine its karyotypic macrostructure, and to physically map the location of the ribosomal genes using *in situ* hybridization. The comparative analysis of our data offers an insight on the chromosome differentiation process and, to a certain extent, the mechanisms involved in the speciation process acting on the *A. scabripinnis* species complex.

## MATERIAL AND METHODS

Specimens of *A. scabripinnis* (90 females and 83 males) from the largest hydrographic system of the Alto Paraná river, Brazil, were studied. According to their origin, samples were

distributed as follows: Tietê river basin – 72 individuals (39 females and 33 males) from the Cascatinha stream, 22°59'23"S 48°25'31"W; 52 (29 females and 23 males) from the Cintra stream, 22°52'35"S 48°28'56"W; 17 (9 females and 8 males) from the Funari stream, 22°53'09"S 48°29'31"W, and Paranapanema river basin – 22 (10 females and 12 males) from the Capão Bonito stream, 22°54'33.91"S 48°30'54.92"W, and 9 (3 females and 6 males) from the Barbosa waterfall, 22°55'22"S 48°32'40"W. After identification and analysis, the specimens were deposited in the fish collection at the museum of the Laboratory of Biology and Fish Genetics, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil.

Chromosome preparations were obtained from anterior kidney cells according to the conventional air-drying technique (Foresti *et al*. 1981). For the identification of the constitutive heterochromatin (C-banding) the basic procedure proposed by Sumner (1972) was used with minor adaptations. The nucleolus organizing regions (NORs) were identified by fluorescent *in situ* hybridization using a labeled 18S rDNA probe and NOR activity was detected using the silver nitrate staining technique (Howell & Black 1980).

Chromosomal mapping of the 18S and 5S rDNA sites was carried out using fluorescent *in situ* hybridization (FISH), according to the procedure established by Pinkel *et al*. (1986), with adaptations. The 18S rDNA and 5S rDNA probes were respectively obtained from the fish *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka & Galetti Jr 2004) and *Leporinus elongatus* Valenciennes, 1850 (Martins & Galetti Jr 1999). The 18S probe was labeled with biotin 14-dATP using nick translation following the manufacturer's instructions (Bionick Labelling System – Invitrogen), the hybridization was detected with avidin-FITC and the signals amplified with biotinylated anti-avidin. The 5S probe was labeled with digoxigenin 11-dUTP (Roche Applied Sciences) using PCR (Polymerase Chain Reaction) and hybridization signals were detected using anti-digoxigenin-rhodamine.

The chromosomes were counterstained with DAPI and analyzed under optical light microscope (Olympus BX61). The images were captured using the Image-Pro Plus 6.0 software (Media Cybernetics). Chromosome morphology was determined based on the ratio of arms length (according to Levan *et al*. (1964), and chromosomes were classified as metacentrics (m), submetacentrics (sm), subtelocentrics (st) and acrocentrics (a) and arranged in karyotype in descending order of size.

## RESULTS AND DISCUSSION

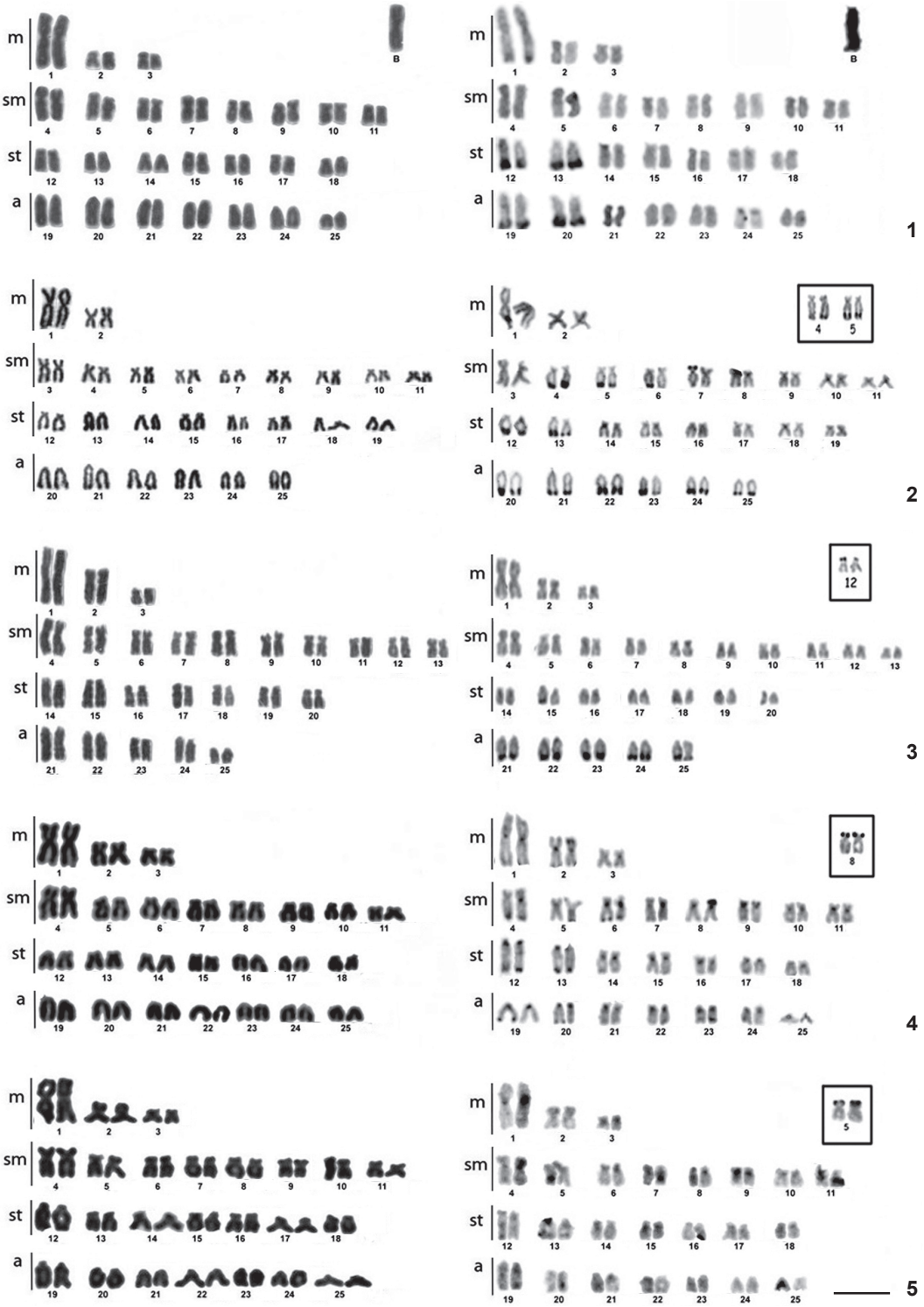
*Astyanax scabripinnis* shows considerable karyomorph diversity in spite of the conserved karyotype chromosomal number. Differences in chromosome morphology and its distribution in the karyotypes, together with differences in chromosome banding patterns and the location of two molecular markers on the chromosomes, allowed the proposition of cytotaxonomic and evolutionary inferences regarding this fish species. The populations of *A. scabripinnis* collected at the Cascatinha stream, the Funari stream, Cintra stream, Barbosa waterfall, and Capão Bonito stream presented a conserved standard diploid number of 50 chromosomes (Figs 1-5). However, striking differences were found in the fundamental numbers and in the distribution of the different chromosomal morphotypes (comparative data are summarized in Figs 6-10 and Tab. I).

The differences seem to be the result of active morphological modification mechanisms that resulted in inter-populational karyotypic differentiation. Therefore, a hypothetical-common-ancestral chromosome formula was likely composed of 6 metacentric, 16 submetacentric, 14 subtelocentric and 14 acrocentric chromosomes is proposed. This proposal is specially based on the conservation of the number of metacentric (three pairs) and the high number of acrocentric chromosomes observed in other species of *Astyanax*.

Consequently, ancestors of the fish from the Funari stream may have had a metacentric and an acrocentric chromosome that were independently rearranged into the current submetacentric and acrocentric chromosomes. In this case, it is possible that the metacentric chromosome became submetacentric while the acrocentric became subtelocentric, both via pericentric inversion. Fish from the Cintra stream, however, seem to have two ancestral acrocentric chromosome pairs independently rearranged into new submetacentric chromosomes, apparently via the occurrence of pericentric inversions. Therefore, it can be suggested that multiple and independent events of non-Robertsonian chromosomal rearrangements, namely pericentric inversions, have also acted on the karyotypic diversification of *A. scabripinnis* populations.

Table I. Descriptive karyotypic and chromosomal data from five populations of *Astyanax scabripinnis* studied in this work.

| Cytotypes | 2n/FN | Number of specimens | | Chromosome | rDNA 18S/5S (number of sites) | Number of B Chromosomes | | NOR (pairs) |
|---|---|---|---|---|---|---|---|---|
| | | Female | Male | Formula | | Females | Males | |
| Cascatinha | 2n = 50, FN = 86 | 39 | 83 | 6m+16sm+14st+14a | 4/2 | 1 or 2 | 0 | 6, 9 |
| Funari | 2n = 50, FN = 88 | 9 | 8 | 4m+18sm+16st+12a | 6/2 | 0 | 0 | 5 |
| Cintra | 2n = 50, FN = 90 | 29 | 23 | 6m+20sm+14st+10a | 6/2 | 0 | 0 | 12 |
| Barbosa waterfall | 2n = 50, FN = 86 | 3 | 6 | 6m+16sm+14st+14a | – | 0 | 0 | 8 |
| Capão Bonito | 2n = 50, FN = 86 | 10 | 12 | 6m+16sm+14st+14a | 6/2 | 0 | 0 | 5 |

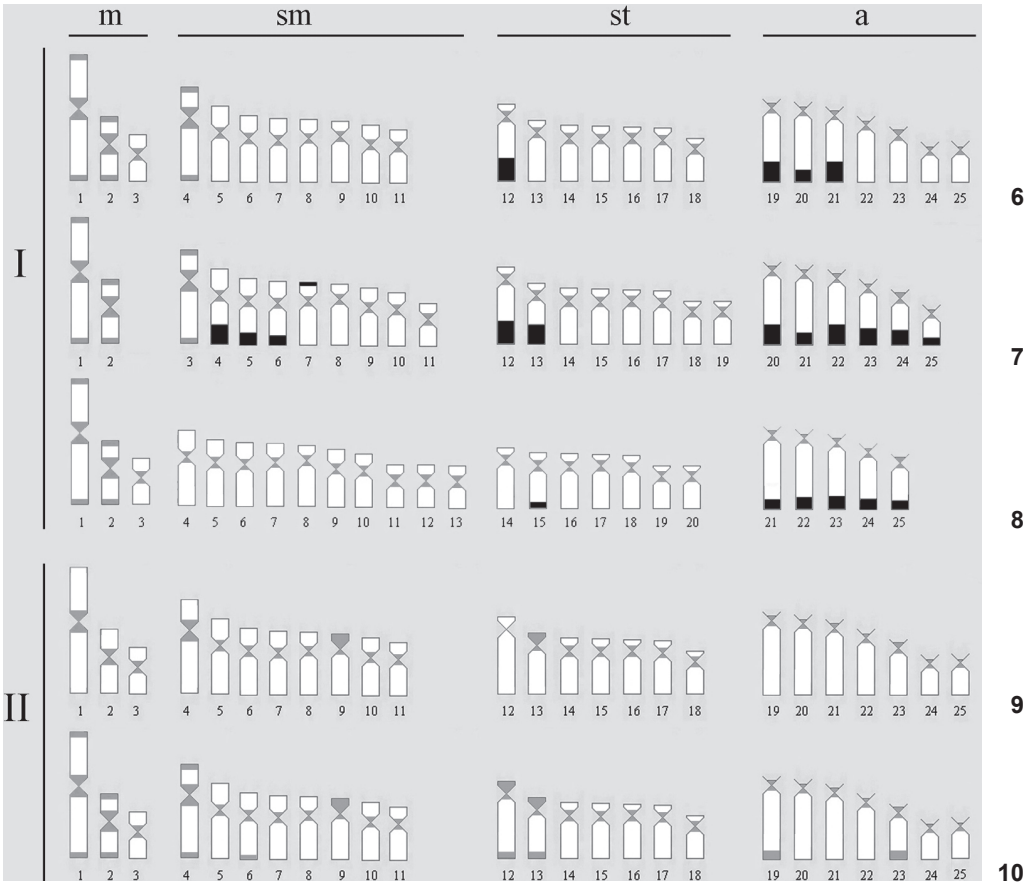



Figures 1-5. *Astyanax scabripinnis* karyotypes from Cascatinha stream (1), Funari stream (2), Cintra stream (3), Barbosa waterfall (4) and Bonito stream (5). Chromosomes were stained with Giemsa (left) and sequentially subjected to C-banding (right). In the boxes are chromosome pairs that show active nucleolus organizer regions, as reflected by signals after silver nitrate staining. Scale bar: 10 μm.

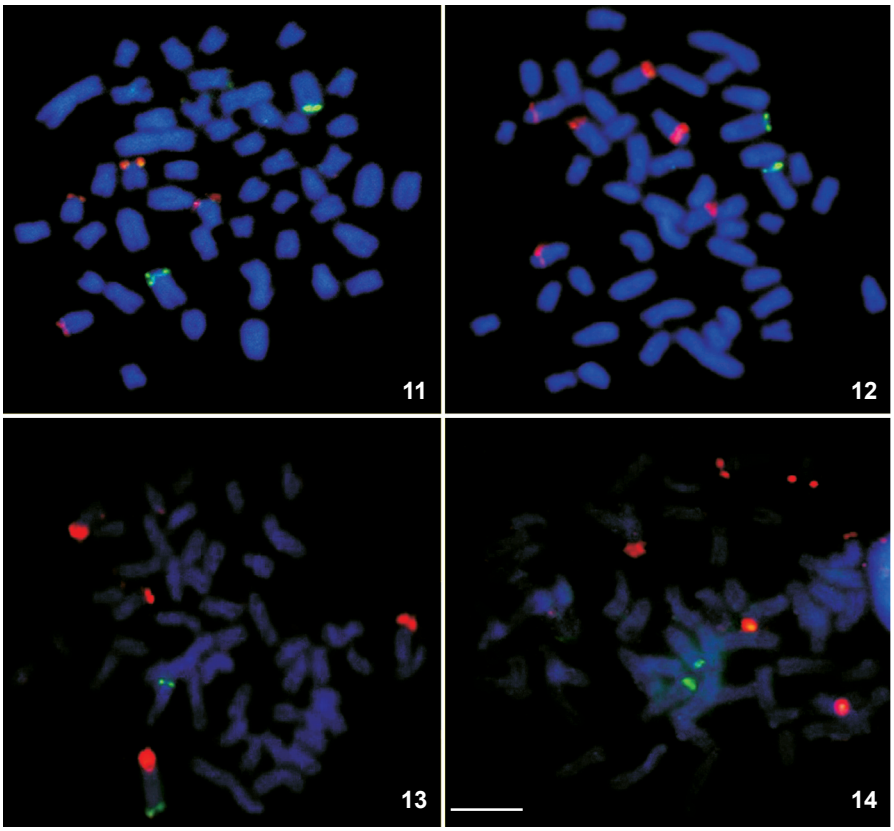
In addition, the results clearly support the hypothesis proposed by Moreira-Filho & Bertollo (1991), which suggests that *A. scabripinnis* should be considered a species complex, with five different and well defined cytogenetic entities distributed among the allopatric populations. The present findings allows concluding that differences in karyotype macrostructure, represented by differentiation in the chromosome formula and in the fundamental number, are common events in allopatric populations of *A. scabripinnis*, reinforcing the position assumed by the abovementioned authors in that such karyotypic differences could be interpreted as evolutionary solutions that allow these fish to occupy differentiated habitats such as the headwaters of rivers.

Nonetheless, *A. scabripinnis* exhibit certain genetic plasticity that allows to the origin, maintenance, and posterior fixation of chromosomal rearrangements in the populations. In this sense, Souza & Moreira-Filho (1995) described the presence of independent evolutionary units (cytotypes) of *A. scabripinnis* living in sympatry in the same river. The occurrence of different cytotypes in sympatry suggests that the discussed karyotypic evolution in this species may be a rather dynamic and fast succession of events. However, the karyotype evolution model of *A. scabripinnis* is complex and goes beyond the 'simple' accumulation of chromosomal rearrangements. Indeed, patent quantitative and qualitative differences in heterochromatin content among the studied populations were also identified (Figs 6-14). Specimens from the Cascatinha, Funari and Cintra streams present the largest amount of heavy stained heterochromatin, and the large heterochromatic blocks are located in the telomeric regions of subtelocentric and acrocentric chromosomes, differentiating these three populations from the remaining two (i.e., the Capão Bonito stream and the Barbosa waterfall that show only relatively small amounts of heterochromatin in some telomeric and centromeric chromosome regions).

The population of the Cascatinha stream was polymorphic for the occurrence of supernumerary chromosomes, presenting individuals with none, one, or two large metacentric B




Figures 6-10. Idiogram of the different karyomorphs and C-banding patterns observed in the *Astyanax scabripinnis* populations from the Cascatinha stream (6), Funari stream (7), Cintra stream (8), Barbosa waterfall (9) and Bonito stream (10). (I) Tiete river basin and (II) Paranapanema river basin.




Figures 11-14. FISH with 18S (red) and 5S (green) rDNA probes on DAPI counterstained chromosome spreads of *Astyanax scabripinnis* from the populations of Cascatinha stream (11), Funari stream (12), Barbosa waterfall (13), and Capão Bonito stream (14). Scale bar: 10 µm.

chromosomes, at the frequency of 0.507 in the sample considered, which corresponds to the 37 individuals carrying B chromosomes (28 females and 9 males), out of the 73 specimens analyzed in that population. It is interesting to notice that specimens that carry B chromosomes, which are highly heterochromatic, happen to belong to a population that has high amount of heterochromatin, probably an indicative of the population' tolerance to heterochromatinized DNA.

Differences in the patterns of heterochromatin distribution were also found by Mantovani *et al.* (2000) among *A. scabripinnis* populations. These authors reported within-population polymorphisms (i.e., inter-individual differences) for number and size of the heterochromatic blocks in the individuals analyzed. Some authors suggest that the polymorphism for heterochromatin can be caused by the presence of transposable elements through genomic mechanisms of invasion (Tafalla *et al.* 2006, Slotkin & Martienssen 2007). Such variation in heterochromatic content has been also often reported in other *Astyanax* species (Souza *et al.* 1996, Mantovani *et al.* 2004, Abel *et al.* 2006). Although additional sampling and biogeographic distribution studies are needed, it is interesting to note that the two main patterns of heterochromatin content coincide with the geographic distribution of the *A. scabripinnis* populations studied herein.

Considering the results presented in figures 1-5 and the ancestral common karyotype inferred in figures 6-10, it is apparent that specimens from the populations restricted to the Tietê river basin have either preserved the ancestral heterochromatic content or have accumulated more heterochromatin. Populations belonging to the Paranapanema river basin, however, show fewer and paler centromeric and telomeric heterochromatic sites as another evidence of heterochromatic loss since the common ancestor (Figs 1-10). At the two extremes of the heterochromatin amount distribution, the Cascatinha stream and Barbosa waterfall populations show respectively the highest and lowest detected amounts of heterochromatin, being the latter population apparently very intolerant to heterochromatinized DNA, probably due to restrictions imposed by the habitat conditions. The lowest heterochromatin content in the populations of the Paranapanema river basin could also be due to the occurrence of stochastic mechanisms during the differentiation process, such as genetic drift and founder effect, although these are less likely given that they would require independent loss of heterochromatin in four

chromosome pairs. However, the hypothesis that specimens forming these populations could have originated from a different stock population before giving rise to the populations of the Tiete river basin could not be discarded.

The differences in the number and localization of the NORs in the chromosomes of the specimens from the studied populations were also considered (Figs 1-5 box, Tab. I). Based on the analysis of the transcriptional genes by silver nitrate (Howell 1977, Jordan 1987), inter-population variability in NOR activity has been a common characteristic among several species of *Astyanax* (Ferro *et al*. 2001, Vicari *et al*. 2008, Kavalco *et al*. 2009). Still, NOR activity is not qualified as a strong cytotaxonomical marker in these fishes. Although some active NORs are apparently fixed in each population, polymorphism for the occurrence of other occasionally active 18S genes sites also exists. Probably due to the presence of silent NORs, the physical mapping of ribosomal genes on the chromosomes of *A. scabripinnis* from four populations (the Cascatinha stream, the Funari stream, the Barbosa waterfall and the Capão Bonito stream) showed higher number of 18S genes sites when compared to the Ag-NORs marks, although when present they were found in accordance to these chromosome markers (Figs 11-14, in red). The dispersion of the rDNA18S sites in fish karyotype has been attributed to several mechanisms, such as transposition and duplication events (Vicari *et al*. 2006), and appears to reflect a concerted evolution event.

With signals present on the homologues of two chromosome pairs, marking an interstitial and a telomeric site (Figs 11-14, in green), the 5S rDNA presented a more conserved chromosomal location among these four population samples. The presence of only one 5S rDNA site was identified in some *Astyanax* species such as *A. altiparanae* Garutti & Britski, 2000 (Fernandes & Martins-Santos 2006) and *Astyanax fasciatus* (Cuvier, 1819) (Ferreira-Neto *et al*. 2012). However, in other *Astyanax* species, including different populations of the *A. scabripinnis* complex (Ferro *et al*. 2001, Almeida-Toledo *et al*. 2002, Mantovani *et al*. 2005) and *A. fasciatus* (Ferreira-Neto *et al*. 2012), two chromosome pairs, one metacentric and the other acrocentric, have been commonly reported to show 5S rDNA sites located proximally to the centromere. Such situation seems to evidence a probable synapomorphic feature among the *Astyanax* species.

We found an uncommon chromosome heteromorphism in individuals of the population from the Barbosa waterfall in which a single chromosome presented 18S and 5S rDNA sites, located on the short and long arms, respectively (Fig. 13). Based on the lowest heterochromatin content in this population and on the apparent lack of evident chromosomal rearrangements since the common ancestor, we consider the abovementioned heteromorphism another evidence suggesting that the divergence of the specimens of this population may be dictated by DNA sequence evolution rather than chromosome level rearrangements.

Several approaches have been used to make sense of the complex and variable nature of *A. scabripinnis* species. In order to minimize problems of identification, unnatural inferences about the relationships, and wrong evolutionary series, each approach should take into account the local geomorphological history and biological characteristics of the species and their evolutionary time (Artoni *et al*. 2009). The great diversity in the karyotype macrostructure found in *A. scabripinnis* is considered a common trait of this species (Moreira-Filho & Bertollo 1991) and molecular cytogenetic data have reinforced this observation (Moisés & Almeida-Toledo 2002, Munin *et al*. 2004, Sofia *et al*. 2006). Such diversity is very likely facilitated by the fact that this fish occupies river headwaters and probably became restricted to these environments (Gomes & Azevedo 1960, Britski 1972). The larger rivers likely represent important barriers preventing interbreeding between populations of headwater stretches (Érica P. Caramaschi, unpubl. data). In addition, founder effect, probably enhanced by the disruption of gene flow in populations isolated by local geomorphologic events and evolutionary time (Artoni *et al*. 2009), or sympatric speciation are events that support the increased variability found in the karyotype of A. *scabripinnis*.

## ACKNOWLEDGMENTS

This study was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP process 2008/57067-1), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We thanks to Renato Devidé for technical support.

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Submitted: 14.II.2012; Accepted: 11.IV.2012.
Editorial responsibility: Walter A. Boeger