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Research Article

Comparative cytogenetics among species of the *Astyanax scabripinnis* complex. Evolutionary and biogeographical inferences

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Abstract

Karyotype data are presented for distinct species of the genus *Astyanax* from four rivers belonging to three different hydrographic basins of the State of Paraná, Brazil (Verde River - Tibagi basin, Açungui River - Ribeira basin, and Santo Antônio and Jaguariaíva Rivers - Jaguariaíva basin). Three karyotypic forms were identified, here denominated karyotype A (2n = 50 chromosomes, with 8m+18sm+10st+14a, and thirteen 18S rDNA sites); karyotype B (2n = 50 chromosomes, with 8m+18sm+10st+14a, and four 18S rDNA sites); and karyotype C (2n = 48 chromosomes, with 10m+16sm+10st+12a, and eight 18S rDNA sites). The pattern of constitutive heterochromatin was similar among the three karyotypic forms, with few differences. The 5S rDNA corresponds to a synapomorphic character regarding its number and chromosomal localization. The karyotypic form A occurs in the distribution center of the type locality of *A. paranae*, in the proximities of the town of Castro (Tibagi basin), and may have reached the headwaters of the Ribeira River by the breakdown of geographical barriers. The karyotypic forms B and C are sympatric and syntopic, occurring solely in the Jaguariaíva River basin. Our hypothesis is that the karyotypic form A corresponds to the species *A. paranae* and forms B and C correspond to other species of the *A. scabripinnis* complex.

Key words: karyotype evolution, cytotaxonomy, rDNA sites, biogeography.

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Introduction

The genus *Astyanax* belongs to a group of Neotropical fish widely distributed throughout Central and South America and previously placed in the subfamily Tetragonopterinae (Géry, 1977). Recently, this group was listed as *incertae sedis* in Characidae, due to a lack of consistent evidence of monophyletism (Lima *et al.*, 2003). Thus, cytogenetical/evolutionary studies are of special interest in *Astyanax*, possibly contributing to the elucidation of the interrelationships among its species.

Based on morphological and chromosomal characters, Moreira-Filho and Bertollo (1991) proposed that *A. scabripinnis* should constitute a species complex. Nowadays, it is considered that this complex is composed by approximately 15 species, including *A. paranae* (Bertaco and Lucena, 2006). This species has been usually considered as a subspecies of *A. scabripinnis* (Eigenmann, 1927; Maistro *et al.*, 1998; Garutti and Britski, 2000), probably due to the lacks of precise diagnostic characters (Bertaco and Lucena, 2006).

The type locality of *A. paranae* is the region of Castro, a city in the State of Paraná, Brazil (Eigenmann, 1914 apud Garutti and Britski, 2000). Specimens of the *A. scabripinnis* complex from streams of the State of Paraná, belonging to the Tibagi basin headwaters in the proximities of Castro and to the Ribeira and Jaguariaíva basins, were analyzed in the present study. The main objective was the karyotype characterization, as well as a cytogenetic comparison among the different samples in order to establish possible evolutionary/biogeographical relationships among them.

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Material and Methods

One hundred and thirty *Astyanax* specimens were analyzed: 44 specimens from the Verde River - a tributary of the Tibagi basin, in the Castro region (25° 04' 81" S and

50° 04' 63" W), 17 specimens from the Açungui River - a tributary of the Ribeira de Iguape basin (25° 44' 89" S and 49° 67' 44" W), and 69 specimens from the Cerrado State Park, upstream the Santo Antônio and Jaguariaíva Rivers - Jaguariaíva basin (24° 35' 42" S and 49° 25' 67" W) - (Table 1). All specimens showed characters that diagnosed the *A. scabripinnis* species complex. Testimony samples were deposited at the fish collection of the Museu de Zoologia of the Universidade Estadual de Londrina, Paraná, Brazil (voucher numbers: MZUEL 3700, *A. scabripinnis paranae* from the Verde River; MZUEL 4064, *Astyanax* sp. cf. *A. scabripinnis paranae* from the Açungui River; MZUEL 3702, *Astyanax* sp. from the Santo Antônio River).

Chromosome preparations were obtained from anterior kidney cells using *in vivo* colchicine treatment (Bertollo *et al.*, 1978). Constitutive heterochromatin was visualized by C-banding (Sumner, 1972), as well as by double fluorochrome staining using Chromomycin A_3+DAPI (Schweizer, 1980), which are indicative of GC- and AT-rich regions, respectively.

Nucleolar organizing regions (NORs) were detected using silver nitrate staining (Ag-NORs), according to Howell and Black (1980), and fluorescent *in situ* hybridization (FISH) to locate the 18S rDNA sites on the chromosomes. An 18S rDNA probe (nearly 1,800 bp) generated by PCR of nuclear DNA from the fish *Prochilodus argenteus* (Hatanaka and Galetti Jr., 2004) was used. A 5S rDNA probe from the fish *Leporinus elongatus* (Martins and Galetti, 1999) was employed to map the 5S rDNA sites on the chromosomes. Both probes were labelled with 14-dATP biotin by nick translation following the manufacturer's instructions (Bionick Labelling System - Invitrogen). The FISH signals were visualized according to Pinkel *et al.* (1986) and analyzed in an Olympus BX50 epifluorescence microscope. Chromosome images were captured using the software CoolSNAP-Pro (Media Cybernetics).

Nearly 30 metaphases per specimen were analyzed to determine the diploid chromosome number and the karyotype structure. Chromosomes were classified as m (metacentric), sm (submetacentric), st (subtelocentric), and a (acrocentric), according to Levan *et al.* (1964).

Results

Three distinct karyotypes were identified considering all the specimens analyzed, here named A, B, and C, differing in the number of chromosomes and/or karyotype structure.

Karyotype A

This karyotypic form showed 2n = 50 chromosomes, differentiated into 8m+18sm+10st+14a (Figure 1 A). The constitutive heterochromatin was located in the centromeric region of all chromosomes and in the interstitial or telomeric regions of a few chromosome pairs (Figure 1 B; Table 2). Multiple telomeric Ag-NORs were observed, with intra and inter-individual variations (Figure 2 A, B; Table 2). Thirteen 18S rDNA sites were detected by FISH, invariably in a telomeric position, with a subtelocentric chromosome presenting bitelomeric sites, *i.e.*, NORs in both telomeres, in only one chromosome of the pair (Figure 2 E). The double CMA_3/DAPI staining showed CMA_3-positive and DAPI-negative signals coincident with the 18S rDNA sites (Figure 2 C, D, respectively). The 5S rDNA genes were located in the proximal region of the long arm in two chromosome pairs, one metacentric and one acrocentric (Figure 2 F). This karyotypic form was detected in the specimens from the Verde River (Tibagi basin) and Açungui River (Ribeira de Iguape basin), in the surroundings of the town of Castro. The acrocentric pair 21 was polymorphic for the Verde River population concerning a large heterochromatic block distally located on the long arm, detected in a few heterozygote specimens (Figure 1 B).

Table 1 - Number of *Astyanax* samples with the karyotypic forms A, B, and C. Kar = karyotypic forms; n. f . = number of female specimens; n. m. = number of male specimens.

Rivers	Kar	n. f.	n. m.	Total
Verde	A	31	13	44
Açungui	A	11	6	17
Santo Antônio	B	34	10	44
Santo Antônio	C	13	6	19
Jaguariaíva	C	0	6	6
Total		89	41	130

Karyotype B

This karyotype also presented 2n = 50 chromosomes, organized into 8m+18sm+10st+14a (Figure 1 C). The constitutive heterochromatin was located in the centromeric region of all chromosomes and in the interstitial or telomeric regions of a few chromosome pairs (Figure 1 D; Table 2). Ag-NORs were also telomeric, with intra and inter-individual variations. These variations, however, were less than those found in karyotype A (Figure 3 A, B; Table 2), which was also confirmed by the detection of only four 18S rDNA sites detected by FISH (Figure 3 E). The double CMA_3/ DAPI staining showed CMA_3-positive and DAPI-negative signals in conspicuous sites coincident with the 18S rDNA sites (Figures 3 C, D, respectively). The 5S rDNA genes were located in the proximal region of the long arm in two chromosome pairs, one metacentric and one acrocentric (Figure 3 F). This karyotypic form was found in the specimens from the Santo Antônio River (Jaguariaíva basin).

Karyotype C

Different from karyotypes A and B, the karyotype C presented 2n = 48 chromosomes, arranged into 10m +

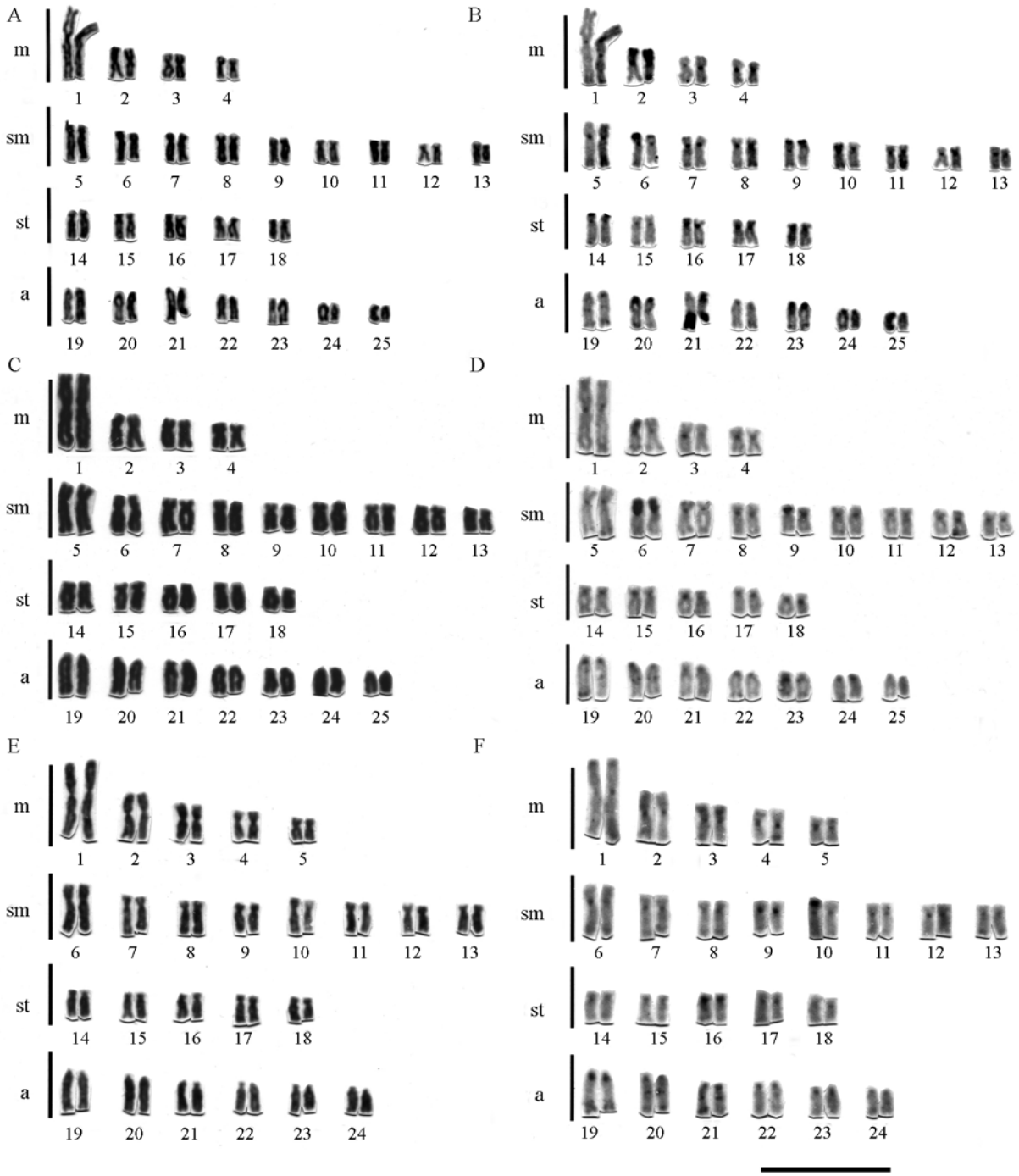

Figure 1 - Karyotypic forms of the *Astyanax scabripinnis* species complex: (A, B) Karyotype A (*Astyanax paranae*) from the Verde and Açungui Rivers; (C, D) Karyotype B (unidentified species) from the Santo Antônio River; (E, F) Karyotype C (unidentified species) from the Santo Antônio River. (A, C, E) conventional Giemsa staining, and (B, D, F) sequential C-banding, respectively. Bar represents 10 μm.

16sm+ 10st + 12a (Figure 1 E). The constitutive heterochromatin was located in the centromeric region of all chromosomes and in the telomeric regions of a few chromosome pairs, besides some conspicuous interstitial bands in acrocentric chromosomes (Figure 1 F). Multiple telomeric Ag-NORs were also observed, with intra and inter-individual variations from two to five sites (Figure 4 A, B; Table 2). Nevertheless, eight 18S rDNA sites were detected by FISH, always in telomeric positions (Figure 4 E). The double CMA_3/DAPI staining showed CMA_3-positive and DAPI-negative signals in conspicuous sites coincident with the 18S rDNA sites (Figures 4 C, D, respectively). The 5S rDNA genes were located near the centromeric region of the long arm in two chromosome pairs, one metacentric and one acrocentric (Figure 4 F). This karyotypic form was found in the Jaguariaíva River population, also occurring in sympatry and syntopy with karyotype B in the Santo Antônio River (Jaguariaíva basin).

Table 2 - Karyotype data of *Astyanax* from the Verde River (Tibagi basin), Açungui River (Ribeira basin), Santo Antônio River (Jaguariaíva basin), and Jaguariaíva River. Kar = karyotypes; Tel heterochr (a) pairs = telomeric heterochromatin in acrocentric pairs; Interst heterochr (a) pairs = interstitial heterochromatin in acrocentric pairs.

Rivers	Kar	2n	Karyotype formulae				Ag-NORs range	N. 18S sites	N. 5S sites	Tel heterochr (a) pairs	Interst heterochr (a) pairs
			m	sm	st	a					
Verde	A	50	8	18	10	14	2-7	13	4	All	19, 20
Açungui	A	50	8	18	10	14	2-10	13	4	19, 20	19, 20
Santo Antônio	B	50	8	18	10	14	1-4	4	4	19, 22, 23	20
Santo Antônio	C	48	10	16	10	12	2-5	8	4	19, 20, 21	20, 21
Jaguariaíva	C	48	10	16	10	12	2-4	8	4	19, 20, 21	20, 21

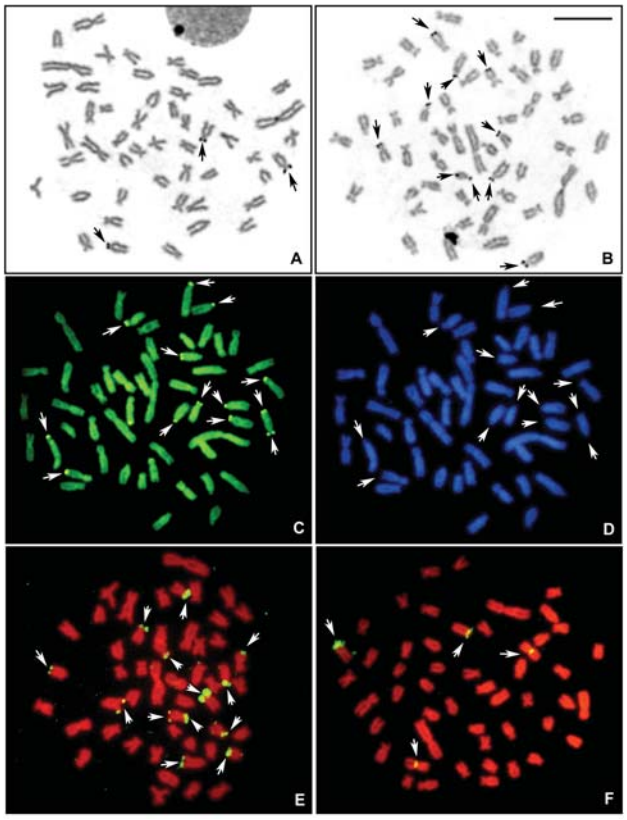

Figure 2 - Metaphases of the karyotypic form A (*Astyanax paranae*) evidencing (A, B) the Ag-NOR bearing chromosomes; (C) Chromomycin A_3 positive or GC-rich sites; (D) sequential DAPI negative or AT-negative sites; (E) the 18S rDNA sites, and (F) the 5S rDNA sites. Bar represents 10 µm.

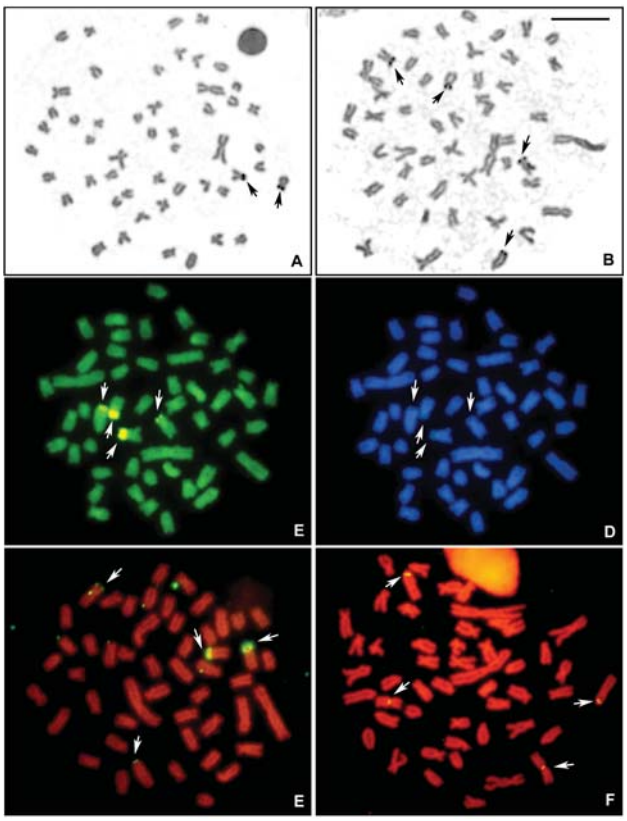

Figure 3 - Metaphases of the karyotypic form B (unidentified species of the *Astyanax scabripinnis* species complex) evidencing (A, B) the Ag-NOR bearing chromosomes; (C) Chromomycin A_3 positive or GC-rich sites; (D) sequential DAPI negative or AT-negative sites; (E) the 18S rDNA sites; and (F) the 5S rDNA sites. Bar represents 10 µm.

Discussion

The diploid chromosome number 2n = 50 was the most frequent one, found in three out the four studied samples (Verde, Açungui, and Santa Antônio Rivers). Indeed, the chromosome number 2n = 50 was also observed in the majority of the populations belonging to the *A. scabripinnis* species complex (Moreira-Filho and Bertollo, 1991; Mantovani *et al.*, 2000; Neo *et al.*, 2000; Maistro *et al.*, 2000; Ferro *et al.*, 2001; Maistro *et al.*, 2001; Moreira-Filho *et al.*, 2001; Ferro *et al.*, 2003).

Although the 2n = 50 karyotypes have similar macrostructures (8m+18sm+10st+14a), with just few variations in the distribution and localization of the heterochromatin, it was possible to differentiate them into two distinct forms concerning the 18S rDNA sites: karyotype A, found in the specimens from Verde and Açungui Rivers, is characterized by thirteen 18S rDNA sites, two of which located in both telomeres of the same chromosome (bitelomeric

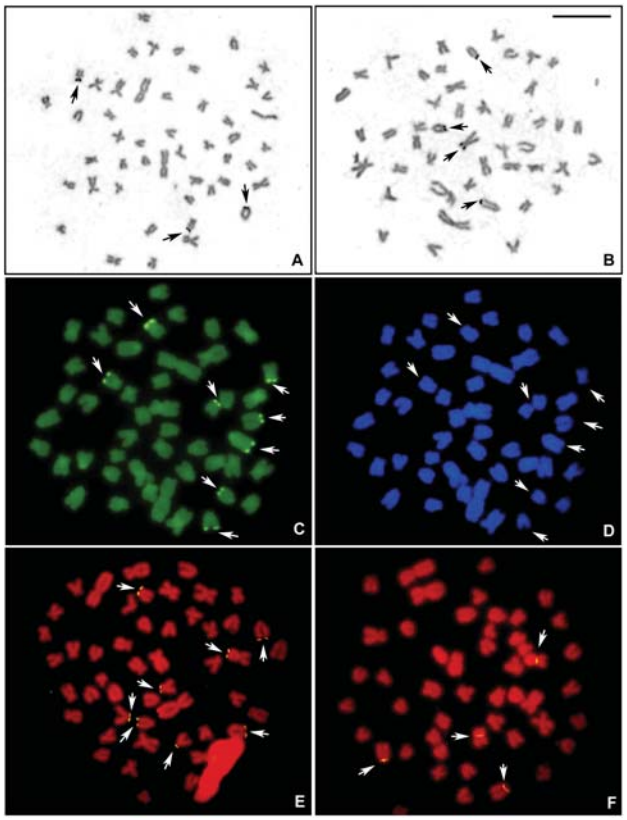

Figure 4 - Metaphases of the karyotypic form C (unidentified species of the *Astyanax scabripinnis* species complex) evidencing (A, B) the Ag-NOR bearing chromosomes; (C) Chromomycin A_3 positive or GC-rich sites; (D) sequential DAPI negative or AT-negative sites; (E) the 18S rDNA sites; and (F) the 5S rDNA sites. Bar represents 10 µm.

NORs); and karyotype B, found in the specimens from Santo Antônio River, characterized by presenting four 18S rDNA sites (Table 2). A third karyotypic form - karyotype C - is characterized by the presence of a smaller diploid number (2n = 48 chromosomes), a differentiated karyotypic formula (10m+16sm+10st+12a), and eight 18S rDNA sites. Specimens bearing this karyotype were found in the Jaguariaíva River, besides its sympatric and syntopic occurrence with specimens with the karyotype B in the Santo Antônio River (Table 2).

The comparative analysis of karyotypes B (2n = 50) and C (2n = 48) of the specimens from the Santo Antônio River suggests that they probably maintain a homeology of their chromosome pairs, except for the absence of two chromosome pairs (1 sm and 1 a) and the presence of a large metacentric pair (pair 2) in karyotype C. The morphological similarity indicates that the metacentric pairs 1, 2, 3, and 4 of the karyotypes A and B must be homeologous to pairs 1, 3, 4, and 5 of the karyotype C, respectively. Thus, the metacentric pair 2 of the karyotype C (2n = 48) may have originated through a translocation event between two small chromosomes, one submetacentric and one acrocentric, from a karyotype with 2n = 50 chromosomes.

The occurrence of 2n = 48 chromosomes has already been described for some specimens of the *A. scabripinnis* complex from the Upper Paraná River basin (Moreira-Filho and Bertollo, 1991; Souza *et al.*, 1996; Mizoguchi and Martins-Santos, 1998; Maistro *et al.*, 2000; Mantovani *et al.*, 2000; Alves and Martins-Santos, 2002). Besides the diploid number, they generally also differ from specimens with 2n = 50 chromosomes by a differentiated subtelocentric pair (Mantovani *et al.*, 2000) or a submetacentric pair (Maistro *et al.*, 2000). However, none of those 2n = 48 specimens presented a metacentric chromosome pair such as the number 2 of the karyotype C. Therefore, the karyotypic form C (2n = 48) from the Jaguariaíva basin would be more closely related to the karyotypic form B (2n = 50) of this same basin than to the other *Astyanax* of the *scabripinnis* complex with 2n = 48 chromosomes (Mantovani *et al.*, 2000; Maistro *et al.*, 2000). None of the sympatric specimens of the Santo Antonio River presented a hybrid karyotypic formula, suggesting a probable reproductive isolation between forms B and C. Furthermore, small differences in the localization of the heterochromatin blocks in the acrocentric chromosomes, as well as the occurrence of a distinct number of 18S rDNA sites (Table 2), agree with the possible existence of two different taxa. These data also suggest a model of allopatric speciation between these two karyotypic forms that would have become sympatric after the breakdown of geographical barriers, as is also proposed for a few other species of the *A. scabripinnis* complex (Souza *et al.*, 1995; Maistro *et al.*, 2000).

Multiple Ag-NORs were observed, but with numerical variations between the distinct karyotypic forms. The 18S rDNA sites were always located in the telomeric regions of the chromosomes, in accordance with the Ag-NORs. The double staining by the GC- and AT-specific fluorochromes, chromomycin A_3 and DAPI, respectively, showed GC-positive and AT-negative signals coincident with the 18S rDNA sites. The occurrence of a subtelocentric chromosome with bitelomeric NORs was verified only in the specimens with karyotype A. This feature, coupled with the higher number of NORs, reinforces a close phylogenetic relationship between the two samples with this karyotypic form, *i.e.*, the specimens from Verde River (Tibagi basin) and Açungui River (Ribeira de Iguape basin). A possible vicariant event by "headwater captures" (term used to designate the incorporation of the headwaters of a given river into another river belonging to an adjacent hydrographic basin), could explain the karyotype conservativeness among the specimens of these two adjacent hydrographic basins, with only few differences in heterochromatin distribution (Table 2), probably resulting from the current gene flow restriction. This model of headwater captures and gene flow restriction is also applicable to other fish species from these same basins, as is the case of *Hoplias malabaricus* (Vicari *et al.*, 2005), and *Geophagus brasiliensis* (Vicari *et al.*, 2006), and also has been used to explain the sympatric occurrence of different *Characidium*

species (Centofante *et al.*, 2001). Our proposition is in accordance with Ribeiro (2006) who considers that the Ponta Grossa Arch represents the most prominent geological feature in the region of Paranapanema, Iguaçu, and Ribeira de Iguape headwaters and stated that "the tectonic activity of the Ponta Grossa Arch could have resulted in a particular accelerated fluvial dynamism between adjacent drainage systems, accelerating the faunal exchange between them".

5S rDNA sites were found in a conserved location, proximally to the centromere of two chromosome pairs, one metacentric and one acrocentric, in all sampled specimens. The maintenance of these two chromosome pairs with 5S rDNA sites in the same position was also verified in other *Astyanax* species, as well as in other species of the *A. scabripinnis* complex (Ferro *et al.*, 2001; Almeida-Toledo *et al.*, 2002; Mantovani *et al.*, 2005), Nevertheless, some *Astyanax* species with only one chromosome pair bearing 5S rDNA sites were already described, such as *A. altiparanae* (Fernandes and Martin-Santos, 2006), and *A. janeiroensis* (Vicari, unpublished data), evidencing a probable synapomorphic feature among the species with two chromosome pairs bearing 5S rDNA.

The karyotype data here presented are important tools for the taxonomy of the *Astyanax* species. In this sense, our proposal is that the karyotypic form A corresponds to *A. paranae*, since it is found in the center of the distribution of its type locality, in the Castro region (Tibagi basin) and reaching, through breakdown of geological barriers, the headwaters of the Ribeira de Iguape basin. On the other hand, the karyotypic forms B and C, found in the Jaguariaíva basin, which are cytogenetically differentiated between themselves and from the form A, although morphologically similar, can represent other species belonging to the *A. scabripinnis* species complex and, if so, indicating that the number of species in this complex is subestimated.

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