

Three sympatric karyomorphs in the fish *Astyanax fasciatus* (Teleostei, Characidae) do not seem to hybridize in natural populations

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Academic editor: V. Gokhman | Received 28 September 2011 | Accepted 3 January 2012 | Published 24 January 2012

Citation: Ferreira-Neto M, Artoni RF, Vicari MR, Moreira-Filho O, Camacho JPM, Bakkali M, de Oliveira C, Foresti F (2012) Three sympatric karyomorphs in the fish *Astyanax fasciatus* (Teleostei, Characidae) do not seem to hybridize in natural populations. Comparative Cytogenetics 6(1): 29–40. doi: 10.3897/CompCytogen.v6i1.2151

Abstract

Ninety individuals of the characid fish *Astyanax fasciatus* (Cuvier, 1819) were collected at Água da Madalena stream (Botucatu, São Paulo, Brazil) and analyzed for diploid chromosome number $2n$ and karyotype composition as well as for the chromosomal location of the 5S and 18S ribosomal DNA (rDNA). Whereas no chromosome differences were associated with sex, three different karyomorphs with diploid chromosome numbers $2n=46$, $2n=48$ and $2n=50$ were found. No intermediate $2n$ numbers were discovered. The $2n=50$ karyomorph showed some differences in 18S rDNA location compared to the two other karyomorphs. Finally, all specimens with the $2n=46$ karyomorph showed the presence of a partly heterochromatic macro supernumerary chromosome, which was absent in all individuals with the two other karyomorphs. All these results suggest that individuals of the three different karyomorphs are not likely to hybridize in the examined populations. Our findings strongly suggest the presence of three separate species (*sensu* biological species concept) easily diagnosed on the basis of differences in the diploid chromosome numbers and other chromosomal markers.

Keywords

fish cytogenetics, chromosome banding, rDNA, sympatric differentiation, B chromosome

Introduction

The genus *Astyanax* (Baird et Girard, 1854) is one of the most diversified among Neotropical characid fishes. Indeed, this genus shows an extensive morphological diversification and a highly complex taxonomy. Moreover, a number of species actually appear to be “complexes of species” with low morphological differentiation but high variation at other levels, e.g. chromosome number and morphology (Morelli et al. 1983). The first *Astyanax* “species complex” was suggested by Moreira-Filho and Bertollo in *Astyanax scabripinnis* Jenyns, 1842 (Moreira-Filho and Bertollo 1991). Other cases were reported in the Neotropical fish *Corydoras aeneus* Gill, 1858 (Turner et al. 1992), *Hoplias malabaricus*, Bloch 1794 (Bertollo et al. 1997), and *Gymnotus carapo* Linnaeus, 1758 (Milhomem et al. 2008).

Astyanax fasciatus is another species that seems to form a “species complex” since the available information points towards the existence of several karyomorphs with the diploid chromosome numbers $2n=45$, $2n=46$, $2n=47$, $2n=48$ and $2n=50$ (Table 1). The karyotype most frequently cited in the literature is $2n=48$ (Table 1), but the known geographical range is actually larger for the karyomorphs $2n=50$ and $2n=46$ (Figure 1).

B chromosomes are supernumerary elements previously reported in the karyotypes of several *Astyanax* species (Moreira-Filho et al. 2004). In *A. fasciatus*, B chromosomes have been reported only for the $2n=46$ karyomorph in a population at the Sao Francisco River basin (Moreira-Filho et al. 2001).

Table 1. Diploid numbers ($2n$) recorded in populations of *Astyanax fasciatus* complex.

Species as originally reported	River/basin	$2n$	B chrom.	Reference
<i>A. fasciatus</i>	Mogi Guaçu river/Paraná	45		14
<i>A. fasciatus</i>	Mogi Guaçu river/Paraná	46		2,4,9,10,11,18
<i>A. fasciatus</i>	Mogi Guaçu river/Paraná	47		14,11
<i>A. fasciatus</i>	Mogi Guaçu river/Paraná	48		11,18
<i>A. fasciatus</i>	Piracicaba river/Paraná	48		7
<i>A. fasciatus</i>	Piracicaba river/Paraná	46		23
<i>A. fasciatus</i>	Tietê river/Paraná	46		5
<i>A. fasciatus</i>	Riacho Águas da Madalena/Paraná	50		23
<i>A. fasciatus</i>	Águas da Madalena stream/Paraná	48		23

Species as originally reported	River/basin	2n	B chrom.	Reference
<i>A. fasciatus</i>	Águas da Madalena stream/Paraná	46	+	23
<i>A. fasciatus</i>	Paranapanema river/Paraná	46		6
<i>A. fasciatus</i>	Paranapanema river/Paraná	50		13
<i>A. fasciatus</i>	Paraíba river/Paraná	48		3,5,9
<i>A. fasciatus</i>	Paíol Grande river/Paraná	48		9,17
<i>A. fasciatus</i>	Barra funda river/Paraná	46		9
<i>A. fasciatus</i>	Passa Cinco river/Paraná	46		9,11
<i>A. fasciatus</i>	Sapucaí river/Paraná	48		12
<i>A. fasciatus</i>	Araguari river/Paraná	46		16
<i>A. cf. fasciatus</i>	Juquiá river/Paraná	48		2
<i>A. fasciatus</i>	Meia Ponte river/Araguaia	46		1
<i>A. prope fasciatus</i>	Córrego Fundo stream/Araguaia	50		8
<i>A. fasciatus</i>	Araras river/Paraná	48		22
<i>A. fasciatus</i>	Patos river/Paraná	48		22
<i>A. fasciatus</i>	Três Bueiros river/Paraná	48		22
<i>A. fasciatus</i>	Almas river/Paraná	48		22
<i>A. prope fasciatus</i>	Tibagi river/Paraná	48		19
<i>A. prope fasciatus</i>	Tibagi river/Paraná	49		19
<i>A. prope fasciatus</i>	Tibagi river/Paraná	50		19
<i>A. fasciatus</i>	São Francisco river/São Francisco	48		7,9,17,22
<i>A. fasciatus</i>	São Francisco river/São Francisco	46	+	7
<i>A. fasciatus</i>	Três Marias/São Francisco	46	+	15
<i>A. fasciatus</i>	Contas river/Leste	48		21
<i>A. fasciatus</i>	Mineiro do Costa stream/Leste	48		21
<i>A. fasciatus</i>	Preto do Costa river/Leste	48		21

References: 1. Jim and Toledo (1975); 2. Morelli et al. (1983); 3. Moreira-Filho and Bertollo (1986); 4. Paganelli (1990); 5. Justi et al. (1990); 6. Reganham and Giuliano-Caetano (1990); 7. Justi (1993); 8. Centofante and Vênere (1995); 9. Heras and Moreira-Filho (1996); 10. Daniel-Silva (1996); 11. Heras and Moreira-Filho (1997); 12. Swerts et al. (1998); 13. Vale and Martins-Santos (1999); 14. Daniel-Silva and Almeida-Toledo (2001); 15. Moreira-Filho et al. (2001); 16. Torres-Mariano and Morelli (2006); 17. Abel et al. (2006); 18. Pazza et al. (2006); 19. Artoni et al. (2006); 21. Medrado et al. (2008); 22. Peres et al. (2009); 23. Ferreira-Neto et al. (present study).

In this study we analyzed the 2n=46, 2n=48 and 2n=50 *A. fasciatus* karyomorphs by examining a high number of individuals living in sympatry. The absence of intermediate chromosome numbers, the restriction of B chromosomes to the 2n=46 karyomorph and the absence of heteromorphism for the 18S rDNA sites (which differ among some karyomorphs) strongly suggest that the abovementioned karyomorphs are not likely to hybridize and are thus three separate species (*sensu* biological species concept).

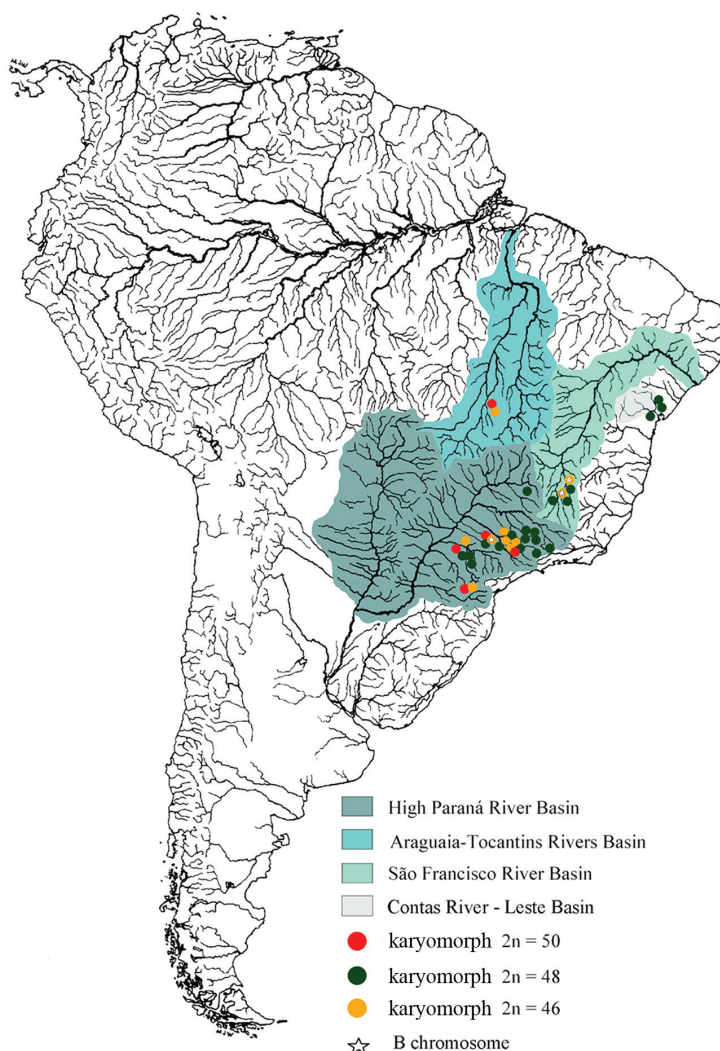


Figure 1. Map of South America highlighting the hydrographic basin of the Paraná, Araguaia-Tocantins, Contas and São Francisco Rivers. The natural distribution of *Astyanax fasciatus* and the presence of B chromosomes are represented according to the legends.

Material and methods

Ninety *A. fasciatus* specimens (56 females and 34 males) were collected at a 3.000 m² area of the Água da Madalena stream, Botucatu, SP, Brazil (22°59'23"S 48°25'31"W). The specimens were anaesthetized in benzocaine (1%) and, after collecting tissue samples for chromosome analysis, fixed in 10% formol and preserved in 70% alcohol. After identification, the specimens were deposited in the Museum of Biology and Laboratory of Fish Genetics, UNESP, Botucatu, São Paulo, Brazil. Chromo-

some preparations were obtained from anterior kidney cells and used for the conventional air drying technique (Foresti et al. 1981). The chromosomal location of active nucleolus organizer regions (NORs) was detected using the silver nitrate staining technique (Howell and Black 1980). Mapping of the ribosomal DNA (rDNA) was performed by fluorescent *in situ* hybridization (FISH) according to Pinkel et al. (1986). The 18S and 5S rDNA probes were obtained from the fish *Prochilodus argenteus* Spix and Agassiz, 1829 (Hatanaka and Galetti Jr. 2004) and *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti Jr. 1999), respectively. The 5SS probe was labeled with biotin 14-dATP by nick translation following manufacturer's instructions (Bionick Labelling System - Invitrogen). Hybridization was detected with avidin-FITC and the signals were amplified with biotinylated anti-avidin. The 18S probe was labeled with digoxigenin 11-dUTP (Roche Applied Sciences) by PCR (Polymerase Chain Reaction) and hybridization signals were detected using anti-digoxigenin-rhodamine.

Metaphase chromosomes were counterstained with DAPI and analyzed under optical light microscope (Olympus BX61). Images were captured using the Image-Pro Plus 6.0 software (Media Cybernetics). To build karyograms, chromosome morphology was determined according to the arm ratio limits established by Levan et al. (1964), and chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a), and were arranged in order of decreasing size.

Results

The cytogenetic analysis of the 90 specimens of *Astyanax fasciatus* revealed the presence of three different karyomorphs in the sample, showing $2n=46$, $2n=48$ and $2n=50$ chromosomes (Table 2 and Fig. 2 a, c and e, respectively), and with fundamental numbers (NF= number of chromosome arms) equal to 84, 86 and 90, respectively. The anatomical sex of the fish, determined by visual examination of the gonads, was not associated with the karyotypic differences, so that males and females were found with all the three cytotypes described here. Interestingly, all the specimens with the $2n=46$ karyomorph showed the presence of one mitotically stable macro B chromosome (Fig. 2 a, b), whereas the specimens of the $2n=48$ and $2n=50$ karyomorphs lacked it. No individual with intermediate odd chromosome numbers was found.

Table 2. Cytogenetic studies in *Astyanax fasciatus* karyotype composition and location of the 5S and 18S rDNA in the chromosomes of the individuals analyzed.

Cytotypes $2n$ / NF	Number of specimens	Chromosome	rDNA 18S/5S	Number of B Chromosomes		NOR
	Female Male	Formulae	location (pairs)	females	males	(pairs)
$2n = 46$, NF=84	12 10	$5m+8sm+6st+4a$	8,15,16,20,21, B/3,20	1	1	9,10
$2n = 48$, NF=86	10 9	$5m+8sm+6st+5a$	8,12,19,20,21/3,20	0	0	9,10
$2n = 50$, NF=84	41 32	$5m+8sm+4st+8a$	5,8,12,18,22/3,21	0	0	9,10

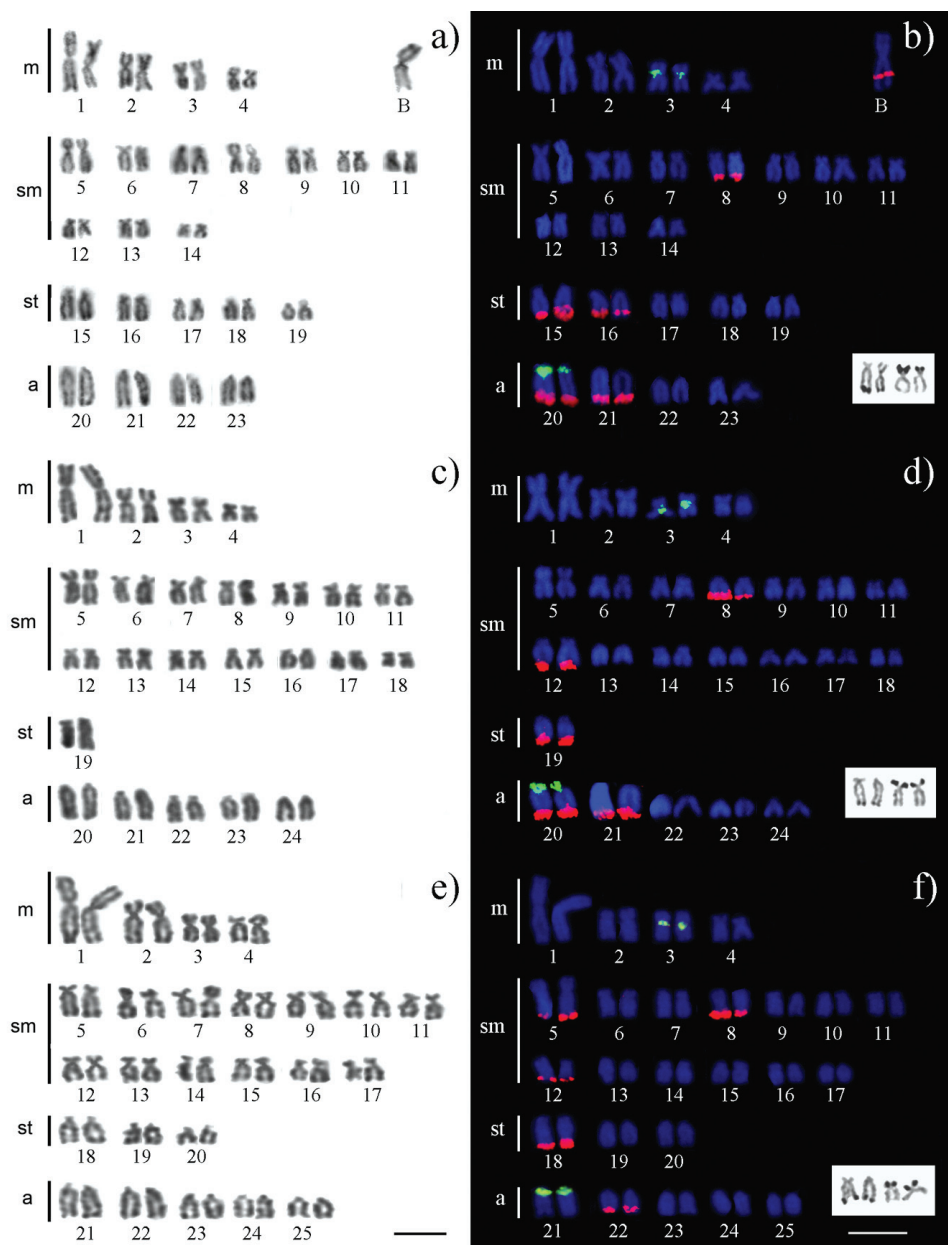


Figure 2. Karyotypes of *Astyanax fasciatus* (2n=46, 48 and 50 chromosomes) deduced after conventional Giemsa staining (a, c, e) and double FISH using 5S (green) and 18S rDNA (red) probes. The chromosomes bearing the Ag-NORs are boxed in b, d and f. Bar = 10 μm.

Physical mapping of the rDNA showed remarkable conservation of the 5S rDNA sites, which were present in two chromosome pairs, i.e. Nos. 3 and 20 (21 in the 2n=50 karyomorph) (Fig. 2 b, d, f).

The silver staining technique revealed the presence, in all cells, of Ag-NORs on two sm chromosome pairs of the three karyomorphs. One Ag-NOR was located at the distal region of the p arm and the other at the q arm (Figs 2 b, d, f, details). FISH analysis, however, showed the presence of 18S rDNA at the distal regions of five chromosome pairs, but no FISH signal was observed on the short arm of any chromosome (Fig. 2 b, d, f). This points towards the presence of a small number of 18S rRNA genes in the short arm of the sm chromosome.

Double FISH showed the presence of a chromosome pair, No. 20 in the $2n=46$ and $2n=48$ karyomorph, carrying both 5S (proximal) and 18S (distal) rDNA, but no chromosome carried both kinds of rDNA in the $2n=50$ karyomorph (Fig. 1f). All six specimens analyzed by FISH were homomorphic for the chromosome No. 20 which carries both rDNA types. Remarkably, FISH mapping showed the presence of an interstitial cluster of 18S rDNA in the long arm of the B chromosome found in the $2n=46$ karyomorph. Nevertheless, this rDNA cluster was never detected by silver staining.

Discussion

Sympatry and syntopy for several cytotypes have been reported in *Astyanax* species, such as *A. scabripinnis* (Souza and Moreira-Filho 1995) and *A. fasciatus* (Pazza et al. 2006, 2008). A review of all the published data on *A. fasciatus* (summarized in Table 1 and Figure 1) indicates that the three karyomorphs reported in this work show broad geographical distribution, and sympatry can be observed for at least two different karyomorphs in four river basins, i.e. High Paraná, Araguaia-Tocantins, Contas and São Francisco. In addition, B chromosomes have been previously reported in two other populations from the São Francisco River basin although, in that case, the number of B chromosomes showed variation among individuals (Justi 1993, Heras and Moreira-Filho 1997, Moreira-Filho et al. 2001). One evidence in support of hybridization events between karyomorphs was reported by Artoni et al. (2006), who found an individual with 49 chromosomes which might be a hybrid between the 48 and the 50 cytotypes in the Tibagi River (High Paraná River Basin, Ponta Grossa, Paraná, Brazil). The other evidence supporting hybridization between karyomorphs was reported by Pazza et al. (2006, 2008) who identified karyomorphs with $2n=45$, 46, 47 and 48 chromosomes in individuals caught along the Mogi-Guaçu river (High Paraná River Basin, Cachoeira de Emas, São Paulo, Brazil). In this last case, the $2n=47$ chromosomes karyomorph could have resulted from hybridization between the 46 and 48 cytotypes, whereas the presence of three individuals with 45 chromosomes could suggest the possible existence of a karyomorph with $2n=44$ in that river.

Our present analysis of a large sample of individuals caught in the same stream shows syntopic occurrence of three *A. fasciatus* karyomorphs with discrete chromosome numbers $2n=46$, $2n=48$ and $2n=50$. The absence of intermediate chromosome numbers, the presence of B chromosomes in only one of these karyomorphs ($2n=46$),

and the absence of apparent heteromorphism for the chromosome 20, suggests that the three karyomorphs do not hybridize in this stream. In such case, the data point out towards the possibility that these three karyomorphs actually correspond to three cryptic species, thus supporting the hypothesis that *A. fasciatus* is in fact a species assemblage, i.e. several species were included under the nominal name of *A. fasciatus* (Artoni et al. 2006).

The B chromosome found in *A. fasciatus* is large and metacentric. Interestingly, both characteristics seem common to all the B chromosomes described *Astyanax* species including *A. scabripinnis* (Salvador and Moreira-Filho 1992, Maistro et al. 1992, Souza and Moreira-Filho 1995, Vicente et al. 1996, Mizoguchi and Martins-Santos 1997, Vicari et al. 2010, 2011), *A. eigenmanniorum* Cope, 1894 (Fauaz et al. 1994), *A. schubarti* Britski, 1964 (Moreira-Filho 2001), *A. fasciatus*, (Moreira-Filho 2001) and *A. bockmanni*, Vari & Castro 2007 (Daniel personal communication). Such morphological similarity would support the hypothesis of a common origin of the *Astyanax* B chromosomes (Moreira-Filho et al. 2001). Nonetheless we show here, for the first time, the presence of rDNA interstitially located in the p arm of the B chromosome in *A. fasciatus* individuals. The apparent absence of rDNA in the q arm may seem incompatible with the isochromosome origin previously suggested for some of these Bs, e.g. in *A. scabripinnis* (Mestriner et al. 2000). However, the presence of a small, bellow FISH sensitivity, amount of rRNA genes is also possible. A genus-wise investigation of the presence and distribution of the rDNA in *Astyanax* species may be very useful for inferring the origin of the B chromosome. To this end, a comparison of rDNA sequences among the A and B chromosomes of several species would be very informative.

Since silver staining of metaphasic chromosomes reveals only those NORs that were active in the previous interphase (Hsu et al. 1975), it seems that the 18S rDNA contained in the B chromosome is usually inactive. Inactivity of the rDNA seems thus a widespread and general feature of the B chromosomes as it was previously reported in several phylogenetically distant species including the grasshopper *Eyprepocnemis plorans* Charpentier, 1825 (Cabrero et al. 1997, Bakkali et al. 2001), the black rat *Rattus rattus* Linnaeus, 1758 (Stitou et al. 2000) and the fish *Haplochromis obliquidens* Hilgendorf, 1888 (Poletto et al. 2010). Nonetheless, active rRNA genes have occasionally been reported in some B chromosomes of species like the grasshopper *E. plorans* (Teruel et al. 2007, 2009) and the rodents *Akodon montensis* Thomas, 1913 and *Oryzomys angouya* Fischer, 1814 (Silva et al. 2004).

The higher number of rDNA clusters (10) than silver stained NORs (4) suggests the inactivity of some rDNAs in most cells. It would therefore be interesting to ascertain whether this phenomenon is facultative or constitutive. Silver nitrate may also bind to other proteins present in the nuclei, implying that some chromosome structures visualized by silver nitrate may not correspond to ribosomal genes (Dobigny et al. 2002). The detection of a higher number of ribosomal genes using FISH against 18S or 28S sequences than using silver nitrate staining is a common result having been reported in *Astyanax scabripinnis* (Ferro et al. 2001), *Salmo trutta* (Pendás et al. 1993),

Colossoma macropomun Cuvier, 1816, *Piaractus brachypomus* Cuvier, 1818 and their interspecific hybrids (Nirchio et al. 2003), *Hyphessobrycon anisitsi* Eigenmann, 1907 (Centofante et al. 2003), *Prochilodus lineatus* Valenciennes, 1836 (Jesus and Moreira-Filho 2003) and *Lebias fasciata* Valenciennes, 1821 (Tigano et al. 2004). Therefore, the chromosome pair that actually has the nucleolus organizer region is probably the first submetacentric pair (the one that shows both FISH and silver nitrate staining signals).

The presence of one B chromosome in all 22 individuals from the $2n=46$ karyomorph is intriguing, since it departs from the usual interindividual variation which characterizes B chromosomes (Camacho 2005). On the other hand, Moreira-Filho et al. (2001) reported the presence of B chromosomes showing interindividual variation in number in 10% of the sample population analyzed at the Sao Francisco River basin. This suggests that the B chromosomes in *A. fasciatus* from the Água da Madalena stream (this report) might be in a state of stabilization. With complete elimination through one sex and complete drive through the other, the population dynamics of *Astyanax* B chromosome resembles the case of germ-line restricted chromosomes in the zebra finch (Itoh et al. 2009). The mechanism behind B chromosome stabilization in populations of the Água da Madalena stream appears therefore to be rather complex and its elucidation requires further population dynamics and chromosome transmission studies.

Acknowledgments

This study was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP (Proc. 2008/57067-1), Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES.

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