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Microsatellite Organization in the B Chromosome and A Chromosome Complement in *Astyanax* (Characiformes, Characidae) Species

Diovani Piscor Patricia P. Parise-Maltempi

Instituto de Biociências, Departamento de Biologia, Laboratório de Citogenética, Universidade Estadual Paulista 'Júlio de Mesquita Filho' (UNESP), Rio Claro, Brazil

Key Words

Astyanax schubarti · Chromosome evolution · Heterochromatin · Mexican blind cavefish · Repetitive DNA

Abstract

The organization of microsatellites in B and sex chromosomes has been linked to chromosomal evolution in a number of animal groups. Here, the chromosomal organizations of $(CA)_{15}$, $(GA)_{15}$, $(CG)_{15}$, $(GACA)_4$, and $(GATA)_8$ microsatellites were examined in several *Astyanax* species with different diploid numbers: *Astyanax mexicanus* (2n = 50 + 1 B chromosome), *A. altiparanae* (2n = 50), *A. marionae* (2n = 48), *A. fasciatus* (2n = 46), and *A. schubarti* (2n = 36). The $(CA)_{15}$ and $(GA)_{15}$ microsatellites were dispersed across the chromosomes of *A. altiparanae* and *A. fasciatus* but were also observed as clusters (CA and GA for *A. altiparanae*, and CA for *A. fasciatus*). In *A. marionae* and *A. schubarti*, the $(CA)_{15}$ and $(GA)_{15}$ microsatellites were dispersed but were also observed as clustered signals and coincident with heterochromatic regions. In all 4 of these species, the $(CG)_{15}$ and $(GACA)_4$ microsatellites were dispersed across chromosomes, and the $(GATA)_8$ microsatellite was co-localized with 5S rDNA. In *A. mexicanus*, the $(CA)_{15}$, $(GA)_{15}$, $(CG)_{15}$, $(GATA)_8$, and $(GACA)_4$ microsatellites were weakly detected and dispersed across the chromosomes of the A complement. On the B chromosome, signals for the different microsatellites were weak, strong, absent, weak, and absent, respectively. The distribution of microsatellites and the locational relationship between microsatellites and 5S rDNA are discussed, and a possible evolutionary pathway is proposed for microsatellites in *Astyanax*.

Microsatellites are excellent markers for the use in studies of sex and B chromosomes [Pokorná et al., 2011; Milani and Cabral-de-Mello, 2014; Palacios-Gimenez and Cabral-de-Mello, 2015]. For example, abundant accumulation of microsatellites was detected in the W chromosome of the lacertid *Eremias velox* [Pokorná et al., 2011], and the authors suggested that accumulation of microsatellites in *E. velox* and other eukaryotic organisms might play an important role in sex chromosome dynamics [Pokorná et al., 2011].

Milani and Cabral-de-Mello [2014] found that, while the majority of examined microsatellites were scattered along the entire length of the B chromosome in *Abracris flavolineata* (grasshopper), conspicuous blocks were apparent and enriched for $(GA)_{15}$ and $(GAC)_{10}$ microsatellites. This suggested that microsatellites could play an important role in B chromosome evolution. In this context, the *Astyanax* genus can be regarded as a good model because of the variation in diploid numbers (e.g.

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Patricia P. Parise-Maltempi
Instituto de Biociências, Departamento de Biologia, Laboratório de Citogenética
Universidade Estadual Paulista 'Júlio de Mesquita Filho' (UNESP)
Av. 24A, 1515, Rio Claro, SP 13506-900 (Brazil)
E-Mail parise@rc.unesp.br

Table 1. Microsatellite organization in the chromosomes of the A complement in *Astyanax* species

Species (diploid numbers)	N	Microsatellites					Localities
		$(CA)_{15}$	$(GA)_{15}$	$(CG)_{15}$	$(GACA)_4$	GATA/5S	
A. altiparanae (50)	5	C	C	S	S	P	SP, Brazil
A. fasciatus (46)	3	C	S	S	S	P	SP, Brazil
A. marionae (48)	6	Ccb	Ccb	S	S	P	MT, Brazil
A. schubarti (36)	1	Ccb	Ccb	S	S	P	SP, Brazil
A. mexicanus (50)	6	S	S	S	S	Ab	Mexico[a]

Ab = Absent but the GATA repeats showed spreading on the chromosomes of *A. mexicanus*; C = spread and with fluorescence signals clustered; Ccb = spread and with fluorescence signals clustered and coincident with C-band heterochromatin; P = present; S = fluorescence signals spread; SP = São Paulo state; MT = Mato Grosso state. [a] Mexican blind cavefish obtained from an aquarium store in Brazil.

A. schubarti with 2n = 36 and *A. altiparanae* with 2n = 50) and the presence of species possessing B chromosomes [Moreira-Filho et al., 2004].

Recent surveys on chromosomal microsatellite locations showed that organization in sex chromosomes differed between *Leporinus* and *Characidium* species [Poltronieri et al., 2014; Scacchetti et al., 2015]. For example, in W chromosomes of the *Leporinus* species, microsatellites accumulated primarily in heterochromatic regions of the long arms. However, microsatellite distributions differed between the 4 *Leporinus* species, despite the 4 species exhibiting similar patterns of heterochromatin distribution. This suggested that the heterochromatinization process is dynamic, free of selection, and subject to distinctive mechanisms after speciation [Poltronieri et al., 2014].

In this study the chromosomal organization of different microsatellite repeats in *Astyanax* species with different diploid numbers (2n = 36, 2n = 46, 2n = 48, and 2n = 50) was analyzed, and the microsatellite distribution in the B chromosome and A complement of *A. mexicanus* was examined. Possible scenarios for the evolution of microsatellite distribution and chromosomal clustering of GATA repeats and 5S rDNA are discussed.

Materials and Methods

Specimens and Classical Cytogenetics

The following animals were collected: *A. schubarti* and *A. altiparanae* from the Piracicaba river in São Paulo state, Brazil; *A. marionae* from the Rio Claro stream in Mato Grosso state, Brazil; *A. fasciatus* from the Ribeirão Claro river in São Paulo state, Brazil; and *A. mexicanus* (Mexican blind cavefish) from aquarists in Brazil (table 1). Mitotic metaphase chromosomes were prepared using the methods described by Foresti et al. [1981]. Heterochromatin was examined using the C-banding technique described by Sumner [1972].

DNA Extraction and Probe Synthesis

Genomic DNA was extracted from fin samples as described by Sambrook and Russell [2001]. PCR with the following primers was used to amplify the 5S rDNA probe: A (5′-TAC GCC CGA TCT CGT CCG ATC-3′) and B (5′-CAG GCT GGT ATG GCC GTA AGC-3′) as described by Pendás et al. [1994] and Martins and Galetti [1999]. The $(CA)_{15}$, $(GA)_{15}$, $(CG)_{15}$, $(GACA)_4$, and $(GATA)_8$ microsatellites were amplified and labeled with biotin during synthesis as described by Milani and Cabral-de-Mello [2014]. Microsatellites were donated by Prof. Dr. Diogo C. Cabral-de-Mello for use in laboratory experiments.

Fluorescence in situ Hybridization and Fiber-FISH

FISH experiments were performed according to Pinkel et al. [1986], with modifications as per Cabral-de-Mello et al. [2010]. Fiber-FISH was performed as described by Barros et al. [2011], and slides were used in double-FISH experiments with 5S rDNA and $(GATA)_8$ probes. Signals were detected using anti-digoxigenin-rhodamine (Roche, Mannheim, Germany) for digoxigenin and avidin Alexa Fluor 488 conjugate (Invitrogen, San Diego, Calif., USA) for biotin. Slides were mounted with Vectashield Mounting Medium (Vector, Burlingame, Calif., USA) containing DAPI (4′,6-diamidino-2-phenylindole) for chromosome counterstaining. Chromosome and fluorescence signals were visualized with an Olympus BX51 microscope coupled to a digital camera (Olympus model D71). Images were captured using DP Controller software.

Results

Diploid numbers of *A. altiparanae*, *A. marionae*, *A. fasciatus*, and *A. mexicanus* are given in table 1. In *A. mexicanus*, one small acrocentric B chromosome was ob-

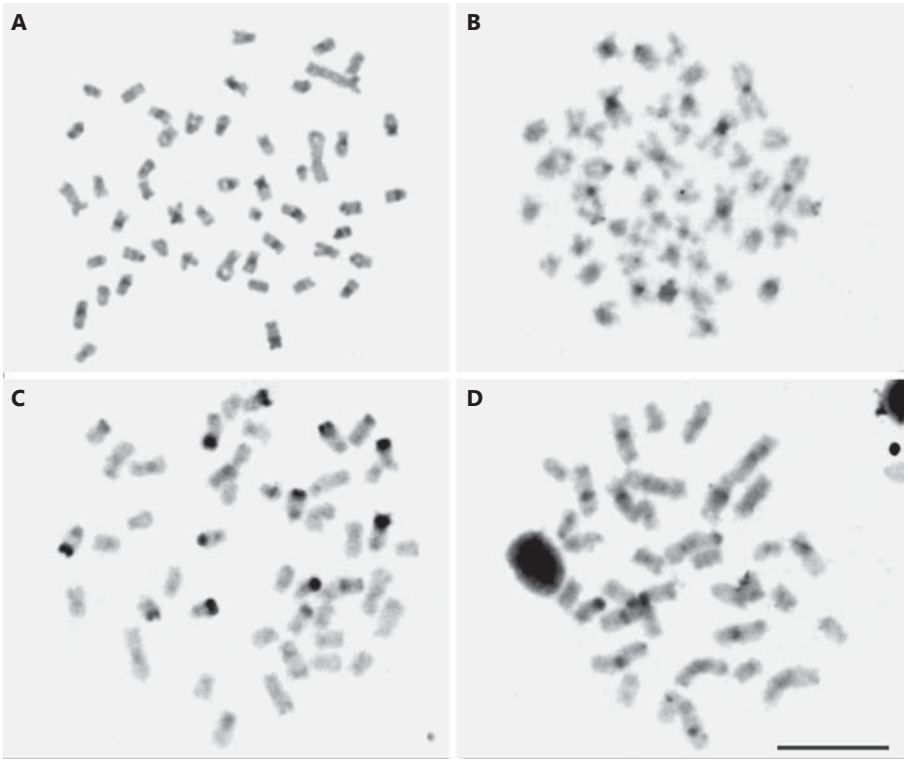

Fig. 1. C-banded metaphases of *A. altiparanae* (**A**), *A. marionae* (**B**), *A. fasciatus* (**C**), and *A. schubarti* (**D**). Bar = 10 μm.

served in all metaphases. Modest blocks of heterochromatin were observed, mainly in the pericentromeric and centromeric chromosomal regions in *A. altiparanae* (fig. 1A), pericentromeric and centromeric regions in *A. marionae* (fig. 1B), terminal regions in *A. fasciatus* (fig. 1C), and pericentromeric and centromeric regions in *A. schubarti* (fig. 1D). *A. mexicanus* chromosomes contained blocks of heterochromatin in the terminal and pericentromeric regions, and the B chromosome was C-band negative.

Microsatellites were distributed in 4 organizational formats:

1 Dispersed and with blocks of $(CA)_{15}$ and $(GA)_{15}$ microsatellites, corresponding to C-band heterochromatin in *A. marionae* and *A. schubarti* (fig. 2; note that the CA and GA repeats show more evident blocks in *A. schubarti*).
2 $(CA)_{15}$ and $(GA)_{15}$ microsatellites were spread across the chromosomes in *A. altiparanae* and *A. fasciatus* and were also found in clusters. However, the clusters did not correspond to C-band heterochromatic regions. CA and GA repeats formed clusters in *A. altiparanae*, but only CA repeats were clustered in *A. fasciatus* (fig. 2).
3 $(CG)_{15}$ and $(GACA)_4$ microsatellites were dispersed across the chromosomes in *A. altiparanae*, *A. marionae*, *A. fasciatus*, and *A. schubarti* (fig. 2).
4 The $(GATA)_8$ microsatellite was co-localized with 5S rDNA on pair 5 in *A. altiparanae*, pair 22 in *A. marionae*, pairs 3 and 22 in *A. fasciatus*, and pairs 3 and 4 in *A. schubarti* (figs. 3, 4; table 1).

In *A. mexicanus*, the $(CA)_{15}$, $(GA)_{15}$, $(CG)_{15}$, $(GACA)_4$, and $(GATA)_8$ microsatellites exhibited weak fluorescence signals on the chromosomes of the A complement (fig. 5; table 1). On the B chromosome, no signal was evident for $(CG)_{15}$ and $(GACA)_4$ microsatellites, $(CA)_{15}$ and $(GATA)_8$ microsatellites produced minimal signals, and $(GA)_{15}$ microsatellite exhibited stronger signals (fig. 5).

Discussion

Four microsatellite organizations were found in chromosomes of different *Astyanax* species with a range of diploid numbers: (1) clustered and coincident with C-band heterochromatin, (2) clustered and non-coincident with C-band heterochromatin, (3) dispersed across the chromosomes, and (4) co-localized with 5S rDNA. These

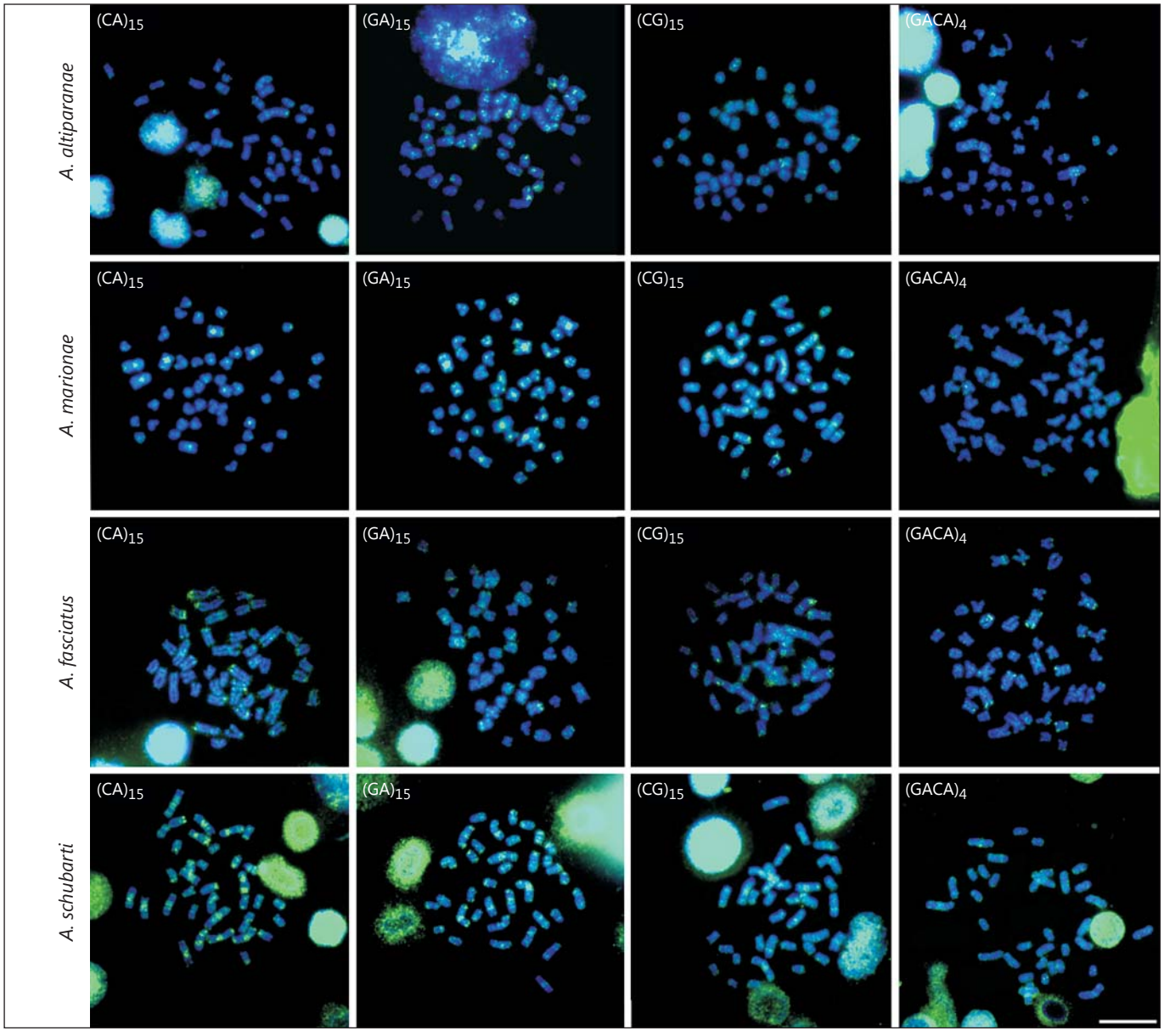

Fig. 2. Microsatellite distribution in chromosomes of *Astyanax* species. Bar = 10 μm.

results suggest that there may be 3 evolutionary pathways related to drive and/or genomic organization of microsatellites in *Astyanax* genomes: (1) the microsatellites can follow a free way to spreading and/or clustering, (2) the heterochromatin can play an important role in the genome organization of the microsatellites, and (3) the co-localization of 5S rDNA-GATA can stabilize DNA structures, acting as 'hot spots' for recombination, as discussed by Merlo et al. [2010].

B chromosomes carrying microsatellite DNA were described previously, for example, in the grasshopper *A. flavolineata* [Milani and Cabral-de-Mello, 2014]. For the authors, microsatellites located on the B chromosome may play an important role in the evolution of these elements. In the present study, *A. mexicanus* individuals contained 0 or 1 B chromosome. Thus, the low rate of recombination in the B chromosome of *A. mexicanus* may facilitate accumulation of microsatellites, as proposed by Charlesworth et al. [2005].

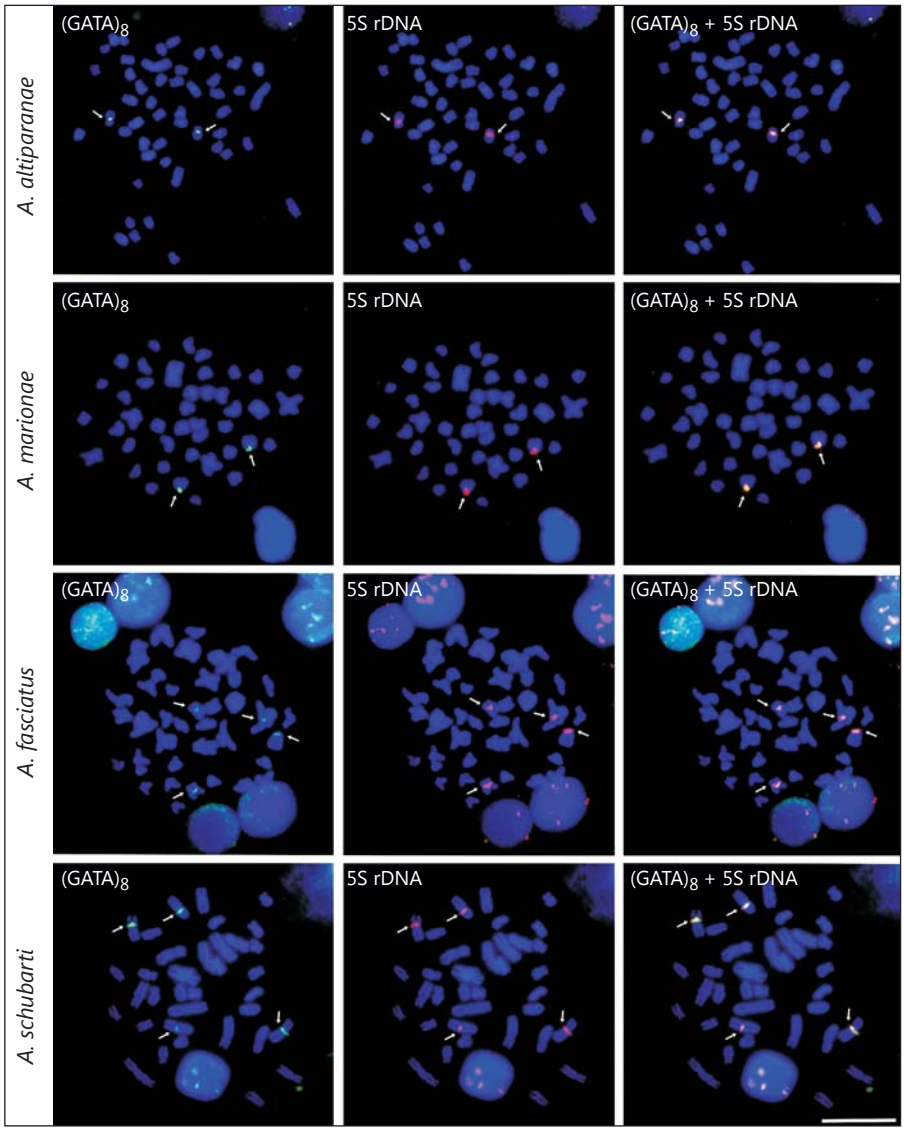

Fig. 3. Chromosomal location of 5S rDNA and GATA repeats in *Astyanax* species. Arrows indicate fluorescence signals. Bar = 10 μm.

Here, we observed chromosomal co-localization of 5S rDNA and the $(GATA)_8$ microsatellite. GATA repeats were found on chromosomes of other animal groups, for example, in the grasshopper *A. flavolineata* [Milani and Cabral-de-Mello, 2014], the fish *Halobatrachus didactylus* [Merlo et al., 2007], snakes *Liasis fuscus*, *Stegonotus cucullatus*, and *Notechis scutatus* [O'Meally et al., 2010], and lacertids *Coleonyx elegans*, *E. velox* [Pokorná et al., 2011], and *Aprasia parapulchella* [Matsubara et al., 2013]. However, in none of these species were GATA repeats co-localized with ribosomal DNA.

Using 2-color FISH, Úbeda-Manzanaro et al. [2010] examined $(GATA)_n$ and 5S rDNA in chromosomes from 4 species of fish of the Batrachoididae family *(Amphichthys cryptocentrus*, *Batrachoides manglae*, *Porichthys plectrodon*, and *Thalassophryne maculosa)*. GATA repeats were not co-localized with or adjacent to 5S rDNA in these species but were dispersed and abundant throughout all chromosomes. According to Úbeda-Manzanaro et al. [2010], GATA sequences cannot be used as chromosomal markers in these fish species. This is in contrast with the results from the present study, which indicate that GATA repeats can act as good chromosomal markers in South American *Astyanax* species.

Scrutiny of DNA sequences showed that microsatellites could be found within the non-transcribed spacers (NTSs) of 5S rDNA in fish [for examples, see Rocco et al., 2005; Pasolini et al., 2006; Pinhal et al., 2009; Merlo et al.,

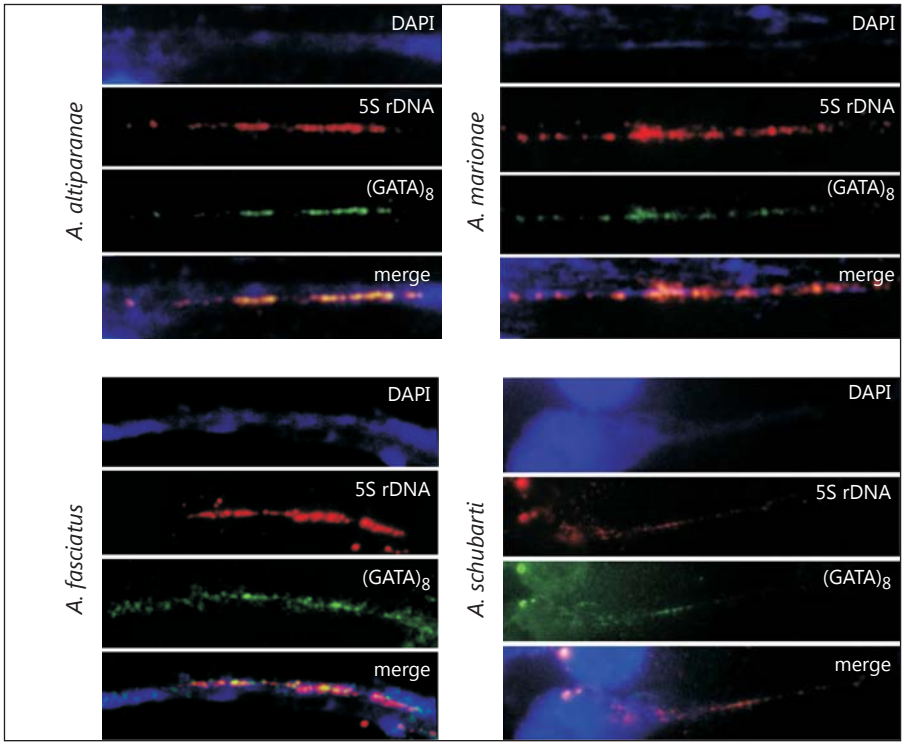

Fig. 4. Fiber-FISH of *Astyanax* chromosomes. The 5S rDNA and $(GATA)_8$ probes are co-localized.

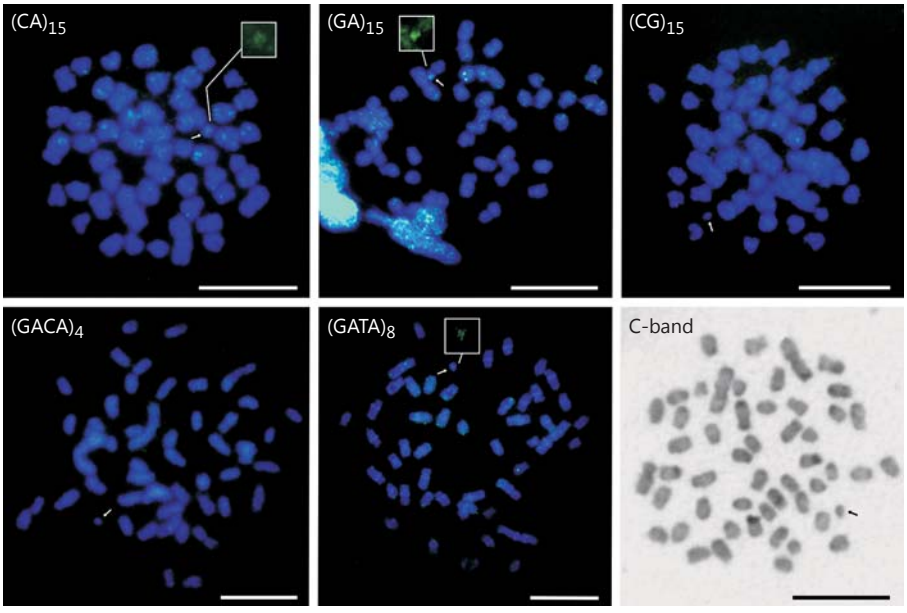

Fig. 5. Microsatellite distribution and constitutive heterochromatin revealed by C-banding in *A. mexicanus*. Arrows indicate the B chromosome, and **insets** show fluorescence signals on the B chromosome. Bars = 10 μm.

2010]. The microsatellite GTT was found within the NTS from 2 species *(Dicentrarchus labrax* and *D. punctatus)* of the Moronidae family [Merlo et al., 2010]. This suggested that the presence of microsatellite repeats favored the maintenance of tandem arrays of multigene families, as proposed by other authors [Liao and Weiner, 1995; Cross and Rebordinos, 2005].

Sequence analysis of 5S rDNA in 2 species of Batoidea, *Taeniura lymma* (Dasyatidae) and *Raja montagui* (Rajidae), revealed microsatellite repeats in the NTS regions of the 5S rDNA, and it was suggested that these repeats might exert an influence on gene regulation [Rocco et al., 2005].

The chromosomal co-location of GATA repeats and ribosomal DNA, which was maintained during evolution in different species with different diploid numbers, suggests an evolutionary advantage for the 5S rDNA-GATA combination in *Astyanax* chromosomes. Furthermore, the absence of 5S rDNA-GATA co-location in *A. mexicanus* indicates that the development of this feature may have occurred relatively recent in the evolutionary history of *Astyanax* species from South America.

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Statement of Ethics

All institutional guidelines for the care and use of laboratory animals were followed. Animals were captured with the permission of the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio; No. 43497-1) and were used for laboratory experiments approved by the Animal Experimental Ethics Committee from Universidade Estadual Paulista (UNESP; protocol No. 2335).

Disclosure Statement

The authors have no conflicts of interest to declare.

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