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RESEARCH ARTICLE

Distinct classical and molecular cytogenetics of *Astyanax marionae* and *A. fasciatus* (Characiformes: Characidae): a comparative study of the organization of heterochromatin and repetitive genes

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Abstract. Genus *Astyanax* is well distributed in Neotropical freshwater environments and its taxonomic position is uncertain, as is the case with other Characidae genera allocated in the group *incertae sedis*. This study aimed to analyse the karyotype of different populations of *Astyanax fasciatus* (Corumbataí River basin) using Giemsa staining, C-band technique, and fluorescence *in situ* hybridization for the H3 histone and 5S rRNA genes, in addition we describe for the first time the chromosomal organization of H3 histone and 5S rRNA genes in *A. marionae* (Paraguay River basin). Chromosomes of three *A. fasciatus* populations were analysed (two with $2n = 50$ and one with $2n = 48$) and the heterochromatin was organized in two forms (blocks with blurred boundaries and distinct blocks). H3 histone and 5S rRNA genes were observed in all the three populations of *A. fasciatus* on two chromosome pairs (one metacentric chromosome showing H3 histone and 5S rRNA gene clusters). In *A. marionae* ($2n = 48$), H3 histone and 5S rRNA genes were observed in one acrocentric chromosome pair (different pairs). Further, differences between karyotypes and heterochromatin, as well as the chromosomal organization of H3 histone and 5S rRNA genes in *Astyanax* species, focussing on chromosome evolution in the group are discussed.

Keywords. H3 histone gene; 5S rDNA; C-band; chromosomes; *Astyanax fasciatus*.

Introduction

Astyanax (Characidae) is one of the genera with the largest number of species in Characidae, containing ~140 valid species (Eschmeyer and Fong 2015). Its wide distribution from the south of USA to central Argentina (Lima *et al.* 2003), occupying a variety of habitats in rivers and streams, makes this group one of the most complex genera of freshwater fishes. Lima *et al.* (2003) allocated many characid genera into *incertae sedis*, e.g. *Hemigrammus*, *Hyphessobrycon*, *Moenkausia* and *Astyanax*. Recently, other authors have also showed that *Astyanax* does not represent a monolyphyletic group (see Javonillo *et al.* 2010; Mirande 2010; Oliveira *et al.* 2011).

Cytogenetic studies of genus *Astyanax* have revealed extensive variation in the diploid number, ranging from $2n = 36$ in *A. schubarti* (Morelli *et al.* 1983) and *A. correntinus* (Paiz *et al.* 2015) to $2n = 50$ chromosomes for most species, e.g. *A. altiparanae* and *A. bockmanni* (Fernandes and Martins-Santos 2004; Kavalco *et al.* 2009, respectively). Further, some species of *Astyanax* may have more than one diploid number e.g., the 'fasciatus complex' which may contain species with chromosomes $2n = 45$ to $2n = 50$ (Centofante *et al.* 2003).

Other cytogenetic papers have shown different patterns of heterochromatin C-band positive in *Astyanax* group (see Artoni *et al.* 2006; Peres *et al.* 2009; Tenório *et al.* 2013; Piscor *et al.* 2015; Piscor and Parise-Maltempi 2016a). For example, Artoni *et al.* (2006) studied three cytotypes of *Astyanax* aff. *fasciatus* (A, B and C) and verified two heterochromatin patterns. According to the authors, cytotypes A and B showed that the heterochromatin mainly

distributed as very conspicuous blocks in the telomeric region of the long arms of acrocentric chromosomes, whereas few chromosomes bearing heterochromatin were observed in cytotype C.

Repetitive sequences were mapped in many *Astyanax* species. Piscor and Parise-Maltempi (2016b) observed the chromosomal location of 5S rRNA and H3 histone genes in eight species and identified similar chromosomes bearing H3 histone, except in *A. schubarti* and *A. mexicanus*. The chromosomal mapping of repetitives are studied here for the first time in *A. marionae*.

Therefore, given the karyotype complexity found in the *Astyanax* genus and considering *A. marionae* and *A. fasciatus* are two species with similar morphological traits, it is necessary to attest whether or not they have similar cytogenetic characteristics. Thus, this study aimed to compare the chromosomes of *A. marionae* with different populations of *A. fasciatus*, and to understand the chromosomal organization of H3 histone and 5S rRNA genes, and of heterochromatin revealed by C-banding.

Materials and methods

Sampling and classical cytogenetics

Three populations of *A. fasciatus* were studied: two individuals from Cabeça river tributary, five from Ribeirão Claro river tributary, and three from the Corumbataí river tributary (Corumbataí river basin, São Paulo, Brazil). The six individuals of *A. marionae* were captured in the Rio Claro stream (Paraguay river basin, Mato Grosso, Brazil). The metaphasic chromosomes were obtained by the methodology of Foresti *et al.* (1981) and stained with Giemsa (10% in phosphate buffer). The morphologies of chromosomes were determined according to the arm's ratio, based on the more common classification system used for fish chromosomes in Brazil: the chromosomes with two arms and arm ratio (AR) of 1–1.7 were classified as metacentric (m), with AR 1.71–3 as submetacentric (sm), and with AR 3.01–7 as subtelocentric (st). Chromosomes with a single arm (AR $>$ 7) were considered as acrocentric (a). Heterochromatin was observed in chromosomes of all the *A. fasciatus* and *A. marionae* individuals using the C-band technique, as proposed by Sumner (1972).

DNA extraction, production of probes, and fluorescence in situ *hybridization*

Genomic DNA was extracted from fin samples of *Astyanax*, as described in Sambrook and Russell (2001). The 5S rDNA probe was prepared using polymerase chain reaction (PCR) with primers described by Pendás *et al.* (1994) and Martins and Galetti (1999) (A, 5′-TAC GCC CGA TCT CGT CCG ATC-3′, and B, 5′-CAG GCT GGT ATG GCC GTA AGC-3′). The H3 histone probe was prepared using PCR with primers described by Cabral-de-Mello *et al.* (2010) (A, 5′-GGC NMG NAC NAA RCA RAC, and B, 5′-TGD ATR TCY TTN GGC ATD AT). The H3 histone genes were amplified using the genomic DNA of *A. fasciatus* and *A. marionae*. The PCR products were sequenced in Korean company (Macrogen) and the sequences were edited and aligned with BioEdit program (Hall 1999). The analysis of similarities to sequences were submitted in website GenBank (https://www.ncbi.nlm.nih.gov/genbank/) for comparison. The H3 histone gene of *A. marionae* was mapped for the first time, sequenced, and deposited in GenBank with the accession number KY389066.

The 5S rDNA probe was labelled using PCR with biotin-14-dATP (Invitrogen, San Diego, USA), and the H3 histone probe was labelled using PCR with digoxigenin-11-dUTP (Roche, Mannheim, Germany). Fluorescence *in situ* hybridization (FISH) was used in all *A. fasciatus* and *A. marionae* individuals according to Pinkel *et al.* (1986) with modifications described by Piscor *et al.* (2013). Chromosomes were counterstained with Vectashield Mounting Medium (Vector, Burlingame, USA) containing DAPI (4′,6-diamidino-2′-phenylindole). Chromosomes and fluorescent signals were visualized with an Olympus BX51 microscope coupled to a digital camera (Olympus model D71), and the images were captured using the DP Controller software.

Statement of ethics

All the institutional guidelines for the care and use of laboratory animals were followed. The animals were captured with permission of Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio (number 43497-1), and used for laboratory experiments approved by the Animal Experimental Ethics Committee from Universidade Estadual Paulista – UNESP (protocol number: 2335).

Results

The karyotype of *A. marionae* showed 8 m, 24 sm, 10 st, 6 a, and the fundamental number (FN) = 90 (figure 1a). C-banded heterochromatin was observed mainly on the centromeric and proximal regions (figure 1b). Clusters of 5S rRNA and H3 histone genes were observed on pairs 22 (a) and 24 (a), respectively (figure 1c).

Populations from tributaries of the Cabeça and Corumbataí rivers showed a diploid number of $2n = 50$ chromosomes and the population from Ribeirão Claro river showed $2n = 48$ chromosomes. Karyotypic formulae for these populations were: 16 m, 12 sm, 6 st, 16 a and FN = 84 for the Cabeça river tributary (figure 2a), 10 m, 20 sm,

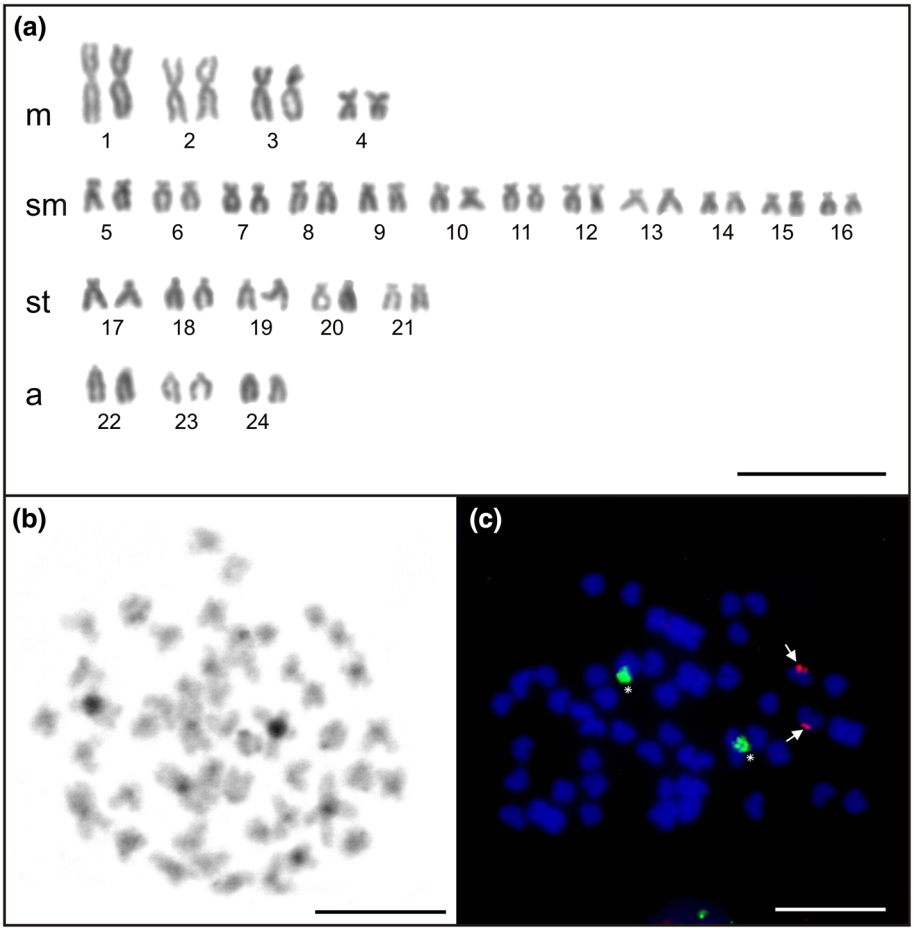

Figure 1. Cytogenetic data of *A. marionae*. (a) Karyotype. (b) C-banded metaphase. (c) Chromosomal location of H3 histone and 5S rDNA clusters. Arrowhead indicates the H3 histone cluster and asterisk indicates the 5S rDNA cluster.

8 st, 10 a and FN = 86 for the Ribeirão Claro river tributary (figure 2c), and 8 m, 26 sm, 6 st, 10 a and FN = 90 for the Corumbataí river tributary (figure 2e). Heterochromatic regions were observed in two organization forms. The first form was noted in the Cabeça and Corumbataí populations, as blocks with blurred boundaries (figure 2, b and f), and the second form was observed in the Ribeirão Claro population, as distinct blocks (figure 2d).

The H3 histone genes (sequenced from DNA of *A. fasciatus* and *A. marionae*) exhibited 95 to 100% similarity with H3 histone sequences of others *Astyanax* species. Gene clusters of the H3 histone were observed on two chromosome pairs in all three populations of *A. fasciatus* (see figure 3). The *A. fasciatus* population from the Cabeça river tributary showed interstitial fluorescent signals on pair 3 (m) and pericentromeric signals on pair 15 (st) (figure 3). The *A. fasciatus* population from the Corumbataí river tributary showed interstitial fluorescent signals on pair 3 (m) and pericentromeric signals on pair 18 (st) (figure 3). The *A. fasciatus* population from the Ribeirão Claro river tributary showed interstitial fluorescent signals on pair 2 (m) and pericentromeric signals on pair 16 (st) (figure 3).

Clusters of 5S rDNA were located on two chromosome pairs (see figure 3). *A. fasciatus* from the Cabeça river tributary showed pericentromeric fluorescent signals on pair 3 (m, same chromosome pair bearing H3 histone genes) and on pair 18 (a) (figure 3). *A. fasciatus* from the Corumbataí river tributary showed pericentromeric fluorescent signals on pair 3 (m, same chromosome pair that bears H3 histone genes) and on pair 21 (a) (figure 3). *A. fasciatus* from the Ribeirão Claro river tributary showed pericentromeric fluorescent signals on pair 2 (m, same chromosome pair that bears H3 histone genes) and on pair 20 (a) (figure 3).

A map of the hydrographic basins and distribution of *A. marionae* and *A. fasciatus*, and representative chromosome pairs bearing H3 histone and 5S rDNA clusters are shown in figure 3.

Discussion

Astyanax fasciatus populations usually differ in diploid number. According to Ferreira-Neto *et al.* (2012) this species can present diploid numbers $2n = 46$, 48 and 50

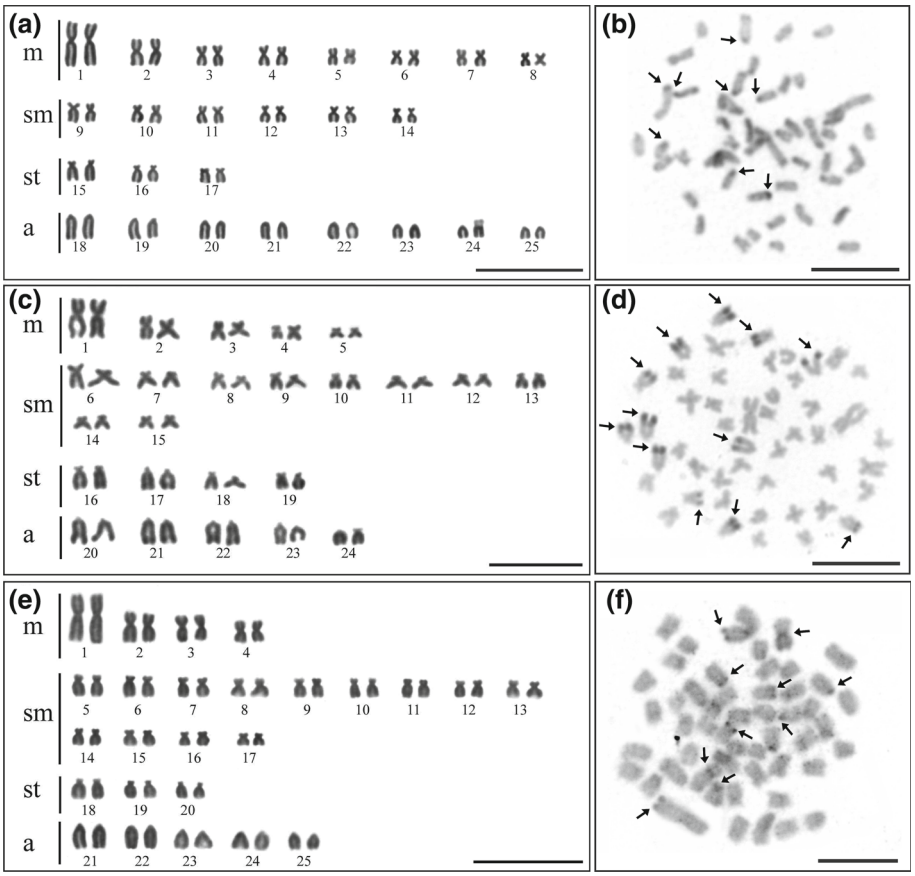

Figure 2. Chromosomes stained with Giemsa and C-banded chromosomes. (a–b) Karyotype and C-banded metaphase of *A. fasciatus* from the Cabeça river tributary. (c–d) Karyotype and C-banded metaphase of *A. fasciatus* from the Ribeirão Claro river tributary. (e–f) Karyotype and C-banded metaphase of *A. fasciatus* from the Corumbataí river tributary. The arrows indicate the blocks of C-band heterochromatin.

chromosomes (the most common number is $2n = 48$). However, previous results have shown karyomorphs with $2n = 45, 47$, and 49 chromosomes, possibly resulting from hybridizations (Artoni *et al*. 2006; Pazza *et al*. 2006). Further, chromosome variations are evident among different populations of the same species, primarily relating to the number and size of heterochromatin blocks (see, for example, Fernandes and Martins-Santos 2003, 2004).

Distinct blocks of heterochromatin have been found in other *A. fasciatus* populations. Pazza *et al*. (2008) analysed populations of *A. fasciatus* from three different sites along the Mogi-Guaçu river (Ouro Fino, Minas Gerais; Cachoeira de Emas, Pirassununga, São Paulo State; Barrinha, Brazil) and observed a similar pattern of constitutive heterochromatin organization on the forms with $2n = 46$ and $2n = 48$ chromosomes (in the telomeric region on long arms of submetacentric, subtelocentric and acrocentric chromosomes, and in the telomeric region on short arms of a submetacentric pair). Similar results were observed in the population from the Ribeirão Claro river ($2n = 48$) studied here.

Moreover, the presence of two heterochromatic organization forms in all three *A. fasciatus* populations analysed in this paper points to the conclusion that distinct evolutionary events may have influenced the organization of heterochromatic segments. According to Peres *et al*. (2009), three *A. fasciatus* populations from the São Francisco river basin (MG) showed $2n = 48$ chromosomes, two of which have shown heterochromatic blocks. The authors suggest that such characteristics may be due to the endemism of populations with discrete heterochromatin blocks. Differing from that, the present work shows three populations that are part of the Corumbataí river basin and are able to maintain contact with each other.

Astyanax marionae presented heterochromatic blocks especially on the centromeric and pericentromeric regions in almost all chromosomes in this study. Krinski and Miyazawa (2014) also evidenced similar heterochromatin location in *A. marionae* and discussed resembling morphological characteristics between *A. marionae* and *A. fasciatus*.

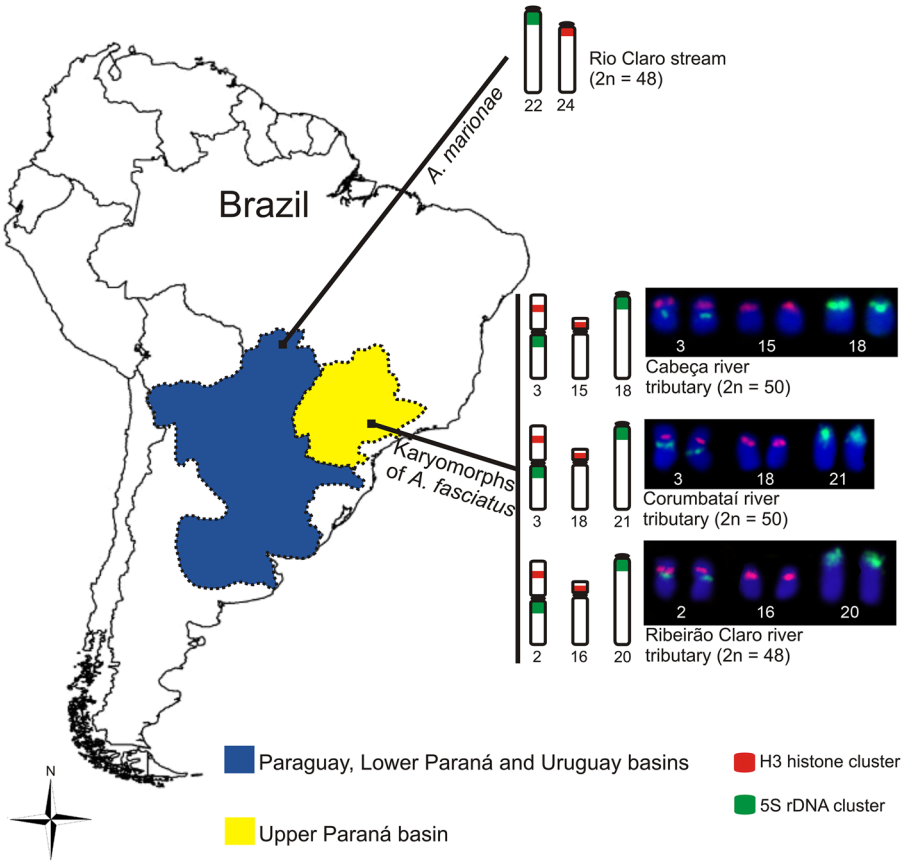

Figure 3. Map of the hydrographic basins and location of repetitive sequences on the chromosomes of *A. marionae* and *A. fasciatus*. Note that the chromosomes bearing H3 histone and 5S rDNA clusters of *A. fasciatus* populations are shown in dark boxes.

On the other hand, our molecular cytogenetic data pointed to two different chromosomal organization patterns of repetitive sequences: (i) one metacentric pair bearing an H3 histone cluster in the interstitial region on the short arm and a 5S rDNA cluster in the proximal region on the long arm in *A. fasciatus*, as one subtelocentric pair with a proximal signal for the H3 histone and one acrocentric pair with a proximal signal for the 5S rDNA are evident for all three populations; (ii) both genes are organized on the proximal regions of two different acrocentric pairs in *A. marionae*.

Therefore, H3 histone sequences may be located on homeologous pairs in both *A. fasciatus* karyomorphs ($2n = 48$ and $2n = 50$) observed in this work, and in the karyomorph with $2n = 46$ evidenced by other authors (Hashimoto *et al.* 2011; Pansonato-Alves *et al.* 2013; Silva *et al.* 2015; Piscor and Parise-Maltempi 2016b). Here, for the first time, location of H3-5S clusters in *A. marionae* is described, showing a particular form of organization. Other peculiar forms have also been observed in *A. jordani* by Silva *et al.* (2015), *A. schubarti* and *A. mexicanus* by Piscor and Parise-Maltempi (2016b).

According to Piscor and Parise-Maltempi (2016b), *A. fasciatus* and other *Astyanax* species (*A. altiparanae*, *A. abramis*, *A. asuncionensis*, *A. bockmanni* and *A. eigenmanniorum*) present H3 histone clusters on two chromosome pairs (one m/sm and one sm/st) with similar morphologies (which contribute to the conservation hypothesis for the H3 histone genes), with the exception of *A. schubarti*, and *A. mexicanus*, that show different forms. In this study, *A. marionae* showed a different system of H3 histone cluster organizations.

In this context, despite presenting similar morphological traits and chromosomal macrostructure as discussed by Krinski and Miyazawa (2014), *A. marionae* and *A. fasciatus* show remarkable differences in the organization of repetitive sequences. In such respect, we suggest that these distinct organization forms of 5S rDNA and H3 histone clusters does not exclude the phylogenetic closeness between them.

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