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Original article

Comparative cytogenetics of *Astyanax* (Teleostei: Characidae) from the upper Paraguay basin

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Astyanax is one of the most abundant and diverse taxa of fishes in the Neotropical region. In order to increase the amount of cytogenetic information for *Astyanax* as well as to exhibit data to subsidize future taxonomic studies, this work analyzed three species of *Astyanax*: two species are cryptic, and are here reported to live in syntopy (*A. abramis* and *A. lacustris*); the first karyotype description for *A. pirapuan* is also presented. Cytogenetic analyzes reveal a diploid number of 2n=50 chromosomes for three species, yet with differences in their karyotype morphology. The physical mapping of 18S rDNA showed up to thirteen sites in *A. pirapuan* and two in *A. abramis* and *A. lacustris.* The physical mapping of 5S rDNA has proven to be an effective marker for the characterization of species of *Astyanax* studied in this work.

Keywords: Cryptic species, Fish, Karyotype, Species complex.

Astyanax é um dos táxons mais representados e diversos na região Neotropical. Com o intuito de aumentar as informações citogenéticas para *Astyanax* e apresentar dados que possam subsidiar estudos taxonômicos futuros, este trabalho traz uma análise citogenética de três espécies de *Astyanax*: duas espécies consideradas crípticas, aqui reportadas em sintopia (*A. abramis* e *A. lacustris*) e a primeira descrição cariotípica de *A. pirapuan*. As análises citogenéticas revelaram 2n=50 cromossomos para as três espécies, com diferença na morfologia cariotípica de cada uma. Foram observados apenas dois sítios de rDNA 18S em *A. abramis* e *A. lacustris* e até 13 para *A. pirapuan*. O mapeamento físico do rDNA 5S se mostrou como um marcador efetivo para a caracterização das espécies de *Astyanax* abordadas neste estudo.

Palavras-chave: Cariótipo, Complexo de espécies, Espécies crípticas, Peixe.

Introduction

Astyanax is the most widely distributed and locally abundant genera living in Neotropical freshwaters (Buckup *et al*., 2007); composed of 155 valid species (Eschmeyer, Fong, 2016) from which 30 were described in the past decade. Six species are reported in the Paraguay basin: *Astyanax lineatus*, *A. pellegrini*, *A. marionae*, *A. lacustris* (=*A. asuncionensis*), *A. abramis* (Britski *et al.*, 2007) and *A. pirapuan* (Tagliacollo *et al.*, 2011).

Owing to the similarity between the morphological characteristics of *Astyanax*, many are considered as cryptic species in which some groups are classified as species complexes because of their taxonomy, as is the case for the "*Astyanax bimaculatus"* complex is which typically shows a horizontally oval black humeral spot, two vertical brown bars, and a black spot in the caudal peduncle that extends onto the caudal fin rays (Garutti, Britski, 2000; Garutti, Langeani, 2009). The "*Astyanax scabripinnis*" complex characterized by having a robust body close to the pectoral fins, massive head, short snout, and a lower number of anal-fin rays (Bertaco, Lucena, 2006).

After taxonomic review of species "*Astyanax bimaculatus*" complex, Lucena, Soares (2016) validates *A. abramis* (Jenyns, 1842), and argues that *A. asuncionensis* Géry, 1972 and *A. altiparanae* Garutti & Britski, 2000 are synonyms of *A. lacustris* (Lütken, 1875).

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Cytogenetic studies on *Astyanax* were stimulated by the work of Jim, Toledo (1975). Several contributions have been made to the study of this group so far that show that, among other characteristics, this genus exhibits a high variability in its diploid number that varies from 2n = 36 *chromosomes* in *A. schubarti* and *A. correntinus* to 2n=52 chromosomes in *Astyanax* sp. (Tenório *et al*., 2013). However, the diploid number of 2n = 50 *chromosomes* is the most frequent for representatives of genus (reviewed from Pazza, Kavalco, 2007).

The term 'species complex' was first used by Moreira-Filho, Bertollo (1991) for describing cryptic species of *A. scabripinnis*. The study showed that populations of this species had different diploid numbers (2n=46, 2n=48 and 2n=50). Two other complexes, *Astyanax fasciatus* and *A. bimaculatus*, were also differentiated on the basis of cytogenetic data The former has a diploid number varying from 2n=45 to 2n=50 chromosomes (Artoni *et al*., 2006; Pazza *et al*., 2006), and the latter a relatively constant diploid number, yet showing differences in the karyotype *composition with variable fundamental numbers (FN) from 76 to 100* (Fernandes, Martins-Santos, 2004).

Species complexes in *Asytanax* have been intensely studied not only regarding the number and morphology of chromosomes, but also regarding other cytogenetic markers. The mapping of ribosomal gene sequences (rDNA 18S and rDNA 5S) by the technique of fluorescence *in situ* hybridization (FISH) has proven to be effective in characterizing species as well as understanding their chromosomal evolution (Mantovani *et al*., 2005; Fernandes, Martins-Santos, 2006a, 2006b; Kavalco *et al*., 2011; Paiz *et al*., 2015; among others).

The location of the 18S rDNA gene is not constant in *Astyanax*, varying from two sites as is the case for *A. lacustris*, *A. abramis*, *A. argyrimarginatus* and *A. asuncionensis* up to seven sites in populations of *A. altiparanae*; all these species are part of the "*Astyanax bimaculatus*" complex (Fernandes, Martins-Santos, 2006a; Peres *et al*., 2008; Ferreira-Neto *et al*., 2009; Tenório *et al*., 2013; Paiz *et al*., 2015). In the "*Astyanax scabripinnis*" complex, 13 sites were observed for this gene in *A. paranae*, and 16 sites in *A. scabripinnis* (Mantovani *et al*., 2005; Vicari *et al*., 2008a). The 5S rDNA gene, in turn, has shown through the FISH technique variable markings on two sites in *A. altiparanae*, *A. lacustris*, and *A. asuncionensis* (Fernandes, Martins-Santos, 2006a; Peres *et al*., 2008; Paiz *et al*., 2015) and on four sites in *A. argyrimaginatus* and *A. abramis* (Tenório *et al*., 2013; Paiz *et al*., 2015). Four sites are observed in *A. paranae* and *A. scabripinnis* (Mantovani *et al*., 2005; Vicari *et al*., 2008a). Although this subject still requires further review, the fact is that *in situ* location of ribosomal sequences may be a valuable tool for helping on the - systematics of *Astyanax* given their variation in location on the chromosomes of this group.

In order to increase the cytogenetic information for *Astyanax* and to produce data that may support future taxonomic studies, the present work is an integrative (cytogenetic, morphological and molecular) study with three species from the upper Paraguay basin. The karyotypic description of *A. pirapuan* Tagliacollo, Britzke, Silva & Benine, 2011 (belonging to the "*Astyanax scabripinnis* complex") are reported for the first time and a comparative study of the cryptic species *A. lacustris* and *A. abramis* (belonging to the "*Astyanax bimaculatus* complex") that occur in syntopy are also reported.

Material and Methods

The 122 specimens of *Astyanax* analyzed were collected from three streams that are part of the upper Paraguay basin in the Brazilian state of Mato Grosso: Soberbo stream (15º11'43.47"S 56º00'00.16"W), Aricá-Mirim stream (15°46'04"S 55°30'44"W), and Cupim stream (15°52'24.30"S 55°28'00.35"W). Voucher specimens were deposited in the Coleção de Peixes da Universidade Federal de Mato Grosso (voucher numbers: CPUFMT 4431-4438).

Chromosomal studies were carried out on 10 female samples and one male sample of *Astyanax lacustris*, 12 females and six males of *A. abramis* from the Soberbo stream as well as on the populations of *A. pirapuan*, 37 females and 16 males from the Aricá-Mirim stream, and 30 females and 10 males from the Cupim stream.

The identification of the specimens was performed following Britski *et al.* (2007) and Tagliacollo *et al.* (2011), which describes *A. pirapuan*. The three species analyzed in this work are: *A. abramis*, *A. lacustris* and *A. pirapuan* (Fig. 1).

Mitotic chromosomes were obtained through the technique of cell suspension, which is routinely used for chromosomal studies on fishes (Bertollo *et al*., 1978). The identification of constitutive heterochromatin was carried out through the technique of C-banding according to Sumner (1972) and the characterization of nucleolus organizer regions (Ag-NORs) was performed using the technique described by Howell, Black (1980).

The technique applied for mapping ribosome sites was fluorescence *in situ* hybridization using 5S and 18S rDNA probes in agreement with the protocol established by Pinkel *et al.* (1986), and later modified by Martins, Galetti Júnior (2001).

The determination of the diploid number in each species was performed through the counting of 30 metaphases per specimen. The best metaphases were photographed using an Olympus BX51 microscope, and the digital mounting of karyotypes was performed using the Pro Image software according to the classification of chromosomal types into metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) on the basis of arm ratio as proposed by Levan *et al*. (1964), with some adjustment to fishes and for determination of the fundamental number (NF), the metacentric,submetacentric and subtelocentric chromosomes were considered biarmed, and the acrocentric uniarmed.

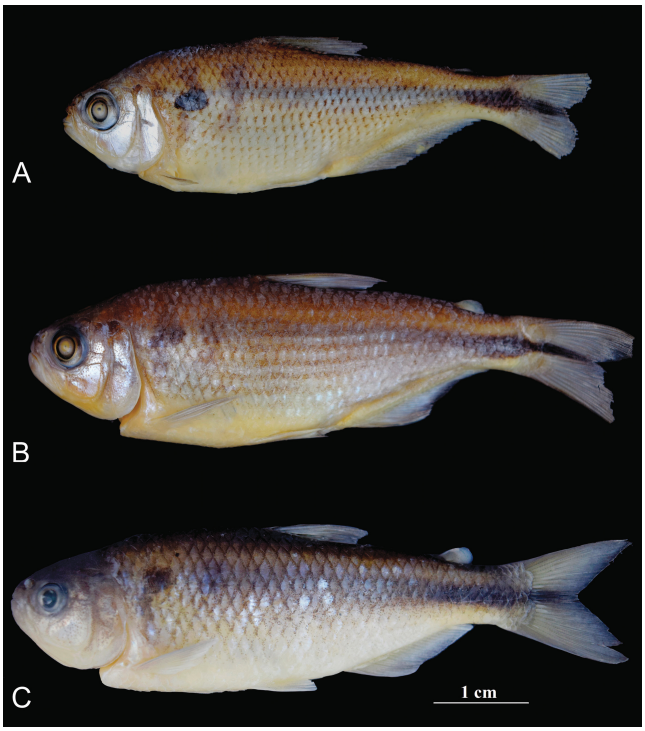

Fig. 1. *Astyanax abramis* - voucher number CPUFMT 4437 Soberbo stream (A), *Astyanax lacustris* - voucher number CPUFMT 4436 Soberbo stream (B) and *Astyanax pirapuan* - voucher number CPUFMT 4433 Cupim stream (C).

Results

All three species of *Astyanax* have the same modal diploid number of 2n=50 chromosomes. Their karyotypes formulas can be summarized as 6m+10sm+18st+16a and NF= 84 for *A. lacustris*, 8m+10sm+26st+6a and NF= 94 for *A. abramis*, and 6m+14sm+14st+16a and NF= 84 for both populations of *A. pirapuan* (Figs. 2A,C,E). Heteromorphic sex chromosomes were not detected.

In *A. lacustris* and *A. abramis*, simple Ag-NORs sites were observed in the terminal region of the short arm of subtelocentric chromosome pairs 13 and 11, respectively (Figs. 2A,C - Box). On the other hand, *A. pirapuan* showed markings on chromosomes one to four, and up to six markings on interphase nucleus. The Ag-NOR were observed in -terminal region of the -short arm of chromosomes 11 and 13. It should be highlighted that three individuals of the population from the Cupim stream presented bitelomeric markings on one of the homologous of chromosome pair 13. In addition, the acrocentric pairs 19 and 22 showed Ag-NORs also in the terminal region of the -short arm and long arm, respectively, in only one of the homologous (Fig. 2E - Box).

Blocks of constitutive heterochromatin are mainly observed in *A. lacustris* on pericentrometric regions of the metacentric chromosomes one and two, submetacentric chromosome six, and acrocentric chromosomes 23 and 25. A prominent marking was observed on the short arm of the subtelocentric chromosome 13 (Fig. 2B), which is coincident with the Ag-NORs. *Astyanax abramis* has karyotypes with low heterochromatin content, which are more specifically localized at the interstitial region of chromosome pair one, in the short arm of the subtelocentric chromosome pair 11 which coincides with the Ag-NORs, pericentrometric regions of subtelocentric chromosome pairs 14, 16 and 17, centrometric regions of chromosome pairs 10, 13, 19, 20 and 22 as well as in the pericentrometric and telomeric regions of acrocentric pairs 23 and 25 (Fig. 2D).

Both the populations of *A. pirapuan* have clearly visible constitutive heterochromatin at the pericentrometric regions of the chromosome pairs 3, 4, 5, 7, 13, 15,16, 18, 20, 22, 23, 24 as well as in the interstitial position of the long arm of pair 1; conpiscuous telomeric blocks occurs on the long and short arms of chromosome pairs 11 and 13, respectively, coinciding with regions bearing Ag-NORs sites (Fig. 2F). The chromosome pair 11 shows polymorphism of heterochromatinin in both populations. Some fishes have heterochromatin in their telomeric regions of the long arm, however others have heterochromatin only in one of the homologous. Furthermore, in places, similar blocks are found on the short arm of whether both chromosomes of pair 11 or only in one of the homologous. Moreover, no heterochromatin blocks were found in some specimens. It is worth mentioning that this condition is accompanied by the presence of 18S rDNA sites in association with heterochromatic regions in this chromosome pair.

The physical mapping of 18S rDNA shows markings on two sites in *A. lacustris* (st number 13), and in *A. abramis* (st number 11) that coincides with Ag-RONs (Figs. 2AC - Box). *Astyanax pirapuan* revealed variation both in the amount (4 to 13 sites) and the position of 18S rDNA in chromosomes (Fig. 3) of individuals from both locations studied. However, despite the variability shown, some markings are constant in the telomeric region of the long arm in chromosomes 11 and 12, and in the telomeric regions of the short arm of pairs 13, 19, 21 and 23. A peculiar feature is that three specimens from the Cupim stream showed bitelomeric Ag-NORs in only one of the homologous of the subtelocentric chromosome pair 13 as confirmed by FISH using an 18S rDNA probe (Fig. 3).

The 5S rDNA sites are found in the pericentrometric region of submetacentric chromosome six in *A. lacustris* (Fig. 4A) as well as in this same position of the following chromosome pairs: submetacentric six, subtelocentric 13, and acrocentric 24, which results in six sites in *A. abramis* (Fig. 4B). On the other hand, the populations of *A. pirapuan* showed sites in the pericentrometric region of chromosome pair 16, for both populations and acrocentric pair 18 - presented 2 sites a pericentromeric and another interstitial to Cupim stream population and interstial for the population Aricá-Mirim stream (Figs. 4C,D).

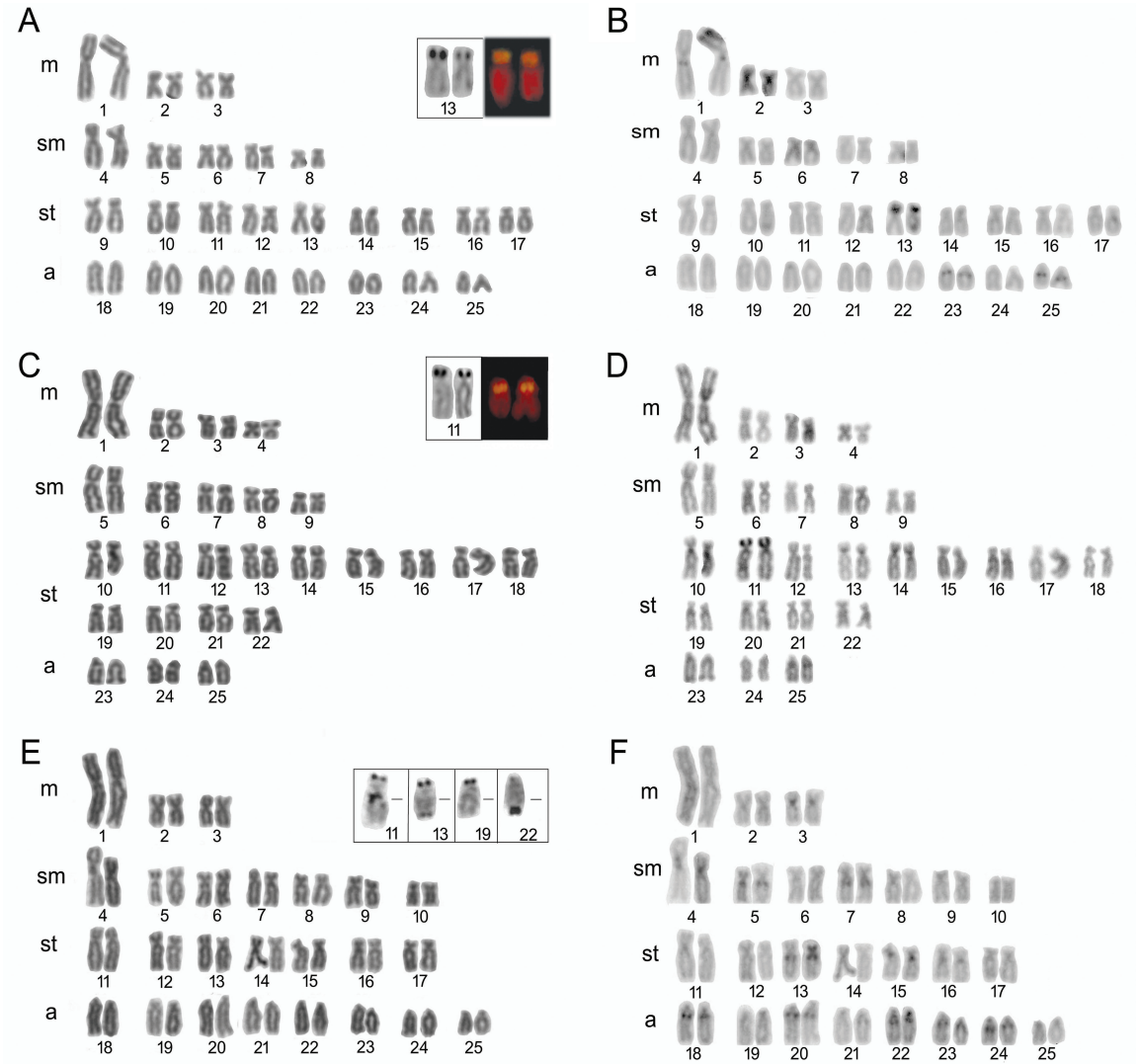

Fig. 2. Karyotypes of *Astyanax abramis* (**A** and **B**), *A. lacustris* (**C** and **D**) and *A. pirapuan* (**E** and **F**) after Giemsa-stained and showing the distribution of constitutive heterochromatin revealed by C-banding. Insets show Ag-NOR and 18S-bearing chromosomes pairs. Scale bar = 10 µm.

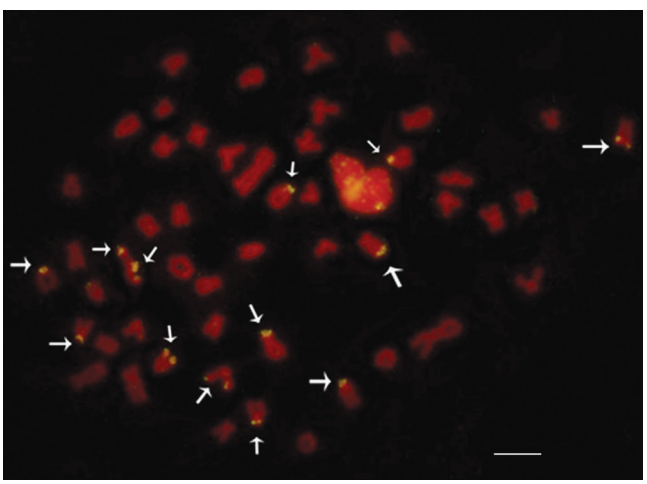

Fig. 3. Metaphase of *Astyanax pirapuan* after fluorescent *in situ* hybridization with 18S probe. Arrows indicate the marked chromosomes. Scale bar = 10 µm.

Discussion

Garutti, Britski (2000), and Bertaco, Garutti (2007) described many species of *Astyanax* to the "*Astyanax bimaculatus*" species complex, which comprises several cryptic species that have a close phylogenetic relationship (Garutti, Langeani, 2009). *Astyanax lacustris* and *A. abramis* are attributed to that complex in this work, and are distinguished by their number of perforated lateral-line scales.

The review of Lucena, Soares (2016) elaborated on the basis of morphological characters establishes that the number of scales for *A. abramis* varies from 42 to 49 while this number varies from 36 to 39 for *A. lacustris*, although rare individuals are known to have from 40 to 41 scales. In the present work the perforated lateral-line scales were counted from specimens of *A. lacustris* and *A. abramis*. We noted variation in number from 33 to 40 scales, and from 35 to 43 scales, respectively.

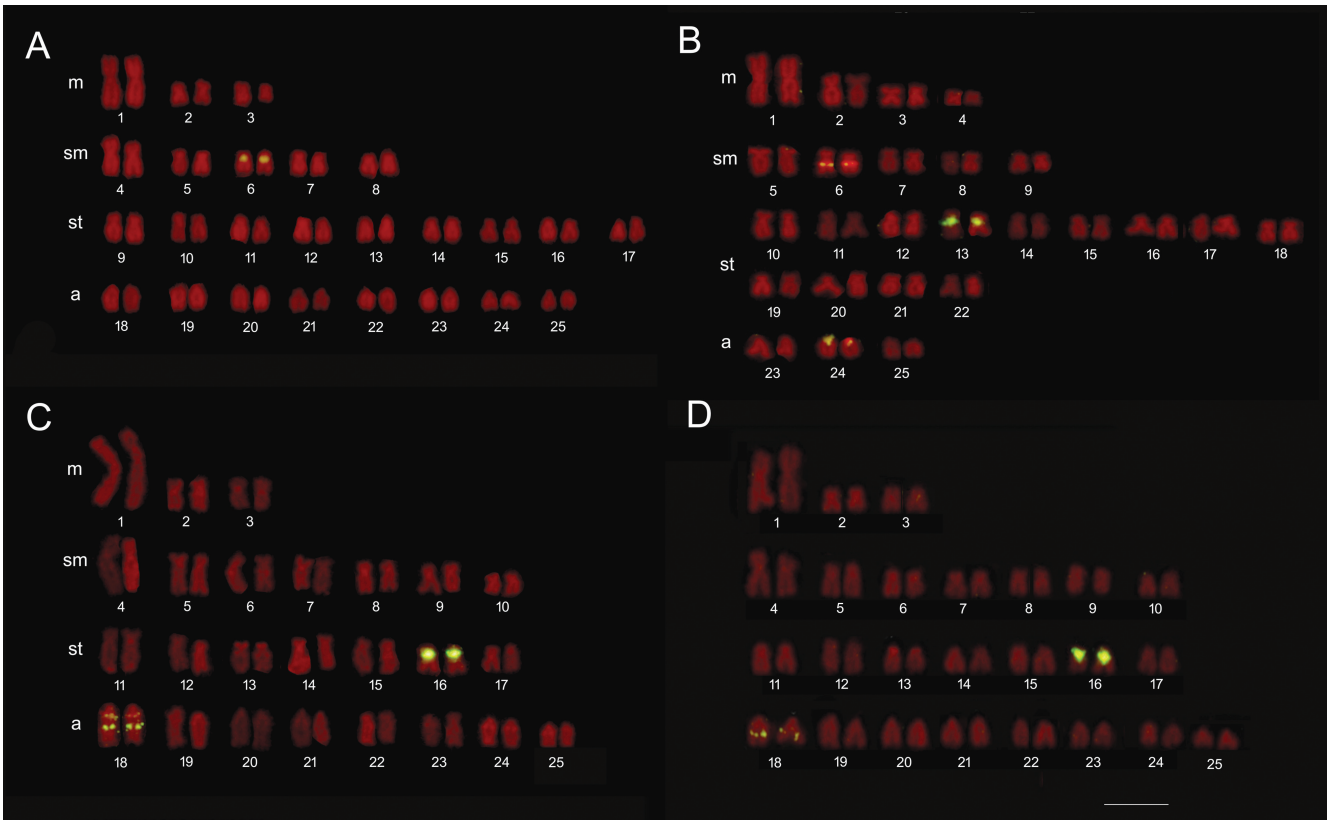

Fig. 4. Karyotypes of *Astyanax abramis* (**A**), *A. lacustris* (**B**) and *A. pirapuan* from Cupim stream (**C**) and Aricá stream (**D**) after fluorescent *in situ* hybridization with 5S probe. Scale bar = 10 μm.

However, *A. lacustris* and *A. abramis* show cytotaxonomic features considered as species-specific with single patterns for each one of them. Although they present the same chromosome number, *A. lacustris* have a higher number of acrocentric chromosomes (16 chromosomes) than *A. abramis* (six chromosomes), which influence on their fundamental numbers (84 and 94, respectively).

The chromosomal location of ribosomal genes helps in the understanding of the evolutionary history of *Astyanax*. The 18S rDNA sites are simple both for *lacustris* and *A. abramis* as well positioned in both species in a subtelocentric chromosome pair, which is likely homeologous. Several species of *Astyanax* share at least a common pair of subtelocentric chromosomes with markings of Ag-NORs/18S in telomeric region of short arm both for species with simple Ag-NORs (Fernandes, Martins-Santos, 2006a; Domingues *et al*., 2007; Peres *et al*., 2008; Peres *et al*., 2012; Barbosa *et al*., 2015) and those with multiple systems (Domingues *et al*., 2007; Vicari *et al*., 2008b; Kavalco *et al*., 2011; Silva *et al*., 2012; Tenório *et al*., 2013; Castro *et al*., 2015). That demonstrates that this subtelocentric chromosome pair and its location pattern for Ag-NORs may represent an ancestral condition for *Astyanax*.

The physical mapping of 5S rDNA is an effective marker for the characterization of *A. lacustris* (two sites) and *A. abramis* (six sites). When comparing this marker behavior to other populations of these two same species, we observe that the number of 5S rDNA sites for *A. lacustris* remains unchanged (Fernandes, Martins-Santos, 2006a; Domingues *et al*., 2007; Peres *et al*., 2008; Ferreira-Neto *et al*., 2009; Kavalco *et al*., 2011; Paiz *et al*., 2015). On the other hand, the population of *A. abramis* collected from the lower Paraná Basin presented only four sites (Paiz *et al*., 2015). Among the species of the "*Astyanax bimaculatus*" complex studied so far, only *A. abramis* and *A. argyrimarginatus* (Tenório *et al*., 2013) have more than two sites (four markings) of 5S rDNA.

Here, we present the first description of the karyotype of *A. pirapuan* ("*Astyanax scabripinnis*" complex) for the Paraguay basin. Populational differences were here highlighted once the population from the Cupim stream may be distinguished by two markings of 5S rDNA on the same arm of the chromosome pair 18, which is usually observed in the homozygous state. This same population shows a polymorphism related to the 18S rDNA sites where some individuals have bitelomeric markings in one of the subtelocentric chromosome pair 13.

In addition, two populations of *A. pirapuan* presented an intra-populational variation in the number of 18S rDNA sites (four to 13 sites). Mantovani *et al*. (2005) also observed an intra-populational variation in 18S rDNA sites for populations of *A. scabripinnis* from the Paraná and São Francisco basins where up to 16 sites are reported. This condition is common for representatives of the "*Astyanax scabripinnis*" complex (Ferro *et al*., 2000; Mantovani *et al*.,

2005; Fernandes, Martins-Santos, 2006b), likely facilitated both by their telomeric position in chromosomes, which makes them more susceptible to chromosomal rearrangement (Mantovani *et al*., 2005) as well as to associations with transposable elements as is the case for *A. bockmanni* (Silva *et al*., 2013).

Opposite of what is observed in most of the species belonging to the "*Astyanax scabripinnis*" complex with respect to heterochromatic regions, *A. pirapuan* shows low heterochromatin content restricted to pericentrometric regions including the presence of small interstitial blocks in some chromosomes. An outstanding feature for most species of the "*Astyanax scabripinnis*" complex is the occurrence of conspicuous heterochromatic blocks, mainly in acrocentric chromosomes (Moreira-Filho, Bertollo 1991; Maistro *et al*., 1998; Souza, Moreira-Filho, 1995; Mizoguchi, Martins-Santos, 1998; Mantovani *et al*., 2000; Souza *et al*., 2007; Tenório *et al*., 2013). The one exception is a population of *A. laticeps* that has a few heterochromatic regions in the karyotype (Rosa *et al*., 2009). Some cases are followed by heterochromatic polymorphism on subtelocentric to acrocentric chromosomes (Mantovani *et al*., 2000; Souza *et al*., 2007) as it is observed in the chromosome pair 11 in *A. pirapuan.*

The data presented in this work show valuable contributions to the cytogenetic studies in the genus *Astyanax*. The karyotype of *A. pirapuan* is described for the first time and corroborates with data already described for most species of the genus. Reinforcing the constant number of chromosomes 2n = 50 with distribution specific for most species. The variation found in the sites for the 18S rDNA reinforces a greater relation of this group with "*Astyanax scabripinnis*" complex. *Astyanax pirapuan* may be distinguished from other species of the *A. scabripinnis* complex by its karyotype formulae, position of 18S and 5S rDNA sites, and low heterochromatin content (except for *A. laticeps*).

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