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Comparative cytogenetics in *Astyanax* (Characiformes: Characidae) with focus on the cytotaxonomy of the group

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Astyanax is a diverse group of Neotropical fishes, whose different forms occupy different environments. This great diversity is also reflected on cytogenetic aspects and molecular markers, which have repeatedly been demonstrated by cytogenetic studies. In order to characterize the karyotype of species of this genus, six species were studied: *Astyanax altiparanae*, *A.argyrimarginatus*, *A. elachylepis*, *A. xavante*, and two new species provisionally called *Astyanax* sp. and *A.* aff. *bimaculatus*. A detailed cytogenetic study based on conventional staining with Giemsa, AgNORs, C-banding, base-specific fluorochromes, and FISH using ribosomal genes 18S and 5S was conducted, aiming to understand some of the chromosomal mechanisms associated with the high diversification that characterizes this group and culminated with the establishment of these species. The results showed 2n = 50 chromosomes for five species and a karyotype with 52 chromosomes in *Astyanax* sp. Small variations in the macrostructure of the karyotypes were identified, which were quite relevant when analyzed by classical banding, fluorochromes, and FISH methods. These differences among *Astyanax* spp. (2n = 50) are largely due to changes in the amount and types of heterochromatic blocks. *Astyanax* sp (2n = 52), in addition to variations due to heterochromatic blocks, has its origin possibly by events of centric fission in a pair of chromosomes followed by minor rearrangements.These results show an interesting karyotypic diversity in *Astyanax* and indicate the need of a review of the group referred as *A.* aff. *bimaculatus* and the description of *Astyanax* sp., including the possibility of inclusion of this unit in another genus.

Astyanax é um grupo bastante diverso de peixes neotropicais cujas diferentes formas ocupam distintos ambientes. Esta grande variabilidade também se reflete em aspectos citogenéticos e moleculares, que têm sido repetidamente demonstrados por meio de estudos citogenéticos. A fim de caracterizar o cariótipo de representantes deste gênero, seis espécies foram estudadas: *Astyanax altiparanae*, *A. elachylepis*, *A. xavante*, *A. argyrimarginatus* e duas espécies novas provisoriamente citadas como *Astyanax* sp. e *A.* aff. *bimaculatus*. Um estudo citogenético detalhado com base na coloração convencional com Giemsa, AgNORs, banda C, fluorocromos base-específicos, e FISH com sondas para genes ribossomais 18S e 5S foi realizado com o objetivo de compreender alguns dos mecanismos cromossômicos associados com a alta diversificação que caracteriza este grupo de peixes e que culminou com o estabelecimento dessas espécies. Os resultados revelaram 2n = 50 cromossomos para cinco espécies e 2n = 52 cromossomos para *Astyanax* sp. Pequenas variações na macroestrutura dos cariótipos foram identificadas e se mostraram relevantes quando analisadas com base nos bandamentos clássicos, coloração por fluorocromos base-específicos e FISH com sondas de DNA 18S e 5S. Esssa diversidade cariotípica detectada indica a necessidade de uma revisão taxonômica no grupo de indivíduos aqui referidos como *A.* aff. *bimaculatus*, inclusive com a descrição de *Astyanax* sp., incluindo a possibilidade de inserção dessa unidade em outro gênero distinto de *Astyanax*.

Key words: C-banding, CMA_3 and DAPI fluorochromes, Fish chromosome, rDNA genes, NORs.

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Introduction

Characiformes comprise the largest number of freshwater scaled fish known in the Neotropical region and Characidae is the largest and most complex family of this order. With 1.040 species (Eschmeyer & Fong, 2013.), this fish family represents more than half of the species of Characiformes, and is a key component of Neotropical freshwater ecosystems (Oliveira *et al*., 2011). According to these authors, the composition, phylogeny, and classification of Characidae groups is currently uncertain, despite significant efforts based on analysis of morphological and molecular data have been undertaken.

According to Garutti & Venere (2009), the last complete *Astyanax* revision founded on morphological characteristics was conducted by Eigenmann (1921, 1927), and the author who most recently treated this genus as a whole was Géry in 1977, who essentially followed Eigenmann. *Astyanax* is one of the most common genera in the freshwaters of Neotropical basins (Bertaco & Garutti, 2007), and is non-monophyletic (Weitzman & Fink, 1983; Garutti & Venere, 2009; Mirande, 2010; Oliveira *et al*., 2011).

Astyanax corresponds to a larger taxonomic unit, with several species sharing similar forms which induce different authors to treat some units as "species complexes" (Moreira Filho & Bertollo, 1991; Garutti & Britski, 2000; Artoni *et al*., 2006). These fishes inhabit many different environments, including the waters from headwater streams, rivers, small and medium-sized lakes, and ponds, which seems to be one of the most important behavioral aspects that remains active in the establishment of the characteristics of species complexes of this genus.

Astyanax is the best cytogenetically studied genus of Characidae. The available data indicate an extensive chromosomal variability and a high degree of polymorphism among different species. However, analyzing the available information, it appears that many species have karyotypes with 2n = 50 chromosomes, as is the case of *A. scabripinnis* (Moreira-Filho & Bertollo, 1991), *A. altiparanae* (Fernandes & Martins-Santos, 2004; Ferreira-Neto *et al*., 2009), and *A. laticeps* (Rosa *et al*., 2009), among others.

Deviations from this value were reported for *Astyanax schubarti*, which reveals smallest chromosome number (2n = 36) described for the genus (Morelli *et al*., 1983, Daniel-Silva & Almeida-Toledo, 2001, 2005); *A. parahybae* with 2n = 48 chromosomes (Kavalco & Moreira-Filho, 2003; Centofante *et al*., 2003), and *A. fasciatus*, which has karyotypes with 2n = 45-48 chromosomes (Pazza *et al*., 2006; Pazza *et al*., 2008a, 2008b).

It is possible to detect "species complexes" referring to three *Astyanax* groups, which show variation in relation to the diploid number and/or karyotype formula. In these complexes, there are outstanding species, such as *A. scabripinnis* (Moreira-Filho & Bertollo, 1991) and *A. fasciatus* (Pazza *et al*., 2006), which show a great variation in both the karyotype macro structure and in the occurrence of supernumerary chromosomes, together with polymorphism in blocks of heterochromatin and nucleolar organizer regions (Souza *et al*., 2007). In *A. altiparanae*, these polymorphisms are also present, being primarily related to the micro and macrostructure of the karyotypes (Fernandes & Martins-Santos, 2004). In this scenario, the cytogenetic data are potentially informative and, although the chromosomal variation is common among species, the mechanisms of fixation of chromosomal rearrangements or even the polarity of characters observed are still a matter of discussion.

Most current works have employed the FISH (fluorescence *in situ* hibridization) technique with different DNA probes to map the chromosomes, in an attempt to resolve cytogenetic dilemmas. This technique has enabled great progress in studies endeavoring to compare different fish species and/or populations (Teixeira *et al*., 2009; Vitorino *et al*., 2011; Martinez *et al*., 2012).

Another interesting issue relates to the fact that the 5S rDNA sites are relatively more constant in number and location in contrast with the 45S rDNA sites. These rDNA sequences occupy interstitial positions, meaning that they need to be most protected of the chromosomal rearrangements, while the 45S rDNA *locus* are typically located in a terminal position on chromosomes, and thus most likely to undergo greater variability (Galetti Jr. & Martins, 2004).

According to Nakajima *et al*. (2012) the presence of 5S rDNA clusters in the interstitial or proximal position was frequently observed for some cichlids, as has previously been observed in several other fish groups. In this way, in relation to the number of these sites, in some cichlids species for example, the average cluster number per genome is slightly higher for 18S rDNA, and the more intense dispersion of these genes seems to be related to their common presence in the terminal regions of the chromosomes. It can be related to the genomic dynamism of terminal regions of the chromosomes that could favor transposition events, leading to the dispersion of segments (Nakajima *et al*., 2012). Although it may occur in *Astyanax* species with 2 or 4 5S rDNA sites (this paper) or even up to six of such sites in *Piabina* (Pazian *et al*., 2012), it seems that there is no standard mechanism that governs the behavior of these sites in different fish species studied so far.

Given the above, it is clear that there are many processes by which the karyotypes of fish may change. Each group can follow different paths, some of which are characterized by high rates of karyotypic diversification while others follow more conservative models in terms of diploid number, chromosome morphology, and banding patterns obtained by several techniques available. But the mechanisms of karyotypic diversification, especially in the genus *Astyanax*, still require many studies. Thus, based on an understanding of these mechanisms, it is believed that it is possible to effectively contribute to a good and broad regional characterization of fish diversicity, especially the ichthyofauna of the streams of the Cerrado bioma (savanna like) in the Central Brazil.

Material and Methods

Six species of *Astyanax* were cytogenetically studied: *A. altiparanae* Garutti & Britski, 2000 from Monjolinho stream (21°59'9.26"S 47°52'52.59"W) (upper Paraná river basin); *A. argyrimarginatus* Garutti, 1999 from Jaraguá stream (15°56'27.18"S 52°15'18.68"W); *A.* aff. *bimaculatus* from Dois de Agosto stream (15°28'34.84"S 51°52'43.87"W); *A. elachylepis* Bertaco & Lucinda, 2005 from Taquaralzinho stream (15°40'42"S 52°17'52"W); *A. xavante* Garutti & Venere, 2009 from Avoadeira stream (15°51'19.66"S 52°15'15.95"W); *Astyanax* sp. from Grande stream (15°46'05"S 52°05'18"W) (Mortes river), all belonging to the Tocantins-Araguaia river basin (Fig. 1). Voucher specimens were deposited in the fish collection of the Laboratório de Biologia e Genética de Peixes (LBP) UNESP, Botucatu (São Paulo State, Brazil) (serial numbers LBP 17108-17115) and Campus Universitário do Araguaia, Universidade Federal de Mato Grosso, Brazil (serial number: ICLMA 635-640).

The specimens were anesthetized and sacrificed by clove-oil overdoses (Griffiths, 2000) and metaphasic cells were obtained through the air-drying technique (Bertollo *et al*., 1978; Foresti *et al*., 1993). C-banding, Ag-NORs, and fluorochrome staining with CMA_3 and DAPI folowed Sumner (1972), Howell & Black (1980) and Schweizer (1976, 1980), respectively. Fluorescent *in situ* hybridization (FISH) was applied to map 18S and 5S rDNA. The probes were labeled with 14-dATP biotin by nick translation, according to the manufacturer's instructions (Bionic Labeling System, Invitrogen). The metaphase chromosomes were treated according to the procedure described by Pinkel *et al*. (1986) and analyzed using an Olympus BX51 epifluorescence microscope. The chromosome images were captured using Olympus DP71 camera and the software Image Pro Express version 6.0 (www.mediacy.com). The final organization of the karyotypes was based on their arm ratios, according to Levan *et al*. (1964), with adjustments for fish cytogenetics (Bertollo *et al*., 1978; Venere & Galetti, 1989; Feldberg *et al*., 1993).

An idiogram was constructed for each species, aiming to summarize the data and facilitate their analysis together. After this step, the information obtained was used for the construction of a matrix of presence and absence, based on 157 characteristics, which were then used for a cluster analysis on the basis of similarities/dissimilarities, using the program Past (Cluster Analysis) based on Euclydean distances.

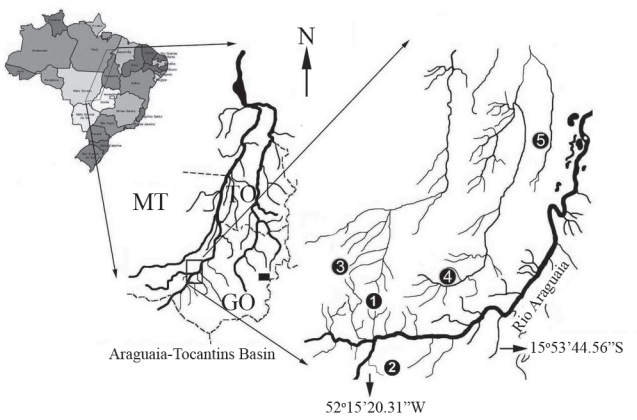

Fig. 1. Collection sites of *Astyanax* species in the Araguaia basin: (1) Avoadeira stream, (2) Jaraguá stream; (3) Taquaralzinho stream, (4) Grande stream, and (5) Dois de Agosto stream.

Results

The diploid number observed for five of the six species was equal to 2n = 50 chromosomes. Only *Astyanax* sp. revealed a divergent diploid number with 2n = 52 and a karyotype formula without the presence of acrocentric chromosomes (Fig. 2). Interspecific karyotypic changes, especially in relation to the amount of subtelocentric and acrocentric chromosomes, were observed among these species, with differences in fundamental numbers (FN) ranging from 88 to 104 in *A. elachylepis* and *Astyanax* sp., respectively (Table 1).

AgNORs were mapped on the short arm of a medium-sized subtelocentric chromosome pair (st) in all species, which occupy different positions in the karyotypes. Besides that pair occupying different positions in the karyotypes analyzed, multiple sites of AgNORs were observed in *Astyanax altiparanae* and *A. xavante* (Fig. 2, box). However, FISH with 18S rDNA probes confirmed a system of multiple NORs only for *A. xavante*, which showed three pairs of chromosomes bearing rDNA sites (Fig. 2, box).

The constitutive heterochromatin distribution pattern (Fig. 3) proved to be species-specific, with some single blocks observed in each of the species studied. Most heterochromatic blocks were located in the pericentromeric and terminal position of the short arms of NOR-bearing chromosomes and in the interstitial regions of m, sm, and st chromosomes, mainly in *Astyanax altiparanae*, *A. elachylepis* and *Astyanax* sp. On the other hand, in *A. xavante* predominate telomeric heterochromatin, including a pair of acrocentric with large blocks of heterochromatin not observed in other species. *A.* aff. *bimaculatus* and *A. argyrimarginatus* presented reduced amount of heterochromatin restricted to the pericentromeric regions and associated with AgNOR sites.

CMA_3 revealed that all blocks corresponding to the AgNORs sites bright more intensely after staining with this fluorochrome, suggesting they are GC^+ regions (Fig. 4).

In addition, several other constitutive heterochromatin blocks revealed to be CMA^+, in opposition to the idea that this fluorochrome would be a good marker to identify only NOR-bearing chromosomes. It is worth emphasizing the large heterochromatic blocks detected in a pair of acrocentric chromosomes in *Astyanax xavante*, which is CMA_3^+ and $DAPI^-$ and are observed only in this species amongst all those species assessed in this work.

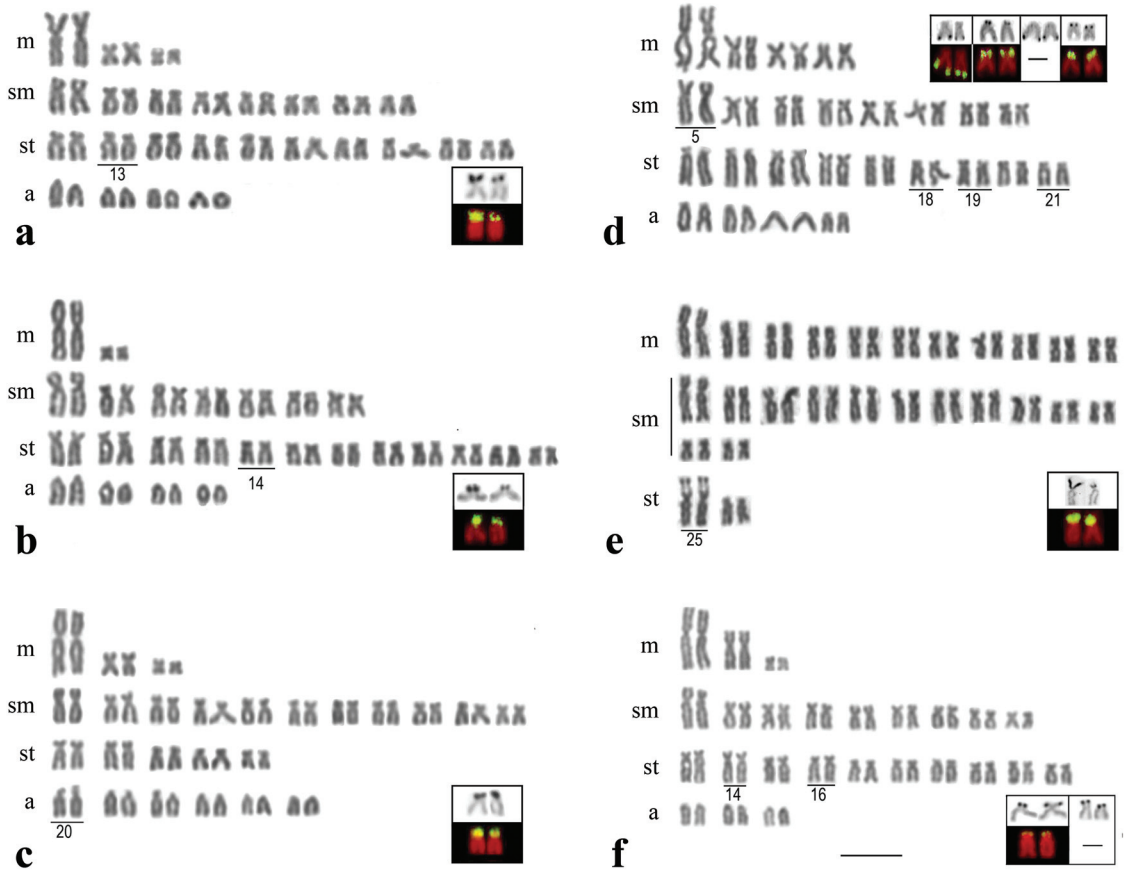

Fig. 2. Karyotypes of *Astyanax argyrimarginatus* (**a**), *A*. aff. *bimaculatus* (**b**), *A*. *elachylepis* (**c**), *A*. *xavante* (**d**), *Astyanax* sp. (**e**), and *A*. *altiparanae* (**f**), after conventional staining with Giemsa. Chromosomes carrying AgNORs and 18S sites (underlined in karyotypes) are highlighted in the box. Bar = 5 µm.

Also in relation to the DAPI fluorochrome (Fig. 5), another interesting event detected in this study was the presence of several interstitial AT-rich heterochromatic blocks, which were equilocally distributed among some chromosomes in *Astyanax argyrimarginatus* and in *Astyanax* sp. In both species, these DAPI-bright heterochromatic blocks were observed always in the interstitial region of the long arm, proximal to the centromere in submetacentric and subtelocentric chromosomes, and in the case of *Astyanax* sp., they were also observed in metacentric chromosomes. In *A*. *argyrimarginatus*, some less conspicuous blocks located in acrocentric chromosomes were also detected.

The localization of 5S rDNA genes revealed the occurrence of two pairs of chromosomes carrying these sites in *Astyanax argyrimarginatus* (submetacentric pair 5 and subtelocentric

Table 1. Studied species, number of specimens, diploid numbers and chromosome types observed in the six *Astyanax* species. 2n = diploid number, NF = fundamental number, m = metacentric, sm = submetacentric, st = subtelocentric and a = acrocentric.

Species	n.º of specimens			nº of cells analized			2n	FN	chromosomal types			
	♀	♂	total	♀	♂	total			M	sm	st	a
A. *altiparanae*	15	10	25	390	60	450	50	94	6	18	20	6
A. *argyrimarginatus*	14	12	26	90	150	240	50	92	6	16	20	8
A. aff. *bimaculatus*	21	4	25	630	120	750	50	92	4	14	24	8
A. *elachylepis*	12	8	20	180	240	420	50	88	6	22	10	12
A. *xavante*	9	2	11	60	270	330	50	92	8	16	18	8
Astyanax sp.	6	5	11	120	200	320	52	104	22	26	4	-

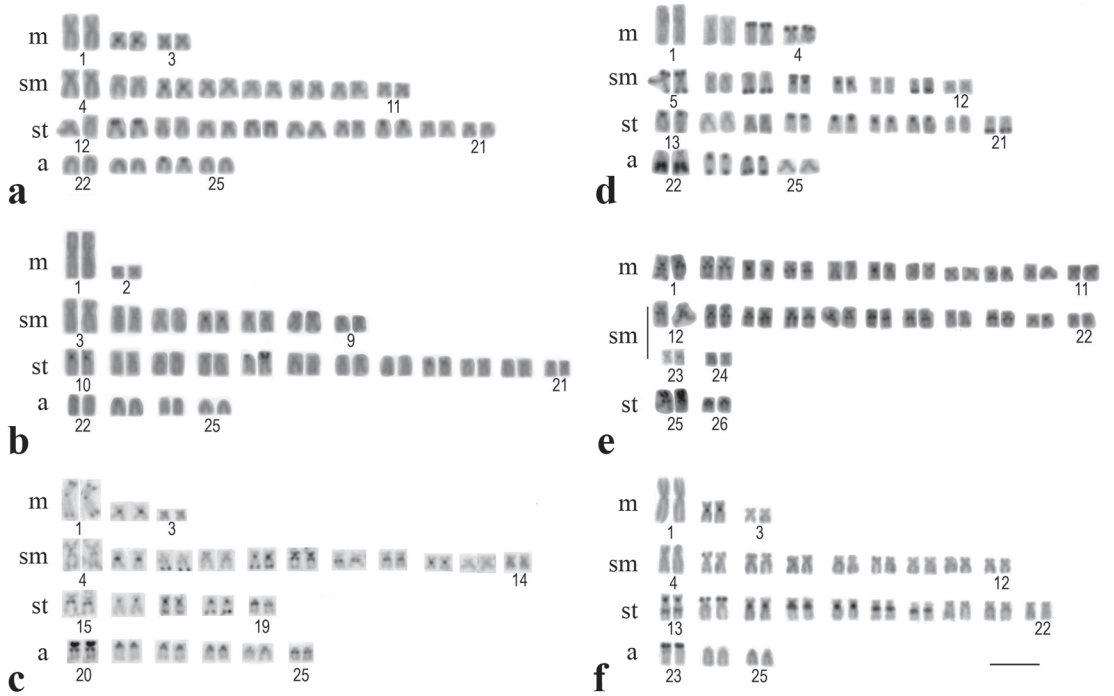

Fig. 3. C-banded karyotypes of *Astyanax argyrimarginatus* (**a**), *A*. aff. *bimaculatus* (**b**), *A*. *elachylepis* (**c**), *A*. *xavante* (**d**), *Astyanax* sp. (**e**), and *A*. *altiparanae* (**f**). Bar = 5 µm.

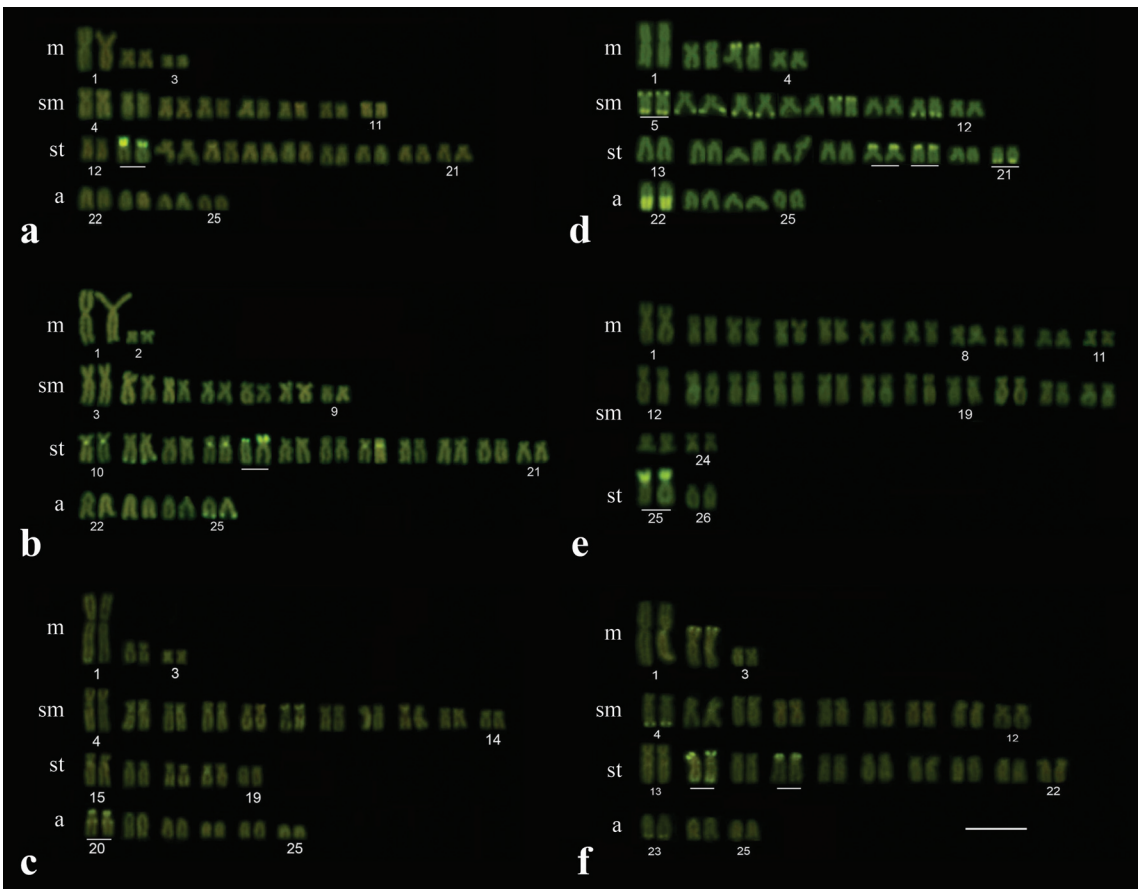

Fig. 4. CMA_3 stained karyotypes of *Astyanax argyrimarginatus* (**a**), *A*. aff. *bimaculatus* (**b**), *A*. *elachylepis* (**c**), *A*. *xavante* (**d**), *Astyanax* sp. (**e**), and *A*. *altiparanae* (**f**). Chromosomes carrying AgNORs and 18S sites are underlined in karyotypes. Bar = 5 µm.

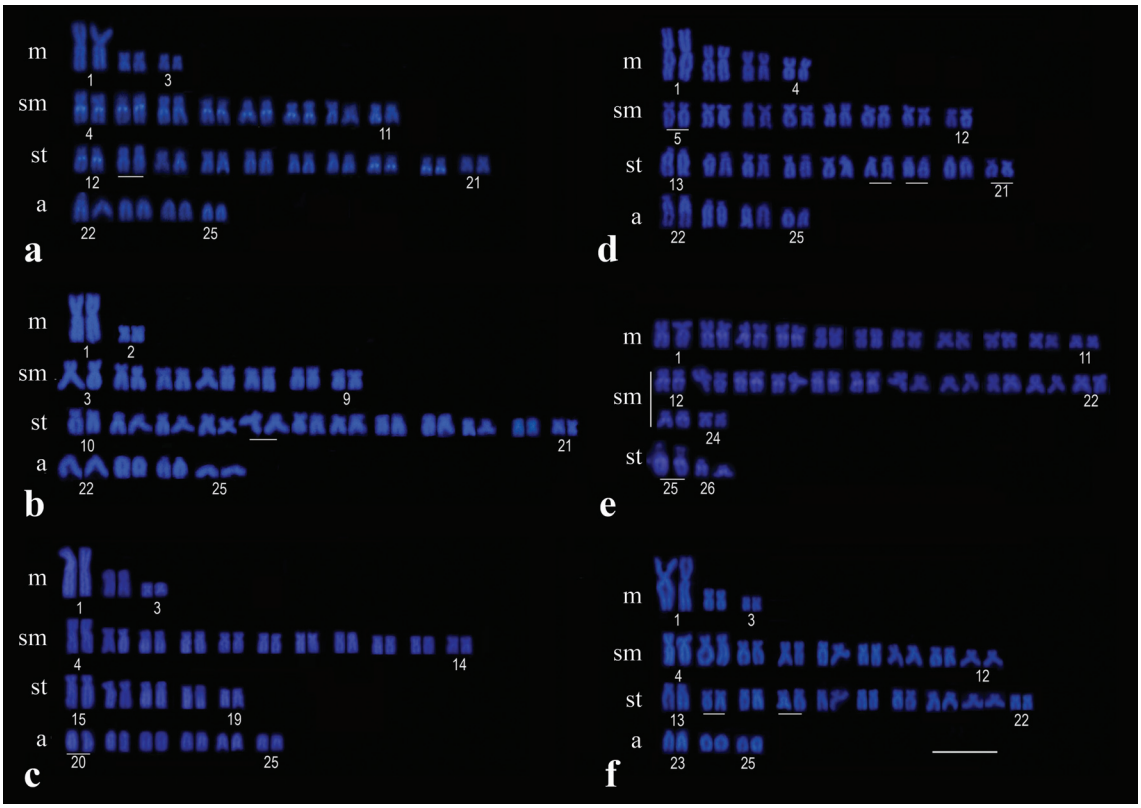

Fig. 5. DAPI stained karyotypes of *Astyanax argyrimarginatus* (**a**), *A*. aff. *bimaculatus* (**b**), *A*. *elachylepis* (**c**), *A*. *xavante* (**d**), *Astyanax* sp. (**e**), and *A*. *altiparanae* (**f**). Chromosomes carrying AgNORs and 18S sites are underlined in karyotypes. Bar = 5 µm.

pair 14), *A*. aff. *bimaculatus* (submetacentric pairs 4 and 5); *Astyanax* sp. (metacentric pair 2 and submetacentric pair 15), and *A*. *altiparanae* (submetacentric pairs 5 and 9). *A*. *elachylepis* and *A*. *xavante* have only one pair of chromosomes bearing these 5S rDNA sites (submetacentric pairs 5 and 6 respectively) (Fig. 6).

For a better visualization of these results, an idiogram representing the haploid karyotypes of the six species with all techniques accomplished is showed in the Fig 7. The cluster analysis based on karyotypic characters enabled the construction of the dendrogram shown in Fig. 8.

Discussion

The diploid number of 50 chromosomes was observed in five species, while *Astyanax* sp. revealed 2n = 52. Fishes of the genus *Astyanax* usually possesses 2n = 50 chromosomes, with exceptions specially found in *A*. *fasciatus* with variations of 2n = 45, 46, 47, 48, and 50 (Pazza *et al*., 2006; Ferreira-Neto *et al*., 2012) and *A*. *scabripinnis* (Moreira-Filho & Bertollo, 1991). This large number of species and/or populations of *Astyanax* with 50 chromosomes, can be considered of great importance for conducting a kinship analysis, since this genus does not represent a natural group of species (Mirande, 2010; Oliveira *et al*., 2011).

Besides the diploid number, several other karyotypic characteristics apparently are shared among species of *Astyanax*. As an example, the metacentric chromosome pair number one seems to be an element shared by most species of this genus, except for *Astyanax* sp. (present paper), whose karyotype is being described for the first time. Another example can be seen in *A*. *fasciatus*, where this chromosome pair was observed with a smaller size (Pazza *et al*., 2008). A macrostructural analysis suggests that this chromosome pair is present in several species of other Characidae genera, suggesting that it represents a primitive feature in the family.

Another pair that seems to be shared by this genus refers to the main NOR-bearing chromosome (a medium-sized subtelocentric pair with NORs located on the short arm), that can be virtually seen in all species examined to date, including *Astyanax* sp. and in a NOR-bearing pair of *A*. *xavante*. Although their position may vary in different karyotypes, studies with fluorescent markers and FISH strengthens the idea that there is a strong similarity between these elements among the various karyotypes studied.

In this paper, differences in interspecific karyotypic macrostructures, especially with respect to the amount of subtelocentric and acrocentric chromosomes, were observed among the species of *Astyanax*, which is easily visualized only by the analysis of fundamental numbers, which ranged from 88 in *A*. *elachylepis* to 104 in *Astyanax* sp.

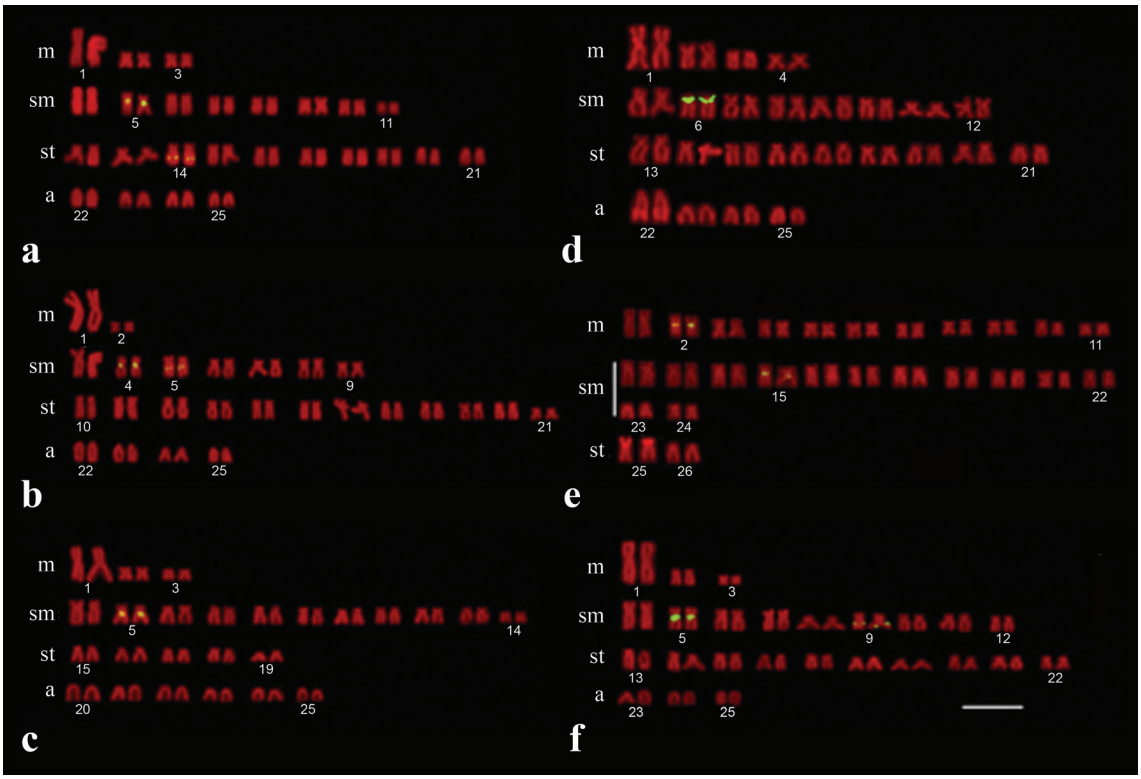

Fig. 6. Karyotypes of *Astyanax argyrimarginatus* (**a**), *A*. aff. *bimaculatus* (**b**), *A*. *elachylepis* (**c**), *A*. *xavante* (**d**), *Astyanax* sp. (**e**), and *A*. *altiparanae* (**f**) after FISH with 5S-DNA probes. Bar = 5 μm.

Such variations with changes in the fundamental numbers and without changes in the diploid numbers indicate that the karyotypic diversification in these groups of organisms are attributable to chromosomal rearrangements due to pericentric and paracentric inversions and/or translocations, which alter the morphology of chromosomes, but do not affect the diploid number. Kantek *et al*. (2007) assert that the variations detected in *Astyanax* sp. D from the Iguaçu River indicated the occurrence of chromosome polymorphisms in some of the homologous chromosome pairs, and that some of those variations were due to paracentric inversions detected by the C-banding technique.

The occurrence of such interpopulation variations in the karyotypic macrostructure in *Astyanax* can be partly explained by the biological characteristics of the species, such as the exploratory adaptive capacity (Orsi *et al*., 2002; Orsi *et al*., 2004), high feeding plasticity, reproductive capacity in various environment, preference for lentic waters and high phenotypic plasticity (Garutti & Britski, 2000). These conditions favor the fixation of regional variations.

In addition to the studies based on Giemsa stained karyotypes, the AgNORs have been widely used as species-specific chromosomal markers for showing a relatively constant location within a species (Almeida-Toledo *et al*., 1998; Venere *et al*., 2008). The occurrence of AgNORs on a single pair of chromosomes (single AgNORs), as well as those distributed in several pairs (multiple AgNORs), are observed in different species of *Astyanax*: *A*. *altiparanae* with 4-7 reported sites (Pacheco *et al*., 2001; Daniel-Silva & Almeida-Toledo, 2001; Fernandes & Martins-Santos, 2004, 2006a, 2006b), *A*. *scabripinnis* with 4-14 sites (Mantovani *et al*., 2000; Mantovani *et al*., 2005; Fernandes & Martins-Santos, 2005), *A*. *fasciatus* with four sites (Daniel-Silva & Almeida-Toledo, 2001; Pazza *et al*., 2006), *A*. *jacuhiensis* with two sites (Pacheco *et al*., 2010), *A*. *laticeps* with three sites (Rosa *et al*., 2009), and *A*. *giton* with 10 and *A*. *intermedius* with 12 small reported sites (Kavalco & Moreira-Filho, 2003).

Most cytogenetically analyzed species present only one pair of homologous NOR-bearing chromosomes. However, according to Pacheco *et al*. (2001), Souza *et al*. (2001); Jorge & Moreira-Filho (2001), Almeida-Toledo *et al*. (2002); Mantovani *et al*. (2005), Fernandes & Martins-Santos (2006a; 2006b), among others, the occurrence of multiple AgNORs is relatively common in some species and/or populations of *Astyanax*. Among the *Astyanax* species studied here, only *A*. *xavante* presented a system of AgNOR multiple sites, which were confirmed as bearers of NORs by FISH with 18S rDNA probes.

In *Astyanax jacuhiensis* (Pacheco *et al*., 2010), *A*. *laticeps* (Rosa *et al*., 2009), and *A*. *fasciatus* (Ferreira-Neto *et al*., 2012), the multiple AgNOR systems were also confirmed by the FISH technique. In *A*. *bimaculatus* from the San Francisco, Doce, and Paraguay rivers, and in *A*. *altiparanae* from the Paraná

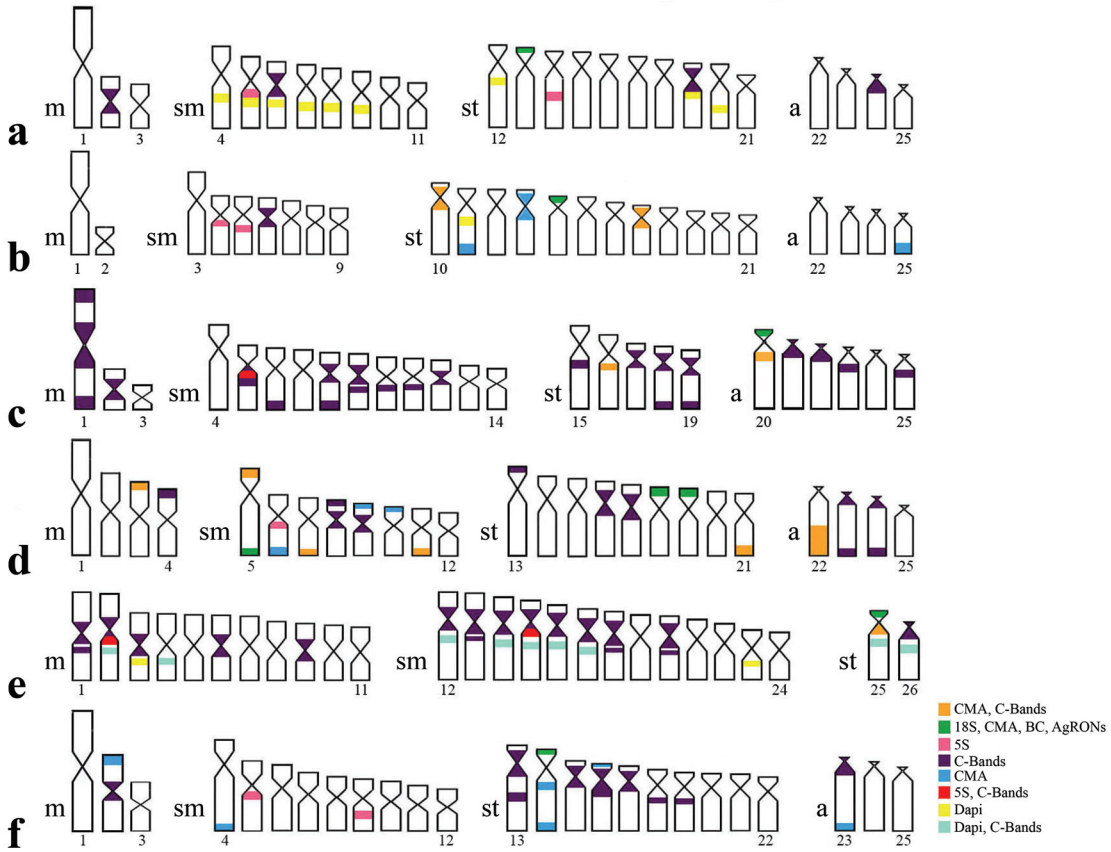

Fig. 7. Idiogram for the six studied *Astyanax* species with data obtained with different methodologies.

River (Fernandes & Martins-Santos, 2004) and high Paraná (Stream Monjolinho), only a pair of AgNORs was evidenced (Peres *et al*., 2008). However, other studies with the same complexes have already reported the occurrence of multiple AgNOR systems in populations of other regions, which have not always been confirmed by the FISH technique.

The heterochromatin presents large amounts of highly repetitive DNA sequences in eukaryotic chromosomes. In this way, as the base pair composition of the constitutive heterochromatin is quite variable between species, base-specific fluorochromes have been useful for the characterization of different heterochromatic blocks. For this reason, the use of *in situ* hybridization with different rDNA probes has been refined enabling the characterization of distinct types of heterochromatin found in chromosomes of a given species (Sumner, 2003; Vicari *et al*., 2008).

The C-banding distribution pattern in the population of *Astyanax* aff. *bimaculatus* here studied corroborates the pattern found in *A. jacuhiensis*, *A. altiparanae*, *A. fasciatus* (Fernandes & Martins-Santos, 2004; Pacheco *et al*., 2010) and *A. bimaculatus*. *A. elachylepis*, *A. argyrimarginatus*, *A. altiparanae*, *A. xavante*, *A.* aff. *bimaculatus* and *Astyanax* sp. show species-specific characteristics, with banding patterns unique to each of them.

Similar occurrences were observed in *Astyanax scabripinnis*, where the constitutive heterochromatin pattern revealed to be one of the main features for discriminating populations (Moreira-Filho & Bertollo, 1991). Another relevant fact is that *A. xavante* also revealed a pair of acrocentric chromosomes with large heterochromatic blocks, similar to that observed in *A. scabripinnis* (Souza, 1996; Mizoguchi & Martins-Santos, 1998; Mantovani *et al*., 2004 among others).

Several of these heterochromatic blocks emerge as GC-rich regions, which vary from species to species, proving to be quite useful for the analysis of the different karyotypes under study. Other GC-rich heterochromatin distribution patterns, which exhibit conspicuous blocks, especially in acrocentric chromosomes, and a diverse origin for different classes of heterochromatin were detected in *Astyanax scabripinnis* (Moreira-Filho *et al*., 1991; Souza *et al*., 2001), *A. fasciatus* (Pazza *et al*., 2008a, 2008b) and *A. janeiroensis* (Vicari *et al*., 2008).

Many of the bands produced by CMA_3 are coincident with heterochromatic blocks in *Astyanax* aff. *bimaculatus*, and also in *A. elachylepis*, *A. argyrimarginatus*, *A. altiparanae*, *A. xavante* and *Astyanax* sp., mainly the pair of chromosomes associated with the AgNORs. There is a close relationship between the nucleolus organizer regions and the

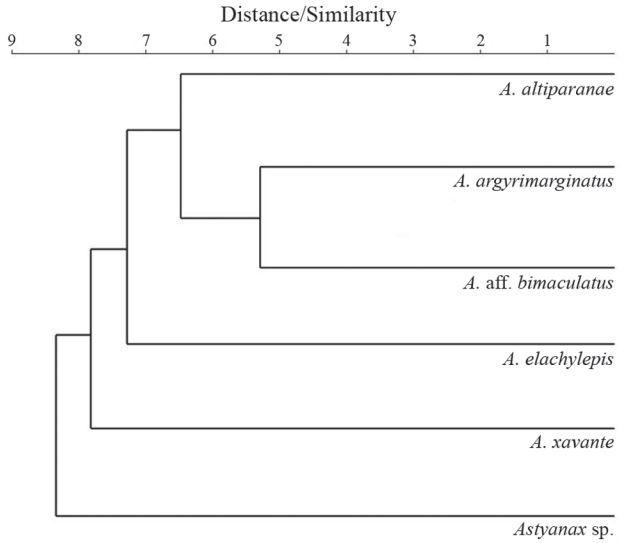

Fig. 8. Dendogram showing the karyotypic similarity obtained by cluster analysis and Euclydean distance coefficient among the six species of *Astyanax* studied.

GC-rich sites, and also with the heterochromatic blocks, although the staining with this specific GC fluorochrome cannot be considered a direct method for determining the location of ribosomal genes (Artoni *et al*., 1999). Nevertheless, this association has been relatively common among fish.

Interestingly, several heterochromatic AT^+ blocks were detected through this study after using the DAPI fluorochrome in the species *Astyanax argyrimarginatus* and *Astyanax* sp., with a localization pattern always maintained in the interstitial region of the long arm, next to the centromere of submetacentric and subtelocentric chromosomes. In the case of *Astyanax* sp., such localization patterns were found in metacentric chromosomes and less evident blocks in acrocentric chromosomes in *A. argyrimarginatus*.

Although the DAPI fluorochrome binds to both types of base pairs (GC and AT), its fluorescence is significantly enhanced by regions rich in AT base pairs and weakened in regions rich in GC base pairs (Lin *et al*., 1977). In fish, the occurrence of these bright bands is not common (Souza *et al*., 2008) and only negative bands coinciding with the GC^+ bands are sometimes noticed, as observed in the first acrocentric chromosome pair in *Astyanax xavante*.

The 5S rDNA sites have proven to be variable among species, as some of which possess only one pair of chromosome bearers of these sites (*Astyanax elachylepis* and *A. xavante*) and others have two pairs (*A. altiparanae*, *A. argyrimarginatus*, *A.* aff. *bimaculatus*, and *Astyanax* sp.) occupying different positions on the karyotypes studied. Thus, the physical localization of these sites on chromosomes also represents an important cytotaxonomic marker, especially when analyzing groups of related species (Martins & Galetti Jr., 2001). In this way Martins & Galetti Jr., (1999) analyzed the 5S rDNA of six species of *Schizodon* (Anostomidae), which proved to be highly conserved in two clusters, one larger and the other smaller in distinct chromosomes.

Based on the similarity dendrogram (Fig. 8) *Astyanax* aff. *bimaculatus* and *A. argyrimarginatus*, together with *A. altiparanae*, are a group of organisms karyotypically very similar. It is noteworthy that according to Garutti (1999), these species are associated with the *A. bimaculatus* complex. Thence, there is consistency between the karyotypic and taxonomic information.

The species *Astyanax* sp. demonstrated a lower degree of similarity compared to the other species, especially regarding the group composed of *A.* aff. *bimaculatus*, *A. argyrimarginatus* and *A. altiparanae*. *A. xavante* also showed high dissimilarity in comparison with the other species. *A. elachylepis* also appears with higher similarity with the group of species related to *A.* aff. *bimaculatus*.

The information provided in this study have proven important for the characterization of *Astyanax* species and could be further explored in a possible detailed taxonomic revision of the genus. Although the diploid numbers are conserved in the organisms studied (except for *Astyanax* sp.), the species showed significant differences either in the karyotypic macro- or microstructure, especially in the chromosomal banding pattern obtained through the different techniques, which allowed the detection of species-specific karyotypes for many of them. *Astyanax* sp. is the most distinct species and *A. xavante* holds several chromosomal characteristics (besides behavioral and morphological affinity) that make it more similar to the *A. scabripinnis* complex.

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