PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia Biologica (Kraków), vol. 62 (2014), No 3

doi:10.3409/fb62_3.193

Cytogenetic and Morphometric Analysis in the Species *Astyanax altiparanae* Garutti & Britski, 2000 (Teleostei, Characidae) from the Iguatemi River Basin, Brazil

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Accepted May 15, 2014

FERNANDES C. A., ROCHA R. H., BAILLY D., GUTERRES Z. R., ALVES D. S., MARTINS-SANTOS I. C. 2014. Cytogenetic and morphometric analysis in the species *Astyanax altiparanae* Garutti & Britski, 2000 (Teleostei, Characidae) from the Iguatemi River basin, Brazil. Folia Biologica (Kraków) **62**: 193-201.

The genus *Astyanax* is relatively common and encompasses various similar taxa forming a highly complex group that is difficult to precisely delimit. The present study aims to analyze cytogenetically and morphologically specimens of *A. altiparanae* belonging to distinct populations of the Iguatemi River Basin, Mato Grosso do Sul State, Brazil, for a better understanding of the evolutionary processes in this fish group. This study analysed 32 specimens of *Astyanax altiparanae* from Iguatemi River basin, MS, Brazil: 24 from the Água Boa stream and 8 from the Santa Maria stream. All specimens showed a diploid number equal to 50 chromosomes with differences in the karyotypic formula and types of chromosomes bearing the NOR between the two localities. The constitutive heterochromatin showed interstitial markings evident in the region of some chromosomes in both populations. In the morphometric analysis, the first three axes were retained for interpretation which together explained 81% of variance, showing morphometric distinction between populations. Chromosomal and morphometric data obtained may be useful for taxonomic and phylogenetic studies in this group of fish.

Key words: neotropical fish, chromosomes, karyotype evolution, PCA, Ag-NOR, constitutive heterochromatin.

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The Characiformes are exclusively freshwater fish distributed in America and Africa, with the greatest diversity in major neotropical watersheds (BUCKUP 1998). Within this order, the family Characidae comprises 1063 valid species and 239 species not yet described, totaling 1302 species (ESCHMEYER & FONG 2014).

Among the Characidae, the genus *Astyanax* is relatively common, comprising 155 valid species (ESCHMEYER & FONG 2014), which consists of small fish known in Brazil as "lambaris". This genus includes several morphologically very similar taxa forming a highly complex group difficult to precisely delimit (MELO 2001). With the exception of *A. latens* and *A. paris*, the species of this genus were recently included within the *Astyanax* clade supported by a single non-exclusive synapomorphy that involves the presence of one or no maxillary teeth (see MIRANDE 2010 for a detailed explanation).

Many species of the genus have been described cytogenetically, with a diploid number ranging from 36 chromosomes in *A. schubarti* (DANIEL-SILVA & ALMEIDA-TOLEDO 2001) to 50 chromosomes in *A. altiparanae* (FERNANDES & MARTINS-SANTOS 2004; DOMINGUES *et al*. 2007; MARTINEZ *et al*. 2012), *A. bockmanni* (FERNANDES *et al*. 2010; HASHIMOTO *et al*. 2011) and *A. scabripinnis*

(MOREIRA-FILHO & BERTOLLO 1991; FERNANDES & MARTINS-SANTOS 2003; MACHADO *et al*. 2012). On the other hand, *A. altiparanae* has shown a conserved diploid number of 50 chromosomes in several populations from different basins, differing in karyotype formula and in the number of chromosomes bearing nucleolar organizer regions (MORELLI *et al*. 1983; FERNANDES & MARTINS-SANTOS 2004; DOMINGUES *et al*. 2007; MARTINEZ *et al*. 2012).

In this sense, morphometric studies can constitute a complementary method for the identification of distinct forms within this complex taxon. Morphometric analysis is based on a set of measurements representing size and shape variation of the individuals by use of numerical data (PATHAK *et al*. 2013). This is a usual approach in evolutionary biology and provides interpretation and accurate comparison of the patterns of variation in quantitative traits (BLACKITH & REYMENT 1971; CAVALCANTI & LOPES 1990; CAVALCANTI & LOPES 1993). In this way, morphometric studies have been commonly integrated with cytogenetic studies conducted in some species of *Astyanax* such as *A. scabripinnis* (MOREIRA-FILHO & BERTOLLO 1991; MIZOGUCHI & MARTINS-SANTOS 1998; MAISTRO *et al*. 1998) and *A. fasciatus* (SHIBATA & ARTONI 2005; ARTONI *et al.* 2006; PAZZA *et al*. 2008). However, for *A. altiparanae* this approach has been rarely employed.

Considering this, the present study aims to analyze cytogenetically and morphologically specimens of *A. altiparanae* belonging to distinct populations of Iguatemi River Basin, Mato Grosso do Sul State, Brazil, for a better understanding of the evolutionary processes in this fish group.

Material and Methods

A total of 32 specimens of *Astyanax altiparanae* were collected in two streams, tributaries of the right bank of the Iguatemi River, Mato Grosso do Sul State, Brazil (Fig. 1). In the Água Boa stream (23°50′ 16,65″/S 54° 20′ 55,54″ W) 24 specimens were captured (10 males, 11 females and 3 with undetermined sex) and in the Santa Maria stream (23°54′50,20′S/54° 17′ 28,76″W) eight specimens were captured (2 males, 4 females and 2 with undetermined sex). All 32 specimens were subjected to karyotype analysis and 22 specimens were subjected to morphometric analysis (14 of the Água Boa stream and 8 of the Santa Maria stream).

Chromosomal analysis

The fishes were identified and deposited in the State University of Mato Grosso do Sul. The fishes were anesthetized by an overdose of clove oil before the evisceration process to obtain the chromosomes (GRIFFITHS 2000). Metaphase chromosomes were obtained from anterior kidney cells using the air-drying technique (BERTOLLO *et al.* 1978). Analysis of the C-positive heterochromatin (C-bands) followed the basic procedure of SUMNER (1972), with some minor adaptations. The nucleolar organizer regions (NORs) were detected by means of silver nitrate staining (Ag-NORs), according to HOWELL and BLACK (1980). About 30 metaphases were analyzed for each specimen and those with a better quality were employed for karyotype analysis. The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) according to their arm ratio

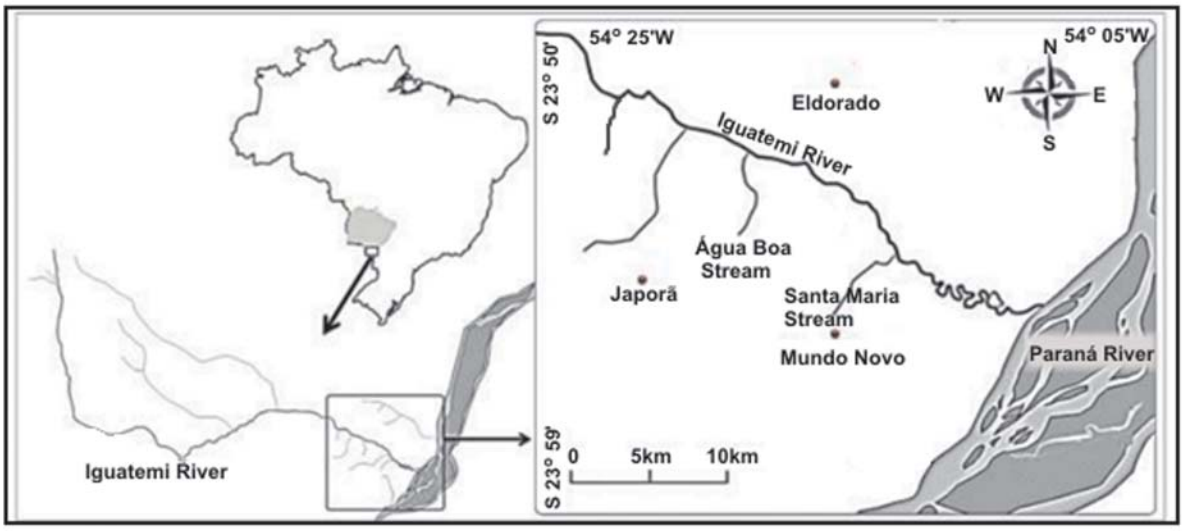

Fig. 1. Localization of the Iguatemi River basin and localization of the streams from which specimens of *A. altiparanae* were obtained.

(LEVAN *et al.* 1964). For the determination of the fundamental number (FN), or number of chromosome arms, the (m), (sm) and (st) chromosomes were considered as bearing two arms and the acrocentric chromosomes only one arm.

Morphometric analysis

To analyze the morphology of the specimens we employed the methodology proposed by STRAUSS and BOOKESTEIN (1982), called truss network, which enables the realization of measures from a combination of anatomical landmarks. The measures of the characters were obtained using digital calipers accurate to 0.05 mm. For morphometric analysis, point-to-point measurements were performed for standard length (SL), pre-dorsal length (PDL), pre-ventral length (PVL), pre-pectoral length (PPL), preanal length (PAL), body height (BH), caudal peduncle height (CPH), caudal peduncle length (CPL), dorsal fin length (DFL), ventral fin length (VFL), pectoral fin length (PFL), anal fin length (AFL), head length (HL), snout length (SNL), upper jaw length (JL), interorbital distance (ID) and orbit diameter (OD).

To assess the morphometric distinction between individuals from the Água Boa and Santa Maria streams, all the results of the morphometric measures were summarized through a Principal Component Analysis (PCA) using the software PC-ORD 4.0 (MCCUNE & MEFFORD 2007). Was adopted the axes selection criteria of Kaiser-Guttman (JACKSON 1993), i.e. axes with eigenvalues greater than 1.0 should be retained for interpretation of the ordination. To assess whether the means of the scores of the PCA axes differ between the analyzed populations, a univariate analysis of variance (ANOVA one-factor) was performed. In this analysis the scores were the dependent variable and the streams were the factors. Finally, Pearson correlations were calculated in order to check the level of association between morphometric variables and the scores of the PCA axes. All statistical analyses were carried out in the software Statistica 7.0 (STASOFT 2005). The level of significance was $P \leq 0.05$.

Results

Chromosomal analysis

The specimens of *A. altiparanae* from Água Boa and Santa Maria streams presented a diploid number of 50 chromosomes, differing only in the karyotype formula and in the fundamental number of arms. The population of Água Boa stream presented a karyotype composed of 6m+24sm+10st+10a and a fundamental number equal to 90 for both sexes (Fig. 2A). The population of Santa Maria stream pre-

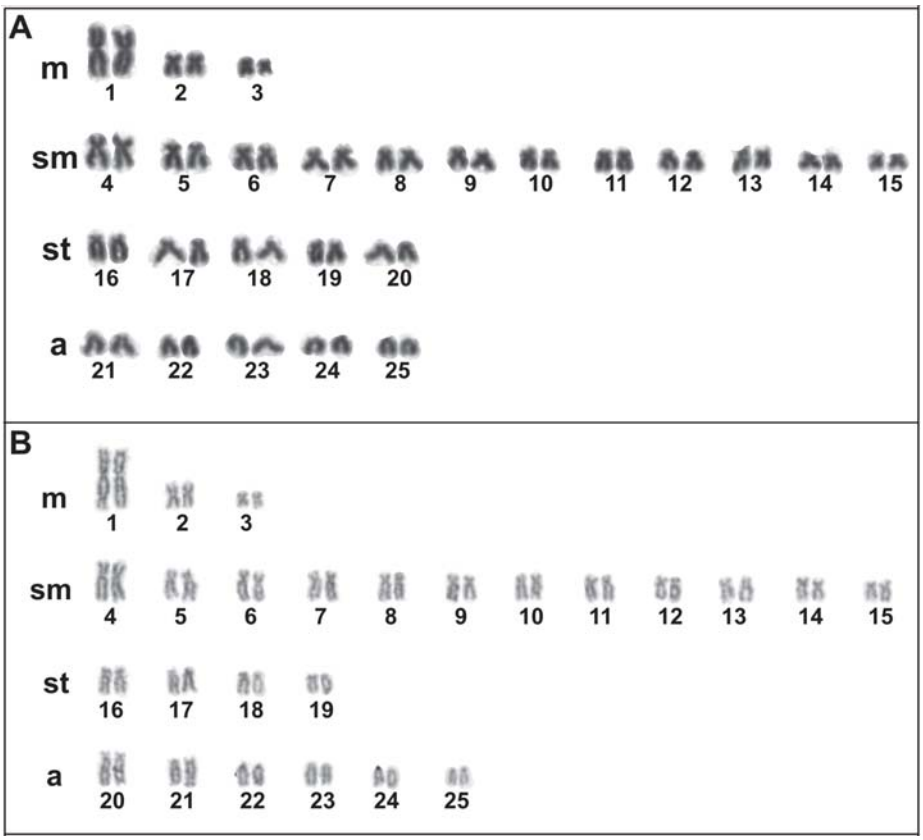

Fig. 2. Giemsa conventional karyotypes of *Astyanax altiparanae* from Água Boa (A) and Santa Maria (B) streams.

sented a karyotype composed of 6m+24sm+8st+12a and a fundamental number of 88 for both sexes (Fig. 2B).

Silver nitrate analysis showed one to four chromosomes marked for both populations. For the population of the Água Boa stream, the markings were on the short arm of pair 16 (Fig. 3A), coincident with the secondary constriction displayed in one of the homologues (Fig. 2A) and markings on the short arm of a pair of subtelocentric chromosomes were also observed (Fig. 3B). For the population of the Santa Maria stream, markings were evident on the short arm of pair 20 (Fig. 3C), coincident with the secondary constriction visualized in both homologues. The markings presented size heteromorphism between homologous chromosomes and markings on the long arm of a pair of subtelocentric chromosomes were also noted (Fig. 3D).

The two populations of *A. altiparanae* showed weak heterochromatic blocks in telomeric and centromeric regions in few chromosomes and evident interstitial markings in several chromosomes (Fig. 4). A NOR on the short arm in the chromosome of pair 20 showed a positive C-band with

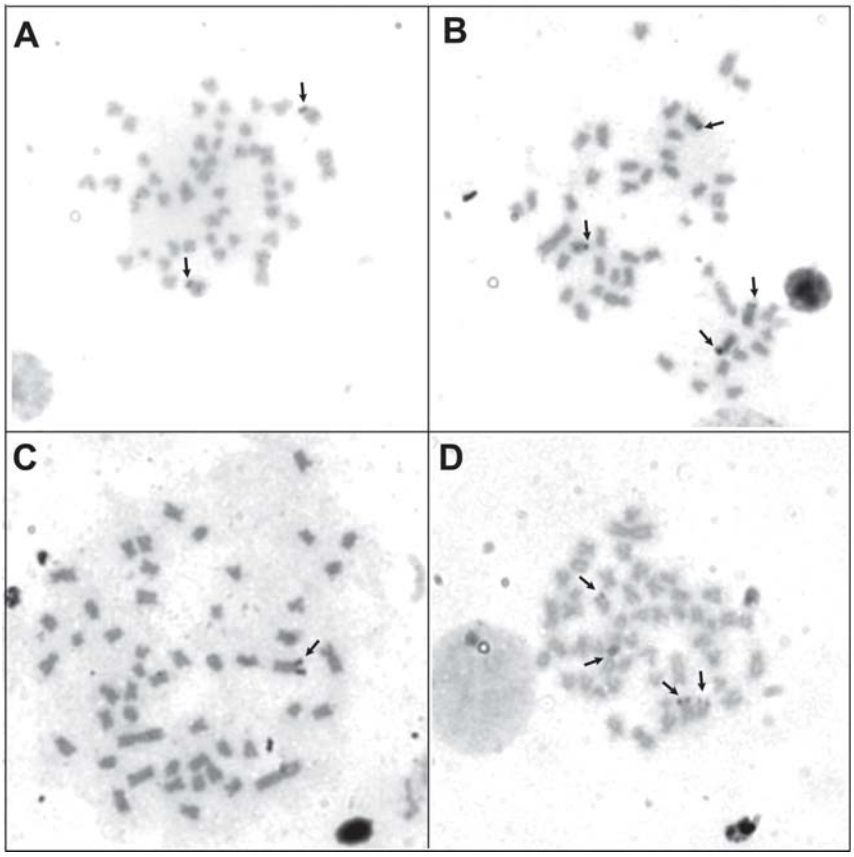

Fig. 3. Metaphase chromosome spreads showing Ag-NORs. Água Boa stream (A, B) and Santa Maria stream (C, D). Arrows indicate Ag-NORs.

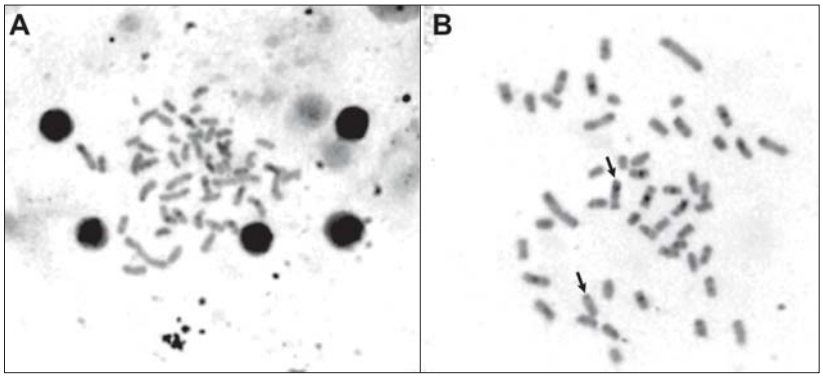

Fig. 4. C-banded metaphase of *A. altiparanae* from Água Boa stream (A) and Santa Maria Stream (B). Arrows indicate pair 20 with size heteromorphism between homologous chromosomes.

size heteromorphism between homologous chromosomes from Santa Maria stream.

Morphometric analysis

The first three axes of the PCA presented eigenvalues greater than 1.0 and were retained for ANOVA. Together they explained 81% of the variation. However, the results of the ANOVA revealed that means of axes 1 ($F = 1.14$; $p = 0.30$) and 3 ($F=0.70$; $p = 0.41$) were not significantly different between the two populations. Only the means of the scores of axis 2 showed significant differences ($F = 16.17$, $p = 0.0007$). Thus, this was the axis retained for interpretation. Therefore axes 1 and 3 were not considered (Fig. 5). The ANOVA assumptions (normality and homogeneity of the dependent variable) were met.

The CPL ($r = 0.78$) and JL ($r = 0.75$) variables correlated significantly and positively with the scores of axis 2. Higher values of these variables are present in the specimens from Água Boa stream (Table 1). The results of the ANOVA for axis 2 indicated that the populations of the two streams are different in morphometric characteristics dictated by JL and CPL (Fig. 5).

Table 1

Values of the Pearson correlations between morphometric variables and the axes of the PCA for the populations of *A. altiparanae*. Bold values are significant

Medidas	PCA 1	PCA 2*	PCA 3
SL	-0.97	-0.11	0.09
PDL	-0.98	-0.09	0.12
PVL	-0.83	-0.14	0.10
PPL	-0.88	0.02	0.01
PAL	-0.81	0.03	-0.23
BH	-0.96	-0.09	0.11
CPH	-0.80	-0.12	0.41
CPL	-0.40	**0.78**	-0.33
DFL	-0.85	-0.16	-0.22
VFL	-0.77	-0.03	-0.22
PFL	-0.90	-0.01	-0.22
AFL	-0.83	-0.24	-0.36
HL	-0.87	-0.18	0.30
SNL	-0.45	0.40	0.68
JL	-0.59	**0.75**	0.02
ID	-0.95	0.08	-0.06
OD	-0.85	-0.05	-0.09

*Axis that presented means of the scores significantly differed between the two populations.

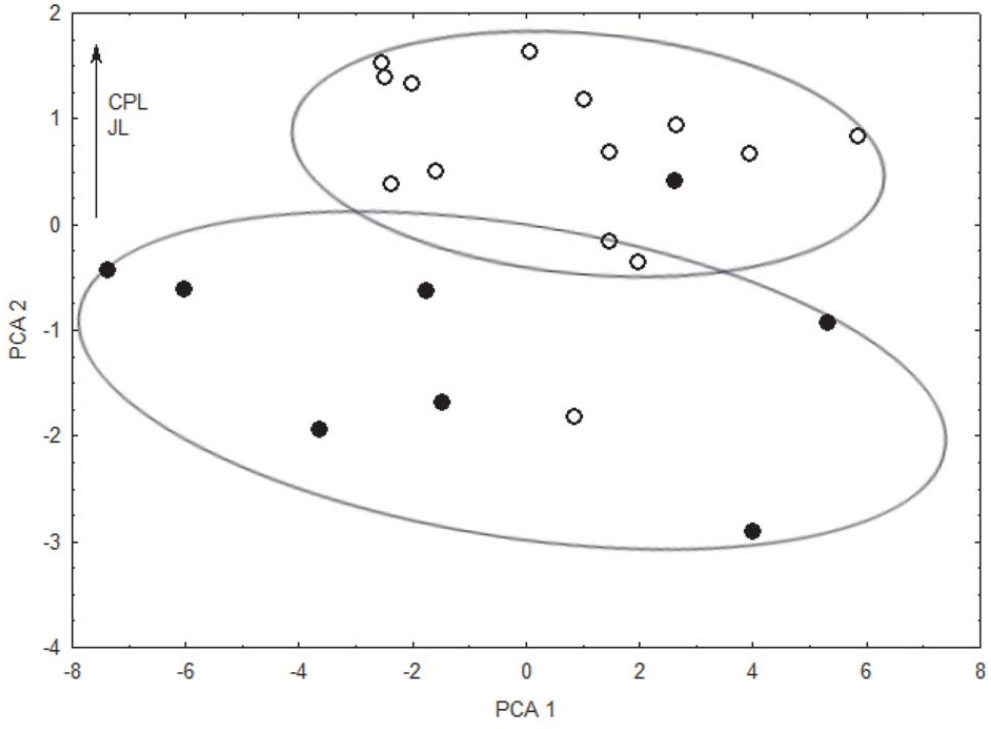

Fig. 5. Dispersion of the scores of the first two axes of the PCA. Empty circles represent individuals from the Água Boa stream population and solid circles represent individuals from the Santa Maria stream population. JL = upper jaw length and CPL = caudal peduncle length.

Discussion

Karyotypic comparisons in *Astyanax altiparanae*

The karyotypic data obtained from both populations of *Astyanax altiparanae* in this study showed the same diploid number, but with different karyotypic formulas, mostly varying in the number of acrocentric chromosomes. Table 2 shows the results of the 26 karyotype analyses obtained from of *A. altiparanae* by different authors for different localizations. Of these, 100% showed a diploid number of 2n=50 chromosomes, but with different karyotype formulae, thus evidencing conservatism with regard to diploid number. The FN of the analyzed populations ranged between 86 for the Claro River, and 100 for the population from Três Bocas stream. For the latter population, the karyotype formula differed from the others due to the absence of acrocentric and submetacentric chromosomes (Table 2). The interpopulational variation in the karyotype described here, as in previous studies, may partially be explained due to biological features of *A. altiparanae*. This species is able to reproduce at sites with distinct environmental characteristics, favoring successful colonization of new habitats, and providing a wide geographical distribution of the species from the upper Paraná River basin. These populations are not morphologically homogeneous (GARUTTI & BRITSKI 2000). Thus, biological features could facilitate the maintenance of chromosomal rearrangements, especially pericentric inversions that alter the karyotype formula without changing the diploid number.

Table 2

Cytogenetic data in *Astyanax altiparanae*

Location	Basin	2n	FN	m	sm	st	a	Bs	References
Mogi Guaçu	A	50	88	10	24	4	12	0	MORELLI *et al.* 1983
Tibagi River	A	50	92	6	28	8	8	0	DOMINGUES *et al*. 2007
Iguaçu River	A	50	94	6	30	8	6	0	DOMINGUES *et al*. 2007
Campo Novo River	A	50	92	12	18	12	8	1	HASHIMOTO *et al*. 2008
Tręs Bocas Stream	A	50	100	40	0	10	0	0	TAKAHASHI *et al*. 1995
Tręs Bocas Stream	A	50	100	28	0	22	0	0	TAKAHASHI *et al*. 1995
Tręs Bocas Stream	A	50	100	34	0	16	0	0	TAKAHASHI *et al*. 1995
Paranapanema River	A	50	88	10	22	6	12	0	DANIEL-SILVA & ALMEIDA-TOLEDO 2001
Mogui-Guaçu River	A	50	88	6	12	20	12	0	MARTINEZ *et al*. 2012
Tietę River	A	50	88	6	12	20	12	0	MARTINEZ *et al*. 2012
Batalha River	A	50	92	10	16	16	8	0	HASHIMOTO *et al*. 2011
Keçaba Stream	A	50	88	6	26	6	12	0	FERNANDES & MARTINS-SANTOS 2006
Paraná River	A	50	88	6	26	6	12	0	FERNANDES & MARTINS-SANTOS 2006
Índios River	A	50	90	6	30	4	10	0	FERNANDES & MARTINS-SANTOS 2004
Paraná River	A	50	88	6	26	6	12	0	FERNANDES & MARTINS-SANTOS 2004
Tatupeba Stream	A	50	88	6	26	6	12	0	FERNANDES & MARTINS-SANTOS 2006
Maringá Stream	A	50	88	6	26	6	12	0	FERNANDES & MARTINS-SANTOS 2006
Claro River	A	50	90	10	26	4	10	0	PACHECO *et al*. 2001
Claro River	A	50	88	10	24	4	12	0	PACHECO *et al*. 2001
Claro River	A	50	86	10	22	4	14	0	PACHECO *et al*. 2001
Paraná River	A	50	88	10	22	6	12	0	DANIEL-SILVA & ALMEIDA-TOLEDO 2005
Monjolinho Stream	A	50	94	12	18	20	6	0	TENÓRIO *et al*. 2013
Tietę River	A	50	88	8	20	10	12	0	KAVALCO *et al*. 2011
Paranapanema River	A	50	86	8	22	6	14	0	KAVALCO *et al*. 2011
Água Boa Stream	A	50	90	6	24	10	10	0	present study
Santa Maria Stream	A	50	88	6	24	8	12	0	present study

A: Paraná Basin; FN: fundamental number; m: metacentric; sm: submetacentric; st: subtelocentric; a: acrocentric; Bs: Presence of extra chromosomes.

The silver nitrate impregnation revealed that both populations of *A. altiparanae* have a multiple NOR system with markings in terminal regions of the chromosomes, frequently observed for this species in different watersheds (FERNANDES & MARTINS-SANTOS 2004; PACHECO *et al.* 2011).

The variation in the sizes of NORs observed between homologous chromosomes of pair 20 from the Santa Maria population is a relatively common feature among some groups of fish, including *A. altiparanae* (FERNANDES & MARTINS-SANTOS 2004; PACHECO *et al.* 2011). Two hypotheses can be used to explain this size heteromorphism. This first hypothesis is that greater gene activity in one of the homologous chromosomes would result in a large amount of stained proteins in a chromosome, since only proteins are stained with silver nitrate. On the other hand, it can be suggested that transposition events, duplication and/or deletion or unequal crossing-over in the region (FERNANDES & MARTINS-SANTOS 2004) may have increased the number of rDNA genes in only one of the homologues responsible for the size heteromorphism of NOR. The latter has been the most commonly accepted hypothesis, whereas in this study the NOR of pair 20 was C-band positive with size heteromorphism, indicating that the amount of constitutive heterochromatin in this region (DNA spacer in 45S rDNA) is higher in one of the homologous chromosomes.

Regarding the constitutive heterochromatin, a greater number of heterochromatic blocks conspicuous in interstitial regions was observed in the two populations of *A. altiparanae*. The default location of the interstitial heterochromatin appears to be a characteristic of this species since this pattern was noted in different populations of *A. altiparanae* (FERNANDES & MARTINS-SANTOS 2004; NETO *et al*. 2009).

Morphometric comparisons in *Astyanax altiparanae*

The results of morphometric analysis revealed that the individuals of *A. altiparanae* of the Água Boa and Santa Maria streams presented significant differences in the caudal peduncle length (CPL) and jaw length (JL), i.e, two distinct groups are discerned by these variables, with a very low overlap if axis 2 is considered. Differently, DOMINGUES *et al.* (2007), analyzed *A. altiparanae* populations belonging to the Tibagi and Iguaçu River basins and found a great overlap of individual scores of ordination axes, indicating that the two populations did not exhibit relevant morphological differences.

Cytogenetic studies associated with morphometric analysis have also been described for other species of the genus. ARTONI *et al*. (2006) evaluated four distinct populations of *A. fasciatus* in the upper Tibagi River basin and reported two groups distinguishable in the first axis: the Furna 2 population was discriminated from the others (Tibagi River, Dourada lagoon and Cará-cará River) because it presented the greatest head length and eye diameter, while the caudal peduncle depth, body depth and pre-dorsal distance were larger than the other three studied populations. Similarly, PAZZA *et al*. (2008) observed that two populations of *A. fasciatus* of the Paraná River basin presented both karyotypic and morphological differences. Already SHIBATA & ARTONI (2005) examined four populations of *A. fasciatus* from Tibagi River basin and reported that minor morphometric differences separated the Furna 2 and Lagoa Dourada populations from the others (Furna 1 and Tibagi River).

MIZOGUCHI and MARTINS-SANTOS (1998) used canonical variable analysis to assess the morphometric data of *A. scabripinnis* and revealed that the four populations under analysis were entirely discriminated from each and also reported that standard length, rostrodorsal distance, eye diameter and snout length contributed the most to this pattern. MOREIRA-FILHO & BERTOLLO (1991) discriminated four of seven studied *A. scabripinnis* populations and MAISTRO *et al*. (1998) discriminated five of nine *A. scabripinnis paranae* populations studied by canonical variable analysis. The populations that could not be discriminated by morphological analysis in these two studies were differentiated on the basis of cytogenetic data.

The populations examined in the present study revealed both cytogenetic and morphometric differentiation. Thus, it is possible to conclude that a combination of morphological and chromosomal studies is a valuable approach to show that in some natural populations (as in the case of the present study) karyotypic diversity can be accompanied by tenuous phenotypic alterations.

Acknowledgements

The authors thank the Brazilian agency Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT) for financial support. Work by D. BAILLY is supported by CAPES post doctoral fellowship.

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