



## Cytogenetic and morphological diversity in populations of *Astyanax fasciatus* (Teleostei, Characidae) from Brazilian northeastern river basins

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### Abstract

In the present work, morphometric and cytogenetic analyses were carried out in populations of the fish *Astyanax fasciatus* (Characidae) from Contas and Recôncavo Sul River basins (State of Bahia, Brazil), providing new data on the genetic structure of this species along the region. Based on morphologic measurements, we observed that populations from the same hydrographic basin were more similar to each other (Contas and Preto do Costa Rivers), and remarkably divergent from Recôncavo Sul (Mineiro Stream), as indicated by clustering analysis. Cytogenetic data revealed a same diploid number for all populations ( $2n = 48$ ), but distinct karyotype formulae ( $8M+24SM+12ST+4A$ ,  $FN = 92$  in Contas River,  $8M+24SM+10ST+6A$ ,  $FN = 90$  in Preto do Costa River, and  $8M+18SM+16ST+6A$ ,  $FN = 90$  in Mineiro Stream). Ag-NORs were identified at telomeres on a subtelocentric chromosome pair, although multiple ribosomal sites have been detected in some specimens from Contas River. These results show that *A. fasciatus* populations from northeastern river basins are well differentiated and present peculiar cytogenetic features when compared to populations from other regions. Therefore, the apparent chromosomal plasticity of this species, likely to represent a complex of cryptic forms, is corroborated. Finally, we demonstrated that morphological features can be successfully used to support other sources of genetic information.

*Key words:* canonic variables, karyotype, morphometry, NOR.

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### Introduction

The small characin fish *Astyanax fasciatus* (Characidae, *Insertae sedis*) was formerly described by Cuvier (1819) as a typical species from “Brazilian rivers”. Ever since, systematic studies have highlighted that *Astyanax* representatives compose distinct species complexes of controversial taxonomy (Reis *et al.*, 2003). So far, the genus comprises 92 valid species and recent descriptions are about to increase this number (*e.g.* Bertaco and Lucena, 2006; Melo and Buckup, 2006).

Cytogenetic studies in South American freshwater fishes have been extremely useful to reveal inter- and intra-specific genetic differences, bringing out relevant data on cryptic species and the biodiversity evolution in the neotropical region (Almeida-Toledo *et al.*, 2000).

The chromosomal data available on distinct Brazilian populations of *Astyanax fasciatus* have shown a remarkable diversification of cytotypes and the occurrence of specific variants. The predominant diploid numbers of this

species are  $2n = 46$  and  $2n = 48$ , but they can range from 45 to 48 and present quite differentiated chromosome formulae (Morelli *et al.*, 1983; Centofante *et al.*, 2003; Pazza *et al.*, 2006, among others). These results, supported by morphological studies (Melo and Buckup, 2006), suggest that the species currently referred as *Astyanax fasciatus* should be divided into other nominal evolutionary units.

It must be pointed out that most genetic reports on neotropical fish in Brazil are restricted to the main river basins from southern and southeastern regions, whereas minor and isolated river basins are still underrepresented. Thus, essential knowledge on patterns of genetic diversity of several species and populations and their relationship to the evolutionary history or environmental peculiarities of each basin remain unknown.

The northeast of Brazil comprises a small-scale hydrographic system when compared to other Brazilian regions. This condition is mainly related to a dominant semi-arid climate over regional rivers, with the exception of some coastal basins located south Bahia and at Maranhão Gulf on the northwestern boundary. Although some rivers, such as Contas and Paraguassu (State of Bahia) are permanent, due to both soil features and location of headwaters at

Diamantina Plateau, they present intermittent tributaries (Rosa *et al.*, 2003).

In order to increase the information on the genetic diversity of *A. fasciatus* along the northeastern region of Brazil, morphometric and cytogenetic analyses were performed in three populations from distinct hydrographic basins in the State of Bahia. These are the first data on genetic structure of this species in the region.

## Material and Methods

### Sampling sites

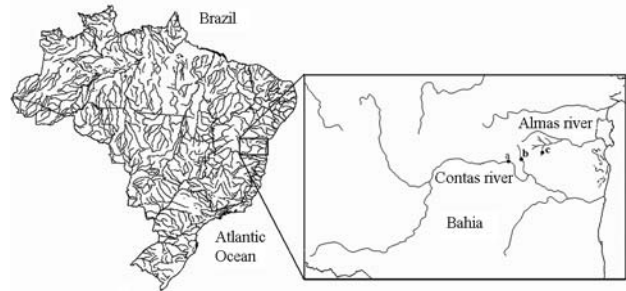
Specimens of *A. fasciatus* were collected by using set gill and drift nets at three collection sites: the main channel of Contas River, Preto do Costa River, and Mineiro Stream (Figure 1). The former is the main river of Contas hydrographic basin and represents one of the most important water systems in the State of Bahia. Its headwaters are located at Diamantina Plateau (about 1,500 m above sea level) and the river flows over 620 km before reaching the Atlantic Ocean. Samples from Contas River were obtained at its middle part, in the city of Jequié (13° 51' 51" S/40° 04' 54" W, 216 m high). Preto do Costa River (13° 45' 84" S/39° 56' 47" W, 423 m above sea level) is located at the transitional Middle Contas sub-basin, and it is a left margin tributary of Contas River, close to the first collection site (28 km apart). Mineiro Stream (13° 60' 54" S/39° 41' 54" W, 285 m above sea level) belongs to Recôncavo Sul basin, and its sampling site was situated in the city of Itamari, Mineiro county, located 63 km from Jequié.

The fish samples were transported to the laboratory and kept in separated tanks prior morphometric and cytogenetic analyses. Each specimen was identified and deposited in the fish collection from Universidade Estadual do Sudoeste da Bahia (BA, Brazil).

### Morphometric studies

Measurements were taken from 30 individuals per population, comprising 15 morphometric characters. Nine of them were based on Lagler *et al.* (1977): standard length (SL), head length (HL), body height (BH), caudal peduncle height (CPH), interorbital width (IOW), ocular diameter (OD), preanal length (PL), predorsal length (PdL), and head height (HH). The other characters were the distances between dorsal and pectoral fins (DPD), pectoral and pelvic fins (PPD), pelvic and anal fins (PAD), dorsal and anal fins (DAnD), anal and adipose fins (AAD), and dorsal and adipose fins (DADd), taking the base of the first fin ray as a reference (or the anterior insertion, in the case of adipose fins).

The evaluation of inter-population genetic diversity was accomplished through canonic variables. Grouping analyses were performed using the generalized Mahalanobis  $D^2$  distance as a dissimilarity measurement and, subsequently, the Tocher optimization method was applied



**Figure 1** - Hydrographic map of Brazil showing in detail the main rivers for the Contas and Recôncavo Sul basins (State of Bahia) and collection sites of this study: (a) Contas River, (b) Preto do Costa River, and (c) Mineiro Stream.

(Sneath and Sokal, 1973). Statistical analyses were performed with SAS (1997). In addition, data matrices were used in a size-free canonical discriminant analysis - CDA (Reis *et al.*, 1990). This analysis consists of removing the effects of within-group size variation by regression.

The following statistical model was used:

$$y_{itr} = \mu_t + P_{it} + e_{itr}$$

where  $t = 1, 2, \dots, 15$ ;  $i = 1, 2, \text{ and } 3$ ;  $r = 1, 2, \dots, 30$ .  $y_{itr}$  = value observed in character  $t$ , at repetition  $r$ , in population  $i$ ;  $\mu_t$  = mean  $t$  character value;  $P_{it}$  = effect of the population  $i$  on character  $t$ ;  $e_{itr}$  = effect of random error associated with the observation  $y_{itr}$ .

### Cytogenetic studies

Thirty-five individuals (one male and 34 females) of *A. fasciatus* from Contas River, 37 animals (six males and 31 females) from Preto do Costa River, and 25 individuals (10 males and 15 females) from Mineiro Stream were cytogenetically analyzed.

Mitotic stimulation was performed on the collected specimens 72 h prior the cytogenetic preparation, as described by Molina (2001). Metaphase chromosomes were obtained from kidney cells according to Bertollo *et al.* (1978). The slides were stained with Giemsa at 5% for conventional analyses. Nucleolar organizer regions were detected by silver nitrate staining (Ag-NOR) (Howell and Black, 1980). The best metaphases were photographed in Imagemlink Kodak ISO 25 for chromosome measurements, karyotyping, and NOR analysis. The chromosomal pairs were arranged in decreasing size order and classified as metacentric (M), submetacentric (SM), subtelocentric (ST), and acrocentric (A) (Levan *et al.*, 1964).

## Results

### Morphometric analyses

The results of canonic variables, eigenvalues, and percent variance for each analyzed character are shown in Table 1. The first two canonic variables were able to ex-

plain 100% of the total variation existing in the original data, distributed into 61.9% for the first variable and 38.1% for the second one.

The less representative features in the genetic diversity of the studied populations were BH, AAD, PdL, and IOW, which displayed the highest coefficients in the canonic variable of lowest importance (CV2, Table 2). Less important characters are either invariant or redundant, *i.e.*, they are represented by other highly correlated characters (individually or grouped) (Cruz and Carneiro, 2003).

Clustering analysis among populations was based on the dissimilarity values shown in Table 3. These numbers represent the estimated Mahalanobis  $D^2$  distances between each pair of populations. Through Tocher optimization method, populations were clustered into two groups: one composed of populations from the Contas River basin (Pre-

to do Costa and Contas Rivers) and the other comprising the population from the Recôncavo Sul basin (Mineiro Stream) (Figure 2).

### Cytogenetic analyses

The three *A. fasciatus* populations presented a modal diploid number of 48 chromosomes for both males and females. However, structural differences were observed when comparing the karyotypes from each locality. Specimens from Contas River presented a karyotype formula composed of 8M+24SM+12ST+4A (FN = 92), while individuals from Preto do Costa River and Mineiro Stream presented, respectively, 8M+24SM+10ST+6A (FN = 90) and 8M+18SM+16ST+6A (FN = 90) (Figure 3).

Active NOR sites were located at the terminal region of the long arms of a large pair of ST chromosomes in all samples (Figure 3, inbox). Additionally, some individuals from Contas River also showed Ag-NOR marks on the short arms of a small pair of SM chromosomes and, sporadically, of a M chromosome (Figure 3a).

**Table 1** - Canonical variables (CV<sub>i</sub>), eigenvalues ( $\lambda_i$ ), and percent variance (% $\sigma^2$ ) obtained from analysis of 15 morphometric measurements in three *Astyanax fasciatus* populations from Contas River (Contas and Preto do Costa Rivers) and Recôncavo Sul basins (Mineiro Stream), State of Bahia, Brazil.

CV <sub>i</sub>	$\lambda_i$	% $\sigma^2$	accumulated % $\sigma^2$
CV1	2.2273	61.9	61.9
CV2	1.3661	38.1	100.00

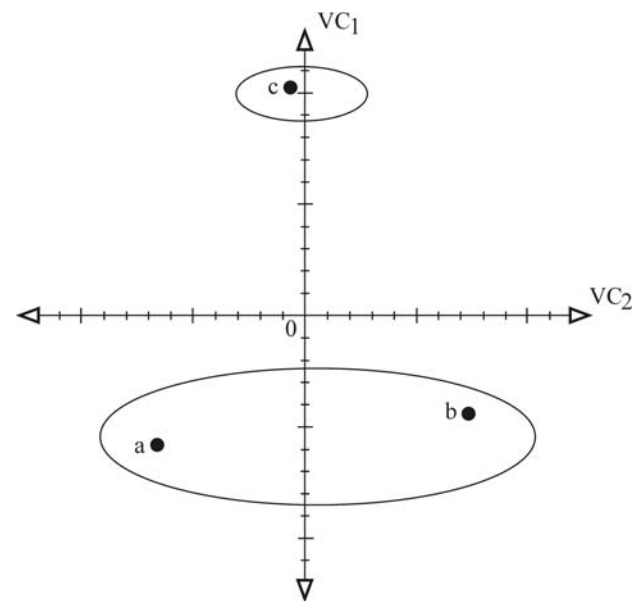
**Table 2** - Analysis of canonic variables (CV) based on morphometric data of *Astyanax fasciatus* populations from two collection sites in the Contas River basin (Contas and Preto do Costa Rivers) and one site in the Recôncavo Sul basin (Mineiro Stream), State of Bahia, Brazil. For description of measurements see Material and Methods.

Measurement	CV1	CV2
SL	-0.573	0.700
HL	1.588	0.225
BH	1.081	-1.833*
CPH	0.078	-0.142
IOW	0.445	0.941*
OD	-0.130	0.072
PL	-1.080	-0.453
HH	-0.217	0.341
PdL	0.286	1.058*
DPD	-0.185	0.174
PPD	-0.411	0.290
PAD	0.277	-0.064
DAnD	-0.137	0.304
AAD	-0.661	-1.328*
DAdD	-0.633	-0.090
Accumulated variation	0.619	0.381

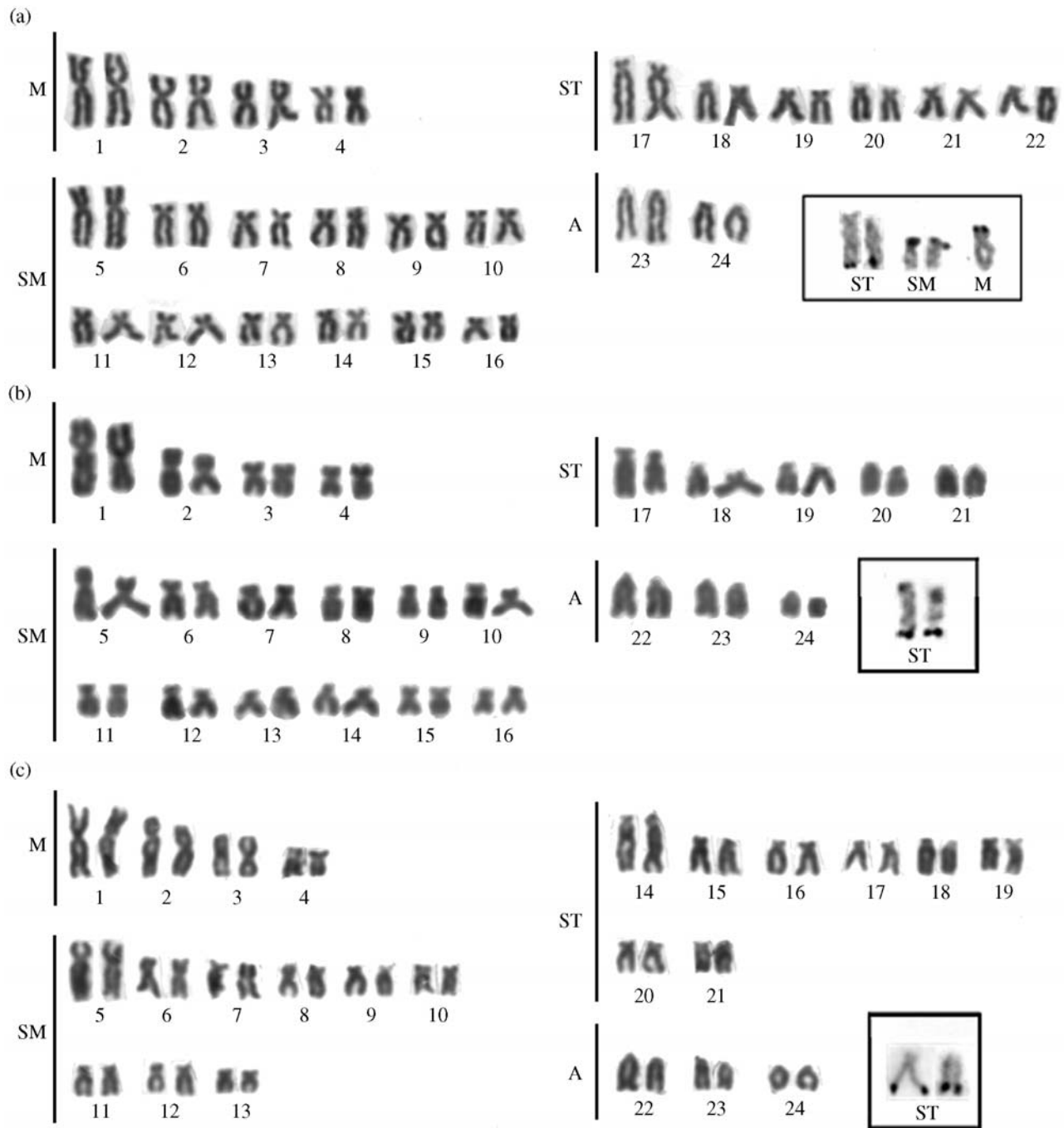
\*Less important characteristics on the inter-population differentiation.

**Table 3** - Mean interpopulation distances (Mahalanobis distance estimates based on morphometric data) of *Astyanax fasciatus* in two sites of the Contas River basin (Contas and Preto do Costa Rivers) and one site of the Recôncavo Sul basin (Mineiro Stream), State of Bahia, Brazil.

Site	Preto do Costa River	Contas River	Mineiro Stream
Preto do Costa River	0		
Contas River	7.95	0	
Mineiro Stream	11.29	12.00	0



**Figure 2** - Graphic dispersal and clustering by Tocher optimization method of three *Astyanax fasciatus* populations in relation to the first two canonic variables: (a) Contas River, (b) Preto do Costa River, and (c) Mineiro Stream.



**Figure 3** - Giemsa-stained karyotypes and NOR-bearing chromosomes after silver nitrate staining (inbox) of *Astyanax fasciatus* from Contas River (a), Preto do Costa River (b), and Mineiro Stream (c).

## Discussion

### Morphometric data

The complex patterns of morphometric variation in animals require the use of multivariate statistical analyses (Cavalcanti and Lopes, 1993). In fish, this approach is facilitated since the characters usually present a well-defined hydrodynamic conformation, turning them into a suitable

material for morphometric studies (Cavalcanti and Lopes, 1991).

According to Cruz and Carneiro (2003), such studies must include a number of variables to explain, at least, 80% of the observed variation. In the present work, the first two canonic variables were sufficient to evaluate the morphometric differentiation among populations, since, together, they explained 100% of the original variation.

Clustering analyses are often used to divide an original set of observations into distinct groups, based on their similarities or dissimilarities (Cruz and Regazzi, 2001). Analyzing the dissimilarity values and grouping results, we observed that *A. fasciatus* populations from the Contas River basin (Contas and Preto do Costa Rivers) display a clear morphometric differentiation from the population of the Recôncavo Sul basin (Mineiro Stream). Populations within the Contas River basin were more similar, reflecting their lower degree of isolation. In contrast, the separation between hydrographic basins was evident, placing the population from Recôncavo Sul apart from the others (Figure 2).

### Cytogenetic data

Cytogenetic studies in populations of *A. fasciatus* have revealed a remarkable numerical and structural karyotype variability, as well as the coexistence of different cytotypes in a single river basin (Centofante *et al.*, 2003; Pazza *et al.*, 2006, among others). The data from the present work increase the wide karyotype diversity of *A. fasciatus*, since the cytotypes herein described differ from those previously reported for other hydrographic river basins (Table 4). These results reinforce the apparent chromosomal plasticity of this species, as suggested by Pazza *et al.* (2006).

Moreover, the karyotype pattern observed in the present work permitted to identify a structural interpopulation variability in *A. fasciatus*. Despite the maintenance of the diploid number ( $2n = 48$ ), specific chromosome formulae were established for each locality. The differences found among populations (and basins) might have been caused by gene flow constraints related to the presence of natural obstacles and/or historical events. In the case of the population from Mineiro Stream, the present results can be correlated to its location in a distinct hydrographic basin, following a typical isolation model.

Although belonging to the same river basin, the genetic separation of the individuals from Contas and Preto do Costa Rivers could be influenced by environmental factors, since the latter presents several small waterfalls, likely able to restrain the dispersal of individuals from one site to another. Reports of karyotype diversity among local neotropical fish populations associated with environmental peculiarities are quite common, particularly within *Astyanax* species (Moreira-Filho and Bertollo, 1991; Almeida-Toledo *et al.*, 2000). Nevertheless, a higher genetic similarity is expected when comparing populations from the same hydrographic basin. Indeed, a comparison of karyotype macrostructure among the three studied populations presupposes a higher number of chromosomal rearrangements in the differentiation of *A. fasciatus* from Mineiro Stream (Recôncavo Sul basin) than between populations from the Contas River basin.

Analysis of nucleolar organizer regions has also indicated chromosomal differences between *A. fasciatus* from

**Table 4** - Cytogenetic data on populations of *Astyanax fasciatus* from Brazil.

Collection site, state	2n	Karyotypic formula	Reference*
Contas River, BA	48	8M+24SM+12ST+4A	1
Preto do Costa River, BA	48	8M+24SM+10ST+6A	1
Mineiro Stream, BA	48	8M+18SM+16ST+6A	1
Mogi-Guaçu River, SP	48	8M+22SM+12ST+6A	2
Mogi-Guaçu River, SP	48	12M+20SM+8ST+8A	3
Mogi-Guaçu River, SP	47	12M+19SM+10ST+6A	2
Mogi-Guaçu River, SP	47	12M+20SM+10ST+5A	2
Mogi-Guaçu River, SP	47	12M+21SM+10ST+4A	2
Mogi-Guaçu River, SP	47	13M+20SM+8ST+6A	3
Mogi-Guaçu River, SP	46	14M+20SM+10ST+6A	4
Mogi-Guaçu River, SP	46	13M+20SM+8ST+5A	3
Mogi-Guaçu River, SP	46	12M+20SM+10ST+4A	2
Mogi-Guaçu River, SP	46	12M+20SM+8ST+6A	3
Mogi-Guaçu River, SP	45	12M+20SM+10ST+3A	2
Mogi-Guaçu River, SP	45	13M+19SM+10ST+3A	5
Piracicaba River, SP	46	12M+20SM+10ST+4A	6
Passa-Cinco River, SP	46	14M+18SM+10ST+4A	3
Jacaré-Guaçu River, SP	46	12M+20SM+8ST+6A	3
Barra-Funda Stream, SP	46	12M+20SM+8ST+6A	3
Paiol Grande Creek, SP	48	12M+20SM+12ST+4A	7
Sapucaí River, MG	48	8M+18SM+14ST+8A	8
Juquiá River, SP	48	10M+24SM+12ST+2A	4
Paraibuna River, SP	48	8M+18SM+12ST+10A	6
Porta Stream, MG	48	8M+10SM+24ST+6A	6
Porta Stream, MG	48	10M+14SM+14ST+10A	3
São Francisco River, MG	46	6M+24SM+8ST+8A	6
São Francisco River, MG	46	8M+22SM+8ST+8A	3

\*1 - Present work, 2 - Pazza *et al.* (2006), 3 - Heras (1998), 4 - Morelli *et al.* (1983), 5 - Daniel-Silva and Almeida-Toledo (2001), 6 - Justi (1993), 7 - Centofante *et al.* (2003), 8 - Swerts *et al.* (1998).

northeastern regions and other basins. Active NORs have frequently been detected on the short arms of SM chromosomes and, occasionally, on the telomeres of ST-A chromosomes in most southeastern populations of *A. fasciatus* (Pazza *et al.*, 2006). On the other hand, the populations analyzed in the present study usually presented NORs at the terminal regions of the long arms of a submetacentric pair, suggesting that the three populations share a common origin. The reason(s) for the fixation of a differentiated active NOR pattern in *A. fasciatus* populations from the northeast of Brazil remain(s) unclear, but a putative adaptive role might be inferred.

Multiple NORs have been reported in this species, ranging from one to ten NOR-bearing chromosomes (Pazza, 2005). However, in the present work, most silver-stained metaphases revealed single NORs, excepting some individuals from Contas River that presented up to five

marked chromosomes. Further studies, involving base-specific fluorochrome staining and *in situ* hybridization with ribosomal probes should be carried out in order to verify whether this condition extends to the other populations or characterizes a population marker.

Apart from evidence of inter-population divergence, the cytogenetic analyses in *A. fasciatus* revealed some similarities in features amongst karyotypes from distinct localities. For instance, the presence of, at least, four metacentric chromosome pairs and a remarkably larger first pair is common to all  $2n = 48$  populations studied so far. The presence of unchanged and homeologous chromosome pairs within dynamic karyotypes was previously reported in *Astyanax* species (Daniel-Silva and Almeida-Toledo, 2001). Such conserved chromosomes can be potentially used for phylogenetic inferences within and between related groups, helping elucidate inter- and intra-specific relationships.

### Final Remarks

Based on morphometric and cytogenetic analyses, the present data present the first information about genetic diversity in the characin *A. fasciatus* from typical northeastern river basins. Both morphological and chromosomal studies were congruent. Similarly, RAPD-PCR, meristic, and morphometric analyses in five samples of *Astyanax aff. bimaculatus* separated by waterfalls and dams along Doce River basin have shown a concordance between molecular and morphological data (Paiva *et al.*, 2006). Microsatellite, allozymes, mtDNA, and morphometric studies in sympatric populations of the catfish *Clarias gariepinus* and *C. anguillaris* have all confirmed the presence of distinct species (Agnès *et al.*, 1997). These data reveal that, in the lack of more expensive and/or advanced technologies, morphological features can be successfully used to support other sources of genetic information.

Besides interpopulation differences, we also describe new cytotypes of *A. fasciatus*, corroborating the extensive karyotype variability in this species. Despite a probable misclassification of chromosomal morphology that might have led to an increased karyotype variation amongst populations studied by distinct authors, it is clear that *A. fasciatus* presents an accentuated plasticity. It is also demonstrated that even within small and closely related hydrographic basins, distinguishable genetic features can be assigned to fish populations. It must be pointed out that Mineiro Stream is located in a typical Atlantic rain forest region, whereas Preto do Costa River is situated in a transition between semi-arid and Atlantic rain forest, and the studied portion of Contas River is in a semi-arid region. Possibly, such environmental differences might characterize distinct selective pressures acting on each population, which would play a major role in phenotypic aspects. As a matter of fact, morphometric data revealed striking differences among populations when compared to cytogenetic results. However, further genetic studies involving refined

cytogenetic and/or molecular techniques along these river basins may eventually demonstrate a higher differentiation among populations.

Our results, taken together with previous reports on both cytogenetic and morphological analyses (*e.g.*, Centofante *et al.*, 2003), indicate the existence of well-differentiated populations of *A. fasciatus* along Brazilian hydrographic systems. The level of divergence between them suggests the occurrence of a species complex, widespread throughout distinct river basins. A similar situation has been described in several neotropical fishes, particularly within *Astyanax* (Moreira-Filho and Bertollo, 1991; Melo and Buckup, 2006), showing that more detailed taxonomical reviews should be carried out in order to provide a reliable scenario about the diversity of the Brazilian ichthyofauna.

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