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Research Article

Restriction fragment length polymorphisms of mitochondrial DNA among five freshwater fish species of the genus *Astyanax* (Pisces, Characidae)

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Abstract

Restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) was employed to characterize species and populations of *Astyanax*, a Neotropical freshwater fish genus. Samples of five species, *A. altiparanae*, *A. fasciatus*, *A. lacustris*, *A. scabripinnis paranae* and *A. schubarti*, from the Upper Paraná and São Francisco river basins were analyzed. Two out of the ten restriction enzymes employed generated species-specific mtDNA patterns for each of the five species. MtDNA exhibited considerable polymorphism within and among populations. All populations sampled showed relatively high values of haplotype diversity. Geographically localized haplotypes were detected for *A. altiparanae* and *A. fasciatus* from the Upper Paraná and São Francisco basins. The relationships between populations are discussed.

Key words: mtDNA, RFLP, *Astyanax*.

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Introduction

The freshwater fish genus *Astyanax* (Pisces, Characiformes) is widely distributed over the Neotropical region. It comprises more than one hundred nominal species and subspecies, many of which undescribed so far (Garutti and Britski, 1997). In view of the number of species, distribution and taxonomy, *Astyanax* has been considered one of the most complex genera of characids in South America, (Zanata, 1995).

Five *Astyanax* species are commonly found in the Brazilian Upper Paraná and São Francisco river basins, presenting the following geographic distribution: *Astyanax altiparanae* and *A. schubarti* are found in the Upper Paraná river basin; *Astyanax fasciatus* inhabits most of South-American rivers; *A. lacustris* is restricted to the São Francisco river basin, and *A. scabripinnis paranae* is found in the headwaters of small rivers and streams of the Upper Paraná system (Britski, 1972; Garutti and Britski, 2000).

Previous cytogenetic studies of *Astyanax* revealed a high karyotypic variability between and within species (Morelli *et al.*, 1983; Daniel-Silva, 1996; Maistro *et al.*, 2000). However, there is still very little information available regarding the genetic diversity, population structure and evolutionary relationships among the species comprised by the genus *Astyanax*. Also, there is no evidence of monophyly for the genus *Astyanax*, believed to encompass different independent evolutionary lineages (Weitzman and Fink, 1983; Zanata, 1995; Weitzman and Malabarba, 1998).

Mitochondrial DNA (mtDNA) has proven to be a useful molecular marker for evolutionary studies in animal populations, because of its predominantly maternal inheritance, relatively rapid base substitution rate, lack of recombination, and easy isolation (Avise *et al.*, 1987; Wolstenholme, 1992).

MtDNA provides efficient molecular markers for the study of population structure, geographic variation and species characterization (Agnèse *et al.*, 1997; Graves, 1998). Hynes *et al.* (1989) detected diagnostic mtDNA restriction patterns which could be used as genetic markers for the discrimination of two different stocks of brown trout. RFLP analysis of mtDNA also proved to be effective in distinguishing among three different species of catfish in the Arabian Gulf, two of which could hardly be differentiated based on morphological traits alone (Simsek *et al.*, 1990).

In the present study, we employed restriction fragment length polymorphism analysis of mitochondrial DNA to assess the genetic variability and to further characterize species and populations of *Astyanax* from the Upper Paraná and São Francisco river basins. This work represents a pioneering effort to employ molecular markers on the population level in the genus *Astyanax*.

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Material and Methods

Samples from populations of *Astyanax altiparanae*, *A. fasciatus*, *A. lacustris*, *A. scabripinnis paranae* and *A. schubarti*, from the Upper Paraná and São Francisco hydrographic basins, Brazil, were analyzed (Figure 1; Table I).

Total genomic DNA was extracted from liver tissue, using slight variations of the standard phenol:chloroform protocol (Sambrook *et al.*, 1989), and digested with 10 restriction endonucleases: *Bam*HI, *Bcl*I, *Bgl*I, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Pvu*II, *Sca*I and *Xba*I, all of which recognized 6 nucleotide cutting sites. These enzymes were selected from a previous survey of 27 restriction enzymes, based on the number and sizes of the resulting fragments. Digestions were carried out as specified by the supplier (Gibco BRL).

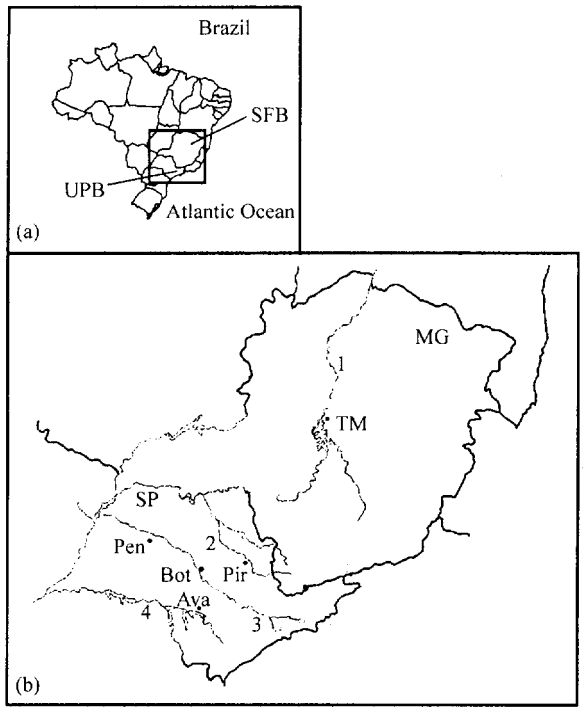

Figure 1 - Maps showing the collection sites of the *Astyanax* specimens. (a) Map of Brazil with hydrographic basins sampled. UPB = Upper Paraná basin; SFB = São Francisco basin. The insert is detailed in (b). (b) Sampling sites (sample designations are listed in Table I). São Francisco river (1); Mogi Guaçu river (2); Tiête river (3); Paranapanema river (4).

Restriction fragments were separated by electrophoresis in 0.8% agarose gels, and transferred by Southern blot (Southern, 1975) onto a positively charged nylon membrane. Membranes were hybridized with a digoxigenin-labeled probe containing the total purified mitochondrial genome from *Piaractus mesopotamicus* (Pisces, Characiformes) oocytes. Hybridization and detection procedures were performed according to the manufacturer's protocols (DIG DNA Labeling and Detection Kit - Boehringer Mannheim). Molecular weights of the fragments were estimated by comparison to *Hind*III λ DNA digests (Gibco BRL). The average size of the mitochondrial genome was estimated by the sum of the restriction fragment sizes produced by each enzyme.

To each different fragment pattern produced by a restriction endonuclease a single capital letter was assigned in order of appearance. Thus, each composite mtDNA haplotype was designated by a 10-letter code. MtDNA restriction sites were inferred from restriction fragment patterns, considering each enzyme separately. A presence/absence restriction site matrix was constructed for each composite haplotype.

Nucleotide (π; Nei and Tajima, 1981) and haplotype (h; Nei, 1987) diversity within populations, as well as nucleotide sequence divergence among pairs of haplotypes (δ; Nei and Li, 1979) and populations (δ; Nei, 1987), were estimated using the REAP software package (McElroy *et al.*, 1992).

Results

The 10 enzymes used yielded three patterns for *Pvu*II, four for *Bam*HI and *Bgl*I, five for *Eco*RI, six for *Bcl*I, seven for *Eco*RV and *Hind*III, eight for *Sca*I and *Xba*I, and eleven

Table I - Sampling sites, sample sizes, and mtDNA haplotype and nucleotide diversity within populations of *Astyanax*.

Species	Sampling sites	Sample designation	Sample size (n)	Haplotype diversity (h)	Nucleotide diversity (π)
A. altiparanae	Mogi Guaçu river (Pirassununga, SP)	Pir	9	0.416	0.0009
	Paranapanema river (Avaré, SP)	Ava	10	0.711	0.0020
	Tietê river (Penápolis, SP)	Pen	11	0.472	0.0018
A. fasciatus	Mogi Guaçu river (Pirassununga, SP)	Pir	13	0.769	0.0023
	Paranapanema river (Avaré, SP)	Ava	10	0.733	0.0022
	Tietê river (Penápolis, SP)	Pen	5	0.900	0.0041
A. lacustris	São Francisco river (Três Marias, MG)	TM	6	0.733	0.0018
A. scabripinnis paranae	Tietê river (Botucatu, SP)	Bot	12	0.166	0.0011
A. schubarti	Mogi Guaçu river (Pirassununga, SP)	Pir	18	0.640	0.0024

Table II - Estimated sizes (kb) of mtDNA restriction fragments in the five *Astyanax* species analyzed.

Enzyme	Restriction fragments	Enzyme	Restriction fragments
*Bam*HI		*Eco*RV	
A/A'	10.3, 2.5, 2.3/10.3, 2.8, 2.3	A	10.9, 4.2, 1.9
B	8.6, 7.4	B	10.2, 2.3, 2.1, 1.9
C	11.3, 5.2	C	14.6, 1.9
D	15.8	D	12.7, 3.8
*Bcl*I		E	17.2
A	6.5, 5.5, 4.2, 0.5*	F	6.0, 6.0, 4.5
B	6.9, 3.1, 2.6, 2.0, 1.5, 0.5*	G	12.0, 4.5
C	4.5, 3.1, 2.6, 2.5, 2.0, 1.5, 0.5*	*Hind*III	
D	10.4, 3.1, 2.6, 0.5*	A	7.9, 3.6, 1.7, 1.4, 0.7, 0.5, 0.2*
E	7.3, 3.1, 3.1, 2.6, 0.5*	B	9.3, 3.6, 1.7, 0.7, 0.5, 0.2*
F	6.9, 3.1, 2.6, 2.5, 1.5	C	4.0, 3.6, 3.2, 1.7, 1.4, 0.7, 0.7*
*Bgl*I		D	4.0, 3.6, 3.2, 1.7, 1.4, 1.1, 1.1*
A/A'	13.7, 3.0/13.7, 3.5	E	9.4, 2.2, 1.8, 1.8, 0.7
B	8.9, 4.7, 2.4, 1.5	F	9.4, 4.3, 1.8, 0.7
C	11.4, 4.7, 1,5	G	8.2, 4.1, 1.8, 1.0, 0.7, 0.2*
D	9.7, 4.7, 1.7, 1.4, 0,1*	*Pvu*II	
*Dra*I		A	5.8, 4.5, 4.5, 1.2
A	5.2, 2.7, 2.5, 1.7, 1.6, 1.1, 0.5*	B	5.4, 4.5, 4.5, 1.2, 0.4*
B	5.2, 3.1, 2.7, 1.7, 1.6, 1.1	C	5.8, 4.5, 3.0, 1.5, 1.2
C	5.2, 2.6, 1.9, 1.8, 1.6, 1.1, 0.8*, 0.5*	*Sca*I	
D	7.2, 2.7, 2.0, 1.8, 1.1, 0.6*, 0.5*	A	10.6, 3.9, 2.2
E	5.2, 2.7, 2.6, 1.5, 1.2, 1.2, 1.1	B	10.6, 3.0, 2.2, 0.9
F	5.2, 3.8, 2.7, 1.5, 1.2, 1.1	C	5.9, 4.2, 3.0, 2.2, 0.9
G	5.2, 2.7, 2.1, 1.5, 1.2, 1.2, 1.1, 0.5*	D	14.5, 1.5, 0.8
H	5.2, 2.7, 2.6, 2.1, 1.5, 0.8, 0.4*	E	10.6, 3.9, 1.5, 0.8
I	5.2, 2.7, 2.1, 1.6, 1.5, 1.1, 0.8, 0.4*	F	10.1, 4.5, 1.4, 0.6
J	5.2, 3.8, 2.7, 1.5, 1.1, 0.8, 0.4*	G	10.1, 4.5, 2.0
K	5.2, 3.8, 2.7, 2.7, 1.1	H	14.5, 2.0
*Eco*RI		*Xba*I	
A	11.9, 4.2	A/A'	7.3, 4.3, 2.7, 1.0/7.3, 4.3, 3.2, 1.0
B	9.6, 4.2, 2.2	B	7.3, 2.7, 2.1, 1.8, 1.0, 0.6*
C	11.9, 2.8, 0.8, 0.6	C	7.3, 4.3, 3.8
D	7.1, 4.6, 2.8, 0.8, 0.6	D	7.3, 4.3, 2.0, 1.0, 0.6*
E	11.9, 3.3, 0.8	E	6.1, 5.7, 2.9, 1.1
		F	5.7, 4.9, 4.3, 1.1
		G	6.1, 5.7, 4.3
		H	5.1, 4.3, 3.4, 1.8, 0.9

*Inferred fragments.

for *Dra*I (Table II). *Dra*I and *Hind*III produced species-specific mtDNA fragment patterns in all five *Astyanax* species studied (Figure 2). The mtDNA haplotype of each individual was derived from the combined restriction patterns of the 10 enzymes used in this survey.

The average size of the mitochondrial genome was estimated as being 16.0 kb for *A. altiparanae*, 16.4 kb for *A. fasciatus*, 16.1 kb for *A. lacustris*, 16.4 kb for *A. scabripinnis paranae*, and 16.5 kb for *A. schubarti*. Enzyme *Dra*I was disregarded in the size estimates, because it produced too many cuts in the *Astyanax* mtDNA. The presence of an unknown number of small fragments, which are difficult to detect, may account for the difference in size found among these species (Table II).

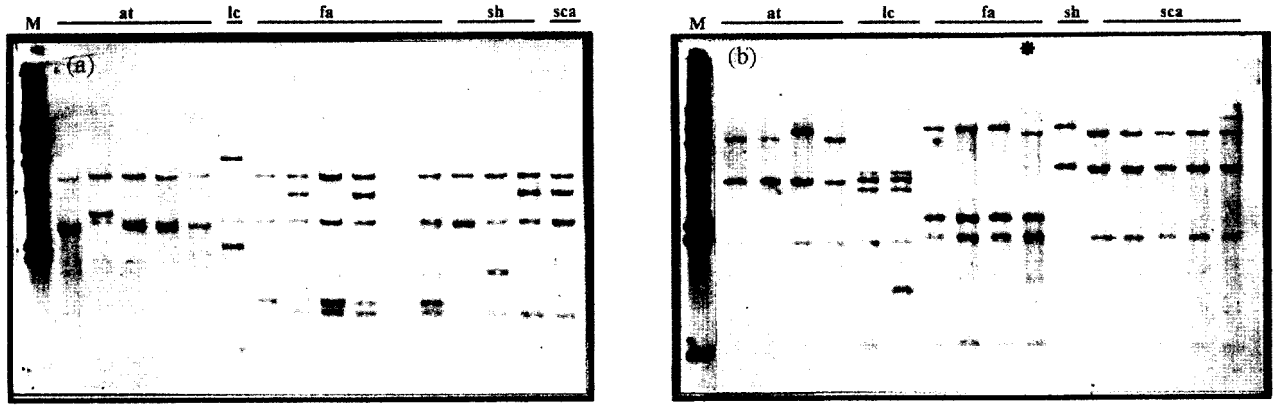

Figure 2 - Species-specific mtDNA fragment patterns produced by digestion with the restriction endonucleases *Dra*I (a) and *Hind*III (b). *Astyanax altiparanae* (at); *A. lacustris* (lc); *A. fasciatus* (fa); *A. scabripinnis paranae* (sca); *A. schubarti* (sh). λ DNA *Hind*III was used as molecular size standard (M); **Hind*III pattern found in the two specimens of *Astyanax fasciatus* from Três Marias not included in the analysis (see Discussion).

A mitochondrial DNA size variation of approximately 400-500 bp, as revealed by the patterns of three restriction enzymes, *Bam*HI, *Bgl*I, and *Xba*I (patterns A and A'), was observed in specimens of *A. lacustris* when compared to specimens of *A. altiparanae*.

Nine different mtDNA haplotypes were detected in *A. altiparanae* populations (Figure 3). Eight out of the nine haplotypes were population-specific. For a single haplotype (*at*1) high frequencies were found in both Pirassununga and Penápolis populations. Pairwise sequence divergence estimates (*d*) among these haplotypes ranged from 0.21% to 0.85% within populations, and from 0.42% to 1.33% among populations. Haplotype and nucleotide diversities in *A. altiparanae* populations are presented in Table I; estimates of nucleotide divergence among populations (δ) are presented in Table III.

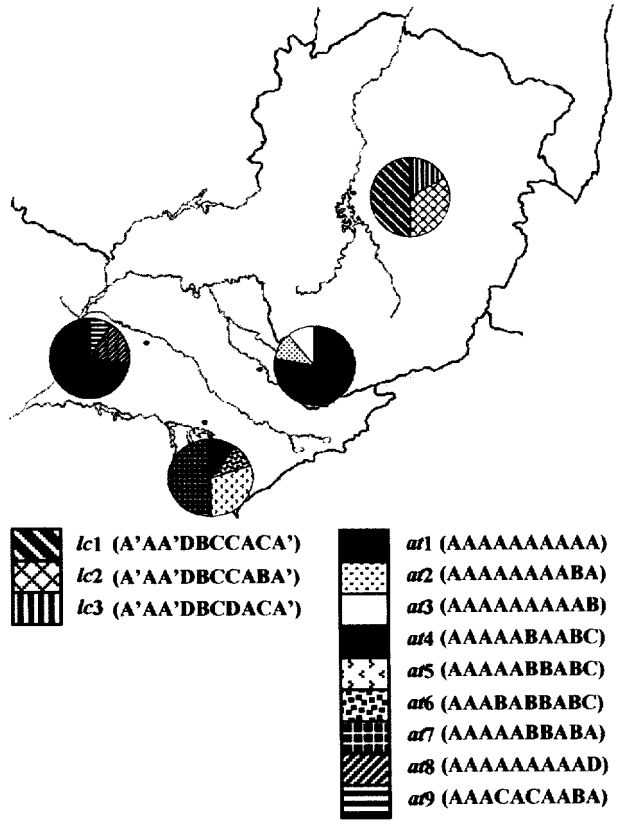

Figure 3 - Geographical distribution of mtDNA haplotypes in *A. altiparanae* (*at*1- *at*9) and *A. lacustris* (*lc*1- *lc*3). Composite haplotypes are denoted by capital letters in the following order: *Bam*HI, *Bcl*I, *Bgl*I, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Pvu*II, *Sca*I, and *Xba*I. Haplotypes A and A' differ from each other only in mtDNA length.

Within *A. fasciatus* populations, nine different haplotypes were detected (Figure 4). Six (*fa*1, *fa*5, *fa*6, *fa*7, *fa*8, *fa*9) out of the nine haplotypes were population-specific, and the remaining three haplotypes (*fa*2, *fa*3 and *fa*4) were shared by two sampling sites (Pirassununga and Penápolis). Pairwise sequence divergence estimates among these haplotypes ranged from 0.20% to 0.62% within populations, and from 0.42% to 1.71% among different populations. Haplotype and nucleotide diversities in *A. fasciatus* populations are presented in Table I, and the nucleotide divergence among populations is found in Table III.

A single population of *A. lacustris* was sampled in Três Marias, MG, São Francisco river basin. Pairwise sequence divergence estimates among the three mtDNA haplotypes found in this population varied from 0.21% to 0.42% (Figure 3). Haplotype and nucleotide diversities are shown in Table I.

In the single *A. scabripinnis paranae* population analyzed, two haplotypes (*sca*1 and *sca*2; d = 0.69%) were detected. In the *A. schubarti* population sampled in Piras-

Table III - Estimates of nucleotide sequence divergence (δ) (%) among populations of *A. altiparanae* (above the diagonal) and *A. fasciatus* (below the diagonal). Sample designations are as in Table I.

Population	Pir	Ava	Pen
Pir	-	0.6112	-0.0006
Ava	1.0026	-	0.6244
Pen	-0.0281	0.9411	-

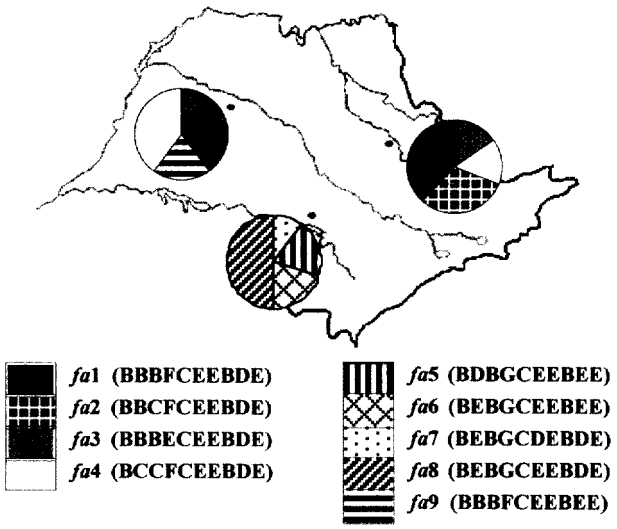

Figure 4 - Geographical distribution of mtDNA haplotypes in *Astyanax fasciatus*. Composite haplotypes are denoted by capital letters in the following order: *Bam*HI, *Bcl*I, *Bgl*I, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Pvu*II, *Sca*I, and *Xba*I.

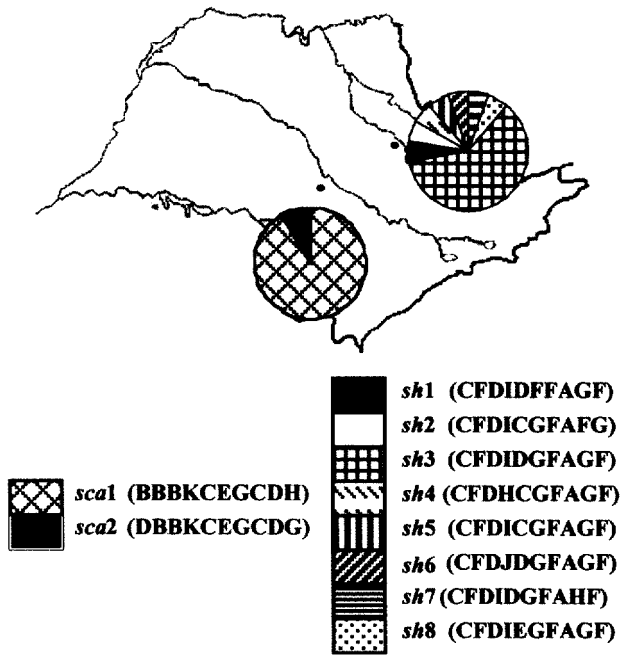

Figure 5 - Geographical distribution of mtDNA haplotypes in *Astyanax scabripinnis paranae* (*sca*1-*sca*2) and *A. schubarti* (*sh*1-*sh*8). Composite haplotypes are denoted by capital letters in the following order: *Bam*HI, *Bcl*I, *Bgl*I, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Pvu*II, *Sca*I, and *Xba*I.

sununga, eight mtDNA haplotypes were found (Figure 5). Divergence values among these haplotypes (sh1-sh8) ranged from 0.20% to 0.85%. Haplotype and nucleotide diversities for *A. scabripinnis* and *A. schubarti* are presented in Table I.

Discussion

The present data on the restriction fragment length of the mitochondrial DNA in *Astyanax* indicate that an expressive number of point mutations occurred in the mtDNA molecule during the evolutionary history of the five species inhabiting the two river basins here analyzed, many of them comprising populations living in sympatry. These mutations are reflected by the 31 haplotypes detected after digestion with 10 selected restriction enzymes. In fact, previous studies employing cytogenetic analysis in this group also showed differences in chromosome number and morphology, in both species and population (Morelli *et al.*, 1983; Daniel-Silva and Almeida-Toledo, 2001). *Astyanax schubarti* presented 2n = 36 chromosomes; *A. fasciatus* presented 2n = 46 chromosomes in populations from the Paraná basin, and 2n = 48 chromosomes in populations from the São Francisco basin (Morelli *et al.*, 1983; Oliveira *et al.*, 1988; Daniel-Silva and Almeida-Toledo, 2001). Although *A. altiparanae* presented a fixed number of 2n = 50 in all populations analyzed, the chromosome formula was sometimes quite distinct (Daniel-Silva, 1996). The *A. scabripinnis* populations studied presented a high variability in chromosome number (2n = 46, 48 and 50) and formula (Moreira-Filho and Bertollo, 1991; Souza *et al.*, 1995; Daniel-Silva, 1996). Variable, usually species-specific, patterns of constitutive heterochromatin blocks were found in these *Astyanax* species. Thus, the C-banding pattern associated with chromosome number and formula resulted in species-specific karyotypes for these four species (Daniel-Silva and Almeida-Toledo, 2001).

In the present study, digestion with *Dra*I and *Hind*III allowed to detect species-specific mtDNA patterns for the five species of *Astyanax* under study. Furthermore, intraspecific variability was observed in all species herein analyzed.

In *A. altiparanae*, out of the nine haplotypes identified, eight were population-specific, and only one was shared by two populations. Nine different haplotypes were also detected in *A. fasciatus*, six of them being private and three shared by two populations. Both in *A. altiparanae* and *A. fasciatus*, the most common haplotypes were found in samples from Pirassununga and Penápolis, both collected in the Upper Paraná basin. Moreover, in both species an extremely low δ value between Pirassununga and Penápolis was observed. This result may suggest either a recent time of separation of these two populations, or the occurrence of gene flow between them. The fact that *Astyanax* species are capable of short-distance migrations (V. Garutti, pers. comm.), and that Pirassununga and Penápolis are connected by the Paraná river, although situated approximately 330 km apart, allows us to postulate the occurrence of gene flow via the stepping-stone model (Kimura, 1953). According to Slatkin (1987), the exchange between populations of a few individuals per generation would be sufficient to prevent the accumulation of a significant genetic drift between geographically distant sites. However, in order to test the hypothesis of a "stepping-stone" gene flow occurring

between Pirassununga and Penápolis, it would be necessary to sample individuals from intermediate populations.

On the other hand, the presence of non-shared haplotypes between samples from Avaré (Paranapanema river) and Pirassununga/Penápolis (Mogi Guaçu and Tietê rivers, respectively), both in *A. altiparanae* and *A. fasciatus*, suggests the absence of gene flow and, consequently, a geographical isolation of these populations.

Haplotype diversity was found to range between moderate and high values in all populations, with the exception of the *A. scabripinnis* population from the Tietê river, which presented low *h* values. Taking into account that *A. scabripinnis* is a headwater species composed of small populations which are most likely isolated from each other, the low haplotype diversity could be explained by a reduction of genetic variability due to genetic drift. Moreover, the headwater habitats are more stable, therefore leading to a more uniform genetic composition. As for the other species, the sporadic occurrence of low haplotype diversity in both *A. altiparanae* and *A. schubarti* could be due to the predominance of one particular haplotype in the sample. In fact, the *h* value indicates that, in spite of their diversity, the mtDNA haplotypes are not very divergent from each other.

The size of the mitochondrial genome estimated for the five *Astyanax* species is in accordance with values previously reported for other fish species (Billington and Hebert, 1991). When comparing the mtDNA genome sizes of *A. altiparanae*, from the Upper Paraná river basin, and *A. lacustris*, from the São Francisco river basin, there seems to be a variation of about 500 bp, the genome of the latter being the longer one. This profile is suggested by the *Bam*HI, *Bgl*I and *Xba*I restriction patterns. The digestions were repeated, in order to prevent technical artifact. Differences in mtDNA length could be due to addition/deletion of sequences of nucleotides or to duplications of tandem repeats in the control region of mtDNA. Size variations of 500 bp or less are typically found in the mtDNA control region, and are common in lower vertebrates (Bermingham *et al.*, 1986; Moritz *et al.*, 1987). They may also be due to the low detection sensitivity of the DIG kit. As it is based on a color reaction instead of a chemiluminescent or radioactive reaction, which are more sensitive, small fragments of 500 bp or less may go undetected with this protocol. To further investigate this matter, the control region of both species will have to be sequenced.

The results reported here regarding the mitochondrial genome differences found in *A. altiparanae* and *A. lacustris* are in agreement with morphological data. Both *A. altiparanae* (Upper Paraná basin) and *A. lacustris* (São Francisco basin) were formerly ascribed to the species *A. bimaculatus*, having only recently been recognized and described as distinct species belonging to the *bimaculatus* species complex, a group comprising at least 15 species and subspecies that share a number of morphological traits (Garutti and Britski, 2000).

MtDNA restriction patterns obtained for two *Astyanax fasciatus* individuals from Três Marias, MG (São Francisco river basin), indicated the presence of one haplotype that is distinct from the others detected in the Upper Paraná basin (data not shown). Due to the small sample size (N = 2), data from these individuals were not included in the analysis, in order to avoid any bias concerning the diversity indices. However, the fact that this haplotype was not observed in any of the other populations of *A. fasciatus* sampled in the Upper Paraná basin might suggest some degree of isolation among populations of these two hydrographic basins. According to Menezes (1988), the Upper Paraná and São Francisco river basins are believed to have separated during the late Tertiary, originating a barrier to gene flow. In fact, cytogenetic and morphological studies have reported marked differences among populations of other fish species from these two basins (Almeida-Toledo *et al.*, 1993; Shibatta and Garavello, 1993). Garutti and Britski (2000) consider the *Astyanax fasciatus* species of the Upper Paraná basin as being different from those of the São Francisco basin. According to these authors, *A. fasciatus* comprises a complex of related forms, and further analyses are likely to result in the description of several new species, as occurred with the *bimaculatus* group.

According to Garutti (1995), the genus *Astyanax* has a compartmentalized distribution, suggesting a remarkable endemism: there is a different form for each basin and, within each basin, there are several different forms with relatively restricted geographical distributions.

Thus, although the genus *Astyanax* presents a wide geographical distribution in the Neotropical region, most populations seem to be partially or totally isolated from each other. The results obtained so far suggest that random genetic drift and gene flow may be among the main forces affecting the geographical distribution of haplotypes in *Astyanax* species.

Molecular markers generated by RFLP analysis of mitochondrial DNA were very informative to characterize *Astyanax* species, and to detect intraspecific genetic variability. The data presented provide a strong base for further analyses concerning population structure and evolutionary relationships within this group of Neotropical fishes.

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