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Primary Research Paper

Population genetic structure of *Astyanax scabripinnis* (Teleostei, Characidae) from an urban stream

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Abstract

Despite the great anthropogenic interference on urban streams, information is still scarce about the genetic variability and structure of native fish populations inhabiting such streams. In the present study, random amplified polymorphic DNA (RAPD) markers were used to analyze genetic variability and structure of populations assigned to the Neotropical fish species *Astyanax scabripinnis* from an urban stream located in Londrina, Paraná State, southern Brazil. Thirty individuals of this species were collected from three sites throughout the upper Cambé stream. A total of 10 primers amplified 159 loci, of which 128 (80.5%) were polymorphic. Each of the three populations showed very similar proportions of polymorphic loci, which ranged from 63.5 to 64.8%. Unbiased genetic distances varied from 0.0612 to 0.0646. $Theta_p$-test values indicated moderate to high genetic differentiation among individuals from different localities. The number of migrants varied from 1.34 to 1.46, suggesting a low gene flow between populations. The genetic similarity among all individuals studied ranged from 0.424 to 0.848. The results suggest that populations of *A. scabripinnis* in Cambé stream are undergoing genetic differentiation.

Introduction

In the past 30 years, advances in molecular technology have greatly increased the number of DNA-based markers capable of revealing genetic variation in many species (Isabel et al., 1999). Random Amplified Polymorphic DNA (RAPD) is a simple and straightforward PCR-based technique, based on amplification of discrete regions of genome by using arbitrary primers (Williams et al., 1990). In recent years, several authors have used RAPD markers to evaluate genetic variability and structure of a variety of species of Neotropical fish, in South American rivers (Almeida et al., 2001; Wasco & Galetti Jr, 2002; Leuzzi et al., 2004; Matoso et al., 2004). However, studies making use of molecular markers to evaluate genetic structure in Neotropical fish populations in urban streams are still lacking.

In urban areas, where aquatic ecosystems are constantly suffering the discharges of effluents produced by human activities (Paul & Meyer, 2001), populations of fish have to adapt frequently to environmental changes. In Londrina city, in northern Paraná State, the Cambé stream is a very disturbed ecosystem, which receives a great assortment of effluents along its course. Previous chemical water analyses of the upper reaches of Cambé stream showed large amounts of metals, mainly lead and aluminum (Yabe and Oliveira,

1998). In addition, Winkaler et al. (2001) showed that feral fish from these upper areas present impaired health and more recently, Lemos et al. (2005) demonstrated that its water induces DNA damage, detected by comet assay. However, so far, there is no information about the genetic variability and structure of native species of fish in this stream.

The freshwater fish genus *Astyanax* Baird and Girard (Pisces, Characidae), commonly named lambari, is widely distributed over the Neotropical region. It comprises more than one hundred nominal species and subspecies (Garutti & Britski, 2000). In South American rivers, species of this genus are among the most important components of the food-web, with a significant participation in the diet of large fish (Prioli et al., 2002).

Astyanax scabripinnis (Jenyns) is a subtropical fish, commonly found in the headwaters of small rivers and streams of the Upper Paraná River Basin, inhabiting the water-column (Britski, 1972; Garutti & Britski, 2000; Veregue & Orsi, 2003). This species presents great morphological diversity (Souza et al., 1995), mainly in terms of standard length, body height and snout length (Moreira-Filho & Bertollo, 1991), showing normally omnivorous feeding habit and low longevity (Barbieri, 1992a, b; Veregue & Orsi, 2003).

Among the Neotropical freshwater fish, *A. scabripinnis* has proved to be an interesting biological material in studies on natural populations due to its distribution limited to the headwaters in most of the Brazilian hydrographic basins (Souza & Moreira-Filho, 1995). In the last few years, several aspects of the biology of *A. scabripinnis* have been studied by a number of authors (Moreira-Filho & Bertollo, 1991; Barbieri, 1992a, b; Veregue & Orsi, 2003). Moreira-Filho & Bertollo (1991) demonstrated that *Astyanax scabripinnis* constitutes a complex of species composed of local populations showing morphological and cytogenetic diversities. In consequence of this fact, cytogenetic studies have been carried out on several local populations of *A. scabripinnis* from different Brazilian regions (Moreira-Filho & Bertollo, 1991; Souza & Moreira-Filho, 1995; Maistro et al., 2000). Nevertheless, except for the study performed by Moysés & Almeida-Toledo (2002), which employed restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA to assess the genetic variability and to characterize species and populations of five species of *Astyanax*, including *A. scabripinnis*, from Upper Paraná and São Francisco river basins in Brazil, information about the genetic variability and structure of *A. scabripinnis* is scarce.

The study of genetic variability is of prime importance for genetic approaches to fish conservation (Carvalho, 1993). According to Haig (1998), the most important contribution that conservation geneticists can make to the assessment of the viability of populations is to determine the relative amounts of genetic diversity within and among populations. Moreover, the knowledge of genetic structure of populations is also essential for the implementation of management programs (Solè-Cava, 2001).

Thus, the aim of this study was to investigate the genetic variability and structure of populations of *A. scabripinnis* inhabiting the Cambé stream, by applying RAPD markers.

Material and methods

Astyanax scabripinnis specimens were collected from three sites along the upper Cambé stream (between coordinates 23°15′ S–51°14′ W and 23°18′ S–51°17′ W), using manual fishing tackle (scoop-net and sieve). This stream is approximately 25 km long and constitutes the main hydrological basin of Londrina, a city of 500,000 inhabitants in Paraná State, Southern Brazil (Fig. 1). Sampling sites comprised the headstream (A), and two other sites (B and C) not very distant from the source (Fig. 1). The study included 12 individuals collected from site A, 11 from B and 7 from C, totaling 30 individuals analyzed. To get these numbers of fish, 6 samplings were performed in site A, 4 in site B and 8 in site C. Repeated samplings were made at the studied sites and fishing methods was improved, even so it was not possible to increase the number of *A. scabripinnis* in each sampling site. There were other fish species in the study area, but unfortunately mainly exotic species such as *Poecillia reticulata*, which was the main species at site A, and *Oreochromis niloticus* at sites B and C. Thus, in order to reduce possible biases in the analysis due to the limited number of fish collected, particularly in site C, a great effort

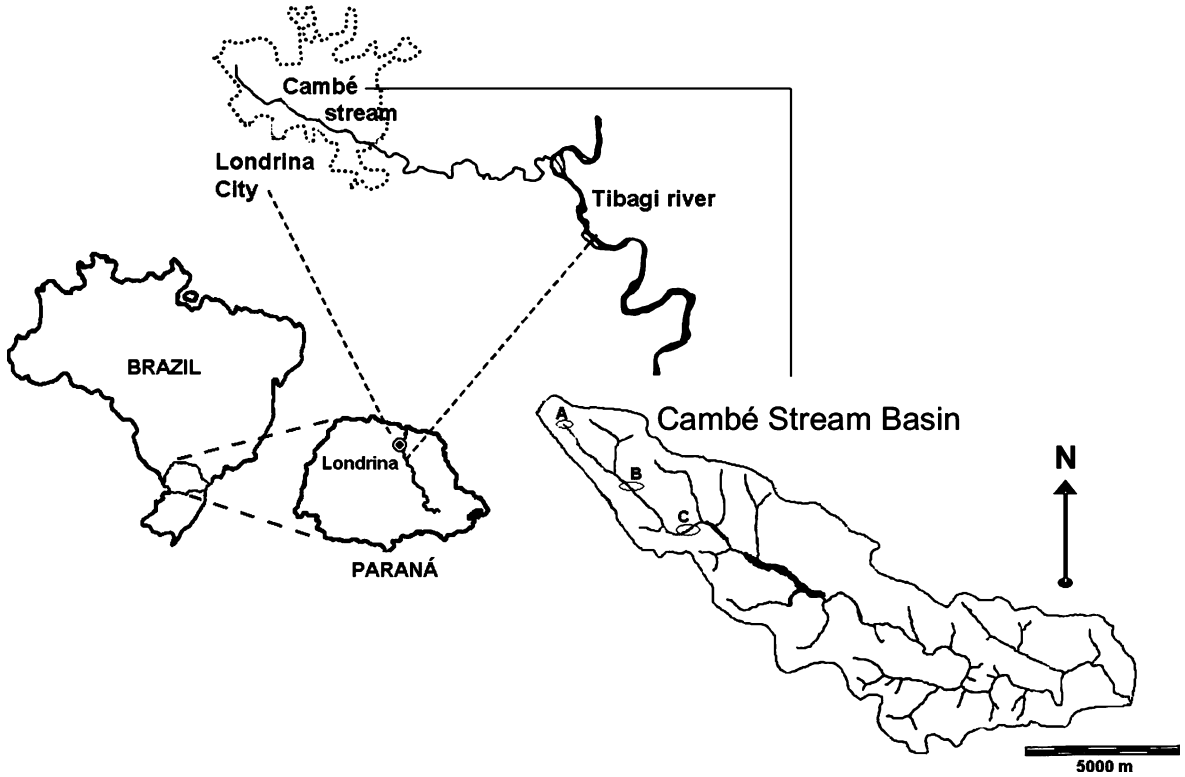

Figure 1. Overview maps indicating the location of Londrina City in Paraná State southern Brazil and the positions of the three collecting sites along the upper Cambé stream.

was made to analyze a high number of loci (Nei, 1978).

Muscles samples were removed from fish immediately after capture and kept at −20 °C until use. After identification some individuals were preserved in ethanol (70%) as voucher specimens.

At the sampling sites, the temperature, dissolved oxygen, pH and conductivity of the water were measured and water samples were collected and analyzed for organic matter, sulphate, phosphate, nitrite and nitrate using procedures described in APHA (1998).

DNA extraction and RAPD analysis

DNA was extracted from the muscle of fish following the procedure described by Almeida et al. (2001). DNA concentration was determined in a DyNA Quant 200 fluorimeter (Hoefer), using the dye Hoechst 33258, and diluted to a standard DNA concentration of 5 ng/μl. All isolates were then either used immediately or stored at −20 °C.

The RAPD profiles were generated from total genomic DNA as described by Williams et al. (1990). Amplification reactions were performed in a total volume of 15 μl containing 15–25 ng of template DNA, 250 μM dNTP (Pharmacia), 0.25 μM of 10-nucleotide primer (Operon Technologies, Alameda, CA, USA), 4.5 mM $MgCl_2$ and 1 U of DNA polymerase (Embrapa, Brazil) in the reaction buffer supplied. For the RAPD analysis, 20 decamer oligonucleotides (kit OPW) were used as random primers in RAPD screening, of which 10 were selected, that produced a good number of amplified bands and patterns of reproducible fragments. Control reactions were run with all components except genomic DNA and none of the primers used yielded detectable amplified products in these reactions. DNA amplifications were carried out in a thermal cycler (MJ Research PTC-100) and the amplification protocol consisted of 4 min at 92 °C followed by 40 cycles of 40 s at 92 °C, 1.5 min at 40 °C, and 2 min at 72 °C. The last round of amplification

was followed by an additional extension at 72 °C for 5 min.

Samples of 15 μl of amplification products were assayed by electrophoresis run at 3 V cm^{-1} in 1.4% agarose gels with TBE buffer (0.89 mM Tris (pH 8.30, 0.89 mM boric acid, 2 mM EDTA) diluted 1:20 (v:v). Gels were then stained with ethidium bromide, photographed under UV light using T-Max 100 Kodak film and scored visually for band presence and absence.

Data analysis

For each water parameter analyzed differences among the sampling sites were tested for significance by one-way ANOVA and multiple range tests (Student–Newman–Keuls procedure) where appropriate. Means were considered significantly different where $p < 0.05$.

Comparative analyses were carried out by placing all samples from the three sites on the same gel, for intra and inter-population analyses. The RAPD marker profiles were determined by direct comparison of the amplified DNA electrophoresis profiles, and each band was analyzed as a binary variable (band presence or absence). Only RAPD bands that were scored unequivocally were counted in the analysis. Each locus was treated as a two-allele system, with only one of the alleles per locus being amplifiable by the PCR. It was also assumed that marker alleles from different loci did not co-migrate to the same position on a gel, and that populations were under the Hardy–Weinberg equilibrium (Lynch and Milligan, 1994).

The TFPGA 1.3 software (Miller, 1997) was used in the following calculations: (a) estimation of genetic variability from the proportion of polymorphic loci ($\bar{P}$), using the 95% criterion; (b) average heterozygosity ($\overline{He}$); (c) Nei's unbiased genetic distance (D) (Nei, 1978); and, (d) estimation of gene frequency divergence among populations by the theta$_p$-test, as described by Weir & Cockerham (1984), performing 1000 interactions in order to find an approximate 95% confidence interval.

Genetic similarity dendrogram among the different groups of individuals analyzed were constructed by applying the Jaccard (J) coefficient and the UPGMA grouping method, using the NTSYS-PC package (Rohlf, 1992). To evaluate the robustness of the groupings formed the bootstrap analysis, with 1000 replications, was performed using the Bood software program (Coelho, 2000).

Results

Physical and chemical data on the water obtained at the sampling sites are given in Table 1. Dissolved O_2 and water temperature did not differ significantly among sites. All other variables showed a trend to increase in sites located downstream the source (site A). The concentrations of nitrite and nitrate were significantly higher at sites B and C and conductivity was significantly higher at site C, in comparison to sites A and B.

The 10 primers used for the *A. scabripinnis* RAPD analysis amplified 159 loci, 129 (81.3%) of which were polymorphic. A RAPD electrophoretic profile for one of the selected primers is shown in

Table 1. Water parameters at sampling sites on the Cambé stream during collection times

Parameters	Site A	Site B	Site C
Dissolved O_2 (mg O_2 l^{-1})	6.33 ± 0.27	7.35 ± 0.95	7.13 ± 0.82
Temperature (°C)	20.18 ± 1.89	22.42 ± 2.01	20.86 ± 2.53
pH	6.47 ± 0.21^a	7.34 ± 0.45^b	7.47 ± 0.48^b
Conductivity (μS cm^{-1})	66.50 ± 22.63^a	96.71 ± 16.98^a	231.88 ± 69.68^b
Organic matter (mg l^{-1})	29.02 ± 14.34	31.18 ± 11.30	51.18 ± 28.08
Sulfate (mg l^{-1})	2.84 ± 2.16	3.21 ± 2.19	4.90 ± 1.89
Phosphate (mg l^{-1})	0.74 ± 0.39	0.67 ± 0.26	1.40 ± 1.14
Nitrite (μg l^{-1})	2.70 ± 1.40^a	21.50 ± 7.80^b	44.40 ± 27.90^b
Nitrate (mg l^{-1})	0.19 ± 0.11^a	0.90 ± 0.38^b	0.72 ± 0.31^b

Values are mean ± SD (n varied from 4 to 8). Different superscript letters indicate significant differences ($p < 0.05$).

Figure 2. The number of fragments per primer ranged from 10 to 23. The proportions of polymorphic loci observed in the three groups of individuals sampled at the different sites were: 64.8% (A), 64.2% (B) and 63.5% (C), while the values of heterozygosity $\bar{H}e$ estimated for *A. scabripinnis* were: 0.2433 (site A), 0.2518 (B) and 0.2296 (C), therefore revealing very similar measures of genetic variability.

The $theta_p$ values were significantly different from zero for all population pairs (Table 2), indicating genetic differentiation among them. The number of migrants per generation (*Nm*) ranged from 1.34 (between the populations A and C) to 1.47 (populations A and B).

Estimates of Nei's (1978) genetic distances for *A. scabripinnis* ranged from 0.0612 to 0.0646 (Table 2). The highest value found was between fish from the points A and C.

The genetic similarity among all individuals of this species, estimated by Jaccard's coefficient, ranged from 0.424 (pair 4A–11B) to 0.848 (pair 11b–2C). In site A the values of similarity among pairs of individuals ranged from 0.480 (pair 3A–8A) to 0.743 (pair 6A–9A), in site B from 0.495 (3B–11B) to 0.757 (8B–9B), and in site C from 0.505 (2C–6C) to 0.822 (5C–7C). The mean genetic similarity and standard deviation (SD) of the populations from sites A, B and C were, respectively: 0.598 (SD = 0.050), 0.622 (SD = 0.061) and 0.600 (SD = 0.067), while the mean values of genetic similarities between pairs of populations were: A–B = 0.574 (SD = 0.047); A–C = 0.543 (SD = 0.043); and B–C = 0.558 (SD = 0.050).

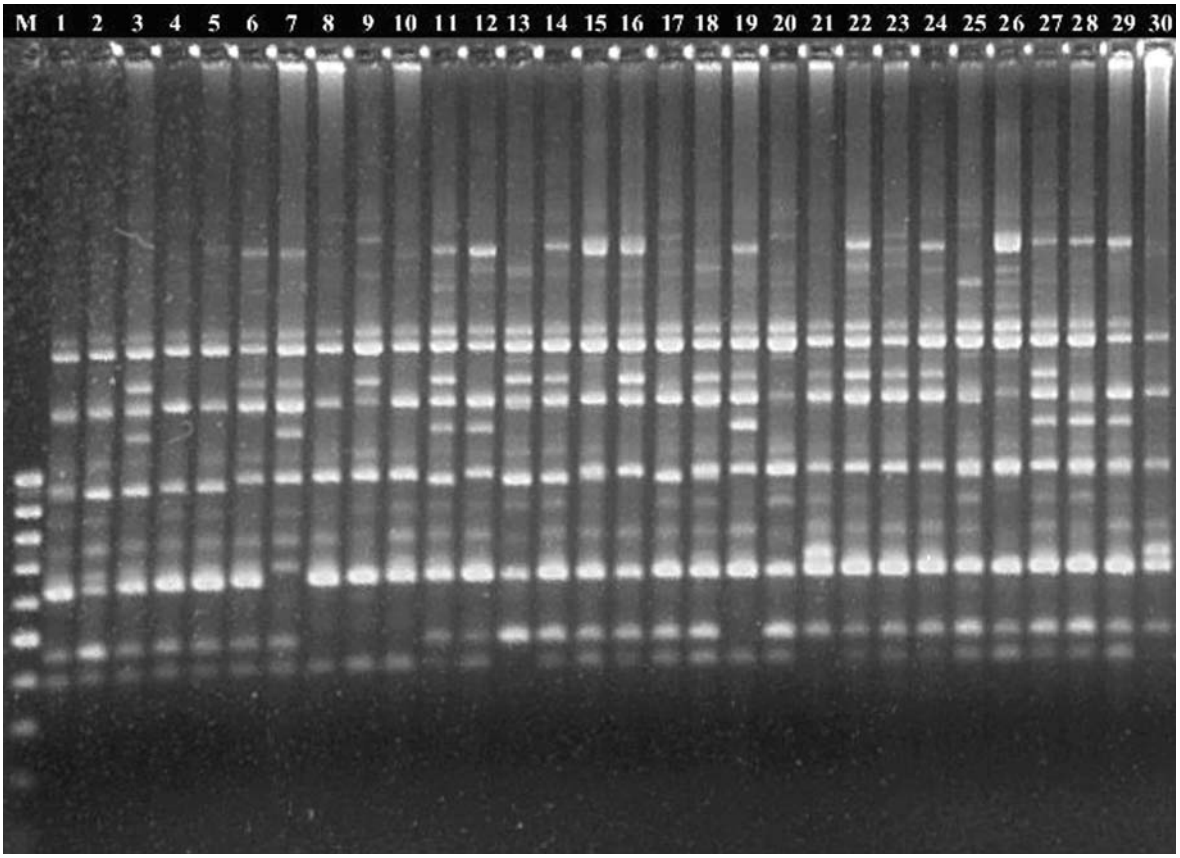

Figure 2. DNA polymorphism of the 30 individuals of *Astyanax scabripinnis* from the three sites along the upper Cambé stream, amplified with primer OPW3. Column M, 100 bp molecular weight marker; columns 1–12, fish collected at site A; columns 13–24, fish from site B; columns 25–31, fish from site C.

Table 2. Estimation of gene frequency divergence among populations ($theta_p$-test), number of migrants per generation (N_m) and Nei's (1978) unbiased genetic distances (D), with Lynch and Milligan (1994) correction, between pairs of *A. scabripinnis* samples from three different sites (A, B and C) on the Cambé stream. Jackknife values, employed to assess the statistical significance of the calculated $theta_p$ value, are also shown

Pairs of Populations	$Theta_p$-test				*D*
	$Theta_p$	Jackknife	χ^2	N_m	*Nei (1978)*
A–B	0.1452*	0.1457 (0.0292)	6.68	1.47	0.0612
A–C	0.1566*	0.1570 (0.0256)	5.95	1.34	0.0646
B–C	0.1459*	0.1461 (0.0204)	5.25	1.46	0.0613

*$p < 0.05$; ( ) = standard deviation; $d.f._{.thetap} = 1$.

The analyses of RAPD marker profiles for *A. scabripinnis* showed that individuals from site C cluster together, excepting fish 1C and 6C (Fig. 3). The most part of other fish grouped together, but subdivided into two small clusters: one containing 11 individuals from the headstream (A) and, the other clustering almost all individuals from site B, plus the individuals 12A and 1C (Fig. 3). However, bootstrap values for the UPGMA tree were generally very low for the major branches and moderate to high bootstrap values (>50) were only given for minor branches, in most cases indicating a tendency for samples of the same site to cluster together.

Discussion

Physical and chemical variables are essential in the assessment of water quality and in this study they indicated that the water quality decreases at the sampling sites located further from the source of the Cambé stream. At sites B and C an increased loading of nutrients was indicated by nitrite and

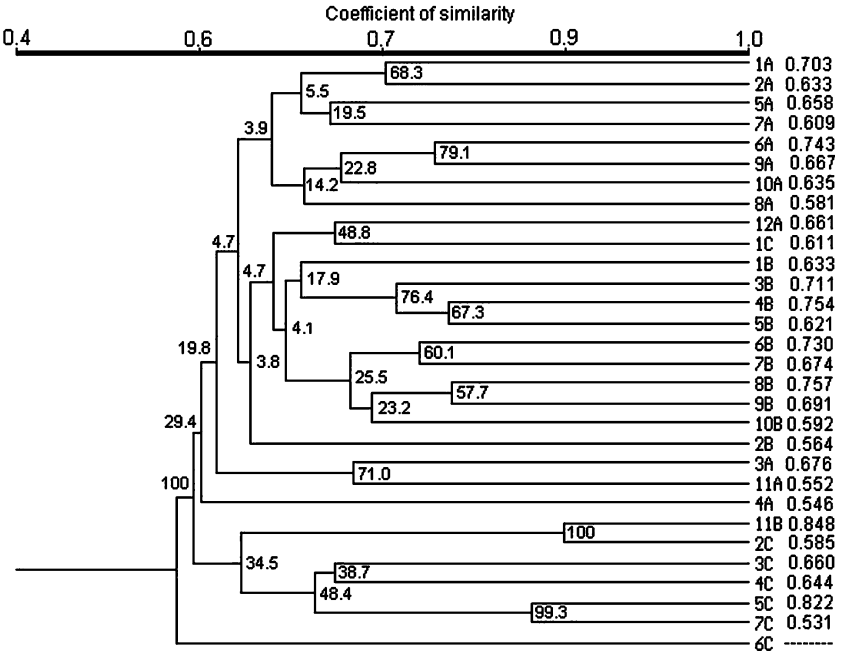

Figure 3. Dendrogram of genetic similarity constructed using the Jaccard coefficient and the UPGMA method for *Astyanax scabripinnis* from the three sites (A, B and C) along the upper Cambé stream. Numbers at the nodes represent bootstrap generated by 1000 replications. The similarity measures of consecutive pairs of individuals listed on the dendrogram are shown in the column on the right.

nitrate concentrations, suggesting a higher discharge of effluents into the stream at these sites. The conductivity at site C was higher than at other sites and values above 100 μS cm^{-1} have been reported at polluted sites (CETESB, 2001) and could reflect poor water quality (Olsen et al., 2001). Even so, none of the three sampling sites could be considered as reference, as all areas in Cambé stream, including its headwaters (site A), have been impacted by human activity.

The fish *A. scabripinnis* inhabits preferentially 'rapid' waters of small tributaries showing a distribution limited to the sources of streams (Britski, 1972) with a decreasing frequency from the up to the downs streams (Souza & Moreira-Filho, 1995). This fact, in addition to the decreased water quality, could partially explain the low frequency of *A. scabripinnis* in site C from Cambé stream.

Genetic variability among different populations and also between individuals within populations is essential to environmental changes (Ryman et al., 1995; Solé-Cava, 2001). Most natural populations are characterized by high levels of genetic variation (Solé-Cava, 2001). When the gene pool of a population narrows and loses genetic plasticity, it becomes increasingly susceptible to changing environmental conditions and hence more vulnerable to extinction (Guttman & Berg, 1998). For many fish species it has already being demonstrated that a link exists between the level of genetic variability of local populations and the kind of environmental disturbance they undergo (Bickham et al., 2000; Cimmaruta et al., 2003).

A number of studies with different species of Neotropical fish, using RAPD markers, have found levels of genetic variability, estimated by proportion of polymorphic loci ($\bar{P}$), ranging from 22.2 to 60% (Almeida et al., 2001; Chiari & Sodré, 2001). In a recent study, Leuzzi et al. (2004) using these markers to study another lambari species also found in Paraná Basin, detected levels of $\bar{P}$ above 70% for populations of *Astyanax altiparanae* collected along the Paranapanema River, a Neotropical aquatic ecosystem that has suffered human intervention due to the building of several hydroelectric dams. Thus, despite the impaired conditions of the Cambé stream, the values of genetic variability found for *A. scabripinnis* in this study, ranging from 63.5 to 64.8%, apparently did not diverge from those, obtained by using RAPD markers, described in the literature for Neotropical fish. In part, these results could explain the tolerance of this species to physico-ecological variations (Souza & Moreira-Filho, 1995).

According to Bickham et al. (2000), disturbed ecosystems can either cause an increase in genetic variation, resulting from new mutations, or a decrease in genetic variation, resulting from population bottlenecks or selective sweeps, in natural populations. For example, Murdoch & Hebert (1994) observed that mtDNA haplotypes diversity of brown bullhead fish (*Ameiurus nebulosus*), assayed by RFLP method, was consistently lower in contaminated rivers than at clean sites. Similar conclusion was found by Nadig et al. (1998) using RAPD technique to assess the nuclear variability in redbreast sunfish (*Lepomis auritus*) from mercury-contaminated sites. On the other hand, Theodorakis & Shuggart (1997) showed a higher degree of RAPD-based genetic diversity, estimated by applying a similarity index, in mosquitofish (*Gambusia affinis*) populations living in contaminated sites relative to the reference sites. However, in the present study it remains unclear which of the two cases apply to the three populations of *A. scabripinnis* from the Cambé stream since no reference population was examined.

The levels of average heterozygosity ($\overline{H}e$) found for all populations of this species of lambari, which ranged from 0.2296 to 0.2518, were higher than the range estimated by allozymes analysis from subpopulations of other species of teleosts in disturbed streams (Foré et al., 1995a, b; Cimmaruta et al., 2003). However, this discrepancy could result from intrinsic differences between these molecular markers, since the RAPD technique frequently produces dozens of loci to be screened, because each primer typically produces multiple bands (Bickham et al., 2000), while allozyme products are less numerous.

F_{st} is a standardizing measure of degree of genetic differentiation among populations, which ranges from 0 (no differentiation) to 1 (no alleles shared) (Hartl & Clark, 1997). According to Wright (1978), values of F_{st} ranging from 0.05 to 0.15 are indicative of moderate population genetic structuring, while values from 0.15 to 0.25 indicate high genetic structuring. Thus, the analysis of the theta$_p$-test, which is a estimator of F_{st}, obtained for *A. scabripinnis* suggest moderate differentiation

between the population at site B and each of the others sites, and high differentiation between populations from sites A and C (Table 2). The highest value of Nei's (1978) genetic distance found between fish from the sites A and C corroborates the results obtained with $theta_p$-test.

Wright's distance models (Foré et al., 1995a) and empirical data (MacArthur et al., 1992) predict that populations from adjacent sites should be more similar genetically because of the greater probability of gene flow. In fact, the results found confirm this idea, since higher genetic diversity was observed between *A. scabripinnis* population pairs A–C, geographically more distant (Fig. 1), while lower genetic differentiation was detected between geographically less distant populations pairs that is A–B and B–C (Table 2). Besides, considering that *A. scabripinnis* is a species that normally shows an allopatric distribution in small populations (Souza et al., 1995), forming separate populations that are able to migrate down, but very seldom do it (Landini et al., 2002), a higher genetic dissimilarity could be expected between fish from sites A and C.

It is also expected that, in urban streams, several factors other than distance, mostly related to environmental stress, would influence the genetic structure of populations. Foré et al. (1995a, b) using allozymes markers to evaluate the allele and genotypic frequencies in populations of two species of fish, bluntnose minnows (*Pimephales notatus*) and central stoneroller (*Campostoma anomalum*), suggested that the water quality altered the distribution of alleles in disturbed streams. Over again, the present results do not allow draw any conclusion about this fact, considering that no reference population was surveyed.

One of the problems in population structure analysis is to estimate the amount of gene flow, which is the most important determinant of the population structure, since it controls to what extent each local population of a species is an independent evolutionary unit (Slatkin, 1993). If gene flow among nearby populations is intense, they evolve together, while, if there is small flow, each population evolves almost independently. Thus, high differentiation is expected among populations when low gene flow ($N_m < 1$), while if $N_m > 1$, gene flow is potentially strong enough to prevent substantial differentiation due to drift and natural selection (Tremblay & Ackerman, 2001). As shown in Table 2, the number of migrants per generation (N_m) were very close to 1, ranging from 1.34 (between the populations A and C) to 1.47 (populations A and B), and suggest low gene flows between populations, insufficient to impede genetic differentiation. Besides, it is believed that estimates of gene flow, which are generally based on models that assume that populations are in equilibrium, grossly overestimate the true number of migrants under real conditions (Widmer & Schmid-Hempel, 1999). Since, gene flow is not necessarily equivalent to N_m (Tremblay & Ackerman, 2001), the values of N_m obtained could be overestimated, and therefore, the gene flow might be not occurring between populations of this lambari species.

On the other hand, estimates of among-population differentiation using RAPD fingerprints can produce biased results (Isabel et al., 1999). According to these authors, in sample sizes inferior to 10 individuals the biased effects produced were even more inflated. It follows that the analyses of genetic differentiation between populations of *Astyanax scabripinnis* should be interpreted very carefully, since values of $theta_p$ could be overestimated and, consequently, the real amount of differentiation among populations may be less than these values suggest. Therefore, although *Astyanax scabripinnis* populations, at the three collection sites on the Cambé stream seemed genetically diverse, increasing the sample size would give greater confidence in the results.

The bootstrap analysis do not support the major branches of dendrogram, which showed low bootstrap values, preventing any consistent conclusion about that clusters joining a larger number of individuals. Besides, the branch lengths separating the main clusters are very short, indicating comparable values of similarity among all individuals analyzed. However, the analysis of dendrogram to some extent seems to reproduce the genetic differentiation among the three populations, since some clusters formed by individuals of the same site showed reliable bootstrap values, reflecting a high robustness of these branches (Fig. 3). The cluster formed by individuals from the headstream may be due to the recognized preference of this species for this habitat (Britski, 1972). Likewise, the small cluster comprising mainly fish from site C could reflect a limited ability of *A. scabripinnis* to explore niches more

distant from the headstream, resulting in greater structuring in this population.

Overall, while a genetic structuring was detected in the three populations of *A. scabripinnis* from Cambé stream, it is not possible to discriminate between types of mechanisms (e.g., selection, genetic drift or bottleneck effects) responsible for this genetic differentiation. The inconsistencies and lack of conclusive results were mostly associated with a lack of reference site, reduced samples size and insufficient knowledge about the life history of the species studied. Thus, future researches should include a more extensive examination of the water quality and of the genetic structure of *A. scabripinnis* populations, including a higher number of fish, inhabiting disturbed and undisturbed streams to get a clear understanding of whether and how the anthropogenic interference along the Cambé stream can affect the genetic structure of this species.

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