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Research Article

Genetic diversity of *Hypostomus ancistroides* (Teleostei, Loricariidae) from an urban stream

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Abstract

In this study, random amplified polymorphic DNA (RAPD) markers were applied to analyze the genetic diversity of samples of the Neotropical catfish *Hypostomus ancistroides*, collected from four sites (S1, S2, S3 and S4) along an urban stream in Southern Brazil. The 11 primers used in RAPD analysis amplified 147 loci, 76 (51.7%) of which were polymorphic. The proportions of polymorphic loci observed in the four samples were: 29.93% (S1), 31.97% (S2), 23.81% (S3) and 38.77% (S4). The average heterozygosity within sampling localities ranged from 0.1230 to 0.1526 and unbiased genetic distances ranged from 0.0253 to 0.0445. AMOVA partitioned 90.85% of the total variation within samples and 9.15% among samples. Excepting for the sample pair S1-S2 ($\phi_{ST} = 0.02784$; $p > 0.05$), all others pairwise ϕ_{ST} values were significantly greater than zero, indicating moderate genetic differentiation among catfish samples from four localities. The relative low levels of genetic variation detected in all groups studied could be related to different factors, including the sedentary habit of these fish, which can be eroding the genetic variation of *H. ancistroides* from each locality.

Key words: Loricariid fish, genetic diversity, RAPD, Siluriformes.

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Introduction

In the last decades the conservation of the genetic diversity has emerged as one of the central issues in conservation biology (Bickham *et al.*, 2000) considering its value for the sustainability of populations (Solé-Cava, 2001; Avise 2004). Simultaneously, advances in molecular techniques increased the availability of different DNA-based markers, which have become efficient tools in conservation genetic studies (Haig, 1998; Sunnucks, 2000; Avise, 2004). Random amplified polymorphic DNA (RAPD) is a simple and straightforward PCR-based technique, which uses arbitrary primers for amplification of discrete regions of genome (Williams *et al.*, 1990). RAPD markers have been used to evaluate the genetic diversity in numerous organisms (Cooper, 2000; Ali *et al.*, 2004; Torezan *et al.*, 2005; Bickel *et al.*, 2006) and studies on genetic conservation of fish populations from South American rivers have successfully applied such markers to access the genetic diversity of different fish species (Almeida *et al.*, 2001, 2003; Dergam *et al.*, 2002; Wasko and Galetti Jr, 2002; Leuzzi *et al.*, 2004; Matoso *et al.*, 2004; Wasko *et al.*, 2004).

Nevertheless, studies of genetic diversity of Neotropical fish populations from urban aquatic ecosystems are still scarce (Sofia *et al.*, 2006). Urban sprawl affects the environment in a myriad of ways at multiple levels of biological organization (Blair, 2004) and considering that cities are typically located near large water bodies, rivers and estuaries, or along coastlines (Melles *et al.*, 2003), more attention should be directed to these aquatic ecosystems in urban areas. Moreover, conservation biologists throughout the world have focused mainly on the protection of “natural” ecosystems, while little importance has been placed on urban areas or urban biodiversity overall (Blair, 1996).

In South American rivers, *Hypostomus ancistroides* (Ihering 1911) is a common fish species in streams of the upper Paraná basin, in South and Southeastern Brazil (Lemes and Garutti, 2002; Shibatta *et al.*, 2002; Casatti, 2005), its occurrence also being reported in urban streams (Oliveira and Bennemann, 2005). Usually, this fish occupies microhabitats composed of rocks and foliage on the bottom of streams and it behaves typically as an algae-eating fish,

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showing grazing habit and nocturnal activity (Lowe-McConnell, 1999; Casatti, 2002). This species belongs to the family Loricariidae (Siluriformes) that includes species ecologically diverse and widely distributed, and commonly known as armored catfishes (Suzuki *et al.*, 2000; Artoni and Bertollo 2001).

Species of *Hypostomus* are benthic fishes, mainly herbivorous or detritivorous (Casatti, 2002), exhibiting non-migratory and nest/brood guard behavior (Suzuki *et al.*, 2000). Such behavioral traits make these fish good candidates for approaches taking into account both genetic variation and genetic structure, since very little gene flow between populations would be expected (Zawadzki *et al.*, 2005). Recently, low levels of genetic variability, assessed by allozyme markers, have been reported for different species of *Hypostomus* (Paiva *et al.*, 2005).

Despite the large distribution of *H. ancistroides* in Brazilian rivers and its significance in the fish assemblage structure of many Brazilian streams (Casatti, 2005; Oliveira and Bennemann, 2005) little is known about the genetic diversity of this Neotropical species. Particularly in urban streams, studies of population genetic diversity of fishes can provide valuable information for outlining of appropriate conservation actions for the management of these threatened ecosystems and their natural populations. In this context the present research used RAPD markers to assess the genetic variation and population structuring of the catfish *H. ancistroides* from a tropical urban stream located in the metropolitan area of Londrina city, Southern Brazil.

Material and Methods

Study area

The Cambé stream and its tributaries constitute the main hydrological basin of Londrina, a city of 500,000 inhabitants, located in the north of the Paraná State, Southern Brazil (Figure 1). This approximately 25 km long stream crosses the entire city, receiving diffuse and point source discharges of industrial, domestic and agricultural wastes along the length of its course (Almeida *et al.*, 2005). *Hypostomus ancistroides* were caught at four sites along the Cambé stream (S1, S2, S3 and S4) located within the urban area of the city of Londrina (Figure 1). Distances between different sampling sites are shown in Table 1.

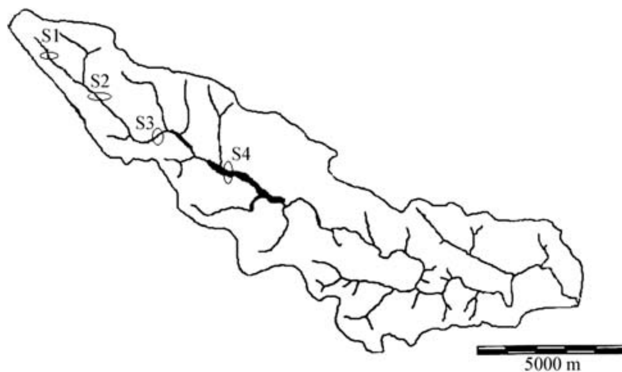

Figure 1 - Location of the study area showing the four sampling sites (S1, S2, S3 and S4) along the Cambé stream, Paraná State, Brazil (Modified from Sofia *et al.*, 2006).

Materials and sampling methods

A total of 46 specimens of *H. ancistroides* were collected using manual fishing tools: 12 at site 1 (S1), 11 at site 2 (S2), 11 at site 3 (S3) and 12 at site 4 (S4). In order to minimize possible biases in the analysis due to the reduced sample size employed, a great effort was made to analyze a large number of loci (Nei, 1978; Kraus and Peakall, 1998).

Fish were brought alive to the laboratory, where they were anaesthetized with benzocaine (0.1 g. L^{-1}), killed by cervical section and then, muscle samples were removed from fish and kept at -20 °C until use. After identification some individuals were preserved in ethanol (70%) as voucher specimens.

DNA extraction and amplification

Genomic DNA from the muscle of fish was sampled according to Almeida *et al.* (2001). DNA concentration was determined in a DyNA Quant 200 fluorometer (Hoefer), using the dye Hoechst 33258, and diluted to a standard DNA concentration of 5 ng μL^{-1}. All isolates were then either used immediately or stored at -20 °C.

Amplifications were performed as described by Sofia *et al.* (2006). Final reaction volumes were 15 µL and contained 15-25 ng of template DNA, 250 µM dNTP (Pharmacia Biotech), 0.25 µM of ten-nucleotide primer (Operon Technologies, Alameda, CA, USA), 4.5 mM of $MgCl_2$ and 1 U of DNA polymerase in the reaction buffer supplied. Since RAPD technique is sensitive to changes in reaction conditions (*e.g.* primer, $MgCl_2$, dNTP concentrations etc; Chiappero and Gardenal 2001), the same exact reaction conditions were used for all samples. For the RAPD analysis, 46 decamer oligonucleotides (from kits OPA, OPAM, OPW) were used as random primers in RAPD screening; 11 oligonucleotides that produced a good number of amplified bands and patterns of reproducible fragments were selected for analyses. 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

Table 1 - Geographic distances between the four sampling sites (S1, S2, S3 and S4).

Sites	S1	S2	S3	S4
S1	***			
S2	0.76 km	***		
S3	3.71 km	2.95 km	***	
S4	6.99 km	6.24 km	3.42 km	***

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 on 1.4% agarose gels with TBE buffer (0.89 mM Tris, 0.89 mM boric acid, 2 mM EDTA pH 8.3) diluted 1:20 (v:v), run at $3V.cm^{-1}$ and stained with ethidium bromide. Agarose gel images were documented under UV light using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 290, and scored visually for band presence and absence.

RAPD analysis

RAPD marker profiles were determined by direct comparison of the amplified electrophoretic profiles of DNA from each individual, and each band was analyzed as a binary variable (band presence or absence). Comparative analyses were carried out by placing all samples from the four sites on the same gel, for intra and inter-sample analyses. Only RAPD bands that could be scored unequivocally were considered in the analyses.

The RAPD technique generally produces dominant multilocus markers, but can also generate co-dominant markers which most probably arise from small insertions or deletions inside the amplified region (Williams *et al.*, 1990). Statistical methods developed for co-dominant markers have been modified for use with RAPD markers. For the purpose of this study, each locus was treated as a two-allele system, with only one of the alleles per locus being amplifiable by PCR. It was also assumed that marker alleles from different loci did not comigrate to the same position on a gel, and that populations were under the Hardy-Weinberg equilibrium (Lynch and Milligan, 1994). Also, to avoid distortion of results due to problems regarding reproducibility (Williams *et al.*, 1990), only reproducible patterns were included in the data analysis. In the present study, reproducibility of the results was tested through additional RAPD reactions and no variation was found.

The TFPGA 1.3 software (Miller, 1997) was used in the following analyses: i) genetic variability estimated by the proportion of polymorphic loci ($\overline{P}$), using the 95% cut-off criterion; ii) average heterozygosity (*He*); iii) genetic diversity determined using the Shannon's index (I_{Sh}); iv) unbiased genetic distance and identity (Nei, 1978); v) a dendrogram based on the genetic distance-values between four sample pairs and a bootstrap analysis (1000 permutations) to test the robustness of the produced branches.

To evaluate the apportioning of genetic variation within and among sampling localities, an analysis of molecular variance (AMOVA) was performed using the software Arlequin version 3.0 (Excoffier *et al.*, 2005), which is based on the original AMOVA adapted for dominant data (Stewart and Excoffier, 1996). Variance components based on a matrix of squared Euclidean distances obtained from RAPD phenotypes were tested statistically by nonparametric permutational procedures using 1000 permutations. Arlequin 3.0 was also used to estimate the genetic diversity based on the index of nucleotide diversity (π_n), which is the probability that two randomly chosen homologous nucleotides are different (Excoffier *et al.*, 2005). To examine the relationship between genetic distance and the ϕ_{ST} values found for different sample pairs, the Spearman coefficient was used.

Finally, RAPD data were analyzed using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) package (Rohlf, 2000) to determine the similarities among individuals. Similarities were estimated using the Jaccard's (*J*) coefficient.

Results

The 11 primers used for the *H. ancistroides* RAPD analysis reproducibly amplified 147 loci, 76 (51.7%) of which were polymorphic. The result obtained with one of these primers, OP-W18, is shown in Figure 2. The number of fragments per primer ranged from 6 for OP-W6 to 24 for OP-AM9 with a mean number of 13.36 bands per primer.

Table 2 shows the different estimates of genetic variation obtained for the samples of *H. ancistroides* from sites S1, S2, S3 and S4 at Cambé stream. The lowest measure of genetic variation was found for fish from site 3. All measures of genetic variation used corroborated in detecting the lowest estimation of genetic variation for sample from site 3. Except for nucleotide diversity (π_n), the other three estimates indicated the highest level of genetic variation for sample from site 4: $\overline{P}$ (38.77%), average *He* (0.1526) and I_{Sh} (0.2230).

Coefficients of variation for errors associated with genetic similarity among *H. ancistroides* genotypes, estimated by Jaccard coefficient, were calculated for different numbers of RAPD markers (Coelho, 2000). In the array between 95 and 147 amplified fragments, the coefficients of variation for errors showed little variation, ranging from 4.98 to 3.97% (Figure 3), indicating that further increase in the number of markers probably would not result in an important reduction in the coefficient of variation. Additionally, values of coefficients of variation below 5% can be considered acceptable for this type of analysis.

Genetic similarity among all individuals estimated by Jaccard's coefficient varied from 0.722 (individuals 12 from site 1, and 3 from site 2) to 0.951 (individuals 7 and 8 from site 1). At site 1, the values of similarity among pairs of individuals ranged from 0.724 to 0.951; at site 2, from 0.763 to 0.933; at site 3, from 0.830 to 0.944, and at site 4, from 0.761 to 0.902. Mean genetic similarity and standard deviation (SD) of the samples from sites 1, 2, 3 and 4 were, respectively: 0.860 (SD = 0.045), 0.841 (SD = 0.036),

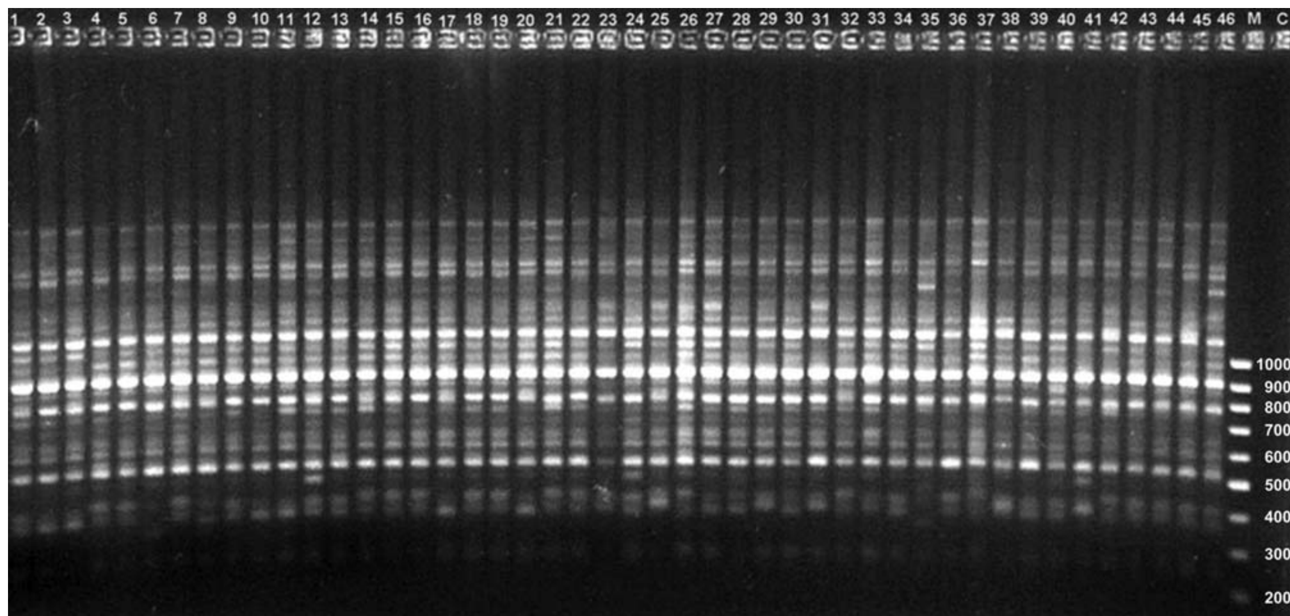

Figure 2 - RAPD profiles, amplified with primer OP-W18, of 46 *H. ancistroides* collected from sites S1 to S4. Column M, molecular weight marker (Biotools) in bp; Column C, control; columns 1-12, fish collected at site 1; columns 13-23, fish collected at site 2; columns 24-34, fish collected at site 3; columns 35-46, fish collected at site 4.

Table 2 - Proportion of polymorphic loci ($\overline{P}$), average heterozygosity (*He*), Shannon's diversity index (I_{Sh}) and nucleotide diversity (π_n) for the samples of *H. ancistroides* from sites S1, S2, S3 and S4 on the Cambé stream; ( ) = standard deviation.

Samples	$\overline{P}$	*He*	I_{Sh}	π_n
S1	29.93%	0.1230	0.1797 (± 0.2714)	0.1251 (0.0690)
S2	31.97%	0.1398	0.2038 (± 0.2853)	0.1379 (0.0759)
S3	23.81%	0.1022	0.1488 (± 0.2584)	0.0906 (0.0502)
S4	38.77%	0.1526	0.2230 (± 0.2835)	0.1270 (0.0700)

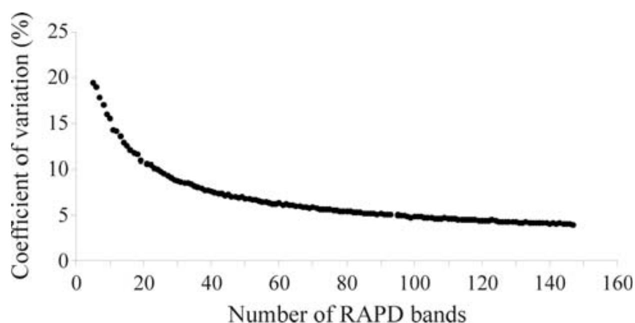

Figure 3 - Relationship between different number of RAPD bands and coefficients of variation (%) for errors associated with genetic similarity among *H. ancistroides* genotypes, based on Jaccard coefficient.

0.894 (SD = 0.021) and 0.804 (SD = 0.033), while the mean values of genetic similarities between pairs of samples were: S1-S2 = 0.845 (SD = 0.039); S1-S3 = 0.861 (SD = 0.039); S1-S4 = 0.833 (SD = 0.042); S2-S3 = 0.856 (SD = 0.030); S2-S4 = 0.826 (SD = 0.032), and S3-S4 = 0.852 (SD = 0.028).

Estimates of Nei's (1978) pairwise unbiased genetic distances for *H. ancistroides* ranged from 0.0253 to 0.0445 (Table 3), and unbiased genetic identity varied from 0.9565 to 0.9750. The highest value of genetic distance (0.0445) was found between fish from sites 2 and 3.

AMOVA partitioned 90.85% of the total variation within sampling localities, and 9.15% (ϕ_{ST} = 0.09153) among samples (Table 3). Except for the pairwise S1-S2 (ϕ_{ST} = 0.02784; $p > 0.05$), the pairwise ϕ_{ST} values were all significantly greater than zero at $p < 0.0001$ (Table 4), indicating some divergence between different sample pairs of *H ancistroides* analyzed. Cluster analysis based on the genetic distance (Nei, 1978) among *H. ancistroides* from four localities grouped sites S1 and S2 (0.0253), and sites S3 and S4 (0.0330) together, supported by bootstrap values of 83.1% and 59.8%, respectively (Figure 4). The comparison of levels of genetic distance with the ϕ_{ST} differences between sample pairs showed no significant relationship between these genetic parameters (r_S = -0.143; $p > 0.05$).

Discussion

In a recent review Ali *et al.* (2004) emphasized the applicability of RAPDs for population studies of fish, their effectiveness in detecting polymorphism between and among different fish populations, and the establishment of genetic relationships among fish populations. In the current study, the values of $\overline{P}$ observed in *H. ancistroides* ranged from 23.81% to 38.77%; these values were lower than those detected for a number of Neotropical fish species from the Paraná River basin which showed values of $\overline{P}$ estimated by RAPD technique from 51.9 to 75.0% (Almeida *et al.*, 2001,

Table 3 - Analysis of molecular variance (AMOVA) using 147 RAPD loci, partitioning genetic variation within and between samples of *H. ancistroides* from four sites on the Cambé stream.

Source of variation	df	Sum of squares	Variance components	% of variation
Among samples	3	32.579	0.50704	9.15
Within samples	42	211.356	5.03229	90.85
Total	45	243.935	5.53933	

Fixation index - ϕ_{ST}: 0.09153.

Table 4 - Estimation of Nei's (1978) unbiased genetic distance (*D*) and identity (*I*), with Lynch and Milligan (1994) correction, between pairs of *H. ancistroides* from four different sites (S1, S2, S3 and S4) on the Cambé stream. AMOVA (ϕ_{ST}) pairwise differences and p-value are also shown.

Pairwise samples	*D*	*I*	ϕ_{ST} pairwise difference	p-value
S1-S2	0.0253	0.9750	0.02784	> 0.05
S1-S3	0.0394	0.9614	0.10546	< 0.0001
S1-S4	0.0351	0.9655	0.11943	< 0.0001
S2-S3	0.0445	0.9565	0.05765	< 0.0001
S2-S4	0.0409	0.9599	0.10321	< 0.0001
S3-S4	0.0330	0.9676	0.12425	< 0.0001

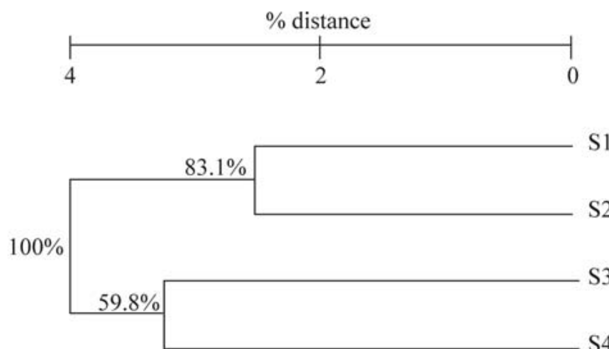

Figure 4 - Dendrogram of genetic dissimilarity (%), based on Nei's (1978) genetic distance, for *H. ancistroides* populations from sites S1, S2, S3 and S4 on the Cambé stream. Numbers at the nodes represent bootstrap generated by 1000 replications.

2003; Leuzzi *et al.*, 2004; Sofia *et al.*, 2006). Although it has been suggested that the forces that regulate the levels of genetic variability of loricariids inhabiting small streams are variable and complex (Zawadzki *et al.*, 2004), the low levels of genetic variation found for this species at Cambé stream could be reflecting, to some extent, an endogamic processes among individuals from each locality since *H. ancistroides* shows a sedentary habit. Also, some authors have attributed low levels of genetic variation in aquatic organisms to the disturbed conditions of streams (Foré *et al.*, 1995; Bickham *et al.*, 2000; Belfiore and Anderson, 2001). In fact, Winkaler *et al.* (2001) showed that wild fish from the upper reaches of Cambé stream present impaired health and Lemos *et al.* (2005) demonstrated by the comet assay that its water induces DNA damage. More recently, Camargo and Martinez (2006) showed that the Neotropical fish *Prochilodus lineatus* confined in these areas presented functional alterations in response to water contamination. However, the present results do not allow drawing any conclusions about this fact, since no reference population was surveyed in this study.

An allozyme genetic variability analysis has revealed the occurrence of a wide variation in heterozygosity (*He*) values for populations of different *Hypostomus* species (Zawadzki *et al.*, 2005). Actually, while low *He* values (from zero to 0.028) have been observed for populations of three species of *Hypostomus* (Paiva *et al.*, 2005), relatively higher levels of heterozygosity (*He* = 0.082 and 0.052) were reported for populations of *H. ancistroides* from Itaipu Reservoir and Corumbá River (Zawadzki *et al.*, 2005). The levels of *He* observed in all samples of *H. ancistroides* from Cambé stream were even higher (0.1022 to 0.1526) than those mentioned by Zawadzki *et al.* (2005). However, this discepancy could be partially explained by the inherent differences between RAPD and allozyme techniques, since allozymes detect only about 1/3 of actual amino acid variation and do not detect synonymous changes, while RAPDs have the potential to detect practically all changes, independent of whether they cause amino acid changes or not.

In this study, the values of genetic similarity estimated between pairs of catfish from the same site, as well as between sample pairs of catfish from different sites were elevated (0.722 to 0.951). These high values of similarities might be reflecting the low levels of genetic variation showed by different catfish populations analyzed here.

According to Wright (1978), theoretical values of F_{ST} ranging from 0.05 to 0.15 are indicative of moderate genetic differentiation between pairs of populations. On the basis of the ϕ_{ST} estimates and at the level of sampling attained in this study, except for the samples of *H. ancistroides* from sites S1 and S2 which did not show differentiation between them (ϕ_{ST} = 0.02784; p > 0.05), all other sample pairs were significantly divergent among each other, showing moderate genetic differentiation values (Table 4). Apparently, these findings are in accordance with the prediction of Zawadzki *et al.* (2005) who pointed out that limited gene flow would be expected between populations of *Hypostomus* because of their sedentary habit.

The highest value of Nei's unbiased genetic distance (Nei, 1978) found between the pairwise comparison S2-S3 did not coincide with the ϕ_{ST} estimates which was the lowest significant value of ϕ_{ST} detected (Table 4). However, this discrepancy between two parameters could be due to intrinsic differences in these genetic estimates. Considering that RAPDs are generally dominant markers, this characteristic prevents direct estimations of allele frequency, and can bias calculations of genetic diversity and population differentiation (Lynch and Milligan, 1994; Isabel *et al.*,

1999). Thus, the analysis of molecular variance (AMOVA) which is not influenced by the dominant nature of RAPD, has been chosen by several authors for investigating the partitioning of dominant marker variation (Ross *et al.*, 1999; Cooper, 2000; Díaz *et al.*, 2001).

The expansion of urban areas worldwide makes our understanding of aquatic ecosystems in urban environments extremely important. Genetic approaches offer powerful tools for examining the current status of populations, for inferring the history of population changes, and for anticipating future population directions (Belfiore and Anderson, 2001). Thus, the present results may well contribute to future management of the populations of *H. ancistroides* from Cambé stream.

Acknowledgments

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