Genetics and Molecular Research

Fine-scale genetic structure patterns in two freshwater fish species, *Geophagus brasiliensis* (Osteichthyes, Cichlidae) and *Astyanax altiparanae* (Osteichthyes, Characidae) throughout a Neotropical stream

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ABSTRACT. Streams are very important environments for Neotropical freshwater fish fauna, and possess a high number of species. These small drainages are also highlighted by their intrinsic biological and physicochemical features; however, knowledge on the genetic distribution of fish in these drainages is limited. Therefore, in the present study, RAPD (random amplified polymorphic DNA) and microsatellite markers were used to analyze population differentiation and gene flow

of *Astyanax altiparanae* and *Geophagus brasiliensis* from three sites (high, medium, and low) throughout the Penacho stream (about 32 km long), which is a Neotropical stream. Both markers revealed higher levels of genetic diversity levels for *A. altiparanae* ($\overline{P}$: 90.05; H_S: 0.350) compared to *G. brasiliensis* ($\overline{P}$: 30.43; H_S: 0.118), which may be related to the particular biology of each species. AMOVA revealed significant genetic variation among populations of each species. All pairwise Φ_{ST} values were significant, ranging from 0.020 to 0.056 for *A. altiparanae* samples, and from 0.065 to 0.190 for *G. brasiliensis* samples. Bayesian clustering analysis corroborated these results and revealed clusters of both *A. altiparanae* (two based on RAPD data) and *G. brasiliensis* (two based on RAPD data and three on microsatellite data). Gene flow estimates showed that there were similar rates of migration among *A. altiparanae* samples and low rates of migration among some *G. brasiliensis* samples. These results suggest patterns of fine-scale genetic structure for both species in the Penacho stream. This information may enhance knowledge of Neotropical streams and may be useful for future management and conservation activities.

Key words: RAPD; Microsatellite; *Astyanax altiparanae*; *Geophagus brasiliensis*; South America

INTRODUCTION

Streams are responsible for a large proportion of Neotropical freshwater fish diversity (Castro et al., 2003). However, these drainages also show high environmental sensitivity, mainly due to their low water volumes and their fragile ichthyofauna, which is composed mostly of small fishes (Luiz et al., 1998). Despite their small size, Neotropical streams show great diversification in their spatial and temporal dimensions, which help to maintain variations in the local microhabitats and stratification of regional faunas (Winemiller et al., 2008). They are also being constantly altered by anthropogenic impact, which prevents longitudinal, lateral, or vertical connectivity throughout the flow (Vrijenhoek, 1998). Considering the importance of these drainages, several studies have investigated fish diversity, species distribution patterns, and anthropogenic interferences in Neotropical streams (Castro et al., 2003; Costa et al., 2013). However, few studies have investigated population genetics in fish streams (Sofia et al., 2006, 2008), revealing a gap in knowledge on Neotropical hydrographic systems.

Esteves and Aranha (1999) noted that due to specific biological and physicochemical features, most of the knowledge obtained in larger rivers cannot be extrapolated to streams. Indeed, studies conducted in stream fishes from the North hemisphere have revealed important information about the patterns of fine-scale genetic structure (Carlsson et al., 1999). However, comparisons between temperate and Neotropical streams are difficult, mainly because they have many distinct features, including species composition, riparian vegetation type, water temperature, precipitation patterns, and evolutionary histories (Boulton et al., 2008).

Interestingly, findings from a limited number of population genetic studies conducted on Neotropical stream fishes also suggest fine-scale genetic structure (Sofia et al., 2006, 2008). Indeed, knowledge on the genetics of species, such as levels of genetic diversity, population

differentiation, and rates of gene flow, are essential for understanding and conserving freshwater ecosystems (Geist, 2011). However, the limited number of such studies highlights the need to obtain more genetic information, involving different species, habitat features, and anthropogenic interferences.

The Paraná River basin is the second largest drainage in South America and one of the most strongly impacted by anthropogenic activities (Agostinho et al., 2008). The fish fauna composition of its streams is one of the best studied (Castro et al., 2003; Costa et al., 2013), with similar patterns of species distribution observed in different drainages. Species of different orders, such as *Astyanax* genus (Characiforms), *Hypostomus* genus (Siluriformes), and *Geophagus* genus (Perciformes), are among the most common and widely distributed in these streams (Castro et al., 2003; Costa et al., 2013). Thus, population genetics could be simultaneously studied in different species, allowing distinctions to be made among general aspects of genetic distribution and, in particular, of each species.

Astyanax altiparanae and *Geophagus brasiliensis* are frequently found in streams of the upper Paraná River basin (Castro et al., 2003; Costa et al., 2013). *A. altiparanae* (a species of lambari) prefers marginal environments and surface lotic stretches, making short migrations during the reproductive period without parental care (Suzuki et al., 2002). Conversely, *G. brasiliensis* is a sedentary species and it is found in greater abundance in lentic and benthonic habitats. During the reproductive period, individuals of this species form couples, build nests, and exhibit parental care (Kullander, 2003).

Molecular markers, especially those derived from PCR, have contributed greatly to population genetic studies of Neotropical fish fauna (Pamponet et al., 2008; Garcez et al., 2011; Ferreira et al., 2015). Therefore, the aim of the present study was to use microsatellite and RAPD (random amplified polymorphic DNA) markers to analyze population genetics of two fish species, *A. altiparanae* and *G. brasiliensis*, which are distributed throughout the Penacho stream, Upper Paraná River. Thus, this study sought to obtain information that may contribute to knowledge on the genetic distribution of fishes in Neotropical small drainages.

MATERIAL AND METHODS

Study sites and samplings

The Penacho stream is a small drainage of the upper Paraná River basin, which is located in the third plateau of Paraná State (Brazil), and is about 32 km in length. Its source and mouth are in the municipality of Ribeirão do Pinhal, flowing into the Laranjinha River (Figure 1).

The Laranjinha River is the main tributary of the Cinzas River basin, and the latter comprises one of the major watersheds of the Paranapanema River, upper Paraná River. Over time, the Pehacho stream has suffered various anthropogenic interferences, including deforestation of riparian vegetation, contamination by domestic sewage, and the constant use of their margins for agriculture and livestock activities (Costa et al., 2013).

A total of 33 fish species were registered along the Penacho stream, belonging to 7 orders and 12 families, highlighting the wide distribution of *A. altiparanae* and *G. brasiliensis* (Costa et al., 2013). Thus, individuals of both species were sampled in three equidistant sites (16 km) along the Penacho stream: upper (UPE - 23°22'33.0'S, 50°19'45.8"W), middle (MPE - 23°23'47.65"S, 50°22'53.17"W) and lower (LPE - 23°23'35.4"S and 050°25'34.6"W) sections.

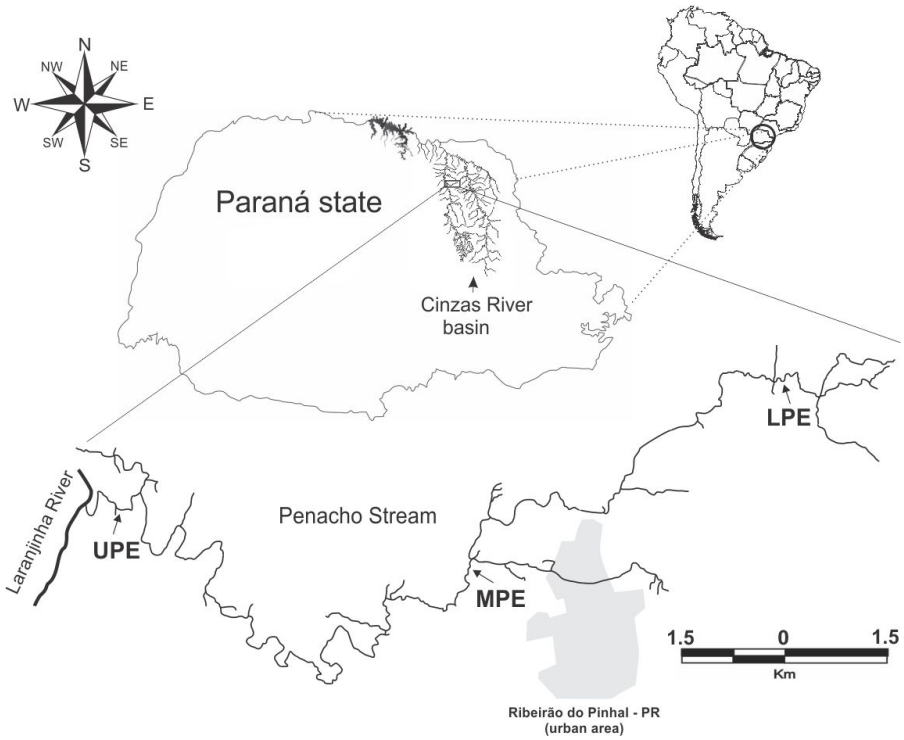

Figure 1. Map of the Penacho stream in Paraná State, Brazil, showing *Astyanax altiparanae* and *Geophagus brasiliensis* sampling sites. UPE, MPE, LPE = upper, middle, lower Penacho Stream, respectively.

From February 2009 to March 2010, seven bimonthly samplings were performed at each site under license No. 23360/IAP (Instituto Ambiental do Paraná). Sampling and catching were performed in a similar way at each site, following the methodology used by Costa et al. (2013). Next, samples of muscle and rayed fin were obtained and stored in microtubes containing 70% alcohol at -20°C. In total, 168 individuals were used in genetic analyses, including 86 *A. altiparanae* and 82 *G. brasiliensis* individuals. Some specimens were deposited in the Londrina State University Zoology Museum (MZUEL) fish collection, under catalog numbers: MZUEL 6451 (*G. brasiliensis*) and MZUEL 6453 (*A. altiparanae*).

DNA extraction and quantification

Genomic DNA was obtained from fin and muscle tissues used an NaCl protocol as previously described (Bardakci and Skibinski, 1994). Samples were quantified using a fluorimeter (Qubit™ - Invitrogen, Carlsbad, CA, USA) and diluted to 5 ng/μL.

Microsatellite markers

Seven polymorphic loci were used for *A. altiparanae*; Asty15, Asty26, Asty23, Asty24, Asty04, Asty16, and Asty12 (Zaganini et al., 2012) and eight were used for *G. brasiliensis*;

Gbra16, Gbra17, Gbra21, Gbra25, Gbra47, Gbra55, Gbra62, and Gbra63 (Ferreira et al., 2013). For *A. altiparanae*, 86 samples were used for genotyping (33 at UPE, 25 at MPE, and 28 at LPE), along with 82 *G. brasiliensis* samples (29 at UPE, 23 at MPE, and 30 at LPE).

For genotyping on an automated sequencer, the forward primer of each locus was prepared according to the method described by Schuelke (2000), which allow the labeling of PCR products with fluorescent molecules (FAM, HEX, NED, or PET). PCR was performed as previously described (Ferreira et al., 2013) with specific annealing temperatures as described for each primer (Zaganini et al., 2012; Ferreira et al., 2013). The electrophoresis of PCR products was performed in an automated sequencer, ABI PRISM 3500-XL (Applied Biosystems, Foster City, CA, USA), using the GeneScan 600 Liz (Applied Biosystems) molecular weight marker. Genotypes were determined manually using the GeneMarker 1.85 software (Soft Genetics, State College, PA, USA).

RAPD markers

To analyze both species, 20 primers were tested from OPD, OPW, OPA, OPD, and OPB kits (Operon Technologies Inc., Alameda, CA, USA), and primers that showed consistent patterns, high intensity, and high numbers of fragments were selected for further analysis. Equal numbers of specimens were analyzed for both *A. altiparanae* (25 at UPE, 25 at MPE, and 25 at LPE) and *G. brasiliensis* (25 at UPE, 25 at MPE, and 25 at LPE).

PCR was performed in a final volume of 15 µL, containing 15 ng DNA, 0.33 µM primer, 3.3 mM $MgCl_2$, 1 U Taq Polymerase (Invitrogen), 1X reaction buffer, 250 µM dNTP (Amresco), and ultrapure water. All reactions were accompanied by a negative control. Only reproducible electrophoretic patterns were analyzed. Amplification was performed in a Peltier Thermo-Cycler PCT-100 with the following program: 4 min at 96°C; 40 cycles of 40 s at 92°C, 1.5 min at 40°C, and 2 min at 72°C, and a final extension of 5 min at 72°C. The PCR products were separated by electrophoresis on a 1.4% agarose gel (0.89 M Tris, 1 mM EDTA, 0.89 M boric acid, pH 8.3) run at 3 V/cm, and visualized by ethidium bromide staining and UV illumination. Images were photographed with a digital system Canon Power Shot A 620 with a 7.1-megapixel resolution and the LPix Image software. In this way, the electrophoretic patterns were analyzed as binary variants and transcribed to a binary matrix for computational analysis.

Genetic analyses

Genetic diversity and demographic analyses

Genetic diversity from RAPD markers was determined using dBoot v. 1. 1 (Coelho, 2001) to assess the coefficient of variation (CV%) of the number of amplified markers, providing a reliability parameter at the level of 5%, and PopGen v. 1.31 (Yeh et al., 2000), to calculate the proportion of polymorphic loci ($\overline{P}$) and Nei's genetic diversity (H_S). Genetic diversity based on microsatellite markers was estimated using the PopGen v. 1.31 program (Yeh et al., 2000) to calculate the number of alleles (A), average number of alleles per loci (N_A), effective alleles (N_E), private alleles (N_P), observed heterozygosity (H_O), and expected heterozygosity (H_E). The Fstat v2.9.3 program (Goudet, 2001) was used to calculate the allelic richness (R_A), and inbreeding index (F_{IS}), and GenePop v.1.2 (Raymond and Rousset, 1995) was used to test for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium. P values were subsequently adjusted by sequential Bonferroni correction.

The BOTTLENECK 1.2.02 software (Piry et al., 1999) was used to detect genetic bottleneck signatures from each population using two tests: Wilcoxon sign-rank test, which detect recent bottlenecks from excess heterozygosity in the following microsatellite evolution models; Infinite Allele Model (IAM), Stepwise Mutation Model (SMM), and Two-Phase Model (TPM - with 90% SMM and 10% IAM), considering a P value of <0.05. A second test, the mode shift test, identifies signs of recent bottlenecks from changes in the distribution of allele frequencies.

Population structure and gene flow

Analysis of molecular variance (AMOVA) was performed in Arlequin 3.11 (Excoffier et al., 2007) for both markers. Using the same program, pairwise Φ_{ST} values among populations were obtained. Significance estimates were conducted based on 10,000 permutations. Correlations between geographic distance and genetic differentiation, comparing the linearized Φ_{ST} values and geographic distances between the sites studied, were conducted from a Mantel test, using the TFPGA v.1.3 program (Miller, 1997).

Bayesian clustering analysis was performed for both markers using Structure v.2.3.3 (Pritchard et al., 2000). For this, we used the admixture model, which assumes that individuals may have ancestries mixed with genotypic contributions from different populations (Pritchard et al., 2000). Estimates of *K* (number of clusters) were obtained from simulations carried out by varying *K* from one to six (*K* = 1-6), running 20 replicates for each value of *K*, according to the method described by Evanno et al. (2005). Each replicate was implemented by running 10,000 Markov Chain Monte Carlo (MCMC) iterations discarded as burn-in, followed by 100,000 MCMC. Structure Harvester 0.6.7 (Earl and VonHoldt, 2011) was used to coordinate the results and infer the most likely number of clusters (*K*) from Δ*K* statistics (Evanno et al., 2005).

Gene flow estimates were performed for the microsatellite data from a Bayesian analysis using the BayesAss 1.3 program (Wilson and Rannala, 2003). This analysis determined recent migration rates and the direction of gene flow.

RESULTS

Genetic diversity and demographic analyses

A. altiparanae: RAPD marker

A total of 171 loci were obtained from eight RAPD primers (OPD-3, OPW-3, OPW-4, OPW-5, OPW-6, OPW-7, OPW-9, and OPW-19). The number of loci analyzed was sufficient, showing a CV% curve stable below 5%, indicating that the results had high reliability. The lowest values for the proportion of polymorphic loci ($\overline{P}$ = 84.21%) and Nei's genetic diversity (H_S = 0.338) were obtained for the MPE population, while the highest values were found in the LPE ($\overline{P}$ = 90.05%) and UPE (H_S = 0.350) populations (Table 1).

A. altiparanae: microsatellite markers

In the microsatellite analyses (SSR - simple sequence repeats), 132 alleles were obtained (a mean number of 87.6 per population). The *A* per sample ranged from 80 (MPE) to

94 (LPE). The lowest H_O was obtained for MPE (0.690) while the highest was found for UPE (0.698). H_E values ranged from 0.809 (UPE) to 0.852 (LPE). The highest mean N_A = 13.429, N_E = 7.709, and R_A = 12.523) were obtained for the LPE sample while the lowest values for these indices were obtained for the MPE (R_A = 11.111 and (N_A = 11.429) and UPE (N_E = 6.623) samples. The N_P ranged from 8 (MPE) to 25 (LPE) (Table 1).

Table 1. Genetic diversity of *Astyanax altiparanae* and *Geophagus brasiliensis* at three sites of the Penacho stream based on RAPD (random amplified polymorphic DNA) and microsatellite markers.

Species		Microsatellites										RAPD	
	Local	N	*A*	R_A	$\bar{N}_p$	$\bar{N}_A$	$\bar{N}_E$	H_O	H_E	F_{IS}	*N*	$\bar{P}$	H_S
A. altiparanae													
	UPE	33	89	11.409	22	12.714	6.623	0.698	0.809	0.112*	25	88.30%	0.350
	MPE	25	80	11.111	8	11.429	6.386	0.690	0.839	0.185*	25	84.21%	0.338
	LPE	28	94	12.523	25	13.429	7.709	0.691	0.852	0.138*	25	90.05%	0.345
G. brasiliensis													
	UPE	29	36	4.361	2	4.500	3.124	0.490	0.578	0.141*	25	20.86%	0.086
	MPE	23	48	5.939	6	6.000	4.221	0.581	0.729	0.167*	25	30.43%	0.117
	LPE	30	44	5.288	8	5.500	3.759	0.665	0.717	0.081	25	28.69%	0.118

*Significant values F_{IS} ($P \leq 0.05$). *N* - number of individuals analyzed, $\bar{P}$ - proportion of polymorphic loci, H_S - Nei's genetic diversity, *A* - total number of alleles found per population, R_A - allelic richness, N_p - number of private alleles, $\bar{N}_A$ - number of alleles, $\bar{N}_E$ - number of effective alleles, H_O - observed heterozygosity, H_E - expected heterozygosity, F_{IS} - index of inbreeding. UPE, MPE, LPE = upper, middle, lower Penacho Stream, respectively.

After applying the sequential Bonferroni correction, four loci in UPE (Asty15, Asty26, Asty23, and Asty04), two loci in MPE (Asty24 and Asty04), and four loci in LPE (Asty15, Asty24, Asty4, and Asty12) showed significant Hardy-Weinberg disequilibrium. Significant linkage disequilibrium was detected in the MPE sample between the Asty15 and Asty12 loci. All F_{IS} values were significant, and ranged from 0.112 (UPE) to 0.185 (MPE) (Table 1).

G. brasiliensis: RAPD marker

In total, 115 loci were obtained from eight RAPD primers (OPA07, OPB04, OPD02, OPD08, OPD10, OPD11, OPD18, and OPW20. The CV% curve was also stable below 5% for this number of loci analyzed. The proportion of $\bar{P}$ ranged from 20.86% (UPE) to 30.43% (MPE). The lowest value for H_S was obtained for UPE (0.086) whiles the highest was obtained for LPE (0.118) (Table 1).

G. brasiliensis: microsatellite marker

Sixty-one alleles were found, ranging from 36 (UPE) to 48 (MPE) with a mean number of 42.6 per population. The lowest H_O and H_E values were obtained for UPE (H_O = 0.490 and H_E = 0.578), while the highest values were obtained for LPE (H_O = 0.665) and MPE (H_E = 0.729). The highest mean N_A = 6.000, N_E = 4.221, and R_A = 5.939 were obtained for the MPE population, while the lowest values for these indices were obtained for the UPE (N_A = 4.500, N_E = 3.124, and R_A = 4.361) populations.

A sequential Bonferroni correction was performed on the results of the HWE test and significant deviations were found at three loci in the UPE (Gbra25, Gbra62, and

Gbra55) and three loci in MPE (Gbra25, Gbra55, and Gbra21) populations. Significant linkage disequilibrium was detected at one loci combination in UPE (Gbra25-Gbra62), one combination in MPE (Gbra25-Gbra55), and two combinations in LPE (Gbra63-Gbra17 and Gbra47-Gbra63). Significant F_{IS} values were obtained for all samples. The MPE population showed the highest value (0.167) and LPE showed the lowest value (0.081) (Table 1).

Analysis of microsatellites using BOTTLENECK failed to detect any distortion of allele frequencies in the mode shift test (the distribution was L-shaped in all samples). However, significant heterozygote excess (bottleneck signal) was observed for *G. brasiliensis* in the MPE and UPE populations, as determined using the IAM and TPM models in the Wilcoxon sign-rank test (Table 2).

Table 2. Results of bottleneck tests for seven microsatellite loci in *Astyanax altiparanae* and eight in *Geophagus brasiliensis*.

Samples	N	Wilcoxon sign-rank test						Frequency distribution of alleles
		IAM[a]		TPM[b]		SMM[c]		
		H_D/H_E	$\bar{P}$	H_D/H_E	$\bar{P}$	H_D/H_E	$\bar{P}$	
A. altiparanae								
UPE	33	1/6	0.148	4/3	0.812	7/0	1.000	L-shaped
MPE	25	1/6	0.148	4/3	0.765	6/1	0.980	L-shaped
LPE	28	1/6	0.054	6/1	0.960	6/1	0.972	L-shaped
G. brasiliensis								
UPE	29	1/7	0.097	2/6	0.125	2/6	0.156	L-shaped
MPE	23	1/7	0.003[c]*	2/6	0.039[c]*	2/6	0.156	L-shaped
LPE	30	0/8	0.001[c]*	2/6	0.013[c]*	2/6	0.156	L-shaped

*Significant values under the Sign/Wilcoxon sign-rank tests ($P < 0.05$). Wilcoxon sign-rank tests for excess heterozygosity, and mode shift test for patterns of allele frequency distribution. N - number of individuals analyzed, H_E - number of loci showing heterozygosity excess, H_D - number of loci showing heterozygosity deficiency, $\bar{P}$ - proportion of polymorphic loci. Normal L-shaped distribution - non-bottlenecked population. [a]Infinite Allele Model (IAM); [b]Two Phase Model (TPM) (90% SSM); [c]Stepwise Mutation Model (SSM). UPE, MPE, LPE = upper, middle, lower Penacho Stream, respectively.

Genetic structure and gene flow

Results of AMOVA for the two markers revealed that most of the genetic variation was within populations (87.49-97.90%) for both *A. altiparanae* and *G. brasiliensis*. However, significant genetic variation was also obtained among populations. RAPD and microsatellite markers revealed 3.23% and 2.09% variation among *A. altiparanae* populations, respectively. For *G. brasiliensis*, 10.82% (RAPD) and 12.51% (SSR) variation was observed among populations (Table 3).

All pairwise Φ_{ST} values were significant. For *A. altiparanae* Φ_{ST} values ranged from 0.020 (MPE x LPE - SSR) to 0.056 (UPE x LPE - RAPD), while Φ_{ST} values for *G. brasiliensis* ranged from 0.065 (MPE x LPE - RAPD) to 0.190 (UPE x MPE - SSR) (Table 4).

Bayesian clustering analysis (Structure) using the data obtained for *A. altiparanae* from RAPD and SSR markers, indicated from ΔK that the most probable cluster number was $K = 2$ (Figure 2A). Graphic representation of microsatellite data does not show a well-defined cluster. However, graphic representation of the RAPD data showed two probable clusters (Figure 2B).

In *G. brasiliensis*, ΔK indicated $K = 2$ for RAPD data and $K = 3$ for microsatellite data (Figure 2C). Three probable clusters can be seen in the graphic representation of microsatellite data. On the other hand, for the RAPD data, the UPE population formed a different cluster to the other samples (MPE and LPE) (Figure 2D).

Table 3. Analysis of molecular variance (AMOVA) for *Astyanax altiparanae* and *Geophagus brasiliensis* populations studied in three sites of the Penacho stream, showing the percentage variation among and within populations for the two molecular markers used.

Source of variation	Percentage of variation			
	G. brasiliensis		*A. altiparanae*	
	SSR	RAPD	SSR	RAPD
Among populations	12.51*	10.82*	2.09*	3.23*
Within populations	87.49	89.68	97.90	96.77

*Significant values ($P < 0.05$).

Table 4. Pairwise genetic differentiation (Φ_{ST}) between samples from three sites studied along the Penacho stream.

	Geophagus brasiliensis				*Astyanax altiparanae*		
	UPE	MPE	LPE		UPE	MPE	LPE
UPE	-	0.135*	0.158*	UPE	-	0.024*	0.056*
MPE	0.190*	-	0.065*	MPE	0.021*	-	0.025*
LPE	0,110*	0.079*	-	LPE	0.046*	0.020*	-

*Significant values ($P < 0.05$). Above diagonal - pairwise Φ_{ST} from RAPD (random amplified polymorphic DNA) data. Below diagonal - pairwise Φ_{ST} from microsatellite data. UPE, MPE, LPE = upper, middle, lower Penacho Stream, respectively.

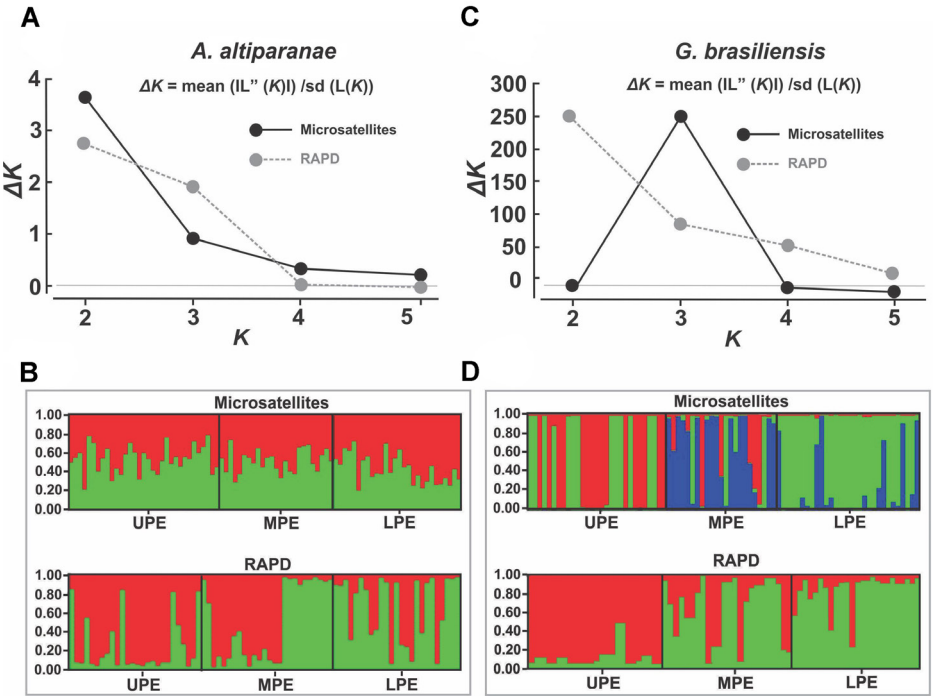

Figure 2. Results of Bayesian analysis (Structure). Number of *K* groups based on the Δ*K* statistic of Evanno et al. (2005) for *Astyanax altiparanae* (**A**) and *Geophagus brasiliensis* (**C**). Graphical representations of results obtained using RAPD and microsatellite markers for *A. altiparanae* (**B**) and *G. brasiliensis* (**D**). Each column represents a different individual and the colors represent the probability membership coefficient of that individual for each genetic cluster. UPE, MPE, LPE = upper, middle, lower Penacho Stream, respectively.

Gene flow estimates based on microsatellite data indicated similar migration rates among *A. altiparanae* samples, ranging from 9.5% (MPE to UPE) to 10.6% (MPE to LPE). In contrast, gene flow estimates for *G. brasiliensis* ranged from 0.9% (UPE to LPE) to 8.4% (UPE to MPE), highlighting a few migration events to UPE (Figure 3).

The Mantel test revealed no significant values between geographic distances and estimates of genetic differentiation (pairwise Φ_{ST}).

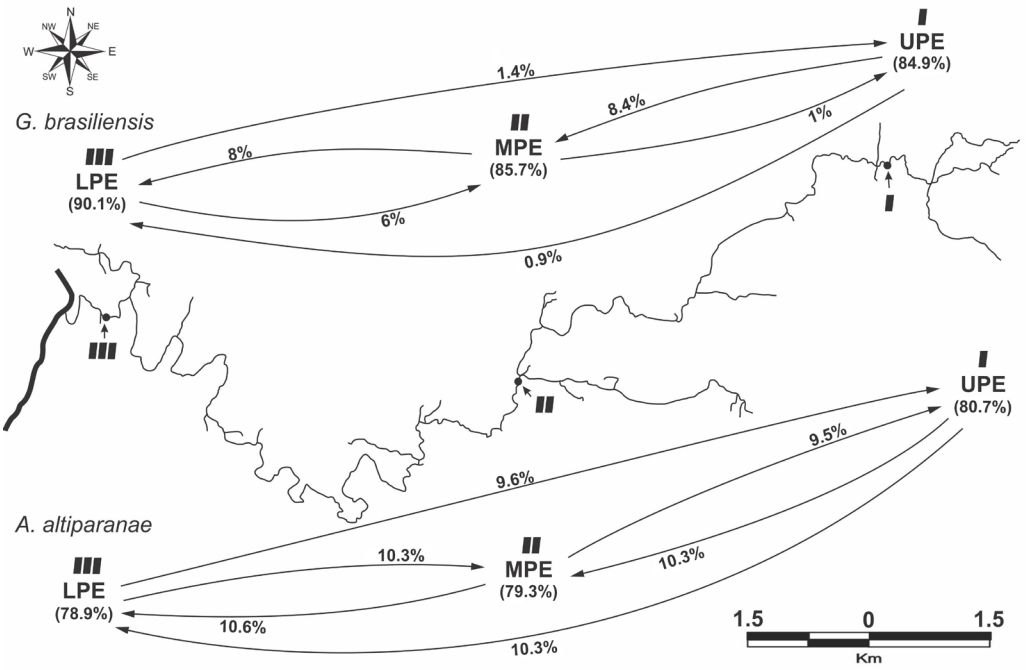

Figure 3. Estimates of gene flow based on Bayesian inferences of migration rates using BayesAss. UPE, MPE, and LPE populations of *Astyanax altiparanae* and *Geophagus brasiliensis*. The proportion of non-migrants is indicated by values in brackets below the name of each site. Migration estimates and the direction of gene flow are represented by arrows. UPE, MPE, LPE = upper, middle, lower Penacho Stream, respectively.

DISCUSSION

Genetic diversity and demographic analyses

In the present study, different levels of genetic diversity were obtained between the fish species *A. altiparanae* ($\overline{P}$ = 84.21-90.05% and H_E = 0.809-852) and *G. brasiliensis* ($\overline{P}$ = 20.86-30.43% and H_E = 0.578-0.729). These results are similar to those previously reported for these species (Leuzzi et al., 2004; Zaganini et al., 2012; Ferreira et al., 2015). Indeed, RAPD and microsatellite markers also revealed high levels of genetic diversity in populations of *A. altiparanae* from other drainages of the upper Paraná River basin, including the Grande River (H_E = 0.799) (Zaganini et al., 2012), Paranapanema River ($\overline{P}$ = 72.65%) (Leuzzi et al., 2004), Tietê River, Keller River, and Pirapó River (Prioli et al., 2002). Similarly, high levels

of genetic diversity were obtained for other species of the *Astyanax* genus, such as *Astyanax bimaculatus* in the Doce River basin, Brazil (Paiva et al., 2006), *Astyanax scabripinnis* in the Cambé stream, upper Paraná River, Brazil (Sofia et al., 2006), and *Astyanax aff. bimaculatus* in the Contas River, Brazil (Pamponet et al., 2008).

In the case of *G. brasiliensis*, the levels of genetic diversity obtained from microsatellite markers were similar to those previously reported for this species within the Laranjinha River basin (Ferreira et al., 2013, 2015). As noted by Ferreira et al. (2015), the diversity of *G. brasiliensis* in this stream appears to be consistently higher than that observed along the Laranjinha River. Although genetic information for *G. brasiliensis* remains limited, the levels of genetic diversity obtained for this species in the present study are consistent with those commonly reported for fish with sedentary behavior (Sofia et al., 2008). Indeed, migratory freshwater fishes generally exhibit higher levels of genetic diversity than those with limited displacement (Garcez et al., 2011; Ferreira et al., 2015).

In particular, low mobility capacity is a common feature of Neotropical stream fishes, mainly due to their small size (Castro et al., 2003). However, it is important to consider that some characteristics of *A. altiparanae*, such as the short reproductive migrations and absence of parental care (Suzuki et al., 2002), may permit greater mobility along the flow than the sedentary habits and parental care of *G. brasiliensis* (Kullander, 2003). Thus, although intrinsic factors may also be important, the mobility of each species seems to be the major determinant for the higher level of genetic diversity in *A. altiparanae*.

According to Garutti (1988), the low water volume in headwater regions does not favor the development of diversified microhabitats; therefore, headwaters do not support a large number of individuals. UPE is located in headwaters of the Penacho stream and, consequently, has different characteristics compared to the other sampling sites studied, mainly being of a smaller size. Thus, sedentary behavior and parental care of *G. brasiliensis* (Kullander, 2003), in combination with a smaller effective population size in UPE, could explain the lower diversity obtained for this sample. In contrast to the UPE population, microsatellite data for other *G. brasiliensis* samples (MPE and LPE) showed signs of recent bottlenecks.

Interestingly, the Penacho stream receives waters from the Pinhal stream in its middle section (MPE). The Pinhal stream is a small tributary that crosses the urban area in the municipality of Ribeirão do Pinhal, into which sewage was released for decades (Costa et al., 2013). Thus, despite the neutrality of the markers used, such a scenario suggests a possible relationship between signs of genetic bottlenecks and anthropogenic interferences in MPE and LPE. Indeed, a reduction in environmental quality can affect the diversity of freshwater drainages, including the genetic diversity of the species (Geist, 2011).

In the case of *A. altiparanae*, the levels of genetic diversity were high and homogeneous along the Penacho stream. In particular, these results suggest that the maintenance of genetic diversity in *A. altiparanae* results from its flexibility and capacity to adapt to different environments (Leuzzi et al., 2004), including its potential for mobility and the maintenance of large populations (Suzuki et al., 2002; Leuzzi et al., 2004).

Genetic structure and gene flow

Overall, the results of this study suggest fine-scale genetic structure of *G. brasiliensis* and *A. altiparanae* in the Penacho stream. Although, similar patterns have been observed for fish in temperate (Carlsson et al., 1999) and Neotropical streams (Sofia et al., 2006, 2008), the

present study is the first to discuss particular aspects of small drainages on the distribution of genetic diversity of Neotropical stream fishes. Indeed, such knowledge helps us to understand the evolutionary history of this species, and is essential for the conservation of biodiversity in freshwater ecosystems (Geist, 2011).

Microsatellite and RAPD markers showed significant levels of genetic diversity among populations of *G. brasiliensis* (RAPD = 10.82% and SSR = 12.51%) and *A. altiparanae* (RAPD = 3.23% and SSR = 2.09%), although the latter has shown smaller values than the prior. The pairwise differences (Φ_{ST}) obtained for *G. brasiliensis* samples were greater than those obtained for *A. altiparanae*. Moreover, rates of gene flow to *A. altiparanae* were higher than those to *G. brasiliensis* and Bayesian cluster analyses showed more evident population subdivisions to *G. brasiliensis*. Thus, our results suggest that the biology of these species strongly influences their level of population differentiation. *A. altiparanae* performs short reproductive displacements (Suzuki et al., 2002), while *G. brasiliensis* is sedentary (Kullander, 2003), highlighting differences in the mobility potential of these species, a factor that may greatly influence genetic structure patterns. Different fish species can share the same river system, but their behavior dispersion and their unique life histories can result in different rates and patterns of gene flow (Vrijenhoek, 1998).

Although separated by small geographic distances, *G. brasiliensis* exhibited genetic differentiation (pairwise Φ_{ST}) from high-to-moderate along the Penacho stream. These results were corroborated by Bayesian cluster analyses, which demonstrated the formation of three groups based on microsatellite data; one group for each sample, and two based on RAPD data, with the UPE samples forming a group and the MPE samples forming another. Gene flow analysis showed that UPE tends to receive few migrants from MPE and LPE, which still have some bidirectional gene flow. Similar patterns of fine-scale genetic differentiation were previously obtained for *Astyanax scabripinnis* (Sofia et al., 2006) and *Hypostomus ancistroides* (Sofia et al., 2008) within a stream of the same watershed. However, these studies did not focus their discussion on the features of small drainages. Ferreira et al. (2015) studied *G. brasiliensis* along the main channel of the larger Laranjinha River, and found smaller differences than those observed in the present study, even in samples separated by more than 100 km. Other studies in larger Neotropical drainages have shown that genetic differences tend to be greater when the flow is interrupted by physical barriers (Paiva et al., 2006; Pamponet et al., 2008). However, depending on the species biology and drainage conditions, no or little differentiation can be obtained even over long distances (Garcez et al., 2011). Thus, short geographical distances, such as those among the sampling sites in the present study, could suggest the absence of genetic differentiation; however, this pattern was not observed for any of the studied species.

Pairwise differences (Φ_{ST}) obtained for *A. altiparanae* range from low, between the closest samples (Φ_{ST} = 0.020), to moderate (Φ_{ST} = 0.065), among more distant samples. Corroborating these data, a large number of private microsatellite alleles (N_P) was obtained for samples from the UPE (22) and LPE (25) populations. In a drainage of the same basin (Laranjinha River), a genetic study was conducted in five fish species (Frantine-Silva et al., 2015) including *A. altiparanae*. Higher levels of differentiation were not found in that study compared to the present one, even for populations that have been separated by a small hydroelectric power plant for decades. Furthermore, although the microsatellite data in the present study showed similar gene flow rates and failed to show defined groups among *A. altiparanae* samples, RAPD data showed a pattern that corroborates the results obtained for *G. brasiliensis*, indicating higher genetic differentiation estimates to UPE sample.

Despite the fact that the correlation analysis between geographical and genetic

differences did not show significant values for either species, the patterns obtained, mainly for *A. altiparanae*, suggest genetic differentiation due to isolation by distance (IBD). Thus, gene flow along the drainage would be maintained from the Stepping-Stone Model, whereby the exchange of alleles occurs preferentially between neighboring demes, and is rarer between distant demes (Kimura and Weiss, 1964). A similar pattern has been previously found for the genus *Astyanax* (Moysés and Almeida-Toledo, 2002) and *G. brasiliensis* (Ferreira et al., 2015); however, the data obtained for *G. brasiliensis* suggest the influence of additional factors in the distribution of genetic diversity throughout the Penacho stream.

Streams generally undergo gradual changes in their characteristics along the flow towards the downstream, showing higher mesohabitats, a majority of species, lower number of barriers to displacement in the lower regions, and more stable habitats (Winemiller et al., 2008). Thus, besides behavioral features and geographical distance, the drainage structure can have a major influence on genetic differentiation patterns in streams (Hughes et al., 2009). This also includes constant anthropic alterations to Neotropical streams, since small drainages are highly sensitivity to these impacts (Luiz et al., 1998).

Overall, the results of the present study suggest that patterns of gene flow and genetic structure in fish in Neotropical streams are distinct from those obtained in higher drainages. Indeed, the main determinants of these results could include particular features found in small drainages. Although the intensity of genetic differentiation differs between *G. brasiliensis* and *A. altiparanae*, both species showed fine-scale genetic structure throughout the Penacho stream. Same species, analyzed using the same markers in a larger drainage of the same watershed (separated by large distances and influenced by a physical barrier), showed smaller differences than those found in the Penacho stream. In addition, these results showed that the genetic diversity of both species is heterogeneously distributed in the Penacho stream, indicating that measures aiming to conserve genetic diversity should consider the entire length of the stream. Such information could be of great importance for the management and conservation of these drainages and could contribute to the understanding of evolutionary patterns of fish in Neotropical streams. However, more studies are needed in other streams so that the results can be extrapolated to other small drainages.

Conflicts of interest

The authors declare no conflict of interest.

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