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Genetic structuring of *Salminus hilarii* Valenciennes, 1850 (Teleostei: Characiformes) in the rio Paraná basin as revealed by microsatellite and mitochondrial DNA markers

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Genetic variation of *Salminus hilarii* was assessed by screening microsatellite loci and mitochondrial D-loop DNA across four sampling in the upper rio Paraná basin of Brazil. Genetic diversity – measured as mean expected heterozygosity (0.904) and mean number of alleles across populations (13.7) – was reasonably high. Differentiation of microsatellite allele frequencies among populations was shown to be low but significant by AMOVA Φ_{ST} (0.0192), and high by D_{EST} (0.185). D-loop variation was high, with haplotypic diversity of 0.950 and nucleotide diversity of 0.011. Mitochondrial DNA-based estimates for population differentiation were high, with an overall Φ_{ST} of 0.173. The results of tests of nuclear and mitochondrial variation yielded no unequivocal inference of historical demographic bottleneck or expansion. Genetic differentiation observed among *S. hilarii* populations in the rio Grande may be caused by a combination of historical differentiation and recent gene-flow disruption caused by the dams followed by reproduction of isolated spawning assemblages in mid-sized tributaries of the respective reservoirs. We present spatially more intensive sampling of *S. hilarii* populations across the rio Paraná basin in order to more effectively distinguish between historical and contemporary differentiation.

A variabilidade genética de *Salminus hilarii* foi avaliada por lócus microssatélites e sequências D-Loop do DNA mitocondrial em quatro populações da região da bacia do Alto Paraná. A diversidade genética – medida pela heterozigosidade média (0,904) e número de alelos médios das populações (13,7) – foi razoavelmente alta. A diferenciação das frequências alélicas entre as populações foi baixa, mas significativa pela AMOVA Φ_{ST} (0,0192), e alta pelo D_{EST} (0,185). A variação mitocondrial foi alta com uma diversidade haplotípica de 0,950 e uma diversidade nucleotídica de 0,011. Estimativas de diferenciação populacional baseadas no DNA mitocondrial foram altas, com um valor global de Φ_{ST} de 0,173. Os resultados dos testes da variação nuclear e mitocondrial demonstram nenhuma inequívoca inferência histórica de contração e expansão demográfica. A diferenciação genética observada entre as populações de *S. hilarii* no rio Grande pode ter sido causada pela combinação de diferenciação histórica e interrupção recente do fluxo gênico causada pela construção de barragens seguida por um isolamento reprodutivo de populações em tributários de médio porte dos respectivos reservatórios. Nós apresentamos uma amostragem mais ampla e intensiva de populações de *S. hilarii* ao longo da bacia do alto rio Paraná para se efetivamente distinguir se a diferenciação genética das populações encontrada é histórica ou contemporânea.

Keywords: D-loop, River disruption, STR, Tabarana.

Introduction

Salminus hilarii (Characiformes: Bryconidae) is one of five species of its genus, and is widely distributed in the Paraná, Paraguay and São Francisco rivers basins in Argentina, Brazil, Paraguay and Uruguay (Langeani *et al.,* 2007; Lima & Britski, 2007). *Salminus hilarii* is commonly known as tabarana or dourado branco in Portuguese, and as dorado, plateado, or sábalo in Spanish. Although this species is not targeted in commercial fisheries, it is popular for recreational fishing (Graça & Pavanelli, 2007). *S. hilarii* is an apex predator species and thus can be regarded as an environmental indicator species (Luz-Agostinho *et al*., 2006). Its reproductive period occurs between October and November in lotic environments (Andrade *et al*., 2004; Honji *et al*., 2009), when it undertakes short-distance spawning migrations (Petrere Júnior, 1985; Oldani, 1990).

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Due to its migratory life history and the broad distribution of *S. hilarii* across the sub-basins of the upper rio Paraná basin, populations presumably were connected before any anthropogenic impacts affected their current population genetic structure. Using microsatellite DNA markers and mitochondrial D-loop DNA sequences, we assessed the genetic diversity and population differentiation of *S. hilarii* at four locations in the upper rio Paraná basin against the null hypothesis of no population structure. Because short-distance migration could give rise to differentiation across space, we also tested our data against an alternative hypothesis of simple isolation by distance.

Material and Methods

Sample collection and DNA extraction. A total of 151 *S. hilarii* individuals were sampled between 2008 and 2009 from three rivers located within the upper rio Paraná basin: the upper rio Tietê - UTR (n = 56) at 23º34'36''S 45º58'26''W, the rio Paranapanema - PAR (n = 19) at 23º10'17''S 49°24'57''W; and two sites along the rio Grande: Igarapava - GRI (n = 39) at 19º59'08''S 47º46'08''W and Lavras - GRL (n = 37) at 21º08'34''S 45º02'74''W that are separated by four dams (Fig. 1). Fin clips were taken from each individual and stored in 95% ethanol at -20°C. Total genomic DNA was extracted following the protocol described by Taggart *et al.* (1992), with the exception of the use of STE (0.1 M NaCl; 0.05 M Tris-HCl; 0.01 M EDTA).

Microsatellite amplification and data analysis. The genetic diversity at microsatellite loci was screened using five primer sets (Sh01, Sh05, Sh10, Sh12 and Sh16) designed for *S. hilarii* (Silva & Hilsdorf, 2011). DNA amplifications were performed in a PTC-100 thermocycler (MJ Research, Waltham, MA, USA) with 10 x buffer (100 mM Tris–HCL pH 8.8, 500 mM KCl), 1.5–2.5 mM $MgCl_2$, 2.5 mM dNTPs, 10 µM of each primer, 5 U of *Taq* DNA polymerase (Fermentas Life Sciences, SP, Brazil), 80 ng of template DNA, and sterile water to a final volume of 10 µL. The PCR parameters were as follow: 3 min of denaturation at 94°C; followed by 35 cycles of 94°C for 40 seconds, annealing (at temperatures previously published) for 30 seconds, and 72°C for 1 minute and 30 seconds; and a final extension at 72°C for 8 min. The PCR products were subjected to electrophoresis through a 9% polyacrylamide gel at 6,000 W for 3 h. The DNA fragments were visualized by standard silver staining techniques (Bassam *et al.*, 1991). Microsatellite allele sizing for every individual was estimated manually by two independent individuals who compared the mobility of amplicon bands to those of molecular weight standards (10 bp ladder, Invitrogen) and also to the amplicon of each microsatellite insert as a control using the Alpha Index 6.5 program (AlphaImager™, Alpha Innotech Corporation).

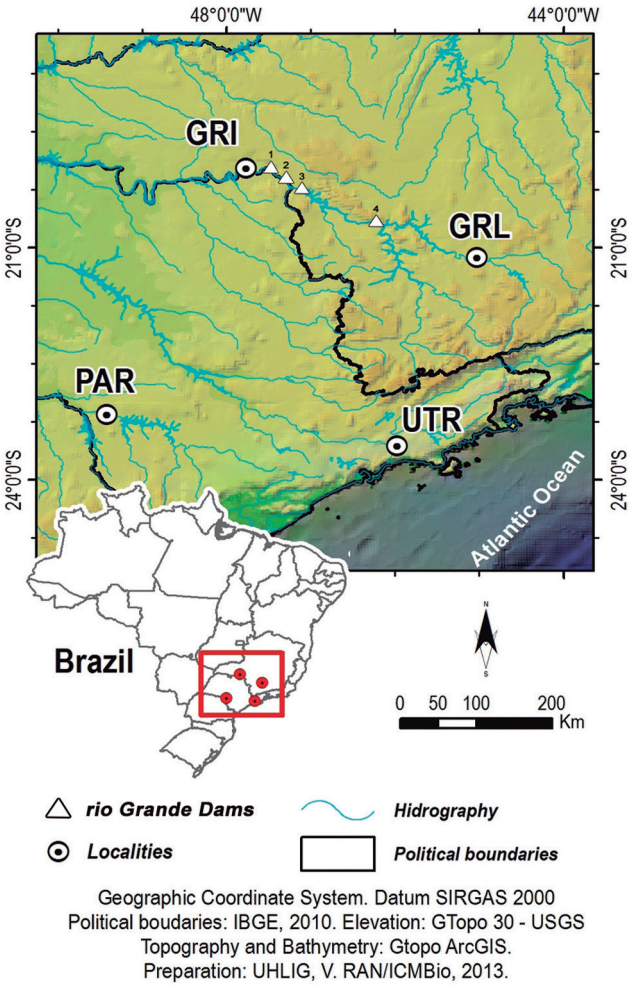

Fig. 1. Sampling sites of *Salminus hilarii* in the upper rio Paraná basin: rio Grande – Igarapava (GRI); rio Grande–Lavras (GRL); upper rio Tietê (UTR); rio Paranapanema (PAR). Power plants dams in the rio Grande: (1) Jaguara (built in 1971, height 71m), (2) Estreito (built in 1969, height 92 m), (3) Marechal Mascarenhas de Moraes (built in 1957, height 69 m), (4) Furnas (built in 1963, 127 m).

Genetic diversity was quantified as the mean number of alleles per locus (A); allelic richness (Ar) per locus for each sample, which takes into account the number of alleles per locus independent of sample size (El Mousadik & Petit, 1996); and gene diversity per locus using hs, an unbiased estimator (Nei, 1987). The inbreeding coefficient F_{IS} quantifying the extent to which heterozygote frequencies deviated from Hardy–Weinberg expected proportions was estimated for each locus and across loci using FSTAT, version 2.9.3.2 (Goudet, 1995). Deviations of genotype frequencies from Hardy-Weinberg expectations (HWE) were calculated by an exact test using the Markov-Chain randomization approach (Guo & Thompson, 1992) with HW-QUICKCHECK (Kalinowski, 2006). Deviations were regarded as significant at the 5% level, employing a sequential Bonferroni correction for multiple tests. The software Micro-Checker (Van Oosterhout *et al.*, 2004) was

used to infer the causes of any possible departures from HWE for null alleles due to misscoring and large-allele dropout. Linkage disequilibrium was examined using the log likelihood ratio statistic (*G*-test) calculated using GENEPOP, version 4.2 (Rousset, 2008) with 10,000 dememorizations, 100 batches, and 5,000 iterations per batch.

Interpopulation genetic differentiation of mitochondrial D-loop DNA sequences was quantified by analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) with Φ_{ST} (Weir & Cockerham, 1984) as the differentiation parameter and 10,000 permutations using Arlequin, version 3.5.1.3 (Excoffier & Lischer, 2010). A sequential Bonferroni adjustment was used to determine the significance levels for simultaneous comparisons (Rice, 1989). Microsatellite genetic differentiation was estimated using the D_{EST} index (Jost, 2008), which quantifies differentiation based on the effective number of alleles rather than on the expected diversity. This estimator improves upon the G'_{ST} method (Gregorius *et al*., 2007) and produces a maximum of unity when populations are entirely differentiated (*i.e*., they share no alleles) and a minimum of zero when no differentiation exists (*i.e*., when populations share all alleles at the same frequencies). D_{EST} was calculated using DEMEtics, version 0.8.7 (Gerlach *et al.,* 2010) using harmonic mean estimate and statistical testing by bootstrapping with 10,000 permutations. D_{EST} genetic distances were used to perform a Mantel test to assess the correlation between geographic and genetic distances between populations using the Isolation by Distance Web Service, version 3.23 (Jensen *et al*., 2005) with 10,000 randomizations.

BOTTLENECK 1.2.02 (Cornuet & Luikart, 1997) was used to assess the possibility of recent reduction in effective population size in each population using the Wilcoxon test (Luikart *et al*., 1998) under the infinite alleles (IAM), stepwise mutation (SMM), and two-phase (TPM) models of mutation. The Wilcoxon test provides relatively high power and can be applied to data sets with few polymorphic loci. For the TPM, a variance of 30, probability of 90% and 1,000 interactions were assumed.

DNA mitochondrial amplification and data analysis. For mitochondrial D-loop DNA sequences, 79 of the samples were amplified and sequenced using the primers: D-Loop L - 5`-AGAGCGTCGGTCTTGTAAACC-3` (Cronin *et al*., 1993) and H16498 - 5`- CCTGAAGTAGGAACCAGATG-3` (Meyer *et al*., 1990). Polymerase chain reaction (PCR) was performed with 3 mM $MgCl_2$, 0.25 mM of each dNTP, 1.0 µM of each primer, 0.5 U of *Taq* polymerase (Fermentas), 3.5 µL of 10× buffer (Fermentas Life Sciences, SP, Brazil), 80 ng of DNA template, and sterile water to achieve a final volume of 25 µL. PCR parameters included: 3 min of denaturation at 94 °C; followed by 30 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 56 °C, and 1 min of elongation at 72 °C; and a final elongation step of 10 min at 72 °C. The PCR products were confirmed using electrophoresis in 1% agarose gels, and subsequently were purified using a GFX™ kit (GE Healthcare, São Paulo, Brazil). DNA sequences were analyzed on an ABI Prism™ 3730 DNA Analyzer (Applied Biosystems) using the BigDye® Terminator v3.1. The quality of the sequences was verified using CodonCode Aligner software, version 3.7.1 (http://www.codoncode.com/aligner/) to generate a consensus sequence. All consensus sequences were aligned using Clustal X, version 2.0 (Larkin *et al.,* 2007) and optimized by eye using Bioedit, version 7.0.9.0 (Hall, 1999).

Genetic variability was quantified as nucleotide diversity (π, Nei, 1987), haplotype diversity (*Hd*, Nei & Tajima 1981) and the number of polymorphic sites (*S*) by DnaSP, version 5.10 (Rozas *et al*., 2003). Tajima's (1989) *D* and Fu's (1997) *F*s statistics were calculated to test the null hypothesis of selective neutrality of mtDNA sequences (Rozas *et al*., 2003) and also to evaluate whether negative, but non-significant values of the *D* and *F*s parameters might indicate population expansion. A haplotype network was built using NETWORK, version 4.1.1.2 (Fluxus Technology, Ltd.) based on the median joining algorithm (Bandelt *et al*., 1999) with a binary matrix of haplotypes.

The DNA sequence mismatch distribution was calculated using Arlequin, version 3.5.1.3 (Excoffier & Lischer, 2010) to compare the distribution of the observed number of differences between pairs of haplotypes to that expected and thereby to assess the possibility of population expansion. A population that has recently undergone rapid demographic growth exhibits as a unimodal mismatch distribution, while a population at demographic equilibrium exhibits a multimodal distribution (Rogers & Harpending, 1992). Additionally, Φ_{ST} (Weir & Cockerham, 1984) was calculated by the analysis of molecular variance (AMOVA; Excoffier *et al*., 1992). To assess genetic structure across the populations evaluated, 10,000 permutations were used to test the significance of the hierarchical differentiation using Arlequin, version 3.5.1.3 (Excoffier & Lischer, 2010). The Φ_{ST} values found by Arlequin were used to implement a Mantel test using the Isolation by Distance Web Service, version 3.14 (Jensen *et al*., 2005) with 10,000 randomizations.

Results

Microsatellite allelic diversity and Hardy-Weinberg equilibrium. Variability at microsatellite DNA loci was high across four populations of *S. hilarii.* A total of 81 alleles were observed at five microsatellite loci. The distribution of alleles across the samples was not uniform; two private alleles were found (one each for loci Sh10 and Sh16) in the downstream sample from the rio Grande at Igarapava. The mean allelic richness per locus per population ranged from a low of 10.98 (± 1.47) in the upstream sample from the rio Grande at Lavras to a high of 12.63 (± 2.30) in the downstream site in the rio Grande at Igarapava (Table 1). The mean observed heterozygosity for each population ranged from 0.93 (± 0.06) to 0.96 (± 0.03) and mean expected heterozygosity from 0.89 (± 0.02) to 0.91 (± 0.01) (Tables 1, 2).

Table 1. Summary statistics for genetic diversity at five microsatellite loci of *Salminus hilarii*: (*A*) number of alleles; (*Ar*) allelic richness; (*hs*) Nei´s genetic diversity; (*Ho*) observed heterozygosity; (H_E) expected heterozygosity; (F_{IS}) inbreeding cœfficient. ns - Not significant, *Significant (P < 0.0125) after Bonferroni adjustment (nominal α = 0.05).

Locus		GRI	GRL	UTR	PAR
Sh01	***A***	14	14	14	16
	Ar	11.57	10.96	11.71	14.69
	hs	0.895	0.900	0.912	0.933
	H_O	0.943 ns	0.892 ns	0.944 ns	0.947 ns
	H_E	0.902	0.900	0.916	0.939
	F_{IS}	-0.054	0.009	-0.035	-0.016
Sh05					
	A	19	13	19	13
	Ar	14.68	11.26	13.08	13.00
	hs	0.931	0.914	0.914	0.833
	H_O	0.972 ns	1.000 ns	0.959 ns	1.000 ns
	H_E	0.931	0.915	0.914	0.887
	F_{IS}	-0.044	-0.095	-0.050	-0.132
Sh10					
	A	12	10	10	10
	Ar	10.78	9.22	9.21	10.00
	hs	0.884	0.860	0.878	0.877
	H_O	1.000*	1.000*	1.000*	0.938 ns
	H_E	0.886	0.865	0.879	0.879
	F_{IS}	-0.131	-0.163	-0.139	-0.069
Sh12					
	A	15	16	13	16
	Ar	12.64	13.65	10.54	14.96
	hs	0.926	0.929	0.878	0.937
	H_O	0.800 ns	0.944 ns	0.944 ns	1.000 ns
	H_E	0.924	0.929	0.876	0.939
	F_{IS}	0.136	-0.016	-0.076	-0.067
Sh16					
	A	13	13	13	11
	Ar	10.70	11.27	10.36	10.50
	hs	0.875	0.913	0.874	0.901
	H_O	0.970 ns	0.838 ns	0.893 ns	0.947 ns
	H_E	0.920	0.912	0.874	0.902
	F_{IS}	-0.055	0.083	-0.022	-0.052
Average					
	A	14.600	13,200	13.800	13.200
	Ar	12.074	11.272	10.980	12.630
	hs	0.902	0.903	0.891	0.896
	H_O	0.937	0.934	0.948	0.966
	H_E	0.912	0.904	0.891	0.909
	F_{IS}	-0.029	-0,036	-0.064	-0.067

Heterozygote excess (*i.e.*, a negative F_{IS} value) was found for all loci within the respective populations, except for loci Sh01 and Sh16 in the upstream collection from the rio Grande at Lavras (Table 1). Notably, the populations did not depart significantly from HWE expectations after sequential Bonferroni correction (P < 0.0125), with the exception of the Sh10 locus in the rio Grande at both Igarapava and Lavras, and in the upper rio Tietê (Table 1). Results of Micro-Checker tests showed no evidence for stuttering due to amplification or scoring error, large allele dropouts, or null alleles. Tests for linkage disequilibrium yielded significant values (P < 0.05) for but 2 of 40 locus pairs, indicating that allelic variation at all loci segregated independently. The two significant values were observed for different pairs of loci, indicating that these values are not a result of physical linkage between the loci.

Differentiation of microsatellite allele frequencies among populations was low but significant by AMOVA Φ_{ST} (0.0192). The overall Jost's (2008) D_{EST} estimate was 0.18548 (P = 0.001), ranging from 0.1269 to 0.2676, which demonstrated moderate to high genetic differentiation among the four local populations (Table 3). Results of the Mantel test for association between Jost´s D_{EST} values and geographic distances indicated no significant isolation-by-distance effect (P = 0.292) among the four local *S. hilarii* populations.

Analyses using Bottleneck 1.2.02 showed significant values (P<0.05) for the upstream rio Grande - Lavras population for all mutational models. Results for the populations from rio Grande – Igarapava and upper rio Tietê were significant only for the infinite alleles and two-phase models, while those for the population from rio Paranapanema were not significant for any of the models. These results suggested that of all the *S. hilarii* populations screened, only the population from the upstream rio Grande at Lavras may have undergone a recent population reduction.

Mitochondrial DNA variation. An average of 547 bp of the mitochondrial D-loop region was sequenced, and 27 haplotypes were observed among the 79 individuals in the four populations (Electronic Supplementary Material 1). Eleven haplotypes were found only in the rio Grande (Lavras and Igarapava) populations, and five haplotypes were found only in the rio Paranapanema and upper rio Tietê populations. The distribution of haplotypes from the respective populations through the haplotype network (Fig. 2) was not random. Haplotypes of only one population, rio Grande – Igarapava (shown in blue) were widely distributed across the network, and showed comparable numbers of observations of eight haplotypes. The rio Grande – Lavras populations (shown in green) exhibited eight haplotypes separated by no more than three mutational steps, with most individuals exhibiting haplotypes H_11 or H_15. The rio Parapanema population (shown in red) exhibited four haplotypes separated by no more than 2 mutational steps. The rio Tietê population (shown in yellow) exhibited seven haplotypes separated by no more than 2 mutational steps, with most individuals exhibiting haplotype H_3.

Table 2. Mitochondrial DNA sampling and descriptive statistics for *Salminus hilarii*. (*N*) = number of sequenced individuals; (*S*) = number of polymorphic sites; (*Hd*) haplotype diversity; (π) nucleotide diversity; (s.d.) standard deviation.

Sampling	*N*	haplotypes	S	*Hd* ± s.d.	π ± s.d.	Tajima *D*	Fu´s *Fs*
GRI	20	8	15	0.847 ± 0.059	0.011 ± 0.001	1.5925 (*P*=0.9553)	1.410 (*P*=0.7635)
GRL	20	10	14	0.911 ± 0.040	0.004 ± 0.001	-1.5277 (*P*=0.0494)	-4.087 (*P*=0.0043)
UTR	20	7	8	0.742 ± 0.095	0.002 ± 0.004	-1.2857 (*P*=0.0930)	-2.356 (*P*= 0.0410)
PAR	19	4	5	0.713 ± 0.058	0.003 ± 0.001	0.7802 (*P*=0.7922)	1.385 (*P*=0.1300)
Total	79	27	32	0.950 ± 0.009	0.011 ± 0.001	-0.1101 (*P*=0.4724)	-0.967 (*P*= 0.4000)

Table 3. Pairwise D_{EST} indices between parenthesis (below diagonal) using microsatellites and mtDNA (above diagonal) between four *Salminus hillari* samples. Statistical significance of Φ_{ST} value was tested based on the exact test based on 10,000 permutations, **P* < 0.05, ***P* < 0.01.

	GRI	GRL	UTR	PAR
GRI	-	0.109*	0.197*	0.219*
GRL	0.127**	-	0,173*	0.187*
UTR	0.137**	0.180**	-	0.272*
PAR	0.267**	0.193**	0.183**	-

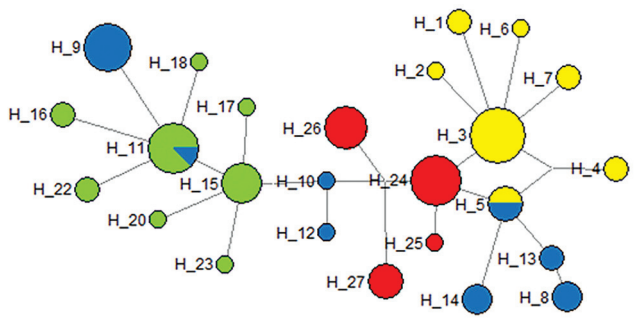

Fig. 2. Unrooted haplotype network for mitochondrial D-loop sequences of *Salminus hilarii* generated by NETWORK 4.1.1.2 based on the median joining algorithm. Sizes of the circled areas are proportional to the frequencies of the haplotypes at issue. Blue circles: rio Grande -Igarapava (GRI); Green circles: rio Grande -Lavras (GRL); Yellow circles: upper rio Tietê (UTR), and Red circles: rio Parapanema (PAR).

Overall genetic variability expressed as haplotype diversity (*Hd*) was 0.950 (± 0.009) (Table 2), and nucleotide diversity (π) was 0.011 (± 0.01) for all regions. Genetic differentiation among the four *S. hilarii* populations was quantified with a global Φ_{ST} of 0.1730 (*P* = 0.000), and all pairwise comparisons showed high population differentiation among the four populations (Table 3). No significant correlation between Φ_{ST} mtDNA values and geographic distance was observed (*P* = 0.173).

Results of Tajima's *D* and Fu's *Fs* tests showed no significant deviations from the null hypothesis of selective neutrality for the rio Grande – Igarapava and rio Paranapanema populations. Mitochondrial DNA sequence variation in the UTR population did not depart significantly from neutrality based on the Fu´s *Fs* test, but did for Tajima´s *D*. The rio Grande – Lavras population was the only one to show a significant departure from the hypothesis of selective neutrality for both tests.

The overall mitochondrial DNA sequence mismatch distribution for *S. hilarii* populations fit the multimodal model, which indicates that the populations collectively have not experienced recent demographic fluctuation and are at demographic equilibrium. However, Fu's *F*s test for demographic expansion within each local population showed significantly negative values for the rio Grande – Lavras and upper rio Tietê populations, suggesting rapid demographic expansion in these populations (Table 2).

Discussion

Within-population genetic variability. Genetic variability at microsatellite DNA markers in four populations of *S. hilarii* was relatively high; mean H_E values of 90.2% and an average of 13.8 alleles per locus were higher than mean values found among 13 freshwater species by DeWoody & Avise (2000). The mean mitochondrial haplotype diversity (*h*) in the four populations (0.955) suggests that there has been a long time for divergence from ancestral haplotypes of the populations evaluated. The nucleotide divergence (1.1%) was lower than the average value of 2.7% among freshwater fishes of the northern hemisphere (Billington & Hebert, 1991) and lower than the average value of 1.5% for 21 Neotropical freshwater fishes (Hilsdorf, 2013). High levels of heterozygosity and genetic variation are the basis for adaptation and survival of fish populations in changing environments (Lieutenant-Gosselin & Bernatchez, 2006; Mitton & Grant, 1984; Wang *et al*., 2002); the microsatellite and mitochondrial variability for these four populations of *S. hilarii* seems sufficient to support ongoing local adaptation.

Populations that have experienced a recent genetic bottleneck exhibit a temporary excess of heterozygosity (Luikart *et al.,* 1998), and rare alleles tend to be observed in heterozygotes much more frequently than in homozygotes, such that heterozygosity may not be lost as fast as allelic diversity (Hartl & Clark, 1997). Results of microsatellite-based bottleneck tests suggested that the rio Grande at Lavras population underwent a recent

population bottleneck. At the same time, this population also showed significant deviation from the null hypothesis of selective neutrality, which may have been caused by different evolutionary forces, such as selective sweep, population bottleneck or recent population growth. The two rio Grande sampling sites (Lavras and Igarapava) are separated by four hydroelectric dams that started operating between 1957 and 1971. None of these dams have fish passage infrastructure, breaking population connectivity for *S. hilarii* and other migratory fishes in this river. Assuming a two-year age-at-maturity (based on our experience breeding the species), the 21-29 generations of *S. hilarii* since construction of the dams may have been too short a time for the signatures of demographic processes (population contraction or expansion) or genetic differentiation (due to selection or drift) to become apparent in molecular genetic data sets. This was the case also for bronze gudgeon *Coreius heterodon* populations separated by dams in the Yangtze River in China (Cheng *et al*., 2013).

The significantly negative values of Fu's *F*s test of mitochondrial DNA variation in the rio Grande – Lavras and upper rio Tietê populations suggest recent demographic expansion. Indicators of different demographic growth patterns observed for the upper rio Tietê population (*i.e*., demographic contraction apparent from nuclear markers, expansion from mitochondrial markers) may be explained by differences in the rates of mutation for mitochondrial and nuclear DNA, or contrasting sex-specific demographic processes (Pilkington *et al*., 2008). Alternatively, the population may have fluctuated demographically. Changing environmental conditions in the upper rio Tietê region, which is toward the headwaters, may explain the low genetic variability and inferred demographic fluctuations in this population. The persistence of *S. hilarii* populations in this region speaks to the resilience of the species, as rivers and tributaries in this region have undergone many man-made environmental changes, including construction of dams for water supply, removal of riparian vegetation and urban and agricultural pollution (Barrella & Petrere Jr., 2003).

Genetic differentiation among populations. Population genetic analyses revealed genetic structure among the populations of *S. hilarii* assessed in this study. Jost's estimator of divergence (D_{EST}) for microsatellites was 0.1858 ($P = 0.001$). Population divergence measured as D_{EST} was high in part because this index captures the fraction of allelic variation that occurs among populations; *i.e*., D_{EST} directly measures the divergence of allelic frequencies among populations regardless of the marker's mutational model or the migration pattern of the species. Thus, the outcomes from D_{EST} suggest that the allele frequency divergence observed would reflect historical genetic structuring of the four populations assessed here, plus any additional effects of recent anthropogenic isolation.

The results generated by comparing the D-loop mitochondrial sequences between the four populations tend corroborate the D_{EST} results. That is, the global Φ_{ST} value for mtDNA of 0.1730 was high ($P = 0.000$). All comparisons among the populations using both marker types were high and significant (Table 3). The haplotype network (median-joining haplotype network) shown in Figure 2 reflects the results of historical mtDNA population differentiation; the haplotype network does not have a star-like shape typical of populations that have both low geographic structure and population expansion (Crandall & Templeton, 1993; Castelloe & Templeton, 1994; Avise, 2000).

The geographic distribution of haplotypes in Figure 2 corroborates the high level of population genetic differentiation of mtDNA haplotypes, as well as differences in allele frequencies at microsatellite loci quantified in the D_{EST} indices. Having found genetic structuring in the rio Paraná system, we recommend surveys of the species across its range.

Conservation implications of genetic structuring. In Neotropical freshwater ecosystems lacking anthropogenic influences, different patterns of distribution of interpopulation genetic variability can occur. Migratory fish species in large ecosystems, such as the Pantanal in the State of Mato Grosso, Brazil, may be under the influence of hydrological factors, such as seasonal flood pulses, that genetically homogenize populations, thereby bringing them close to panmixia (as in pacu *Piaractus mesopotamicus*, Calcagnotto & DeSalle, 2009; Iervorlino *et al*., 2010). In other cases, non-migratory species or those species making but short migrations tend to show more significant levels of population genetic structure (as in Tietê tetra *Brycon insignis*- Matsumoto & Hilsdorf (2009); and spotted sorubim catfish *Pseudoplatystoma corruscans*- Pereira *et al*. (2009); Carvalho *et al*. (2012)). Here, *S. hilarii* populations showed genetic structure among four locations within the rio Paraná basin. While we first expected that contemporary genetic structuring might be the result of isolation by distance (Wright, 1938), no significant correlation between genetic distance and geographic distance was found using either microsatellite or mitochondrial markers. Thus, geographic distance did not explain the current genetic differentiation among the population, suggesting that a stepping stone model of migration (Kimura & Weiss, 1964) may explain the population structure of the four *S. hilarii* populations. More intensive sampling within the upper rio Paraná basinwill be needed to test this hypothesis.

Esguícero & Arcifa (2010) evaluated the effects of the Gaviao Peixoto Dam (constructed in 1900) in the rio Jacaré-Guacu basin on populations of *S. hilarii* that currently live up- and down-stream of the dam using canonical variable analysis of morphometric features, and observed significant differentiation between these two populations.

The authors suggested that lack of connection between up- and downstream populations due to damming led to population fragmentation of *S. hilarii* in the area studied. Similarly, we found strong genetic differentiation (for mtDNA, Φ_{ST} = 0.109; for microsatellites, D_{EST} = 0.1269) between the Igarapava and Lavras populations on the rio Grande, separated from each other by 422 river km and four hydroelectric dams. The genetic differentiation we observed among *S. hilarii* populations in the rio Grande may be a combination of historical differentiation and recent effects of gene-flow disruption caused by the dams followed by reproduction of isolated spawning assemblages in mid-sized tributaries of the respective reservoirs. The contribution of contemporary lack of gene flow to historical differentiation is difficult to assess, although we note that the mean value for the mitochondrial Φ_{ST} value (0.192) was slightly greater than that for the corresponding nuclear metric, D_{EST} (0.181). We suggest spatially more intensive sampling of *S. hilarii* populations across the rio Paraná system in order to more effectively distinguish between historical and contemporary differentiation. Our data set also provides the basis for future studies of temporal variation in genetic variation of *S. hilarii* in the system. Similar studies of *S. hilarii* might be undertaken in other river systems, prioritizing those were dams have been or might be constructed.

The case that dams may have imposed fragmentation upon *S. hilarii* in the upper rio Paraná basin is strengthened by reference to a growing body of literature on the disruptive effects of dams upon gene flow in other riverine fishes. For example, Yamamoto *et al.* (2004) observed reduced genetic diversity (in terms of numbers of alleles and expected heterozygosity) and highly significant genetic differentiation (expressed as F_{ST}) for white-spotted charr *Salvelinus leucomaenis* isolated by 11 dams in Japan; genetic differentiation was negatively related to the habitat available and positively related to the period of isolation of populations above and below the dams. Roberts *et al.* (2013) investigated the range-wide population genetic structure of Roanoke logperch *Percina rex*, a stream fish of the southeastern United States, and found that in the absence of hydrological barriers, gene flow was extensive throughout watersheds. F_{ST} was positively related to the spatial distance and degree of hydrological alteration between sites and negatively related to genetic diversity within sites. While the effect of reservoirs was equivocal, dams strongly influenced differentiation: the effect of a dam on F_{ST} was comparable to that of a between-site distance of over 1200 km of unimpounded river. Accelerated genetic differentiation of fish populations following disruption of a riverine connection has been observed for blue sucker *Cycleptus elongates* (Bessert & Ortí, 2008), three-spined stickleback *Gasterosteus aculeatus* (Raeymaekers *et al.,* 2008), bullhead *Cottus gobio* (Junker *et al.,* 2012) and Yazoo darter *Etheostoma raneyi* (Sterling *et al.,* 2012). Dams are common features of riverine landscapes, and with continuing economic development, their prevalence is expected to increase. The adaptive consequences of the resulting anthropogenically induced divergence are only beginning to be investigated (Waples *et al.,* 2007), and warrant additional research.

Populations of *S. hilarii* in the upper rio Paraná system have but limited if any contemporary gene flow and therefore may be considered "management units" (MUs) (Moritz, 1994). Management units (MUs) are defined as populations that are demographically independent of one another (Allendorf & Luikart 2007), meaning that their population dynamics depend mostly on local birth and death rates, and not on genetically effective migration from other spawning assemblages. Identification of MUs is useful for designing and implementing fishery management actions, such as managing habitat, setting harvest rates, and monitoring population status. MUs generally do not show long-term independent evolution or strong adaptive variation. At an operational level, Moritz (1994) suggested that MUs are populations that have substantially divergent allele frequencies at many loci. The respective populations of *S. hilarii* clearly meet this definition.

In our context, recognition of MUs for *S. hilarii* is critical for establishment of management strategies for monitoring and conserving these populations. Mitigating actions to maintain connectivity among populations upstream and downstream of the dams by fish passage systems has been considered; however, such action has aroused critical discussions on the preservation of the riverine system within the Neotropical region (Pelicice & Agostinho, 2008). Maintenance of genetic diversity in local populations formed after isolation will depend on the conservation of tributaries and environmental integrity to provide effective feeding and reproductive opportunities for the respective demographic units. These measures will contribute to the continued evolution of these populations and the preservation of their genetic resources (Feist *et al*., 2003).

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