Hydrobiologia **474:** 131–137, 2002.

RAPD analysis in the Neotropical fish *Brycon lundii*: genetic diversity and its implications for the conservation of the species

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Received 5 June 2001; accepted 31 January 2002

Key words: Brycon lundii, conservation genetics, genetic variability, random amplified polymorphic DNA (RAPD)

Abstract

Random amplified polymorphic DNA (RAPD) technique was used to examine the genetic variability on an endangered Neotropical fish species, *Brycon lundii*, collected on two regions with distinct environmental conditions in the São Francisco River (Brazil), downstream from a hydroelectric station. Using decamer oligonucleotides as single primers in Polymerase Chain Reaction (PCR), genetic similarity index, mean allele frequency and mean heterozigosity were estimated, revealing variations between samples from the two regions. Moreover, a fragment of about 1200 base pairs was found in 100% of the examined animals collected at the region closer to the hydroelectric dam, while its frequency was much lower (27.3%) within the sample from the second collecting site, 30 km downstream from the dam, indicating a possible correlation between genetic variation and geographical area. A dendogram representing the relationships among genotypes was obtained, demonstrating at least two major clusters of animals. Based on the data, a model of population structuring in *Brycon lundii* is suggested. The described approach holds great promise for further analyses and gives support to biodiversity maintenance and recovery efforts of *B. lundii*.

Introduction

The genus *Brycon* Müller & Troschel, 1844, comprised of around 40 species (Howes, 1982), composes a widely distributed freshwater group throughout all main hydrographic Brazilian systems and represents important fishery resources and hatchery stocks. However, some species have been threatened due to the growing impact of environmental disturbances (Braga, 1982; Ceccarelli & Senhorini, 1996). *Brycon lundii* Reinhardt, 1874, endemic of the São Francisco hydrographic basin, has already been considered endangered (Godoy, 1975; Braga, 1982) due to river pollution, reduction of food resource, and construction of hydroelectric stations (Faria, 1994).

It has been observed that several migratory fish collected at the downstream region closest to the Três Marias hydroelectric station, built in 1960 in the main canal of the São Francisco River in Brazil, are smaller in size and have immature gonads during the spawning season. A distinct condition has been observed 30 km downstream from the dam, where these animals generally are normal-sized and have developed gonads. Although these features were specially observed in *Prochilodus marggravii*, several individuals of *Brycon lundii* collected closer to the Três Marias dam also presented morphological alterations, suggesting that this region presents less favorable conditions to their reproduction, possibly due to, among other factors, environmental modifications caused by the hydroelectric station, as lower water outflow from the reservoir into the river and lower water temperature and oxygenation (Sato et al., 1995). As destruction, alteration and fragmentation of natural environments can cause

an enormous loss of biodiversity (Wilson, 1988; Erlich, 1988; Avise, 1996), information on biology and pattern of genetic variation of *Brycon lundii* can be strongly important to develop efficient conservation strategies for this endangered Neotropical fish species.

In the last years, several molecular markers have been used in studies of genetic diversity and conservation biology (O'Brien, 1994; Avise, 1996), in order to define priorities to the management of threatened species or populations (Moritz, 1994), to develop demographic models of small or fragmented populations (Lacy & Lindenmayer, 1995), and to analyze the fitness value of natural or captive populations (Nunney & Campbell, 1993; Lynch, 1996). Randomly amplified polymorphic DNA (RAPD) is one of the genetic markers that have been employed with these approaches in several studies with fish species (Florian et al., 1995; Dahle et al., 1997; Bielawski & Pumo, 1997; Kuusipalo, 1999; Cagigas et al., 1999) due to its simple and efficient methodology, based on the amplification of several regions of a genome using single arbitrary primers that can detect polymorphism in the absence of specific DNA nucleotide sequence information (Williams et al., 1990; Welsh & McClelland, 1990).

In order to detect the genetic variability of *Brycon lundii* from two regions with distinct environmental conditions in the area of influence of the Três Marias dam in the São Francisco River, some genetic markers were analyzed, through the use of RAPD. The results were extremely useful, not only to characterize *B. lundii* but also to give support to recovery efforts and to the biodiversity maintenance of this species.

Materials and methods

Sample collection

Adult individuals of *Brycon lundii* were caught in two selected locations in the Upper São Francisco River system, downstream from the Três Marias hydroelectric station (Brazil, Minas Gerais State, township of Três Marias) (see Fig. 1a), which had not been subjected to supplemental stocking of fish. The first locality (denominated region A), that presents lower water outflow, temperature and oxygenation, corresponds to the area immediately below the dam to about 30 km downstream in the confluence of the São Francisco River with one of its principal tributaries, the Abaeté River. The second locality (region B) comprehends the area from these rivers' confluence to about 20 km downstream and presents higher water outflow, temperature and oxygenation (Sato et al., 1995) (see Fig. 1b). Eleven individuals from each local were collected during the same reproductive season, when several migratory fish species, including *B. lundii*, exhibit upstream movements in the São Francisco River towards its tributaries to spawn.

Table 1. Random amplified polymorphic DNA (RAPD) primers, corresponding sequences, GC content, and the number of loci associated with each primer. The total number of scorable loci and the number of polymorphic fragments are represented by n_t and n_p, respectively

Primer	Sequence (5′ - 3′)	(G+C)%	Number of loci	
			n_t	n_p
OPP-04	GTGTCTCAGG	60	10	4
OPP-11	AACGCGTCGG	70	11	9
OPP-13	GGAGTGCCTC	70	7	3
OPP-18	GGCTTGGCCT	70	10	2
OPC-02	GTGAGGCGTC	70	9	0
OPK-01	CATTCGAGCC	60	10	0
OPK-14	CCCGCTACAC	70	7	2
OPK-19	CACAGGCGGA	70	8	5
Total			72	25

DNA extraction and amplification

Genomic DNA was extracted from liver using a phenol-chloroform protocol (Sambrook et al., 1989) and the samples were included as accessions at the DNA Library of São Carlos (Laboratório de Citogenética, Universidade Federal de São Carlos, Brazil). A set of eight 10-mer RAPD oligonucleotides (Operon Technologies, Inc.) (see Table 1) were initially tested as single primers on two individuals of each locality to test the effect of DNA, $MgCl_2$, primers, and *Taq* polymerase concentrations, to determine the optimum annealing temperature, and to optimize the reproducibility of the RAPD assay. Further experiments were taken on 11 individuals from each sampling region. Each RAPD-PCR reaction mixture consisted of 1× reaction buffer (10 mM Tris–HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$ – Pharmacia.Biotech), 1.25 mM of each dNTP, 10 pmol random 10-mer primer, 1.5 mM $MgCl_2$, 50 ng genomic target DNA, and 1.25U *Taq* polymerase (Pharmacia.Biotech), in a total volume of 25 μl. Amplifications were carried out in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler with

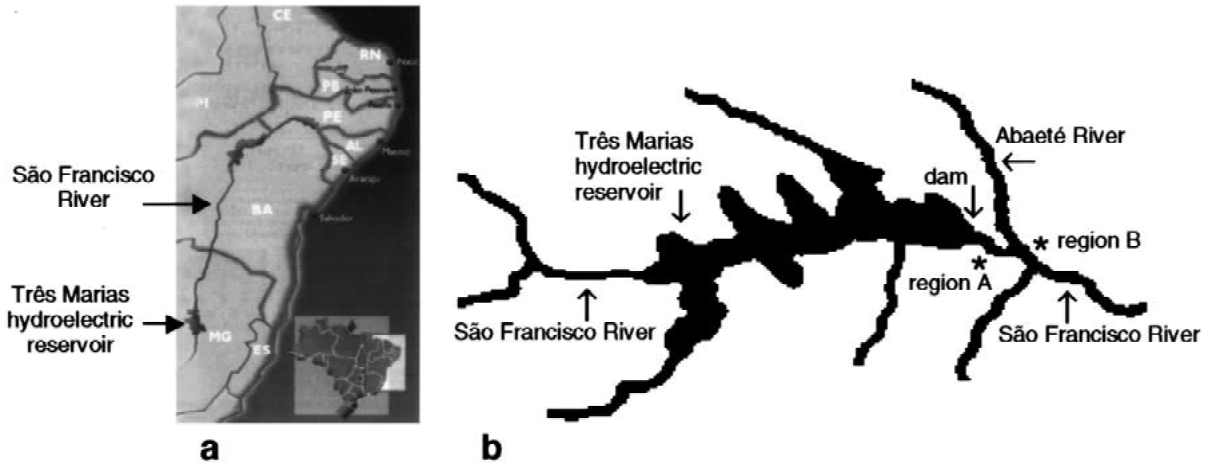

Figure 1. Sampling localities at the São Francisco River. (a) partial map of Brazil; (b) region from the Três Marias hydroelectric dam until 30 km downstream in the confluence of the São Francisco River with the Abaeté River (region A), and region from the confluence of the the São Francisco River with the Abaeté River until 20 km downstream (region B).

the following cycle program: 45 cycles at 94 °C for 1 min, 37 °C for 1 min, and 72 °C for 2 min; and a final extension step at 72 °C for 10 min. A negative control, consisting of all the reaction components except template DNA, was also included to monitor any possible contamination. Reactions products (10 μl) were subjected to electrophoresis on 1.4% agarose gel for 4.5 h at 80 V. DNA bands were visualized after ethidium bromide staining (Sambrook et al., 1989) and molecular weights were estimated using standard DNA markers.

Statistical RAPD analysis

For each genotype, the presence and absence of fragments were scored as 1 or 0, respectively. Although visualization of same-sized DNA fragments on agarose gels does not exclude the possibility that some may contain non-homologous DNA sequences, we assumed that marker alleles from different loci do not co-migrate to the same position on a gel and that each fragment represents a Mendelian locus in which the visible 'dominant' allele is in Hardy-Weinberg equilibrium with a 'recessive' null allele or absent fragment (Lynch & Milligan, 1994). Only reproducible well-marked amplified fragments ranging from 300 to 1600 bp were scored and pairwise comparison of banding patterns was evaluated among samples for a combined data of primers, by calculating an index of genetic similarity using the coefficient method of Jaccard (1901). The genetic variability was expressed by mean allele frequency (Jeffreys & Morton, 1987) and by mean heterozigosity (Georges et al., 1988). In addition, cluster analysis, using the UPGMA method (unweighted pair-group method with arithmetical averages) (Sneath & Sokal, 1973), was carried out, from which a dendogram representing the relationships among genotypes was obtained. Statistical analyses were performed using the NTSYS-PC version 1.70 (Numerical Taxonomy and Multivariate Analysis System) computer program (Rohlf, 1993).

Results

A primary evaluation of the eight oligonucleotides, tested on two individuals from each collecting site, indicated that the primers produced amplification products ranging from approximately 300 base pairs to 3000 base pairs (bp), and that each of them generated an unique band pattern of amplified DNA in *Brycon lundii* (data not shown). Further RAPD-PCR, using 11 individuals from each locality, showed that the total number of fragments that were selected to be used in the analyses (300–1600 bp), generated by each primer, varied from 7 to 11 (as shown in Table 1) with an average of 7.4 bands per individual and primer. The eight primers amplified a total of 72 fragments and, of these, 47 loci (65.3%) were present in all individuals and 25 loci (34.7%) were polymorphic.

Repeated amplifications with a given primer were mostly reproducible. Amplifications using the primer OPC-02 resulted on faint and diffuse bands that seemed to be monomorphic. One other primer (OPK-

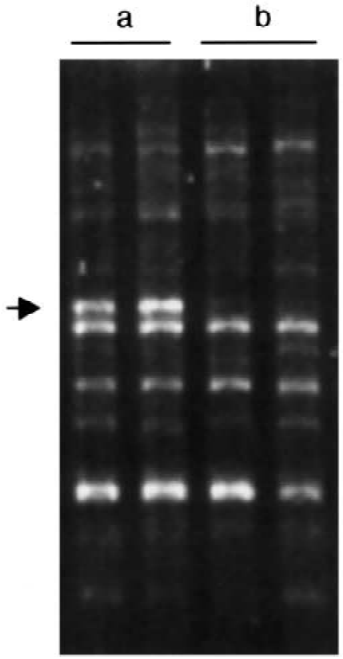

Figure 2. RAPD patterns of two individuals of *Brycon lundii* from region A (a) and two individuals from region B (b), using the primer OPP-18. Arrow denotes a 1200 bp characteristic band of *B. lundii* from region A.

01) also produced a monomorphic banding pattern. Primers OPP-11, OPP-04 and OPK-14 generated extremely high complex banding patterns or variable fragments that were weak and not well separated from each other. The primers OPP-13, OPP-18 and OPK-19, that generated the highest quality banding patterns, with intense stained fragments well separated from other bands, and sufficient variability, were selected for the statistical analyses. Using these three last oligonucleotides, from a total of 25 scorable bands, 15 (60%) were conserved among all individuals, while 10 (40%) amplification products were polymorphic (as shown in Table 1).

All RAPD primers failed to yield a diagnostic band, present in all and only the members of a determined locality. However, a fragment of about 1200 bp (see Fig. 2) was detected, using the primer OPP-18, in all animals from region A, while the frequency of this band was much lower (27.3%) within the sample from region B. Due to its different frequencies in the two sampling localities, this fragment could be denominated as a characteristic band of *Brycon lundii* from region A.

Values of mean similarity index within and between the two sampling localities were obtained by a combination of the primers OPP-13, OPP-18 and OPK-19 (see Table 2). The individuals collected at region A showed a higher mean genetic similarity when compared to the individuals collected at region B. An intermediary mean similarity value was detected between individuals from the two collecting sites, when compared to the values within region A or within region B. The mean allele frequency and the mean heterozigosity (see Table 2) also presented variable values. Individuals from region A presented higher allele frequency and lower heterozigosity, while these results were inverse within animals from region B.

Table 2. Values of mean band similarity index, mean allele frequency and mean heterozigosity within and between sampling localities of *Brycon lundii*, calculated for combined data of primers OPP-13, OPP-18 and OPK-19

Sampling localities	Mean Similarity Index	Mean Allele Frequency	Mean Heterozigosity
Region A	0.84	0.60	0.57
Region B	0.77	0.52	0.64
Region A and region B	0.79	0.54	0.63

UPGMA analyses generated a dendogram indicating relationships among individuals of *Brycon lundii*. Almost all the animals collected at region A were clustered, while the individuals from region B were observed in two distinct groups (as shown in Fig. 3).

Discussion

The obtained results showed that some of the analyzed decamer primers permitted the detection of polymorphic fragments in *Brycon lundii*, revealing different levels of genetic variability within and between region A and region B. Band-sharing-based similarity indices, mean allele frequency and heterozigosity values were sufficient to distinct these two sampling localities, even with the limited survey of individuals and primers used in this study. Similar results, even more expressive, were obtained with the use of minisatellite core sequences as primers in PCR (Wasko & Galetti Jr., unpublished data). Equivalent genetic differentiation was also observed on other fish species, *Prochilodus marggravii*, collected at the same regions in the Upper São Francisco River (Hatanaka & Galetti Jr., unpublished data).

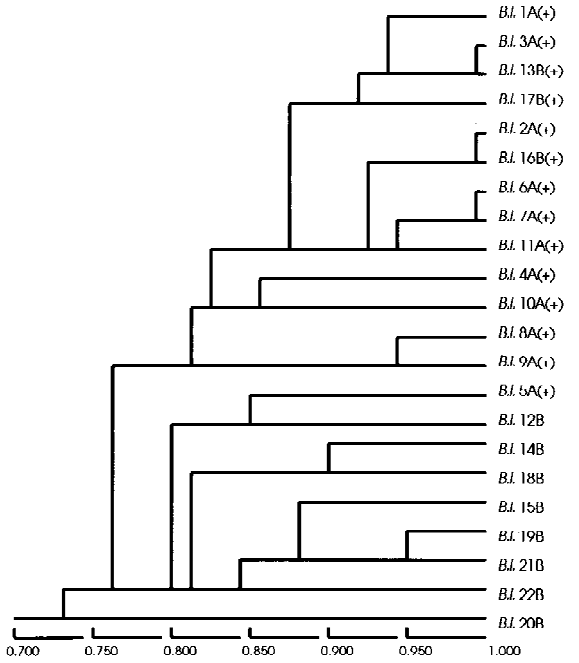

Figure 3. Dendogram showing the genetic relationships among individuals of *Brycon lundii* based on UPGMA cluster analysis using combined data of RAPD primers. *B.l.*A represents individuals collected at region A, and *B.l.*B indicates individuals collected at region B in the São Francisco River. The presence of a 1200 bp fragment, detected with the primer OPP-18, is indicated by the symbol (+).

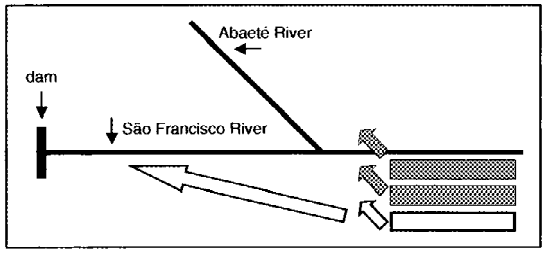

Figure 4. Illustration of a population structuring model of *Brycon lundii* in the Upper São Francisco River. The distinct fish stocks are represented with different arrows.

Despite the fact that no sampling locality-specific genetic marker was found in any of the two analyzed regions, one band of about 1200 bp was detected, using the primer OPP-18, among 100% of the individuals from region A and among only 27.3% of the individuals from region B, indicating a characteristic band from animals collected at region A. The frequency of other fragments also varied between the two sampling localities, although these differences were less expressive. As significant levels of divergence in frequencies of shared markers, assumed to be independent and selectively neutral, can indicate a genome-wide effect resulting from a restriction in gene flow (Wright, 1969), the results reinforce a differentiation between animals collected at region A and B.

The UPGMA analysis permitted clustering individuals from region A closer to each other, indicating a high genetic similarity among them, while individuals from region B were not clustered on a single unit, demonstrating a higher genetic heterogeneity. Considering the existence of a single panmictic population at region B, part of this group could migrate towards region A during the reproductive period and the greater genetic similarity among these animals could be just the result of genetic drift. However, the obtained data could also suggest that the animals collected at region A could either represent an unique stock of *B. lundii*, while individuals collected at region B could represent a faunal mixing, comprehending at least two stocks with genetic differences. Interestingly, some of the animals from region B, exactly the ones that also presented the 1200 bp fragment (characteristic of all the individuals collected at the region A), clustered with the individuals from region A (see Fig. 3) and, therefore, could belong to an unique stock of *B. lundii*. As so, it seems that this fragment, instead of a characteristic band (present in distinct frequencies among populations) could be a diagnostic band (present in all and only the members of a population). However, we cannot ignore the possibility that the detection of this private allele may be due partially to the limited sample sizes. No other characteristic and/or diagnostic fragments were found in *B. lundii*, which is presumable, considering that as genetic differentiation of subdivided populations occurs as result of selection or the random processes of mutation and genetic drift, it is not expected to occur at all loci (Meffe & Vrijenhoek, 1988).

Although we do not claim that the current data could provide a complete picture of the distribution of *Brycon lundii* in the Upper São Francisco River, based on the obtained data, a model of population structuring can be suggested, where the animals collected at region A could represent an unique stock and the animals from region B could comprehend at least two co-occurring populations that present a co-migrating behavior during the spawning season. The animals found at region A seem to represent a small fraction of *Brycon lundii* found in the Upper São Francisco River that migrate towards the Três Marias dam (to region

A), while the remainders would possibly migrate to places with more favorable environmental conditions for reproduction (as shown in Fig. 4).

As previous detailed that region A lacks appropriate conditions for reproduction and that several individuals of *B. lundii* collected at this area present smaller size (Sato et al., 1995), the proposed population structuring scenery could be related to a competition for resources defense (e.g. Krebs & Davies, 1996), which implies that stronger individuals (most of the animals found at region B) occupy areas with better resources and establish territories, leading the remaining individuals to less favorable habitats (region A). Another possible explanation for the population structuring of *B. lundii* can be related to homing behavior and reproductive site fidelity, already described for other migratory fish species (Yoshiyama et al., 1992; Tallman, 1994; Dittman & Quinn, 1996; Hartney, 1996; Wirgin et al., 1997; Hodgson et al., 1998) and that has been associated to differences in allozyme allele frequencies (Varnavskaya et al., 1994), morphology, age, length, egg size, fecundity, and timing of reproduction (Gard et al., 1987; Rogers, 1987; Blair et al., 1993; Quinn et al., 1995). The distinct stocks of *B. lundii* could also present a preference for a particular site and its movement during the spawning period should not be entirely random or simply a passive response to some prevailing freshwater conditions. The observed genetic differences could be due to different evolutionary pathways of breeding populations, while the remaining genetic similarities could represent a putative genetic pool of a common ancestor stock.

The identification of few individuals with the 1200 bp fragment at region B that should be representatives of the stock found at region A, suggests that part of this population seems to find a spawning area at region B, thus enhancing the maintenance of this fish stock as this region presents favorable conditions to the reproduction (taking into account the hypothesis of competition for resources defense), or indicates that these animals were collected just through their migratory route towards the Três Marias dam (taking into account the hypothesis of homing behavior).

The careful selection of appropriate natural stocks, based on genetic criteria, can offer greater potential for success in species-recovery and maintenance programs (Quattro & Vrijenhoek, 1989) and, in the last years, several molecular markers have been used in conservation studies of endangered fish (Ashbaugh et al., 1994; Ferguson et al., 1995; Wirgin et al., 1997; Diaz et al., 1998; Kirchhoff et al., 1999). As *Brycon lundii* has been chosen to integrate a genetic conservation program of Brazilian endangered migratory fish species (Bedore & Godinho, 1999), the region B and, perhaps, the tributaries of the São Francisco River, would be propitious to collect founder stocks as these localities could retain more variability.

The present study proposes a population structuring model of a Neotropical migratory fish on a small geographic area, suggesting the presence of different co-existent gene pools of *Brycon lundii*. It would be instructional to continue the genetic studies in coming years, including analyses of individuals from other localities and at different times along the year, in order to define priority areas in the Upper São Francisco River system to be selected to include different unities of *Brycon lundii* to be used in reproductive and restocking programs.

Acknowledgements

The authors thank Yoshimi Sato (Companhia de Desenvolvimento do Vale do São Francisco) for collecting the specimens. A.P.W. was supported by a fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). This work was also supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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