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PCR primed with minisatellite core sequences yields species-specific patterns and assessment of population variability in fishes of the genus *Brycon*

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Summary

Minisatellite core sequences were used as single primers in polymerase chain reaction (PCR) to amplify genomic DNA in a way similar to the random amplified polymorphic DNA methodology. This technique, known as Directed Amplification of Minisatellite-region DNA, was applied in order to differentiate three neotropical fish species (*Brycon orbignyanus*, *B. microlepis* and *B. lundii*) and to detect possible genetic variations among samples of the threatened species, *B. lundii*, collected in two regions with distinct environmental conditions in the area of influence of a hydroelectric dam. Most primers generated species-specific banding patterns and high levels of intraspecific polymorphism. The genetic variation observed between the two sampling regions of *B. lundii* was also high enough to suggest the presence of distinct stocks of this species along the same river basin. The results demonstrated that minisatellite core sequences are potentially useful as single primers in PCR to assist in species and population identification. The observed genetic stock differentiation in *B. lundii* associated with ecological and demographic data constitute a crucial task to develop efficient conservation strategies in order to preserve the genetic diversity of this endangered fish species.

Introduction

The use of molecular techniques has increased dramatically over the past several years, largely due to the development of the polymerase chain reaction (PCR). The random amplification of polymorphic DNA (RAPD), a methodology that employs a single short primer in PCR (Welsh and McClelland, 1990; Williams et al., 1990), has been widely used in several studies in animals, including the identification of fish species (Dinesh et al., 1993; Borowsky et al., 1995; Sultmann et al., 1995; Partis and Wells, 1996; Callejas and Ochando, 1998) and the detection of population genetic variability in these organisms (Johnson et al., 1994; Caccone et al., 1997; Nadig et al., 1998; Cagigas et al., 1999; Chong et al., 2000). A great advantage of using RAPD in genetic analysis, more than its cost-efficiency and the detection of high levels of polymorphism, is that the procedure is quite simple. However, one drawback of using short oligonucleotides as random primers is the low stringency necessary for the DNA amplification, which can lead to non-reproducible results, limiting its application.

Minisatellites, or variable number of tandem repeats (VNTR), are tandemly repeated DNA sequences dispersed throughout animal and plant genomes, generally consisting of 10–60 base pairs (bp) motifs that vary in the number of units between genotypes (Jeffreys et al., 1985; Nakamura et al., 1988). Each VNTR includes a short, highly conserved region, known as a core sequence (Jeffreys et al., 1985), that can be used as a single primer in PCR to direct the amplification to regions rich in minisatellite repeats, a technique denominated Direct Amplification of Minisatellite-region DNA (DAMD) (Heath et al., 1993). This methodology has been used in some fish, birds (Heath et al., 1993), rice (Zhou et al., 1997), wheat (Somers et al., 1996; Bebeli et al., 1997), bean (Metais et al., 2000), and fungal species (Barroso et al., 2000; Santini and Capreti, 2000) to detect DNA polymorphism through RAPD-like results. As these VNTR core sequences are longer than RAPD primers, this methodology can also be effectively carried out at relatively high stringencies (high PCR annealing temperatures), thus yielding more reproducible results. Furthermore, some isolated DAMD-PCR fragments can also potentially be used to generate genome-specific probes (Heath et al., 1993).

The genus *Brycon* Müller & Troschel 1844 comprises a neotropical migratory fish group of great economic importance for commercial fishing and fish farming in Brazil. Most of the recent genetic data in *Brycon* is related to cytogenetics research (Almeida-Toledo et al., 1996; Margarido and Galetti Jr, 1996; Wasko and Galetti Jr, 2000), which had demonstrated a high karyotype stability in the group, sometimes making it difficult to identify species-specific markers. Moreover, little information exists on biology and pattern of genetic variation of these animals, which could be strongly important to develop efficient conservation strategies as several species, including *B. lundii* endemic in the São Francisco hydrographic basin, have been considered at risk of extinction (Braga, 1982; Ceccarelli and Senhorini, 1996).

The purpose of the present study was to utilize the DAMD-PCR methodology in *Brycon*, not only to characterize and access genetic diversity of some species of the group but also to verify the occurrence of genetic variations in *B. lundii* from two regions with distinct environmental conditions in the area of influence of a hydroelectric dam. The results were extremely useful to improve the genetic data of *Brycon* as well as to give support to recovery efforts and to the biodiversity maintenance of *B. lundii*.

Materials and methods

Sample collection

The three analyzed species of the genus *Brycon* were collected at different hydrographic Brazilian systems. *Brycon orbignyanus*

was collected at Paraná River (township of Porto Rico, Paraná State) and *B. microlepis* was caught at Cuiabá River (township of Cuiabá, Mato Grosso State). Individuals of *B. lundii* were collected during the same spawning season in two distinct regions in the Upper São Francisco River, downstream from the Três Marias hydroelectric dam (township of Três Marias, Minas Gerais State). The first locality (designated region A) corresponds to the area from the dam to 30 km downstream at the confluence of the São Francisco River with one of its principal tributaries, the Abaeté River, and seems to present worse environmental conditions to the reproduction of several migratory fish. The second locality (designated region B) composes the area from the confluence of the two rivers to 20 km downstream; this area presents more favorable conditions to reproduction (Sato et al., 1995; Wasko and Galetti Jr, 2002).

DNA extraction and amplification

Genomic DNA was extracted from the liver, following a phenol–chloroform protocol detailed in Sambrook and Russell (2001) and the samples were included as accessions at the DNA Library of São Carlos (Laboratório de Citogenética, Universidade Federal de São Carlos, Brazil).

Five VNTR core sequences (Table 1) were used as single primers in PCR. Several experiments were initially run to test the effect of DNA, $MgCl_2$, primers, and *Taq* DNA polymerase concentrations. As repeated amplifications with a given primer were reproducible, further experiments were taken on four individuals of *B. orbignyanus*, 11 individuals of *B. microlepis* and 11 individuals of *B. lundii* from each collecting site. Each VNTR-PCR reaction mixture contained 1X reaction buffer (10 mM Tris–HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$; Amersham Pharmacia Biotech), 1.25 mM of each dNTP, 100 pmol of a single primer, 200 ng of template DNA, and 1.25 U *Taq* polymerase (Amersham Pharmacia Biotech), in a total volume of 50 µl. Amplifications were performed in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler with the following cycle program: denaturation at 94°C for 5 min, followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; and a final extension step at 72°C for 5 min. A negative control, consisting of all the reaction components except template DNA, was also included to monitor any possible contamination. The PCR products (6 µl) were then fractionated on 8% polyacrylamide gels for 4–5 h at 80 V and the DNA bands were visualized after silver nitrate staining (Sambrook and Russell, 2001). Molecular weights were estimated using 1 Kb bp ladder as a standard DNA marker.

Statistical analysis

Statistical analyses were performed using the NTSYS-PC Numerical Taxonomy and Multivariate Analysis System (NTSYS-PC) version 1.70 computer program (Rohlf, 1993). Pairwise comparison of banding patterns was evaluated among samples amplified by each primer separately and for the combined data of some primers, by calculating an index of genetic similarity using the coefficient method of Jaccard (1901). Bands ranging from 100 to 1000 bp were scored.

Table 1
Minisatellite core sequences used as single primers in PCR

Primer	Sequence (5′–3′)	Reference
PER1	GACNGGNACNGG	Georges et al. (1988)
INS	ACAGGGGTGTGGGG	Nakamura et al. (1987)
M13	GAGGGTGGNGGNTCT	Vassart et al. (1987)
YN73	CCCGTGGGGCCGCCG	Nakamura et al. (1988)
YNZ22	CTCTGGGTGTCGTGC	Nakamura et al. (1987)

Results

The reproducibility of DAMD-PCR was tested changing different parameters, such as quantity of *Taq* DNA polymerase and genomic template DNA. The high reaction stringency (hybridization temperature of 55°C) greatly limited the amplification of artifacts and consistent amplification patterns were obtained using the minisatellite core sequences as single primers in PCR.

The five primers yielded amplification products in the three analyzed species of the genus *Brycon*. The number and positions of the bands depended on species and primer. Amplifications using the primer YNZ22 resulted on several faint and diffuse bands and the primer YN73 generated a highly complex banding pattern with fragments not well separated from each other (data not shown). Primers PER1, M13 and INS yielded easily scorable fragments (intensely stained and separated from other bands) and species-specific banding patterns in *B. orbignyanus*, *B. microlepis*, and *B. lundii*. The patterns obtained with the primers PER1 and INS can be observed in Fig. 1. Moreover, as amplification products with primers INS and M13 presented high diversity within each species, these oligonucleotides were selected for the statistical analyses of genetic variability. The total number of analyzed bands and polymorphic fragments obtained for each species, using primers INS and M13, are given in Table 2.

The frequency of several amplified fragments differed substantially between the two sampling regions of *B. lundii*. A fragment of about 400 bp (Fig. 2) was detected, using primer M13, in almost all the animals (91%) from region A and on only 27.3% of the individuals collected at region B. Owing to its significantly different frequencies in the two sampling localities, this fragment could be denominated as a characteristic band of *B. lundii* from region A.

Values of mean similarity index within each species and between the two sampling localities of *B. lundii* were calculated for primers INS and M13 separately and for a combination of these two primers (Table 3). Individual primers differed slightly in the amount of detected variation in *B. orbignyanus* and *B. microlepis*. Greater differences were observed in *B. lundii*: the animals collected at region A showed higher mean genetic similarity when compared with the individuals from region B. Intermediate values were observed between the two *B. lundii* sampling regions. Furthermore, the individuals of *B. lundii* from region B presented band-sharing-based indices very similar to those values detected for *B. orbignyanus* and *B. microlepis*.

Discussion

Some minisatellite core sequences (PER1, INS and M13) used as single primers in PCR yielded species-specific fragment patterns in *B. lundii*, *B. orbignyanus* and *B. microlepis*. Although several amplified fragments were shared by all three species, clearly distinguishable bands were observed only in a determined species. Consistent species-specific banding patterns were also visualized in *B. cephalus*, *B. brevicauda*, *B. insignis* and *Brycon* sp. using the primers PER1 and INS

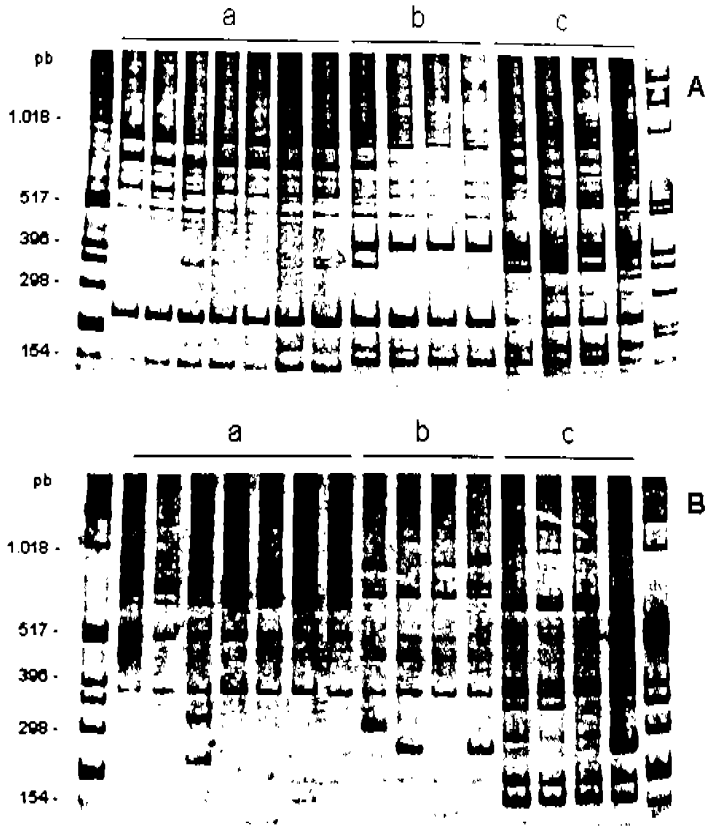

Fig. 1. Minisatellite-PCR patterns of individuals of *Brycon lundii* (a), *B. orbignyanus* (b) and *B. microlepis* (c), using primers PER1 (A) and INS (B). The first and last lanes correspond to 1 Kb molecular weight marker

Table 2
Number of loci associated with each primer in the analyzed species. The total number of loci and the number of polymorphic fragments are represented by n_t and n_p, respectively

	Primer INS Number of loci		Primer M13 Number of loci	
	n_t	n_p	n_t	n_p
Brycon orbignyanus	21	16	24	17
Brycon microlepis	33	31	23	21
Brycon lundii	18	17	12	12

(data not shown). As the genus *Brycon* presents a high karyotype stability, which makes it difficult to identify species-specific chromosome markers (Margarido and Galetti Jr, 1996; Almeida-Toledo et al., 1996), the PCR using minisatellite sequences as single primers represents a potential and simple methodology to identify different species of the group as it generates unique DNA fingerprints.

Although minisatellite core sequences were also used in PCR to reveal species-specific banding patterns in salmonids such as *Salmo salar*, *Oncorhynchus tshawytscha* and *O. mykiss*, and in striped bass such as *Morone saxatilis*, *M. chrysops*, and *Pseudopleuronectes americanus* (e.g. Heath et al., 1993), most of the amplified fragments were not variable within each of these species. On the other hand, several fragments amplified by primers INS and M13 showed an extensive intraspecific polymorphism in *B. orbignyanus*, *B. microlepis* and *B. lundii*. Moreover, a genetic variation could also be observed among individuals of the latest species collected in two regions in the São Francisco River.

Band-sharing-based similarity values of *B. lundii* were significantly distinct between animals collected at region A and region B. Although no specific marker for *B. lundii* from any of the two sampling regions was found, a characteristic fragment of about 400 bp was observed, using primer M13, in almost all the individuals from region A and in only 27.3% of the individuals collected at region B. The frequency of other fragments also varied between animals from the two localities. Significant divergence levels in the frequency of shared markers could be reflecting a restriction in gene flow (Wright, 1969). Similar differences were also detected on samples of *B. lundii* from these two regions with the use of RAPD primers, suggesting the presence of distinct stocks of the species in the Upper São Francisco River (Wasko and Galetti Jr, 2002). An equivalent genetic differentiation was also observed on other migratory fish species, *Prochilodus marggravii*, collected at the same regions in the São Francisco River (Hatanaka, 2001).

Genetic variations have been used as evidence for the existence of population subdivision among fishes from the same river basin (Bielawski and Pumo, 1997; Diaz et al., 1998) and substantial genetic differentiation can be detected even without distinct geographical barriers (Avise and Felley, 1979; White and Turner, 1984). The genetic differentiation observed in *B. lundii* in the present study supports previous RAPD analyses (Wasko and Galetti Jr, 2002), reinforcing a suggested model of population structuring of the species, where the animals collected at region A in the São Francisco River could represent an unique stock and the animals collected at region B could comprehend at least two distinct co-occurring and co-migrating populations during the spawning season (Wasko and Galetti Jr, 2002).

Although band-sharing comparisons among *B. lundii*, *B. orbignyanus* and *B. microlepis* were not performed as it could be possible that common fragments in different species

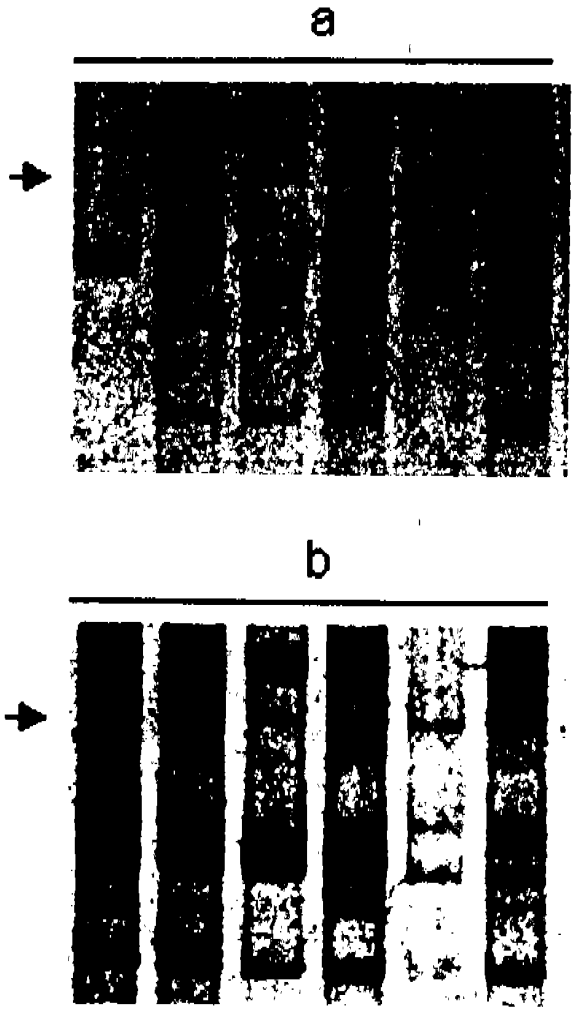

Fig. 2. Minisatellite-PCR patterns of individuals of *Brycon lundii* from region A (b) and from region B (a), using the primer M13. Arrow denotes a 400 bp characteristic band of *B. lundii* from region A

Table 3
Values of mean similarity index among individuals of each species, calculated for each primer separately (INS and M13) and for combined data of these primers

	Mean similarity index		
	Primers		
Species and sampling sites	INS	M13	INS/M13
Brycon orbignyanus	0.45	0.52	0.48
Brycon microlepis	0.38	0.40	0.38
Brycon lundii - within region A	0.75	0.54	0.65
Brycon lundii - within region B	0.51	0.39	0.47
Brycon lundii - within region A and region B	0.58	0.40	0.51

were not related to the same loci, the within-species band-sharing indices could be used as a parameter to estimate an expected common genetic variability level for species of the genus *Brycon*. If so, it is possible that other species of the group also present a population structuring on other hydrographic Brazilian systems, as observed for *B. lundii* from the São Francisco River.

In recent years, fish culture programs of *B. lundii* have been carried out in Brazil (Bedore and Godinho, 1999) to integrate a species-recovery and biodiversity maintenance program. The aim of conservation programs must be the development of an integrated strategy that conserves as much genetic diversity within the species as possible, and ensures the presence of utilizable fish resources (Hansen and Loeschcke, 1994). The identification of different stocks of *B. lundii* could contribute to the selection of individuals that represent a better genetic diversity of the species.

The use of minisatellite core sequences as single primers in PCR were shown to be a simple and efficient method to detect DNA variations in species of the genus *Brycon*, permitting not only the identification of several species but also the estimation of genetic diversity in *B. orbignyanus*, *B. microlepis* and *B. lundii*. Moreover, it was also possible to detect a high level of polymorphism in *B. lundii*, suggesting the occurrence of population subdivision of this species in the Upper São Francisco River. Therefore, the DAMD technique appears to represent a good and easily reproducible means to identify several species of fish and to analyze fish population in general.

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