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Short Communication

Description of novel microsatellite loci in the Neotropical fish *Prochilodus argenteus* and cross-amplification in *P. costatus* and *P. lineatus*

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Abstract

Prochilodus is one of the most important fish resources of South America, in addition to the important role it plays in nutrient cycling of Neotropical rivers. In the present study, we describe the isolation and characterization of nine novel microsatellite loci in *Prochilodus argenteus*. The number of alleles per polymorphic locus varied from 5 (Par76) to 21 (Par85), revealing a total of 116 alleles. The values of observed and expected heterozygosities ranged from 0.629 (Par69) to 0.926 (Par85 and Par86) and from 0.643 (Par66) to 0.931 (Par80), respectively. Furthermore, the ability of these and other previously described microsatellite markers to amplify orthologous loci was tested in two related species, *Prochilodus costatus* and *Prochilodus lineatus*. These loci will be useful for studies of population genetic structure in this group of fishes, and in aiding future genetic mapping studies of *P. argenteus*.

Key words: cross-species amplification, enrichment libraries, microsatellite, *Prochilodus*.

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Family Prochilodontidae constitutes one of the most important fish resources of South America (Bayley and Petrere, 1989), in addition to its important role in nutrient cycling in Neotropical rivers (Flecker, 1996). *Prochilodus* comprises 49 nominal species of which only 13 species are valid (Castro and Vari, 2003). Among the species found in the São Francisco River basin, *Prochilodus argenteus*, popularly known as curimatã-pacu, forms a bulk of the subsistence fishery of the region, although numbers harvested have drastically declined in the last years (Sato and Godinho, 2004).

Microsatellites are polymorphic DNA sequences containing short tandemly arranged repetitions (Tautz, 1989), distributed throughout the genome (Litt and Luty, 1989), and found in all prokaryotic and eukaryotic genomes studied until now (Zane *et al.*, 2002). Due to their high variability, these genetic markers have been widely used in genetic mapping (Knapik *et al.*, 1998; Shimoda *et al.*, 1999; Coimbra *et al.*, 2003) and population structure studies (Hatanaka *et al.*, 2006; Primmer *et al.*, 2006). However, one of the great impediments for the wider use of microsatellites is the need to isolate and characterize these markers via cloning and sequencing of genomic libraries for each species of interest (Angers and Bernatchez, 1997). Nevertheless, once the flanking sequences of microsatellite markers are known, a large number of individuals may be rapidly genotyped.

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Although the Neotropical ichthyofauna is the world's most diversified (Lowe-McConnel,1969; 1987), microsatellite primers have been published only for *Piaractus mesopotamicus* (Calcagnotto *et al.*, 2001), *Astyanax fasciatus* (Strecker, 2003), *Arapaima gigas* (Farias *et al.*, 2003), *Brycon opalinus* (Barroso *et al.*, 2003), *Eigenmannia* sp. (Moysés *et al.*, 2005), *Pseudoplatystoma corruscans* (Revaldaves *et al.*, 2005), *Brycon hilarii* (Sanches and Galetti, 2006) and *Prochilodus costatus* (Carvalho-Costa *et al.*, 2006). The isolation and characterization of microsatellite loci has also been performed in *P. argenteus*, and thirteen loci were previously described for this species (Barbosa *et al.*, 2006). However, for the construction of genetic maps, a large number of genetic markers is necessary. Thus, the present study had the objective of describing novel *P. argenteus* loci in order to aid future genetic mapping studies. Furthermore, the heterologous amplification of these and other previously described loci was tested in two other *Prochilodus* species, specifically *P. costatus* and *P. lineatus*.

Total genomic DNA was extracted from liver tissue of a *P. argenteus* individual using the phenol-chloroform method (Sambrook *et al.*, 1989). A partial genomic library enriched for tetranucleotide loci was constructed following the protocols of Hamilton *et al.* (1999). The DNA was digested with *BstU* I and DNA fragments ranging from 300 to 1200 bp were excised from the gel and purified using the

Wizard SV Gel and PCR Clean-Up System kit (Promega). Enrichment was performed using eight biotinylated oligonucleotides {$(AAAC)_6$ $(AAAG)_6$ $(AATC)_6$ $(AATG)_6$ $(ACCT)_6$ $(ACAG)_6$ $(ACTC)_6$ $(ACTG)_6$} and streptavidin-coated magnetic beads (Streptavidin Magnesphere Paramagnetic Particles, Promega). Clones containing inserts were sequenced using the DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare) and products were resolved on an ABI Prism 377 automated sequencer (Applied Biosystems). The microsatellite-like sequences were analyzed using Tandem Repeats Finder (Benson, 1999) and flanking primers were then designed using the Primer 3 software (Rozen and Skaletsky, 2000).

To analyze if loci were variable up to 30 individuals from two collection sites in the São Francisco River (Brazil) were used. All loci were amplified following the PCR method described by Schuelke (2000), in which three primers are used simultaneously, a fluorescently-marked M13 primer, a forward primer with a fusion of a leading M13 universal sequence, and a reverse primer. PCR reactions were carried out in 10 µL reactions containing 100 ng of DNA, 200 µM dNTPs, PCR buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl; LGC Biotecnologia), 4 pmol of each reverse and 6-FAM or NED M13 (-21) primers and 1 pmol of the forward primer, 1.5 mM $MgCl_2$ and 1 U of *Taq* DNA Polymerase (LGC Biotecnologia). Conditions of the PCR amplification were as follows: 1 cycle at 95 °C (5 min), 35 cycles at 94 °C (30 s), 30 s at the annealing temperature (Table 1) and 72 °C (30 s), followed by 8 cycles 94 °C (30 s), 53 °C (45 s), 72 °C (45 s), and a final extension at 72 °C for 10 min.

For genotyping, the PCR products were subjected to electrophoresis along with GeneScan ROX 350 (Applied Biosystems) internal size standards on an ABI 377 automated sequencer (Applied Biosystems). Allele sizes were assigned using the Genescan and Genotyper 2.5 software packages (Applied Biosystems). Hardy-Weinberg equilibrium (HWE), observed and expected heterozygosities and linkage disequilibrium tests were performed using the software Genepop 3.3 (Raymond and Rousset, 1995).

Of the 96 sequenced clones, 51 (53.13%) contained microsatellite sequences and primers were designed for 31 loci (Par 55 - Par86). Of these, 15 were successfully amplified. Only loci Par61, Par72, Par73 and Par74 presented a monomorphic pattern, *i.e.* the most common alleles presented a frequency superior to 95% (Zou *et al.*, 2005), whereas the others showed high levels of polymorphism. The number of alleles per polymorphic locus varied from 5 (Par76) to 21 (Par85), revealing a total of 116 alleles. The values of observed and expected heterozygosities ranged from 0.629 (Par69) to 0.926 (Par85 and Par86) and from 0.643 (Par66) to 0.931 (Par80), respectively. Significant deviations from the Hardy-Weinberg equilibrium were found in the Par71, Par83 and Par86 loci ($p < 0.05$), which may be explained by the presence of null alleles, mating systems, cryptic population structure and non-random sampling. Summary data for all loci are presented in Table 1.

To test cross-species amplification, three individuals of *P. costatus* and three individuals of *P. lineatus* were used. In addition to the loci isolated in the present study, 13 previously described loci for *P. argenteus* (Barbosa *et al.*, 2006) were also amplified in these species. The markers de-

Table 1 - Characterization of microsatellite loci in *Prochilodus argenteus*.

Locus GenBank accession n.	Repeat motif	Primers (5'→3')	Ta (°C)	Size-range (pb)	N_a	H_O	H_E	p
Par66 DQ524172	$(AACA)_{12}$	F: TCTATAACTGTGGTCGTATG R: GAGGTTTTGAGATCAGTTG	47.0	153-185	9	0.737	0.643	0.528
Par69 DQ524173	$(TTAT)_7(TCAT)_6$	F: AATCTTTTCTAGGCTGTAGG R: GGGAAGTAGAAAGAAGAAAC	55.6	222-256	7	0.629	0.700	0.969
Par71 DQ524174	$(GA)_{24}$	F: TGTCGTCTGAAAGGAGTC R: GAGGTTGTCCATTTTTAGAG	55.6	237-281	17	0.872	0.600	0.000*
Par76 DQ524175	$(CAGT)_{16}$	F: GGGTTACATTACATTCTAGG R: CAAGTCTCTTCTGCTAACTG	53.4	226-242	5	0.702	0.733	0.207
Par80 DQ524176	$(CT)_{37}$	F: CTAACCTACAAACCTCATTC R: CTGTAAAAGCTCCACTTATC	51.3	221-279	17	0.921	0.931	0.850
Par82 DQ524177	$(CT)_{27}$	F: CTCTAACAAGGTGAAACAAC R: TTTAAACTGTAGGCACAGAC	51.3	177-209	14	0.831	0.900	0.437
Par83 DQ524178	$(CACT)_{11}$	F: CATTTTCTAACAGCACTCC R: TTCTTGTTCTCCTGTGTAAC	55.6	264-300	10	0.853	0.897	0.043*
Par85 DQ524179	$(AG)_{25}$	F: CCACTTAATGAGACCACAC R: TTTCATTAGACTCGGTGAG	51.3	227-271	21	0.926	0.925	0.276
Par86 DQ524180	$(GA)_{46}$	F: ATCCGTCTCTATGTGTGTC R: TCTACAGTTACTTGGAGGAC	57.8	135-181	16	0.926	0.740	0.045*

Ta, annealing temperature; bp, base pairs; N_a, total number of different alleles observed; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE test, p values for Hardy-Weinberg equilibrium test; *$p < 0.05$.

veloped for *P. argenteus* amplified and were polymorphic in both species tested. The efficiency of heterologous amplifications was 100% in *P. costatus* and 95.45% in *P. lineatus* (Par34 presented a faint band). Allele sizes for *P. costatus* and *P. lineatus* corresponded to the size range observed for *P. argenteus*. In *P. costatus* the loci Par34 and Par76 were monomorphic, while Par35, Par69, Par71, Par76 and Par82 were monomorphic in *P. lineatus*. All other remaining loci were polymorphic in their respective species (Table 2).

A description of these novel microsatellite loci in *P. argenteus* and the data showing their heterologous amplification in *P. costatus* and *P. lineatus* will provide useful markers that will allow a better understanding of the *Prochilodus* population structure, and will aid future *P. argenteus* genetic mapping studies.

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Table 2 - Sample genotypes with allele sizes in base pairs.

	Prochilodus costatus			*Prochilodus lineatus*		
Locus	231	235	236	91	96	97
Par10*	210/210	182/194	182/192	192/200	200/200	200/200
Par12*	196/196	196/220	188/196	232/232	200/216	200/216
Par13*	242/270	250/260	252/262	258/264	258/288	256/262
Par14*	236/250	220/250	236/250	208/220	250/270	250/270
Par15*	188/188	168/178	162/176	154/182	166/172	150/170
Par21*	185/185	173/185	165/189	173/185	181/185	181/185
Par26*	254/266	302/302	302/302	302/302	298/302	298/302
Par31*	271/271	269/271	271/275	273/275	267/275	271/271
Par34*	350/350	350/350	350/350	FB	FB	FB
Par35*	256/268	256/256	256/256	256/256	256/256	256/256
Par43*	271/283	257/265	273/275	251/255	265/277	247/247
Par53*	176/184	176/188	176/184	NP	NP	188/188
Par54*	226/242	222/222	224/226	224/230	218/222	224/226
Par66	177/181	177/185	169/181	177/181	169/177	169/177
Par69	244/244	236/244	236/244	252/252	252/252	252/252
Par71	253/267	269/275	261/265	275/275	275/275	275/275
Par76	230/230	230/230	230/230	230/230	230/230	230/230
Par80	247/261	227/237	251/253	219/225	239/255	239/257
Par82	179/195	191/193	183/189	195/195	195/195	195/195
Par83	272/282	264/272	272/272	276/280	276/288	276/288
Par85	251/263	243/257	259/267	243/243	NP	225/237
Par86	173/173	189/189	157/171	NP	139/155	139/155

NP = No PCR product, FB = Faint band, * = Locus previously described in Barbosa *et al.* (2006).

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