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Isolation and characterization of 20 polymorphic microsatellite loci in the migratory freshwater fish *Leporinus obtusidens* (Characiformes: Anostomidae) using 454 shotgun pyrosequencing

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Twenty polymorphic microsatellite markers were developed for the Neotropical fish *Leporinus obtusidens* using a next generation sequencing approach and tested in two other characifomes species, *Schizodon platae* and *Prochilodus lineatus*. Microsatellite loci alleles in *L. obtusidens* ranged between 2 and 20 alleles per locus (mean = 5·7), with expected heterozygosity values ranging from 0·097 to 0·956 (mean = 0·578) and observed heterozygosity values ranging from 0·000 to 0·800 (mean = 0·400) in a sample of 20 specimens from the lower Paraná River (Argentina). Most of these markers will be a valuable tool for captive breeding and stocking programmes, as well as for analyses of population connectivity and genetic structure in this broadly distributed Neotropical migratory fish.

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Key words: boga; molecular marker; Neotropical fish; Paraná River; *Prochilodus lineatus*; *Schizodon platae*.

Boga *Leporinus obtusidens* (Valenciennes 1837) (Characiformes, Anostomidae) is a migratory freshwater fish distributed throughout the La Plata and San Francisco Basins in Argentina, Uruguay, Brazil and Paraguay (Froese & Pauly, 2012). This species is an important fishery resource with excellent acceptance in the domestic market and stands as a promising species for aquaculture (Cappato & Yanosky, 2009).The definition of stocks for the conservation and the design of breeding programmes depend on the genetic population studies that are not available today for *L. obtusidens*.

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Microsatellite DNA markers constitute a valuable tool for population and conservation genetics as well as for monitoring pedigree and genetic diversity in the hatchery-reared organisms (Vandeputte *et al.*, 2011). Eight microsatellite markers were developed by Morelli *et al.* (2007) for *Leporinus macrocephalus* Garavello & Britski 1988, which also showed cross-amplification with several other *Leporinus* species including *L. obtusidens*. These molecular markers, however, are insufficient for their use in the population studies and the broodstock evaluations. Therefore, the goal of this work was to develop the first panel of microsatellite markers in *L. obtusidens* using a 454 shotgun pyrosequencing technique. Availability of these markers will allow further genetic diversity studies for *L. obtusidens*. Additionally, the panel was tested in two other Characiformes species.

Microsatellite sequences were identified *in silico* from the raw sequences obtained by 454-Roche next-generation sequencing technology. A whole-genome random shotgun library was performed from one specimen of *L. obtusidens* obtained from the lower Paraná River (Argentina). Total DNA was prepared from a fin clip by phenol–chloroform extraction method (Sambrook *et al.*, 1989) and its quality was assayed by 1% agarose gel electrophoresis. Sequencing was performed in a Roche 454 Genome Sequencer FLX at the sequencing platform of INDEAR (www.indear.com), using 1/16th run. Mitochondrial genome sequences from the set of reads were identified by comparison with the non-redundant (nr) database from GenBank by basic local-alignment search tool N (BLASTn) 2.2.24+ (Altschul *et al.*, 1997) and all of them were removed. Remaining reads were screened for the repetitive sequences using MSATCOMMANDER 0.8.2 (Faircloth, 2008) according to the following criteria: dinucleotide motifs with ≥8 repeats, trinucleotide motifs with ≥5 repeats, tetranucleotide motifs with ≥4 repeats, pentanucleotide and hexanucleotide motifs with ≥3 repeats. The run resulted in a 9·59 Mb spread over 33 731 individual sequence reads with 285 bp average read length. As a result, 2477 microsatellite-containing sequences were identified. Among the different repeat motifs, dinucleotides were the most abundant (62%), followed by tetranucleotide (15%), trinucleotide (14%), pentanucleotide (4%) and hexanucleotide (2%) repeats. This spread is similar to other vertebrate genomes (Liu *et al.*, 1999; Tóth *et al.*, 2000; Serapion *et al.*, 2004). The most common motif found was AC (62%), as in the case of channel catfish *Ictalurus punctatus* (Rafinesque 1818), fugu, *Takifugu rubripes* (Temminck & Schlegel 1850) and other vertebrate genomes (Edwards *et al.*, 1998; Tóth *et al.*, 2000; Serapion *et al.*, 2004). AAT (25%) was the most represented motif in trinucleotides which was also among the most frequently occurring microsatellites in *T. rubripes* genome and the most common trinucleotide motif in Vertebrata introns (Tóth *et al.*, 2000) (Fig. 1).

Primers were successfully designed on the conserved flanking regions for 262 pre-selected microsatellite loci using Batch PRIMER 3 (You *et al.*, 2008). These microsatellites were selected based on the allowance of primer design out of the 2477 identified sequences. In order to avoid cross-amplifications, microsatellite-containing sequences and their reverse and complementary orientations were clustered to identify redundant sequences using Clustal X (Thompson *et al.*, 1997). BLAST algorithm was used to query the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) nucleotide collection to identify putative homologue sequences in other organisms. Forty microsatellites of the 262 pre-selected microsatellites were selected based on the amplicon length distribution between 50 and 500 bp and representation of di, tri, tetra and pentanucleotide repetitive sequences. Each

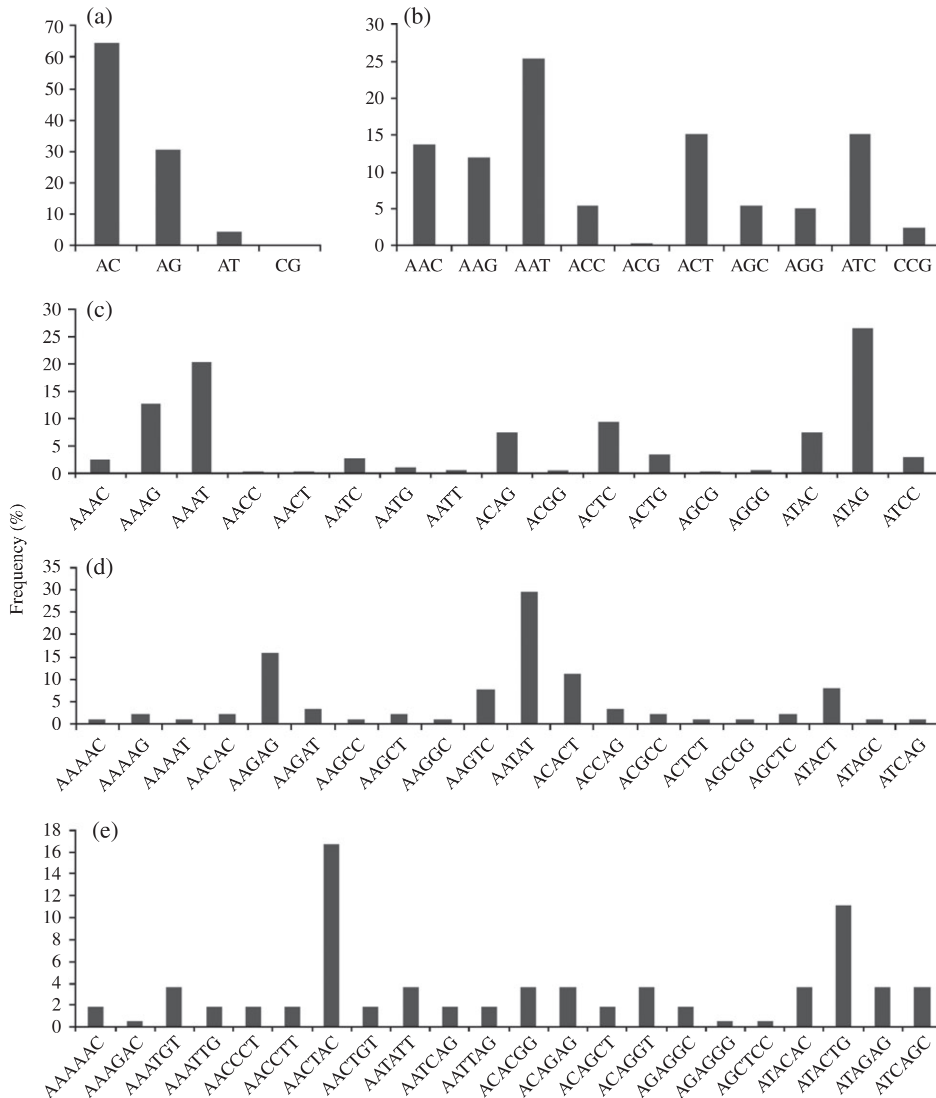

FIG. 1. Frequency distributions (%) of microsatellite motifs in *Leporinus obtusidens*: (a) di, (b) tri, (c) tetra, (d) penta and (e) hexanucleotides.

putative microsatellite locus was initially evaluated for polymerase chain reaction (PCR) amplification using DNA from two individuals. Genomic DNA was extracted from the fin clips using the Chelex Resin procedure (Walsh *et al.*, 1991). Amplicons were checked on 1·5% agarose gels. Loci were amplified in 15 μl volume including 1 μl of template DNA (*c*. 30 ng), 1× PCR Gold Buffer (Applied Biosystems; www.lifetechnologies.com), 1·5 mM $MgCl_2$, 100 μM deoxynucleotide triphosphate (dNTP), 10 pmol of both forward and reverse PCR primers and 0·5 U Amplitaq Gold DNA polymerase (Applied Biosystems). Thermal cycling was performed on a Verity 96-Well Thermal Cycler (Applied Biosystems) as follows: initial denaturation at 95° C

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for 10 min, 35 cycles of denaturation at 94° C for 45 s, annealing temperature (between 56 and 60° C) for 50 s and extension at 72° C for 50 s followed by a final extension step at 72° C for 10 min. Annealing temperatures for each locus are shown in Table I. When no amplification was observed, a refined analysis was carried out at different annealing temperatures and $MgCl_2$ concentrations. A group of 20 microsatellites were finally selected, based on the appearance of only one or two bands in the agarose gels. Forward primer of each primer pair was 5′ end-labeled with the custom fluorescent dyes (6-FAM, NED, PET or VIC provided by Applied Biosystems). The fluorescently labelled PCR products were run on an ABI PRISM 3730 DNA Sequencer (Applied Biosystems) using GeneScan-500 LIZ as an internal size standard and genotyped with GeneMapper version 4.0 (Applied Biosystems). These markers were finally analysed on 20 individuals of *L. obtusidens* collected from the Paraná River (Argentina, Rosario, 32° 54′ 8′′ S; 60° 40′ W). A cross-species amplification assay was performed using five specimens of two other Characiformes species: *Schizodon platae* (Garman 1890) and *Prochilodus lineatus* (Valenciennes 1837). The analysis of total number of alleles (A), expected (H_E) and observed (H_O) heterozygosities and frequency of null alleles at each locus was estimated on all genotyped loci using CERVUS 3.0 (Kalinowski *et al.*, 2007). Statistical analysis was performed using the Hardy–Weinberg equilibrium (HWE) and linkage disequilibria among pairs of loci were tested in Genepop 4.0 (Rousset, 2008). Microchecker 2.2.3 (Van Oosterhout *et al.*, 2004) was used to check for genotyping accuracy (*e.g.* stuttering and large allele drop out) and to detect homozygote excess evenly distributed among homozygote size classes at specific loci, which may also be interpreted as null allele evidence.

The description of the selected 20 polymorphic microsatellite loci isolated from *L. obtusidens* is shown in Table I. No homology with the previously reported markers was observed. These loci were successfully amplified in all samples. Alleles per locus ranged from 2 to 20 (mean ± s.d. = 5·75 ± 4·05), H_E ranged from 0·097 to 0·956 and H_O ranged from 0·000 to 0·800. Significant deviations from HWE ($P < 0·01$) were detected at *LoLAR8*, *LoLAR9*, *LoLAR23*, *LoLAR24* and *LoLAR29* in the tested population using Bonferroni correction for multiple tests. These deviations are probably related to the presence of null alleles, as shown by the high positive F_{IS} values (Table I). Microchecker analysis supported null allele hypothesis showing that there was no evidence for scoring errors due to stuttering or large allele drop out for *LoLAR8*, *LoLAR9*, *LoLAR24* and *LoLAR29*. These markers are not suitable for either population structure analysis or parentage analysis, especially *LoLAR9* and *LoLAR29* which showed severe null allele problems. Evidences of significant linkage disequilibrium was not found between pairs of loci even after Bonferroni correction for multiple tests ($P < 0·001$).

Development of the microsatellite DNA primers requires substantial inputs of time, economic resources and expertise that can be avoided by using available markers for related species. In order to test cross-amplification of *L. obtusidens* characterized microsatellites with other species, two closely related species were selected among several species of Characiformes found in the lower Paraná River (Liotta, 2005): *S. platae* commonly known as boga lisa, and *P. lineatus* commonly named sabalo in Argentina, Paraguay and Uruguay and curimba in Brazil. Both *S. platae* and *L. obtusidens* belong to the Anostomidae, while *P. lineatus* belongs to Prochilodontidae. In addition, *P. lineatus* represents the most extensively exploited resource in the Paraná River. No microsatellites are described for *S. platae*, whereas 18 are characterized

TABLE I. Primer sequences and characteristics of 20 microsatellite loci isolated from *Leporinus obtusidens*

Locus	Motif	GenBank	Size range (bp)	Primer (5′–3′): forward and reverse	T_a (° C)	$MgCl_2$ (mM)	*A*	H_O	H_E	*F* (null)	P_{HWE}	F_{IS}
LoLAR5	$(ATCC)_6$	KC193140	125–145	AACCTCCATCTGACACCCATA GGGACAAAGCGGAGGATAAT	60	1	5	0·750	0·741	−0·0306	0·0411*	−0·0124
LoLAR6	$(CTGTT)_5$	KC193141	202–207	GACGGTGACGAATGTGTGTC TGCAGAAGGCAAAGAACTGA	58	1·5	2	0·529	0·487	−0·0570	1·0000	−0·0909
LoLAR7	$(CTCCG)_5$	KC193142	204–214	CAGAGCTGCTCCATGACTGA CTCATGGGAAGCGAGTGTTT	58	1·5	3	0·200	0·232	0·1298	0·2484	0·1412
LoLAR8	$(ATCT)_{12}$	KC193143	128–202	CTCACGTTCCCTCTCTCACG CGGGCCAAAAACAGAGATAG	60	1	20	0·650	0·956	+0·1813	0·0000**	0·3261
LoLAR9	$(AATT)_4$	KC193144	154–156	TGTGCATATGAAGGCGGTAA ACCCCTAACACATGGAATGG	58	1	2	0·000	0·185	+0·9070	0·0018**	1·0000
LoLAR11	$(AATA)_6$	KC193145	155–183	GGGAAAGGAACTGTTGCATTAT ATTCAGAGCTGATCCCTCCA	56	1·5	4	0·550	0·633	+0·0303	0·0346*	0·1346
LoLAR15	$(TTCA)_7$	KC193146	190–230	CCTCCCACCTCTACCCTTTC GCAGAGGCAGAGTCAGCAG	58	1·5	8	0·800	0·826	−0·0030	0·9692	0·0318
LOLAR17	$(TTAT)_6$	KC193147	212–224	GCTTGTGTGATTCTCCAGCA TGCACGAGCTACATCAAAGG	60	1	4	0·550	0·510	−0·0840	0·4430	−0·0801
LoLAR22	$(CAA)_9$	KC193148	135–156	AGCGAGGAGGAAAACAACAA AGCGCCAACTCTTCACTGAG	58	1	7	0·500	0·751	+0·1930	0·0075*	0·3403
LoLAR23	$(ATC)_6$	KC193149	163–190	ACTCTAAAAGCTCCCGCACA AACCACCAGTGAACCTTTGC	58	1	5	0·500	0·604	+0·0813	0·0005**	0·1757
LoLAR24	$(ATT)_7$	KC193150	141–165	TTAGATCTGCCGGAGTCACC GTGGTCCAGTCTCCCATCAG	60	1·5	3	0·200	0·497	+0·4067	0·0013**	0·6042
LoLAR25	$(TAA)_7$	KC193151	85–94	GTGGAGCAAAGAGGCGTAAG ACACCACCATGCTGCCTTAT	58	1	4	0·550	0·691	+0·1018	0·5974	0·2083
LoLAR27	$(TTA)_9$	KC193152	116–143	TTCAGCTGCATCATTTCAGG	60	1	7	0·700	0·797	+0·0640	0·1082	0·1250

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Table I. Continued

Locus	Motif	GenBank	Size range (bp)	Primer (5′–3′): forward and reverse	T_a (° C)	$MgCl_2$ (mM)	A	H_O	H_E	F (null)	P_{HWE}	F_{IS}
				GCAGAATACGGGAACTAGGC								
LoLAR29	$(AT)_{11}$	KC193153	141–159	TGTTCACATTGCTGTCCTGAG	58	1	3	0·000	0·272	+0·9695	0·0000**	1·0000
				CTTGGCTCTTCCTGATGCTC								
LoLAR30	$(CA)_{11}$	KC193154	245–247	AAACCGTGTCTGTGCCTAAAA	58	1·5	2	0·100	0·097	−0·0164	1·0000	−0·0277
				CTCGTTGTAACCTGAACAGCA								
LoLAR31	$(AC)_{11}$	KC193155	214–232	CACAGCCCCTTGTGTTCTTT	58	1	8	0·800	0·786	−0·0275	0·8854	−0·0184
				CAAGCTCATAACCCTGCAGTC								
LoLAR33	$(AG)_{18}$	KC193156	120–156	CATCTCAGGAGGAAATGACCA	58	1·5	6	0·300	0·492	+0·2629	0·0091*	0·3968
				ATCGCAGTGCTGTCAATATGT								
LoLAR35	$(AC)_{13}$	KC193157	134–184	ATGGCAATGCCTCTCAACAT	60	1	10	0·700	0·709	+0·0070	0·6392	0·0130
				TGGAGATGTTGTGTCTGTAGGG								
LoLAR37	$(TG)_{14}$	KC193158	127–145	TTAGAGCCCAAGCTCTCGAC	60	1	7	0·650	0·679	+0·0196	0·6866	0·0445
				TGGGCCTCGCGTATTTATAG								
LoLAR38	$(TA)_{10}$	KC193159	192–228	TCATGTTGGTCTTGAATGCTG	60	1	5	0·450	0·614	+0·1351	0·0402*	0·2723
				ATGCAGCTTTGTCCGTCTCT								

Number of alleles (A), allelic size range, observed (H_O) and expected (H_E) heterozygosities and Hardy–Weinberg P-values (P_{HWE}) are based on 20 individuals. T_a, annealing temperature; F (null), frequency of null alleles; F_{IS}, inbreeding coefficient estimated following Weir & Cockerham (1984); P_{HWE}, probability of departure Hardy–Weinberg equilibrium (HWE). Asterisks indicate significant departure from HWE (*$P < 0·05$) and significant after Bonferroni correction (**$P < 0·01$).

Table II. Microsatellite cross-amplifications in *Schizodon platae* and *Prochilodus lineatus*

Microsatellite	*S. platae* ($n=5$)	Size range (bp)	*P. lineatus* ($n=5$)	Size range (bp)
LoLAR5	–		–	
LoLAR6	NS		NS	
LoLAR7	NS		NS	
LoLAR8	–		–	
LoLAR9	NS		NS	
LoLAR11	A (5/5)	235	–	
LoLAR15	–		–	
LOLAR17	A (5/5)	184–188	A (5/5)	206–210
LoLAR22	A (5/5)	129–153	A (5/5)	144–156
LoLAR23	A (5/5)	176–368	A (4/5)	205–298
LoLAR24	A (5/5)	106–289	A (3/5)	202–322
LoLAR25	–		–	
LoLAR27	NS		–	
LoLAR29	–		–	
LoLAR30	NS		–	
LoLAR31	A (5/5)	318–324	A (2/5)	73–115
LoLAR33	A (4/5)	144–172	–	
LoLAR35	A (5/5)	158–198	–	
LoLAR37	A (5/5)	126–128	–	
LoLAR38	–		–	

A, positive amplification (the number of samples amplified from the total is given in parenthesis); –, no amplification; NS, non-specific amplification.

for *P. lineatus* (Yazbeck & Kalapothakis, 2007; Rueda *et al.*, 2011). As expected, cross-amplification levels were higher in *S. platae* than in *P. lineatus* (Table II), due to a closer phylogenetic relation between *Leporinus* and *Schizodon* genera than between *Leporinus* and *Prochilodus* genera (Guisande *et al.*, 2012). Nine markers were able to amplify in *S. platae* (*LoLAR11*, *LoLAR17*, *LoLAR22*, *LoLAR23*, *LoLAR24*, *LoLAR31*, *LoLAR33*, *LoLAR35* and *LoLAR37*) and five in *P. lineatus* samples (*LoLAR17*, *LoLAR22*, *LoLAR23*, *LoLAR24* and *LoLAR31*). Products of a similar size range were obtained for most of the loci (Table II).

Leporinus obtusidens is one of the most important fishery resources and export products of the Paraná River. Despite its importance, microsatellites for this species had not been described before. Most of the *L. obtusidens* microsatellites developed here represent a valuable tool for the further genetic diversity and population structure studies in the wild and the hatchery populations of *L. obtusidens*.

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