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TECHNICAL NOTE

Polymorphic microsatellite loci from the endangered Giant Otter (*Pteronura brasiliensis*)

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Abstract We describe the first microsatellite loci isolated from the giant otter (*Pteronura brasiliensis*), an endangered mustelid endemic to South America. Fourteen di- and trinucleotide polymorphic loci were characterised in fourteen individuals from the Pantanal wetlands, Central Brazil. Number of alleles per locus ranged from 2 to 5, and average observed heterozygosity was 0.577. Two loci were in linkage disequilibrium, and one further locus deviated from Hardy–Weinberg equilibrium, probably due to the presence of null alleles. The transferability of these markers to two other mustelids (*Lontra longicaudis* and *Eira barbara*) and to the mephitid *Conepatus semistriatus* was also evaluated. These loci are useful to study the ecology and evolution of these species.

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Giant otters (*Pteronura brasiliensis*) are large semi-aquatic mustelids (sub-family Lutrinae) and one of the few social carnivores endemic to South America. They are considered the most threatened otter species in the world (IUCN 2010) as a consequence of historical hunting (Carter and Rosas 1997), and past and present anthropogenic habitat destruction (e.g. mining and hydroelectric dams, river and land pollution, and over-fishing) (Schenck 1999; OSG IUCN 2010).

Microsatellites are ideal markers to address population structure and kinship issues, thus they are able to provide crucial information for the elaboration of conservation plans for this endangered species. Although microsatellite primers developed for Eurasian and North American River otters (*Lutra lutra* and *Lontra canadensis*, respectively) have been tested in giant otters (Pickles et al. 2009), the use of heterologous primers may lead to underestimation of heterozygosity ("ascertainment bias", Garner et al. 2005). Therefore, we isolated microsatellite loci for *P. brasiliensis* (which are the first microsatellites characterised in otters from Latin America). We also tested their applicability in the mustelids *Lontra longicaudis* (neotropical river otter) and *Eira barbara* (tayra), and in the mephitid *Conepatus semistriatus* (striped hog-nosed skunk).

Genomic DNA was extracted from giant otter skin samples collected with biopsy darts (Ribas et al. in prep.), using a phenol–chloroform protocol (Sambrook et al. 1989). Microsatellites were isolated from an enriched partial genomic library, following the protocol of Bloor et al. (2001). A pool of high-quality genomic DNA (4 μg) was digested with SauIIIA, ligated to phosphorylated double-stranded linker

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oligonucleotides and size selected (between 500 and 1000 bp). DNA fragments were hybridised with biotinylated $(CA)_{12}$, $(CAA)_8$ and $(GATA)_5$ probes, and isolated using streptavidin-coated magnetic beads. The forward linker oligo was used as a primer for enrichment of DNA containing microsatellites. Enriched fragments were then cloned using pGEM-T vectors (Promega) and OneShot TOP10 competent cells (Invitrogen). Recombinant clones were screened for the presence of microsatellite inserts, which was confirmed by two or more amplified products after a PCR primed with the forward linker oligo and (non-biotinylated) microsatellite oligos. Forty-eight positive clones were sequenced in both directions in an ABI3500 sequencer. Sequences were edited using SeqMan (DNAStar).

Twenty-five primer pairs flanking microsatellite regions were designed using WebSat (Martins et al. 2009). We used the tailed primer method (Schuelke 2000), hence, PCR reactions contained three primers: tailed (forward with M13 tail), labelled (M13 with either VIC, NED, PET or 6-FAM fluorescent dyes), and reverse. PCR consisted of 1U GoTaq (Promega), 0.20 mM dNTPs, 2.5 mM $MgCl_2$, 15 μg BSA, 0.2 μM of tailed primer, 0.4 μM of labelled primer, and 0.8 μM of reverse primer, in 15 μl reactions with approximately 20 ng of DNA template. Cycling conditions were: 94°C, 4 min, 30X (92°C, 45 seg; T_a, 45 seg; 72°C, 45 seg), 8X (92°C, 45 seg; 53°C, 45 seg; 72°C, 45 seg), 72°C, 30 min. T_a for all primer pairs was 60°C, except for *Pbra01*, which was 52°C. PCR products were

Table 1 Levels of variability of 14 polymorphic microsatellite loci in the giant otter (*Pteronura brasiliensis*) from Pantanal, Brazil ($n = 14$)

Locus	GenBank accession no.	Primer sequence (5′–3′)	Motif	Size range[a] (bp)	N_a	H_o H_e	Null freq.	PExcl1	PExcl2
Pbra01	JF712852	F: ACCACAAGGGGTTCACTCTAAA	AC (18)	219–225	4	0.357	0.1875	0.871	0.749
		R: TGACCTACTGTCCATTCTGCTG				0.521			
Pbra02	JF712853	F: TCTCCCCATTTTCACTCTGG	AC(9)_AC(16)	401–413	5	0.714	0.0034	0.687	0.509
		R: ACTTTCAGCCTTTGGTGCTC				0.746			
Pbra05	JF712854	F: GGAAAGGGTTGCTGAATGAA	CA(18)	363–375	4	0.714	−0.0159	0.743	0.578
		R: GAGGGTCCTGATGATGGAAG				0.706			
Pbra08	JF712855	F: TACTCTTTTCAGATGCCCCACT	GT(16)	181–191	3	0.571	−0.0051	0.843	0.734
		R: AATATGATGTCTCCCGCACG				0.582			
Pbra09	JF712856	F: CACCTTTCCCTCACTTTTGC	CA(20)	394–400	3	0.429	0.0769	0.899	0.763
		R: TCATCCTTCAGTTATGCCGA				0.466			
Pbra10	JF712857	F: GCCTGACAAGTGATTGCGTA	TG(14)	319–327	3	0.500	−0.1328	0.92	0.798
		R: CCGAACCAGAGGCATAAGAA				0.415			
Pbra11	JF712858	F: GGTTGCCTATGGCTGAGAGA	(TG)(GA)	339–343	3	0.714	−0.1355	0.831	0.685
		R: GGAGCATGTCTTCCGTGATT				0.603			
Pbra14	JF712859	F: AGAAACACACACGGGACACA	AC(10)CA(11)	136–160	3	0.500	−0.1366	0.924	0.818
		R: TTGCTAATGCTGTAGGGGCT				0.405			
Pbra16	JF712860	F: CAGTGCGGGTCATACAAAGA	CTT(8)	327–336	2	**0**	0.8315	0.991	0.938
		R: ACAGAACCAGTCCCTGTTGG				**0.138**			
Pbra17	JF712861	F: AACACCAAAGCAAACCCTTG	TG	336–350	4	0.643	0.0757	0.675	0.499
		R: CCACCACAGAAAGCACAAAA				0.775			
Pbra20	JF712862	F: GCCAGACCATCCAACAAAGT	CA	358–370	4	0.714	0.0144	0.701	0.526
		R: TTTCCTTTCTCCATCCTCCA				0.749			
Pbra21[b]	JF712863	F: GGAAACAACACAGCGGAACT	AC(19)	195–205	4	0.857	−0.078	0.693	0.520
		R: CTGAATGAGACACGCAGGAA				0.759			
Pbra23[b]	JF712864	F: AGATGTTCAGAGAGGCGGAA	TG(17)	171–181	4	0.857	−0.0735	0.686	0.512
		R: GGGTGAGTTGTCGGTTTGTT				0.765			
Pbra24	JF712865	F: GGTGTCTTTGAAGTGGTTAT	TG(13)	313–335	4	0.786	−0.072	0.743	0.578
		R: AGTGGCTTAACGGACTGAGC				0.706			

[a] Allele sizes discounting the tailed extension of the primers

[b] Linked loci

N_a indicates number of alleles observed, H_o observed heterozygosity, H_e expected heterozygosity, *Null freq* estimated null allele frequency, *PExcl1* and *2* probability of non-exclusion of a parent, unknowing both or one of them, respectively, *Bold* heterozygote defficiency

Table 2 Results of cross-amplification tests

Species	N	*Pbra02*	*Pbra05*	*Pbra08*	*Pbra09*	*Pbra11*	*Pbra14*	*Pbra16*	*Pbra17*	*Pbra21*
Lontra longicaudis	2	60	54	–	60	60	58	58	60	52
Eira barbara	2	–	54	54	58	60	60	58	54	52
Conepatus semistriatus	1	–	–	–	–	–	–	–	–	56

Failure of amplification is indicated by a minus. Loci that were successfully amplified are indicated by the corresponding annealing temperature (°C)

pooled, separated in an ABI3500 sequencer and sized using GeneMapper and GS500-LIZ (Applied Biosystems).

During optimisation attempts, five primer pairs were discarded due to PCR failure. The remaining 20 were evaluated for polymorphisms in fourteen giant otters from the Miranda/Vermelho River (UTM- 21 K 502060, 7831592), Pantanal wetlands, Brazil. Six loci were monomorphic, while 14 loci resolved between 2 and 5 alleles and had observed and expected heterozygosities varying between 0–0.857 and 0.138–0.775, respectively (Table 1). Deviations from Hardy–Weinberg and linkage equilibrium conditions were tested using FSTAT (Goudet 1995) and the online version of Genepop (Raymond and Rousset 1995). Locus *Pbra16* presented a clear heterozygote deficiency, possibly caused by the presence of null alleles, as suggested by the high null allele frequency estimated by the software Cervus (Kalinowski et al. 2007). All loci pairs were in linkage equilibrium, except for markers *Pbra21* and *Pbra23*, which were strongly linked ($P < 0.00055$, which remains significant after sequential Bonferroni correction—Rice 1989). The probability of non-exclusion of a false parent was estimated for each locus (Table 1) and also combining the twelve selected markers (i.e. excluding *Pbra16* and *Pbra21*). The estimated proportions of type II errors were small (5.7% without knowing the parents and 0.4% knowing one of them), indicating that the selected loci provide sufficient power for paternity analyses in the species.

All 14 markers were tested for cross-amplification in two individuals each of neotropical otter and tayra, and in one striped hog-nosed skunk, using the optimised conditions detailed in Table 2. Five loci could not be amplified in any of these species (*Pbra01*, *Pbra10*, *Pbra20*, *Pbra23* and *Pbra24*), but seven of them were successfully amplified in the two other mustelids (Table 2).

These markers will contribute to elucidate the giant otter social system and population structure, providing information that will be useful in the elaboration of management and conservation plans for this endangered species.

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