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TECHNICAL NOTE

A panel of new microsatellite loci for genetic studies of antarctic fur seals and other otariids

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Abstract Nine dinucelotide microsatellite loci were developed in the Antarctic fur seal *Arctocephalus gazella*. Each locus possessed between 4 and 9 alleles in a sample of twenty individuals sampled from Bird Island, South Georgia, and expected heterozygosity ranged from 0.52 to 0.84. All but one of the loci conformed to Hardy–Weinberg equilibrium and no evidence was found for genotypic disequilibrium. Additionally, all of the loci successfully cross-amplified in the South American fur seal *Arctocephalus australis*, the New Zealand fur seal *Arctocephalus forsteri* and the Steller's sea lion *Eumetopias jubatus*, and six also yielded products in the more distantly related harbour seal *Phoca vitulina* and walrus *Odobenus rosmarus*. These loci should prove useful for studies of the population genetics of Antarctic fur seals and other important otariid species.

Keywords *Arctocephalus gazella* · Microsatellite · Cross-species amplification · Stock structure · Assignment testing · Heterozygosity-fitness correlation (HFC) · Pinniped

The otariidae (fur seals and sea lions) are an important and charismatic family of marine mammal. Following heavy exploitation by commercial sealers in the eighteenth and nineteenth centuries many species have made a full recovery, but others remain depleted or even in danger of local extinction (e.g. Rosa de Oliveira et al. 2006). Moreover, modern day populations still face numerous threats including environmental pollution, disease, commercial fisheries bycatch, anthropogenic disturbance and climate change (Bonner 1978; Forcada et al. 2005; Robertson and Gemmell 2005; Rosa de Oliveira et al. 2006).

Fortunately, population genetic approaches offer an unparalleled opportunity to address a variety of questions that are important to otariid conservation. For example, microsatellites have been used to assess the stock structure of Steller's and Galapagos sea lions (Hoffman et al. 2006a; Wolf et al. 2008), to assign individual New Zealand fur seals caught in trawl fisheries to their natal populations (Robertson and Gemmell 2005), to track the life histories of individual Antarctic fur seals that cannot otherwise be identified (Hoffman et al. 2006b) and to link genetic heterozygosity to disease resistance in California sea lions (Acevedo-Whitehouse et al. 2003). However, some authors (e.g. Robertson and Gemmell 2005) have identified a need for greater numbers of genetic markers. Here I present nine novel microsatellite loci developed in the Antarctic fur seal *Arctocephalus gazella* and test these for cross-amplification in three other otariid species, the South American fur seal *Arctocephalus australis*, the New Zealand fur seal *Arctocephalus forsteri* and the Steller's sea lion *Eumetopias jubatus*. In addition, to determine the broader utility of these markers across the Pinnipedia, the loci were also screened in the harbour seal *Phoca vitulina* and the walrus *Odobenus rosmarus*.

Total genomic DNA was extracted from tissue samples of three individuals using an adapted Chelex 100 protocol (Walsh et al. 1991) followed by phenol-chloroform purification (Sambrook et al. 1989). Approximately 5 μg of pooled DNA was then digested using the restriction enzyme Sau3A (Roche) and ligated to linkers made by annealing equimolar amounts of Sau-L-A (5′-GCGG TACCCGGGAAGCTTGG-3′) and 5′end phosphorylated

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Table 1 Nine microsatellite loci developed from the Antarctic fur seal *Arctocephalus gazella*

Locus	Primer sequence (5′–3′)	Repeat motif in clone	Size range (bp)	T_1 (°C)	T_2 (°C)	No. of alleles	H_O	H_E	Null allele frequency	HWE *P* value
Agaz-1	F-ACTCATGCCCTGCTTGAAAT R-CAGGAGACTTAGGCCAGCAC	$(CT)_2GT(CT)_3GT(CT)_3GT(CT)_3GT(CT)_5GT(CT)_{12}TA(AC)_{15}$	238–260	50	54	9	0.850	0.840	−0.019	0.403
Agaz-2	F-CCCAAGTTTGACCCTCGATA R-GGAAGGTGGGCCTTAGGTAT	$(AC)_{24}$	230–244	46	48	7	0.950	0.790	−0.123	0.421
Agaz-3	F-CATGAGTGTCCCTTGCAAAA R-TCAGCCTAGGCCAAAAGAAA	$(AC)_9$	204–210	46	48	4	0.600	0.542	−0.063	0.726
Agaz-4	F-GAAGTCCTCCTTTGCTCGAA R-TGTGCACCTGTTGCAGAAGT	$(GT)_3AT(GTGC)_2(GT)_{13}$	194–200	48	52	4	0.400	0.729	0.280	0.008
Agaz-5	F-CTCCGATGAGCTCCAAGAAC R-GAGTCTGAAACCCTCCCTGA	$(GT)_{10}$	192–198	48	52	4	0.700	0.558	−0.126	0.314
Agaz-6	F-TTGTGTGTGCATGCATCTGT R-TGCCCGAGAGGTGTTCTATT	$(GT)_{11}$	174–180	46	48	4	0.550	0.517	−0.044	0.682
Agaz-7	F-AGACCTCTCCAGAGGCATCA R-GGGAGACAGGAGGTGACAGT	$(AC)_{11}(ATGC)_4(AC)_2TC(AC)_3GC(AC)_3$	160–166	46	48	4	0.813	0.732	−0.068	0.365
Agaz-8	F-GGGGAGCCCTGATAGAAATC R-AGATTGATGGCCTGGGAAC	$(AC)_{22}$	136–164	48	52	7	0.650	0.778	0.077	0.240
Agaz-9	F-TTCATGAGTTGCTCTCCTTCTTC R-CATGCCTTGTTTGCAGGTTA	$(GT)_{17}$	198–210	60	65	7	0.900	0.767	−0.093	0.161

Polymorphism characteristics are given for 20 unrelated Antarctic fur seal individuals. T_1 and T_2, annealing temperatures; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, Hardy–Weinberg equilibrium

Sau-L-B (5′-GATCCCAAGCTTCCCGGGTACCGC-3′) oligonucleotides. Fragments in the size range 300–600 bp were then excised from an agarose gel and purified using QIAquick gel extraction spin columns (Qiagen). This fraction was enriched for AC repeat motifs by overnight hybridization at 65°C to Hybond-N membranes (Amersham Biosciences) to which poly(dA-dC).poly(dG-dT) oligonucleotides (Amersham Biosciences) had been bound. After stripping the enriched DNA from the filters, it was polymerase chain reaction (PCR) amplified using 1 μM Sau-L-A (25 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 40 s, extension at 72°C for 40 s). Resulting PCR products in the size range 300–600 bp were then purified using QIAquick gel extraction spin columns (Qiagen), ligated into p-GEM T-easy vectors (Promega) and used to transform DH5α subcloning efficiency competent cells (Invitrogen). After plating cells on selective media (containing ampicillin and x-gal), 96 white colonies were picked and transferred onto new agar plates for future use and also into 50 μl TE. To check for the presence of the insert, these were then boiled for 5 min to release the plasmids from the bacteria and PCR-amplified using the Sau-L-A primer. Next, plasmid DNA was extracted by alkaline lysis and the insert was sequenced using a Big-Dye 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems) and the M13 reverse primer. Reactions were carried out following the manufacturer's instructions. Sequenced products were analysed using an Applied Biosystems 3730xl DNA Analyser. Out of 48 clones sequenced, 31 carried the (AC) repetitive motif and possessed sufficiently long flanking sequences to allow primers to be designed using the program Primer 3 (Rozen and Skaletsky 2000).

Primer pairs were used to amplify microsatellites in twenty unrelated Antarctic fur seal individuals. Microsatellite genotyping was conducted as described by Hoffman and Amos (2005). PCR reactions were carried out in 10 μl reaction volumes containing approximately 10 ng template DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Tween 20, 0.1% gelatine, 0.1% IGEPAL, 60 mM TMAC, 2.5% formamide, 0.1 mM dGTP, 0.1 mM dATP, 0.1 mM dTTP, 0.02 mM dCTP, 4 pmol of each primer, 0.25 units of *Taq* polymerase and 0.01 μCi [α^{32}P]-dCTP. Loci were amplified using the following PCR profile: one cycle of 120 s at 94°C, 45 s at T_1, 50 s at 72°C; ten cycles of 30 s at 94°C, 45 s at T_1, 50 s at 72°C; 25 cycles of 30 s at 89°C, 45 s at T_2, 50 s at 72°C; and one final cycle of 5 min at 72°C (see Table 1 for T_1 and T_2). PCR products were resolved by electrophoresis on standard 6% polyacrylamide sequencing gels and detected by autoradiography. Genepop (Raymond and Rousset 1995) was used to calculate observed and expected heterozygosities and to test for deviations from Hardy–Weinberg equilibrium and for linkage disequilibrium among the 20 Antarctic fur seals genotyped. Null allele frequencies were calculated using the program Micro-checker (Van Oosterhout et al. 2004) following Brookfield (1996).

Nine loci yield clear, polymorphic PCR products in the 20 Antarctic fur seals genotyped (Table 1). Each locus possessed between 4 and 9 alleles, and expected heterozygosity ranged from 0.52 to 0.84. One locus (Agaz-4) deviated significantly from Hardy–Weinberg equilibrium and showed evidence of null alleles being present (Table 1). Tests for linkage disequilibrium yielded one weakly significant P value ($P < 0.05$) which did not remain significant following Bonferroni correction for multiple tests, suggesting that the nine loci are unlikely to be physically linked.

To check whether any of the loci were inadvertently identical to microsatellites previously cloned from other pinniped species, BLAST searches (Altschul et al. 1990 were used to test for flanking sequence homology to the Genbank NR database (http://www.ncbi.nlm.nih.gov) using

Table 2 Cross-species amplification of nine novel microsatellite primer pairs cloned from Antarctic fur seal *Arctocephalus gazella*

Locus	South American fur seal *Arctocephalus australis*	New Zealand fur seal *Arctocephalus forsteri*	Steller's sea lion *Eumetopias jubatus*	Harbour seal *Phoca vitulina*	Walrus *Odobenus rosmarus*
Agaz-1	5 (244–256)	2 (260–266)	3 (216–222)	3 (222–228)	6 (188–248)
Agaz-2	2 (230–232)	4 (218–240)	4 (234–240)	NA	NA
Agaz-3	4 (204–214)	3 (206–212)	1 (206)	3 (198–204)	3 (198–208)
Agaz-4	3 (196–202)	2 (200–202)	2 (192–194)	2 (192–194)	4 (180–196)
Agaz-5	2 (192–194)	1 (192)	1 (192)	1 (182)	NA
Agaz-6	4 (176–182)	3 (182–186)	1 (186)	NA	NA
Agaz-7	5 (156–166)	1 (162)	2 (164–166)	1 (148)	NA
Agaz-8	4 (144–188)	5 (154–184)	3 (176–180)	NA	NA
Agaz-9	4 (202–212)	4 (202–216)	2 (202–204)	2 (204–206)	6 (166–180)

For each species/locus combination, the number of alleles is given together with the allelic size range in parentheses. NA, not amplifying obvious alleles

default matching criteria. No matches were found with previously published pinniped microsatellite clone sequences, suggesting that none of the loci have been previously described.

All of the loci generated PCR products, most of which were polymorphic, in the South American fur seal, New Zealand fur seal and Steller's sea lion (Table 2). This finding is consistent with previous studies showing that pinniped microsatellite loci often cross-amplify in related species Coltman et al. 1996; Gemmell et al. 1997; Davis et al. 2002; Hoffman et al. 2007), and suggests that this panel of loci should prove useful for studying most, if not all, otariid species. In addition, six of the loci cross-amplified in the harbor seal and walrus, indicating limited applicability to more distantly related pinniped species.

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