

PERMANENT GENETIC RESOURCES

Ten novel polymorphic dinucleotide microsatellite loci cloned from the Antarctic fur seal *Arctocephalus gazella*

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Abstract

Ten new dinucleotide microsatellite loci were isolated from the Antarctic fur seal *Arctocephalus gazella*. These markers should prove useful for studying the reproductive ecology of Antarctic fur seals and other related pinniped species.

Keywords: fur seal, heterozygosity, mating system, microsatellite, Otariidae, pinniped, relatedness

Received 26 July 2007; revision accepted 15 August 2007

The Antarctic fur seal *Arctocephalus gazella* is a sexually dimorphic pinniped that breeds on sub-Antarctic islands (Bonner 1968). Detailed genetic analysis of a breeding colony at Bird Island, South Georgia has confirmed behavioural predictions of strong polygyny, with a quarter of all paternities being assigned to fewer than 3% of territorial males (Hoffman *et al.* 2003). Interestingly, heterozygosity appears to be an important component of individual fitness, with males that are relatively heterozygous at nine microsatellite loci fathering more offspring (Hoffman *et al.* 2004), and females appearing to exert choice for males that are most likely to produce heterozygous pups (Hoffman *et al.* 2007a). However, additional markers are required to test whether these observations most likely reflect genome-wide or local effects (Hansson & Westerberg 2002). Here we report the development of primers for polymerase chain reaction (PCR) amplification of 10 novel microsatellite loci in the Antarctic fur seal.

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'-GCGGTACCCGGGAAGCTTGG-3') and 5' end phosphorylated Sau-L-B (5'-GATCCCAAGCT-TCCCGGGTACCGC-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 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 and 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. We then double-checked for the presence of the insert by boiling for 5 min to release the plasmids from the bacteria and PCR-amplifying the insert using the Sau-L-A primer. Next, plasmid DNA was extracted by alkaline lysis and the insert was sequenced using a BigDye 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, 27 carried the (AC) repetitive motif and for 22 clones, primers could be designed in the flanking sequence of the microsatellite using the program PRIMER 3 (Rozen & Skaletsky 2000).

Primer pairs were used to amplify microsatellites in 20 unrelated Antarctic fur seal individuals. Microsatellite genotyping was conducted as described by Hoffman & Amos (2005). PCR amplifications were carried out in 10 µL

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Table 1 Ten novel microsatellite loci developed from the Antarctic fur seal *Arctocephalus gazella*. 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

Locus	GenBank accession no.	Primer sequence (5'-3')	Repeat motif in clone	Size of clone (bp)	T_1 (°C)	T_2 (°C)	No. of alleles	H_O	H_E	Null allele frequency	HWE P value
Ag-1	EU045412	F-TCTGCAATATTTGCCAGAAAAC R-ACCTTCTCCCTCTCCCTCTGC	(GT) ₁₀	102	46	48	8	0.900	0.860	–0.033	0.038
Ag-2	EU045413	F-TGGAGTATCGAGTGTGTAAGTGTG R-AAATGGTCCAGAGGATGTGC	(GT) ₃ AA(GT) ₂ GC(GT) ₅ (GT) ₁₀ G(GT) ₃	212	48	52	7	0.850	0.779	–0.051	0.243
Ag-3	EU045414	F-TGCTCATCAATTACCACTCA R-AGCTACAATTGCCAGACTCTGTGTA	(AC) ₆ AG(AC) ₆	150	46	48	2	0.250	0.224	–0.026	1.000
Ag-4	EU045415	F-AATGCATCTCTGCCATTTC R-GCAGAGGGATCAGGACAAAC	(GT) ₆ GA(GT) ₁₂	159	46	48	11	0.850	0.863	–0.005	0.618
Ag-5	EU045416	F-CATTGAGGCCAAATCAACCT R-TGCCCTCTCAACTGTATTIT	(GC) ₇ (AC) ₁₂	134	46	48	9	0.556	0.824	0.122	0.006
Ag-6	EU045417	F-CCTGAGGCTCTTCTTCTCCT R-CCAGGACCACTGGGAAGTTA	(AC) ₆ AA(AC) ₈	133	48	52	6	0.400	0.646	0.141	0.047
Ag-7	EU045418	F-TTTGAAAAGTGGACAAGCTAGAGA R-GCTCTTCCACCATGGGATT	(GT) ₈ AT(GT) ₁₃	129	46	48	6	0.950	0.790	–0.123	0.800
Ag-8	EU045419	F-GGTGTGGCTTAAGAAATCCA R-ACTGACCTTCGGTGAAGAC	(GC) ₈ (AC) ₃₄	179	48	52	16	0.900	0.942	0.010	0.352
Ag-9	EU045420	F-GTGCACCCCCCTACAAGTAG R-TCATGAGTGTGTTGTGCAGTG	(AC) ₂ TC(AC) ₉	189	48	52	3	0.600	0.555	–0.064	0.114
Ag-10	EU045421	F-TCAATGTTTCCCCACACA R-TCAGATTACGGGGAGA	(AC) ₁₃	189	46	48	6	0.750	0.776	0.004	0.661

reaction volumes containing approximately 10 ng template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 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 U of *Taq* polymerase and 0.01 μ Ci [α ³²P]-dCTP. Loci were amplified using the following PCR profile: one cycle of 120 s at 94 °C, 45 s at T1, 50 s at 72 °C; 10 cycles of 30 s at 94 °C, 45 s at T1, 50 s at 72 °C; 25 cycles of 30 s at 89 °C, 45 s at T2, 50 s at 72 °C; and one final cycle of five minutes 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. GENEPOL (Raymond & 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 following Brookfield (1996) using the program MICRO-CHECKER (Van Oosterhout *et al.* 2004).

Of the 22 primer pairs tested, 10 yielded clear, polymorphic PCR products (Table 1). Each locus possessed between two and 16 alleles, and expected heterozygosity ranged from 0.22 to 0.94. Three of the loci (Ag-1, Ag-5 and Ag-6) deviated significantly from Hardy–Weinberg equilibrium, although these deviations did not remain significant following Bonferroni correction for multiple tests (Hochberg 1988). However, loci Ag-5 and Ag-6 should be treated with caution because of the probable presence of null alleles (Table 1).

Tests for linkage disequilibrium yielded two weakly significant P values ($P < 0.05$) out of 45 pairwise comparisons, none of which were significant following Bonferroni correction, indicating that the 10 loci are unlikely to be physically linked. The primers designed for locus Ag-1 also co-amplified a second locus with three alleles and an observed heterozygosity of 0.42. This additional locus did not deviate significantly from Hardy–Weinberg equilibrium ($P = 0.42$) and was not found to be in linkage disequilibrium with any of the other 10 loci (P values ranged from 0.30 to 0.867).

To check whether any of our loci were inadvertently identical to microsatellites previously cloned from other pinniped species, we used BLAST searches (Altschul *et al.* 1990) to test for homology between our flanking sequences and the GenBank NR database (<http://www.ncbi.nlm.nih.gov>). Default matching criteria were used. In the majority of cases, no matches were found, suggesting that our loci have not previously been described. Interestingly, however, loci Ag-1 and Ag-2 aligned significantly to regions of *Canis familiaris* chromosomes 11 and 17, respectively ($E < 2 \times 10^{-59}$), suggesting possible homology with the dog genome.

Elsewhere, microsatellite loci cloned from several different pinniped species have been shown to readily amplify polymorphic PCR products across the Pinnipedia (Coltman *et al.* 1996; Gemmell *et al.* 1997; Davis *et al.* 2002; Hoffman *et al.* 2007b). Consequently, our new panel of microsatellite loci should prove useful for studying not only Antarctic fur seals, but also other related pinniped species.

Acknowledgements

We are grateful to Bill Amos for providing laboratory facilities and to Jaume Forcada for supplying tissue samples. This work was funded by the Natural Environment Research Council (NERC) and the Antarctic Funding Initiative (AFI). J.I.H. was also funded by the Balfour Fund and Cambridge University VIP scheme. Samples were collected and retained under permits issued by the Department for Environment, Food and Rural Affairs (DEFRA), and in accordance with the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

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