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PRIMER NOTE

Ten novel dinucleotide microsatellite loci cloned from the Galápagos sea lion (*Zalophus californianus wollebaeki*) are polymorphic in other pinniped species

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Abstract

We isolated and characterized 10 novel dinucleotide microsatellite loci from the Galápagos sea lion (*Zalophus californianus wollebaeki*) and tested their amplification utility in four further otariid species (*Zalophus californianus californianus*, *Arctocephalus gazella*, *Arctocephalus australis* and *Eumetopias jubatus*) and three phocid species (*Hydrurga leptonyx*, *Halichoerus grypus* and *Phoca vitulina*). All of the loci amplified polymorphic polymerase chain reaction (PCR) products in at least three species other than the Galápagos sea lion. These markers will be useful for studies of pinniped mating systems, genetic structure and genetic diversity.

Keywords: cross-species amplification, microsatellite, Otariidae, Phocidae, pinniped

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Despite the Otariidae (fur seals and sea lions) comprising almost half of all pinniped species, relatively little is currently known about the reproductive and population biology of this family. This could be partly due to a shortage of genetic markers. For example, genetic studies of otariid mating systems and population structure (e.g. Hoffman *et al*. 2003; Hoffman *et al*. 2006) have to date relied heavily upon the cross-amplification of microsatellite loci developed in the Phocidae. More recently, Hernandez-Velazquez *et al*. (2005) and Wolf *et al*. (2006) have published a number of otariid microsatellites. However, additional loci will facilitate high-resolution analyses such as parentage and assignment testing. Moreover, screening new markers for cross-amplification across a range of otariid species will aid researchers working on different taxa. Here, we report the development of primers for polymerase chain reaction (PCR) amplification of 10 novel microsatellite loci in the Galápagos sea lion (*Zalophus californianus wollebaeki*) and demonstrate their amplification utility across five otariid and three phocid species.

We constructed a microsatellite library enriched for AC_n as described in detail by Wolf *et al*. (2006). Briefly, genomic DNA from five individuals was extracted using the DNeasy tissue kit (QIAGEN) and subject to an enrichment protocol following a modified fast isolation by AFLP of sequences containing repeats (FIASCO) method described in Steinfartz *et al*. (2004). Fragments enriched for (AC) repetitive stretches were cloned using the TOPO TA cloning kit (Invitrogen). Ninety-six clones were randomly selected and sequenced with the T3 primer (5′-ATTAACCCTCACTAAAGGGA-3′) using an ABI PRISM 3100 automated DNA sequencer following the manufacturer's protocols. Sixty-five of these clones carried the (AC) repetitive motif and, for 27 clones, primers could be designed in the flanking sequence of the microsatellite using the program PRIMER 3 (Rozen & Skaletsky 2000). Previously, Wolf *et al*. (2006) showed that eight of these primer pairs amplify polymorphic microsatellite loci in the Galápagos sea lion. Here we extend this work by characterizing 10 of the most promising remaining loci and conducting cross-species amplification trials.

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Table 1 Ten novel microsatellite loci developed from the Galápagos sea lion *Zalophus californianus wollebaeki*. Polymorphism characteristics are given for 20 unrelated Galápagos sea lion individuals

Locus	GenBank Accession no.	Primer sequence (5′–3′)	Repeat motif	T_1 (°C)	T_2 (°C)	No. of alleles (size range)	H_O	H_E	Null allele frequency	HWE *P* value
ZcwA05	DQ836319	F-CACTTCACTTCAGCGTCAGTCT R-CTCTTGGCTCCTACAGACATCGT	$(GT)G(GT)_{25}(AC)_2$	46	48	8 (112–128)	0.700	0.740	0.012	0.550
ZcwA12	DQ836320	F-CCATTCCCCAGGTACATACTTCAG R-AATACAGTTGGGGAGGGTAGGAG	$(AC)CC(AC)C(AC)_{35}$	50	54	10 (196–218)	0.950	0.886	−0.046	0.814
ZcwB03	DQ836321	F-ACCAAGAGAGCAGCCACTGACTAT R-CATAGACCATATGCCCTGTGAG	$(AC)ATCT(AC)_{23}$	48	52	5 (208–216)	0.450	0.723	0.149	0.055
ZcwB07	DQ836322	F-CAGCCAGTTACTCTTCCTCAACTG R-GACACAGCAGAGGTAACACACAAG	$(GT)_{21}$	50	54	4 (194–200)	0.421	0.479	0.031	0.364
ZcwC01	DQ836323	F-GGCCATGCTCATAACTCTTACCTAC R-GATGGCTCTGTATCTGGTAGATCCT	$(AC)_{22}AGG(AC)$ $TT(AG)_2C(AC)_3$	48	52	6 (151–163)	0.600	0.728	0.064	0.414
ZcwE04	DQ836324	F-GCTGCTGTTACCACCTTTGTT R-TAAGAAGACCCAGGATAGAGACCAG	$(GT)_{20}(ATCT)_3$	46	48	5 (128–138)	0.700	0.612	−0.065	0.228
ZcwE12	DQ836325	F-AAGTATACATGGTGGGATGGAGAAC R-CACCTTTGAGAGAACCAAGTACA	$(GT)_{27}$	48	52	7 (166–198)	0.90	0.836	−0.047	0.753
ZcwF07	DQ836326	F-TATTCCTAGAGGGGCAAGTCAAG R-CATTGACTCTCTGAAATGGTGTC	$(GT)_2TT(GT)_{20}$	46	48	5 (148–156)	0.789	0.684	−0.074	0.823
ZcwF09	DQ836327	F-TGTTTATACATGTGGTATGCACCTA R-TCTGTATAACCCAGAGAGGTCCAAT	$(GT)_{24}AT(GT)_2$	50	54	4 (124–130)	0.600	0.655	0.024	0.432
ZcwG04	DQ836328	F-TTGCTGGTGAGTTGGATGAC R-AGAAGAGGGTCCTGTTCACTTG	$(AC)GC(AC)_{25}$	48	52	13 (162–202)	0.800	0.901	0.042	0.343

T_1 and T_2, annealing temperatures; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, Hardy–Weinberg equilibrium.

Microsatellite genotyping was conducted as described by Hoffman & Amos (2005). PCRs were carried out in 10-μL reaction volumes containing approximately 10 ng template DNA, 1× Thermalase buffer (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 tetramethylammonium chloride 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 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; 10 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 final elongation 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. Allele sizes were determined by comparison with laboratory standards of known size. GENEPOP (http://wbiomed.curtin.edu.au/genepop/; Raymond & Rousset 1995) was used to calculate observed and expected heterozygosities and to test for deviations from Hardy–Weinberg equilibrium and linkage equilibrium among the 20 Galápagos sea lions genotyped. Null allele frequencies were calculated following Brookfield (1996) using the program MICRO-CHECKER (Van Oosterhout *et al*. 2004).

Table 1 summarizes the 10 novel microsatellite loci and their polymorphism characteristics for 20 unrelated Galápagos sea lion individuals, all sampled from a single breeding colony (for details of the sampling site, see Wolf *et al*. 2005). Each locus yielded between four and 13 alleles and expected heterozygosity ranged from 0.48 to 0.90. None of the loci showed significant deviations from Hardy–Weinberg equilibrium. However, ZcwB03 should be treated with caution because a high frequency of null alleles was detected at this locus. Tests for linkage disequilibrium yielded three weakly significant *P* values ($P < 0.05$) out of 45 pairwise comparisons, none of which were significant following sequential Bonferroni correction (Hochberg 1988), indicating that the 10 loci are unlikely to be physically linked.

All of the loci amplified polymorphic PCR products in at least three species other than the Galápagos sea lion (Table 2). Cross-amplification was particularly successful among the Otariidae, with eight of the loci being polymorphic in every species tested. However, while most of the loci also amplified PCR products in phocid seals, almost half of these species–locus combinations yielded monomorphic bands and a small number failed to amplify. This pattern is consistent with previous studies (e.g. Davis *et al*. 2002) and suggests that our panel of markers will be most useful for genetic studies of otariid species.

Table 2 Cross-species amplification of 10 novel microsatellite primer pairs developed from the Galápagos sea lion *Zalophus californianus wollebaeki*. Four individuals of each species were tested. For each species/locus combination, the number of alleles is given together with the allelic size range in parentheses

Locus	California sea lion *Zalophus californianus californianus*	Antarctic fur seal *Arctocephalus gazella*	South American fur seal *Arctocephalus australis*	Steller's sea lion *Eumetopias jubatus*	Leopard seal *Hydrurga leptonyx*	Grey seal *Halichoerus grypus*	Harbour seal *Phoca vitulina*
ZcwA05	2 (98–112)	4 (90–114)	6 (106–144)	3 (112–116)	6 (98–108)	1 (90)	3 (88–92)
ZcwA12	4 (196–216)	5 (198–228)	5 (202–212)	5 (174–200)	3 (166–190)	3 (178–184)	3 (180–184)
ZcwB03	2 (210–212)	4 (186–200)	2 (200–206)	3 (188–208)	NA	1 (176)	1 (176)
ZcwB07	5 (192–200)	6 (184–200)	5 (174–196)	1 (196)	1 (200)	1 (202)	1 (200)
ZcwC01	1 (157)	4 (127–151)	2 (123–151)	1 (145)	5 (143–155)	1 (121)	1 (121)
ZcwE04	4 (126–138)	5 (112–130)	6 (128–140)	5 (124–142)	2 (112–114)	NA	1 (112)
ZcwE12	5 (168–184)	4 (180–192)	5 (174–200)	4 (182–192)	3 (162–166)	4 (170–178)	1 (166)
ZcwF07	6 (138–156)	5 (136–150)	5 (140–150)	4 (144–150)	3 (132–138)	2 (142–146)	3 (140–150)
ZcwF09	3 (128–132)	6 (104–120)	4 (106–120)	3 (114–118)	1 (90)	NA	3 (94–98)
ZcwG04	5 (180–196)	4 (172–192)	6 (170–186)	5 (170–180)	1 (168)	6 (174–190)	1 (170)

NA, not amplifying.

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