New polymorphic microsatellite markers for California sea lions (Zalophus californianus)

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Abstract

Nine microsatellite loci were isolated and characterized from California sea lions (Zalophus californianus). In addition, two of five loci tested from harbour seal (Phoca vitulina) produced a single, clear band in Z. californianus, as did one out of five loci from grey seal (Halichoerus grypus) and one out of two loci from elephant seal (Mirounga sp.). No locus tested from South American fur seal (Arctocephalus australis) amplified in Z. californianus. Locus variability was assessed in California sea lions from Los Islotes rookery, Baja California Sur, Mexico. All loci were variable, with allele numbers ranging from three to 12.

Keywords: microsatellites, otariidae, pinniped, Zalophus californianus californianus

Discrepancies between observational and genetic measures of parentage have been shown in some pinnipeds (e.g. Amos et al. 1993; Wilmer et al. 1999). Such differences are likely to be large in California sea lions (Zalophus californianus, Family Otariidae) because they breed in large groups, on uneven terrain or in the water, and individual identification is seldom possible. Microsatellite loci have been developed for phocids, including grey (Halichoerus grypus) (Allen et al. 1995), harbour (Phoca vitulina) (Coltman et al. 1996; Goodman 1997), elephant (Mirounga sp.) (Gemmell et al. 1997), crabeater (Lobodon carcinophagus), leopard (Hydrurga leptonyx) and Weddell seals (Leptonychotes weddellii) (Davis et al. 2002); and for one otariid, the South American fur seal (Arctocephalus australis) (Gemmell et al. 1997). Some have been tested with varying success in otariids, e.g. New Zealand fur seal (Arctocephalus fosteri) and Steller's sea lion (Eumetopias jubatus) (Coltman et al. 1996; Gemmell et al. 1997; Davis et al. 2002), but not in California sea lions. Microsatellite-containing clones were therefore isolated from California sea lions, either from an enrichment protocol library based on Glenn et al. (2000), Hamilton et al. (1999) and Toonen (1997) or a standard library (Paetkau & Strobeck 1994).

A standard microsatellite library was constructed from approximately 150 ng of California sea lion genomic DNA digested overnight with Mbo1 (Gibco BRL). Fragments of 400–1000 bp were selected and ligated into BamHI digested dephosphorylated pUC-18 vector (all Gibco BRL). Competent cells were transformed (Escherichia coli DH5α, Max Efficiency, Gibco BRL) by thermal shock and plated onto agar LB-Ampicillin plates. A total of 1302 positive colonies were picked and re-incubated on new LB-Ampicillin plates. Recombinant colonies were blotted onto nylon membranes (Roche Diagnosis GmbH) and screened by hybridization with a DIG labelled nucleotide probe $(TG)_{10}$ (DIG-High prime kit) (Roche Diagnosis). Positive colonies were identified using the DIG DNA labelling and detection kit (Roche Diagnosis).

Fifty-two positive clones were identified and polymerase chain reaction (PCR) amplified using 20 μL of PCR super mix (Gibco BRL) and 1 μL of 10 μm primer solution (universal M13). Inserts were amplified in a 22.5 μL reaction using an ABI 480 thermocycler (Perkin-Elmer). PCR conditions were: 96 °C for 5 min, then 5 cycles of 96 °C 1 min, 60 °C 1 min, 72 °C 4 min, and 25 cycles of 94 °C 1 min, 43 °C 1 min, 72 °C 4 min, with a 10-min final extension at 72 °C.

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An enriched genomic library was constructed by simultaneous cutting and ligation of California sea lion genomic DNA with RsaI and SNX linkers (Hamilton et al. 1999) incubating at 37 °C for 2 h. This product was amplified in an MJ Research PTC-200 thermocycler under the following conditions: 72 °C 2 min, 95 °C 2 min, followed by 15 cycles of 95 °C 20 s, 60 °C 20 s, 72 °C 90 s, and ending with 72 °C 10 min. The enrichment was carried out with biotinylated oligonucleotides in two separate reactions; one consisting of $(CA)_{10}$, $(AG)_{10}$ and $(GATA)_6$ and the second of $(GACA)_6$. This product was combined with Streptavidin MagneSphere Particles (Promega) at 50 °C. Three initial washes with TBST [25 mm Tris/Tris HCl, 0.15 m NaCl, pH 7.5, 0.05% (w/v) Tween-20] at 50 °C, and a final wash with SSC (30 mm NaCl, 3 mm sodium citrate, pH 7.0), 0.1% SDS were carried out. The product was then eluted in 100 μL of TLE (10 mm Tris, 0.1 mm EDTA, pH 8.0) and PCR recovered in an MJ Research PTC-200 under the following: 94 °C 2 min, 15 cycles of 94 °C 30 s, 60 °C 30 s, and 90 s at 72 °C, ending with 72 °C 30 min. The resulting PCR products were ligated into a pGem-T Easy Vector (Promega) and transformed into JM109 high efficiency competent cells (Promega) by thermal shock. Approximately 1100 recombinant clones were transferred to Hybond-N + membranes (Amersham Pharmacia Biotech) and hybridized with the $(CA)_{10}$ $(AG)_{10}$ and $(GATA)_6$ probes at 56 °C, and with the $(GACA)_6$ probe at 67 °C. Microsatellite-bearing colonies were identified with the CDP-Star chemiluminescent detection kit (Sigma-Genosys Ltd).

Thirty-five positive clones from the standard library and 100 clones from the enriched library were sequenced using the Big Dye kit and resolved on an ABI 377 sequencer (both PE Applied Biosystems). Ten primer pairs from the standard and 36 from the enriched libraries were designed using either primer select of DNAstar (Lasergene) or oligo explorer version 1.1.2 (Kuulasma 2002).

Loci were tested on 58 California sea lion samples collected in 2002 and 2003 at the Los Islotes rookery, located 28 km north-northeast from La Paz, Baja California Sur, Mexico. Loci were amplified in 25 μL reactions [1 μL template DNA, 0.4 μm NH_4 buffer, 2 mm $MgCl_2$, 0.64 mm dNTP, 0.4 μm each primer (forward primer fluorescently labelled), and 0.063 units Taq (Bioline)] under the following conditions: 94 °C for 3 min, 30 cycles of 93 °C 30 s, annealing temperature (T_a, Tables 1 and 2) 30 s, 72 °C 30 s, and then 72 °C 3 min and 15 °C 5 min. PCR products were detected on an ABI Prism 3700 DNA analyser using an internal size standard [GeneScan-500 (ROX)], analysed and sized using genescan and genotyper software (PE Applied Biosystems).

Most microsatellite loci from other pinniped species tested did not recognize homologous sequences in California sea lions (Table 1). A total of nine primer pairs amplified clearly defined bands in the target species, showing intermediate to high levels of variability with three to 12 alleles per locus and expected heterozygosity ranging between 25% and 84% (Table 2). Apart from two heterozygous male individuals, locus ZcCgDh1.16 was monomorphic across our samples

Table 1 Locus, size range, repeat motif of the genotyped allele, diversity statistics and primer sequence of 13 loci developed for Arctocephalus australis (Aa), Halichoerus grypus (Hg), Mirounga sp. (M & BG), and Phoca vitulina (Pv)

Locus	Size range	Repeat motif	T_a	N_A	H_O	H_E	Primer sequences
Hg6.1*	160–174	$(CA)_{11}TA(CA)_{10}$	60	9	0.6154	0.6415	F: TGCACCAGAGCCTAAGCAGACTG R: CCACCAGCCAGTTCACCCAG
M11a†	132–149	NA	55	10	0.7917	0.7999	F: TGTTTCCCAGTTTTACCA R: TACATTCACAAGGCTCAA
Pv9§	174–182	$(GT)_{14}$	55	6	0.4748	0.5118	F: TAGTGTTTGGAAATGAGTTGGCA R: ACTGATCCTTGTGAATCCCAGC
Pv11§	176–184	$(CA)_{20}$	64	5	0.5338	0.6198	F: GTGCTGGTGAATTAGCCCATTATAAG R: CAGAGTAAGCACCCAAGGAGCAG
Aa4‡	201–232	NA	52	1	—	—	
BG‡	240–334	$(GGAAA)_{12}$	60	1	—	—	
Pv3§	117–281	$(GT)_{36}$	—	—	—	—	N/A
Pv10§	119–146	$(GT)_{13}$	—	—	—	—	N/A
Pv17§	128–174	$(GT)_{17}$	—	—	—	—	N/A
Hg0‡	145–175	NA	—	—	—	—	N/A
HgDii*	107–145	$(CA)_{14}$	55	—	—	—	M/B
Hg1.3‡	216–268	$(CA)_{12}$	—	—	—	—	N/A
Hg3.7‡	178–418	$(CT)_{10}(CA)_5CT(CA)_{15}$	—	—	—	—	N/A

*Allen et al. (1995); †Hoelzel et al. (2001); ‡Gemmell et al. (1997); §Goodman (1997).

NA, not available; N/A, no amplification; M/B, multiple bands amplified; T_a, annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

Table 2 Locus, size range, repeat motif of the genotyped allele, diversity statistics, primer sequence and accession no. of nine California sea lion microsatellite loci

Locus	Size range	Repeat motif	T_a	N_A	H_O	H_E	Primer sequences	GenBank Accession no.
ZcCgDh5.8	334–350	$(GT)_{21}$	61	12	0.8676	0.8457	F: GCACTATGCAACTGGGAATA R: ACAGATAGGAGGCTTGGGAT	AY676474
ZcCgDh1.8	173–183	$(GT)_{14}(GC)_2(GT)_8$	56	10	0.7619	0.7735	F: GCACTACTTATTCAGTAGCA R: CCCCACTGTGTTCATTCAAC	AY676475
ZcCgDh3.6	235–253	$(CA)_{16}$	53	7	0.6452	0.6217	F: AATCACACATCCTTAGTCTC R: GCCATAAAATTCTAGGTTG	AY676476
ZcCgDh5.16	254–262	$(GATG)_4(GATA)_2GAT(GATA)_3$ $GAC(GATA)_4GAT(GATA)_{13}$	62	6	0.7165	0.6883	F: TGCTCAGTCACCCTTCTTG R: CAGAAACGTGGAATACACAG	AY676477
ZcCgDh48	236–250	$(TC)_9(AC)_{14}$	56	5	0.5827	0.5601	F: AGGCAAGTATAGGCAGGTGTTTC R: CAGTGTTTGTGTGTCTCATTCC	AY676467
ZcCgDh7tg	267–273	$(TG)_{10}(AG)_{19}$	55	4	0.5188	0.4906	F: TTGGGCATTTAATAATTTTGAC R: AACCAAGACCACAGAAGAAACT	AY676479
ZcCgDh4.7	258–264	$(GT)_{16}(GA)_{15}$	61	4	0.4714	0.4783	F: ACAGCTCAGGAAAACTAAGG R: CCTCATTCACTCACTCATTC	AY676478
ZcCgDhB.14	236–244	$(TGGA)_4GC(GATC)_5$	66	3	0.2520	0.2557	F: TTACACACCTTCCTGGGATAGAG R: ACGATGCCTTGCTCTGTTACC	AY676473
ZcCgDh1.16	165–170	$(TG)_{24}$	63	3	—	—	F: CATAACACTCTCCAGTTCCATC R: TAGCAGCAATGTCCCCAATAG	AY676480

T_a, annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

tested. Data was tested for Hardy–Weinberg equilibrium (HWE) and genotypic disequilibrium with fstat 2.9.3.2. No significant deviations from HWE or genotypic disequilibrium were found ($P > 0.05$).

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