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TECHNICAL NOTE

Isolation and cross-species amplification of novel microsatellite loci in a charismatic marine mammal species, the northern elephant seal (*Mirounga angustirostris*)

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Abstract Due to its demographic history, the northern elephant seal is a charismatic species with a peculiar place in conservation biology. After having being almost exterminated by commercial sealing, and having being repeatedly declared extinct, the species has enjoyed a period of expansion at sustained rate. The low genetic variability produced by the bottleneck is apparently not affecting the viability of the species, but implies practical problems in the application of standard molecular ecology tools due to the lack of polymorphic markers. We developed novel microsatellite markers that, although showing a rather small variability, are a valuable addition to the molecular toolbox that can be used to study the species.

Keywords Microsatellites · Genetic variability · Northern elephant seal · *Mirounga angustirostris*

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The northern elephant seal (*Mirounga angustirostris*; NES) is an important species for conservation biology theories because of its peculiar demographic history, and the genetic consequences of it. The species suffered a significant population bottleneck due to commercial sealing, was repeatedly declared extinct, and was actually reduced to a very small number of individuals (20–100; Weber et al. 2000), concentrated on a single island, Isla Guadalupe (Mexico). The species shows a very low genetic variability in all markers that have been tested, including allozymes (Bonnell and Selander 1974), mtDNA (Weber et al. 2000), minisatellites (Lehman et al. 1993) and MHC loci (Weber et al. 2004). Notwithstanding this sustained depletion of genetic variability, after Mexico and USA government adopted strict protection rules the species enjoyed an impressive recovery (Bartholomew and Hubbs 1960), growing at a sustained rate, and greatly increasing its geographic range (Stewart et al. 1994; Carretta et al. 2011). All together, it seems that the lack of variability is not affecting the viability of the species, at least in the medium term (Weber et al. 2004). The lack of variability has an important negative practical implication: it is difficult to apply to NES the molecular ecology methods that are normally used to study population structure, kinship, paternity, etc., because of the lack of suitable polymorphic markers. In this note we present the development of 9 new polymorphic microsatellite markers that are a valuable addition to the molecular toolbox that can be used to study this species.

We collected skin samples in the Islas San Benito (Baja California, Mexico), taking by surprise small (1–2 g) pieces of inter-digital membrane of the rear flippers of live NES, and storing them in 2.0 ml screw cap tubes with 95 % ethanol kept at ambient temperature (Fabiani et al. 2004). DNA was extracted from skin samples using the

DNEasy Blood and Tissue kit (Qiagen), following the manufacturer mouse-tail protocol. Extracted DNA was checked for degradation by electrophoresis in 1.0 % agarose gels and by NanoDrop ND-1000 (ThermoScientific) measurements of absorbance (DNA concentration between 100 and 300 µg/µl).

We used 6 male and 6 female samples to construct four microsatellite enriched libraries using biotinylated probes

Table 1 Characteristics of the novel loci

Locus/GenBank	Repeat motif	Primers	PCR	Conc.	MgCl$_2$	T$_a$	N	N$_a$	Size
Mang01	(CT)$_2$GT(CT)$_4$…(CT)$_{10}$	GCCTTTGGTTAGGTATCCAG	Good	0.300	1.50	55	22	2	212–216
JQ714261		CACTCTTGAATACTGTAGCCTTG		0.300					
Mang03	(TA)$_5$	GTGGAAAGAGCCAAGATG	Good	0.300	1.50	55	22	1	147
JQ714262		CTCAGCATAGTATCCTCTAGTTCC		0.075					
Mang04	(GA)$_9$	GAGCTCTAGGTTATGATTTG	Good	0.300	1.50	55	22	1	105
JQ714263		CTGCTGCTCTACCAGC		0.075					
Mang05	(GT)$_5$	GTTGTCGTGAGGACTGATG	Good	0.300	1.50	55	22	1	112
JQ714264		GAGATGAGCCTTAAAGAATGG		0.075					
Mang06	(GT)$_9$…(GA)$_{14}$	CTATCACGGAGATGGGTG	Good	0.300	1.50	55	22	2	159–161
JQ714265		CTCAGGAAACCTTCATTGC		0.075					
Mang09	(TATC)$_6$CATC(TATC)$_7$ATC(TATC)$_3$	GGAAGGAGGTGCTATTACTCTC	Good	0.300	1.50	52	22	6	230–242
JQ714266	…(TATC)$_{12}$	CTCATCTCTTGAGGCATCC		0.075					
Mang14	(CT)$_3$GT(CT)$_3$	GAGCCTCCTCTGTAATGG	Good	0.300	1.50	55	22	1	129
JQ714267		GATTCGTGACCAGAAAATC		0.300					
Mang16	(CT)$_7$	CAGAACATCAAACCAAGTGAG	Good	0.300	1.50	59	22	1	227
JQ714268		GGTTCAGTGTCTGCCTTC		0.075					
Mang17	(CA)$_6$TG(CA)$_5$	GAGTGTCACCCTTCCTCG	Good	0.300	1.50	55	22	1	153
JQ714269		GCAAAATGCTGTGTATGAGC		0.075					
Mang21	(GT)$_5$CTCT(GT)$_3$	GATAACTTCTGGGGTGGG	Good	0.300	1.50	55	22	1	132
JQ714270		GCTGAGAAAAAATACTGTAAGATTC		0.075					
Mang23	(CA)$_{11}$	CAGTGACTTCCCCCTCC	Scarce	0.300	1.25	57	22	1	136
JQ714271		GATCACAGGACAGCCTTCAG		0.075					
Mang27	(GT)$_2$GA(GT)$_{16}$	GGAAATGGTATTGTAGTTATGTAGG	Good	0.300	1.50	55	22	2	109–111
JQ714272		CTCCCCCTTCTGCATC		0.075					
Mang33	(CT)$_3$T(CT)$_6$	CCTGGGTGGCTCTTGATT	Good	0.300	1.50	55	22	1	273
JQ714273		TGACACATTACAAAATACTCCA		0.075					
Mang34	(GT)$_7$GCA(TG)$_6$CA(TG)$_4$	GCTGATGGACTGGCATTTTA	Scarce	0.300	1.70	60	22	3	196–200
JQ714274		GTGCTCGCCTCCTCTCCT		0.075					
Mang35	(CA)$_{14}$	ATTGGTTTCTTGATTATGC	Good	0.300	1.50	53	22	2	245–248
JQ714275		ATGCCCGTATCTATTCCT		0.075					
Mang36	(CCAT)$_3$…(CCAT)$_4$…(CCAT)$_2$	GGGGACACAAGCACAAC	Good	0.300	1.50	55	22	2	339–343
JQ714276	…(CCAT)$_5$	CTCAAAGGATGGATAGATAAGC		0.075					
Mang37	(TC)$_4$	GAGCCCCGCATCAGG	Good	0.300	1.50	57	22	1	104
JQ714277		TTTATTTATTTAGAGAGTGTTCGTG		0.075					
Mang38	(GATA)$_4$GATTA(GATA)$_{13}$	GGGGACAGCACAAGGAAG	Good	0.300	1.50	56	21	1	217
JQ714278		GAAGGAATGGGGAAGCCTA		0.075					
Mang41	(GATA)$_3$…(GATA)$_6$GAT(GATA)$_2$GAT(GATA)$_3$	GCCTTTCCTTTCTTTCC	Good	0.300	1.50	53	22	1	276
JQ714279	… (GATA)3…(GATA)3GAT(GATA)10	GTCTCCATAACTGCCTGA		0.075					
Mang43	(GATA)$_2$GAT(GATA)$_{11}$	ACAGGATAGGGAATGGTGA	Good	0.300	1.50	55	21	3	238–246
JQ714280		GGGGAAAGAGGATTGTTC		0.075					
Mang44	(GATA)$_{14}$…(GATA)$_2$GAT(GATA)$_2$	CATCTTACCCAGGAGACAG	Good	0.300	1.50	55	22	3	176–188
JQ714281		GAGACAAGGGATAGGTCA		0.075					
Mang48	(GT)$_{16}$(GA)$_{10}$	AGCCTGTAGCCCTTGT	Good	0.300	1.50	55	22	1	270
JQ714282		GCACCCTTCTGTGTGAG		0.075					

GenBank = GenBank accession number; Primers = primer sequence, forward above and reverse below; PCR = quality of the amplification; Conc. = primer concentration, forward above and reverse below (µM); MgCl$_2$ = magnesium concentration (mM); T$_a$ = annealing temperature (°C); N = number of typed individuals; N$_a$ = number of alleles; Size = allele size range in base pairs excluding the M13 universal tag

$(CT)_{15}$, $(GT)_{15}$, $(CTGT)_6$ and $(GATA)_{10}$, following the protocol of Glenn and Schable (2005). The sequencing of 160 positive clones showed the presence of 89 microsatellite loci (success rate = 55.6 %), 56 % dinucleotides and 30 % tetranucleotides. We designed primers for 48 of these loci (Primer3, Rozen, and Skaletsky 2000).

For the PCR amplification and genotyping we used the universal tag approach of Schuelke (2000). We used the following PCR mix (15 µl final volume): 1X PCR buffer (Promega), 1.0–1.9 (see Table 1 and electronic supplement) mM $MgCl_2$, 200 µM dNTP Mix, 0.3 µM forward primer, 0.3 or 0.075 µM (see Table 1) reverse primer plus M13 universal tag (5′ TGTAAAACGACGGCCAGT 3′), 0.3 µM M13 universal tag plus fluorescent dye (Hex, Fam, Ned), and 0.02 units of GoFlexi Taq DNA polymerase (Promega). We used the following PCR program: 5′ at 95 °C; 35 cycles of 20″ at 95 °C, 20″ at the optimized annealing temperature (see Table 1) and 30″ at 72 °C; 8 cycles of 20″ at 95 °C, 20″ at 47 °C and 30″ at 72 °C; 10′ at 72 °C; hold at 4 °C. PCR products were resolved on a 3730XL Automated Sequencer (Applied Biosystems), and genotyped using GeneMarker 1.85 (SoftGenetics). We genotyped 22 NES individuals. We used the same protocol to optimize PCR conditions and carry out the genotyping for 5 individuals of each of the following pinniped species: southern elephant seal (*M. leonina*, ML), harbor seal (*Phoca vitulina*, PV), California sea lion (*Zalophus californianus*, ZC), and Guadalupe fur seal (*Arctocephalus twonsendi*, AT). Samples of these species were collected during previous research projects carried out by the Authors.

We calculated statistics of microsatellite variation and exclusion propobabilities using custom scripts, we tested the loci for Hardy–Weinberg equilibrium in GenePop4 (Rousset 2008), we verified the presence of null alleles in Microchecker (Van Oosterhout et al. 2004), and we calculated maximum likelihood estimates of genotyping error and null alleles frequency in ML-Null (Kalinowski and Taper 2006).

We were able to optimize PCR conditions and carry out the genotyping for 22 loci (Table 1). We found a low variability, with a mean number of alleles per locus of 1.7 (SD = 1.2, range = 1–6). Only 9 loci (40.9 %) were polymorphic, with a mean number of alleles per locus of 2.8 (SD = 1.3; loci statistics in Table 2). All polymorphic loci were in Hardy–Weinberg equilibrium (all loci test, Fisher's method: $P = 0.89$; Table 2). We were able to cross-amplify most loci in all the species tested (AT 86.4 %, ML 100 %, PV 95.5 %, ZC 81.8 %; Table 1 of the Electronic Supplement). We obtained for all the four species a greater allele range than for NES.

The results of our study were mixed. Although we were able to develop new microsatellite markers for the species, their variability was rather limited, and not greater than the variability observed in microsatellites developed in other species and cross-amplified in NES (unpublished data). Anyway, we think that these new microsatellite loci are a valuable addition to the molecular toolbox that can be used to study northern elephant seals, in particular if combined with the cross-amplification of microsatellites developed for other pinniped species. On a more general ground, our study confirms that NES have an unusually low variability also in microsatellite loci. Observed variability statistics are lower than the ones calculated in most seals and sea lions, and are similar to the ones found in two heavily

Table 2 Statistics of the polymorphic loci

Locus	H_o	H_e	F_{is}	I	N_e	PIC	PE_1	PE_2	PE_3	PID	PID_{sibs}	β	Null	P(HW)
Mang01	0.545	0.495	−0.103	0.676	1.936	0.367	0.183	0.117	0.276	0.371	0.604	0.000	0.000	0.682
Mang06	0.091	0.088	−0.024	0.184	1.094	0.083	0.042	0.004	0.078	0.818	0.916	0.000	0.000	1.000
Mang09	0.636	0.734	0.133	1.419	3.538	0.669	0.475	0.303	0.656	0.103	0.424	0.000	0.006	0.236
Mang27	0.273	0.305	0.104	0.474	1.424	0.253	0.127	0.044	0.207	0.502	0.736	0.000	0.027	0.538
Mang34	0.591	0.559	−0.057	0.928	2.204	0.486	0.291	0.149	0.441	0.231	0.543	0.000	0.000	0.202
Mang35	0.364	0.359	−0.012	0.536	1.541	0.290	0.145	0.062	0.230	0.450	0.695	0.000	0.000	1.000
Mang36	0.273	0.240	−0.132	0.398	1.307	0.208	0.104	0.028	0.176	0.577	0.785	0.000	0.000	1.000
Mang43	0.190	0.182	−0.005	0.383	1.217	0.169	0.086	0.014	0.159	0.650	0.833	0.045	0.000	1.000
Mang44	0.773	0.625	−0.237	1.019	2.570	0.541	0.331	0.186	0.482	0.195	0.500	0.000	0.000	0.216
Mean	0.415	0.399	−0.037	0.669	1.870	0.341						0.005	0.004	
SD	0.230	0.217	0.114	0.387	0.794	0.192						0.015	0.009	
Cumulative							0.881	0.635	0.972	0.000	0.021			

H_o = observed heterozigosity; H_e = gene diversity (expected heterozigosity); F_{is} = inbreeding coefficient; I = Shannon index of information; N_e = number of effective alleles; PIC = polymorphism information content; PE_1 = one parent probability of exclusion; PE_2 = missing parent probability of exclusion; PE_3 = Both parents probability of exclusion; PID = probability of identity; PID_{sibs} = probability of identity among sibs; β = maximum likelihood estimate of genotypic error; Null = maximum likelihood estimate of null allele frequency; P(HW) = exact probability of deviation from Hardy–Weinberg equilibrium bottlenecked phocid species, the Mediterranean monk seal (Pastor et al. 2004) and the Hawaiian monk seal (Schultz et al. 2010) that, contrary to NES, are demographically challenged.

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