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PRIMER NOTE

Ten polymorphic microsatellite loci for the endangered Buena Vista Lake shrew (*Sorex ornatus relictus*)

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Abstract

The ornate shrew (*Sorex ornatus*) is restricted to the vanishing wetlands of California, USA and Baja California, Mexico. Several subspecies of ornate shrews are considered 'mammal species of special concern' in California by the Department of Fish and Game, and one (*Sorex ornatus relictus*) has recently been listed as endangered. Populations of shrews around Buena Vista Lake have been diminished or extirpated due to habitat deterioration and human development. In order to study the patterns of genetic variation in isolated populations of Buena Vista Lake shrews, we developed 10 polymorphic microsatellite loci. There were 6–27 alleles per locus, and the loci had heterozygosity values that ranged from 20 to 80%. In addition, we screened 20 different populations of *S. ornatus*, eight species within two subfamilies of shrews (Soricinae and Crocidurinae), as well as in a mole (Talpidae, *Neurotrichus gibbsii*), to determine if these loci could be informative in other species as well.

Keywords: microsatellite, ornate shrew, primer, *Sorex ornatus relictus*, Soricidae

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Shrews are members of a primitive order of mammals, the Insectivora. Shrews also comprise a significant portion of mammalian species (about 1/3) and are distributed worldwide, excluding Australia, Antarctica and southern South America. Despite this wide distribution, little is known about patterns of differentiation, movements and gene flow in shrews. In this note, we describe microsatellite markers in the Buena Vista Lake shrew (*Sorex ornatus relictus*), which will be used to enhance the resolving power of our data for understanding their historical population structure. *Sorex o. relictus* once occupied the marshlands of the San Joaquin Valley throughout the Tulare Basin. However, it is now thought that their range has been restricted because of the disappearance of wetlands (Maldonado *et al*. 2001).

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Genomic DNA was isolated from tissue samples from liver by overnight digestion with Proteinase K in a digestion buffer containing 1× TNE and 10% SDS (New England Biolabs), followed by extraction with phenol/chloroform/iso-amyl alcohol and ethanol precipitation to yield a final concentration of 1 μg/μL (Sambrook *et al*. 1989). Approximately 20 μg of genomic DNA from several individuals was digested to completion with the restriction enzyme *Sau*3A 1 (New England Biolabs). The digestion product was electrophoresed on a 1% agarose gel, and the fragments from 300 to 600 bp were size selected by excising the piece of agarose containing these fragment lengths and eluting the DNA. Size-selected fragments were then ligated into the Lambda Zap phagemid vector (Stratagene) cut with *Bam*HI restriction enzyme (New England Biolabs) and the vector arms incubated with calf intestinal alkaline phosphatase (New England Biolabs). The ligated products were packaged using a Gigapack II Gold packaging extract (Stratagene), and the library was amplified

in *Escherichia coli*. The genomic library was titred and plated out onto appropriate nutrient media. Plaques were lifted and fixed onto a nylon membrane, which was then screened for the presence of microsatellites by probing with an end-labelled dinucleotide repeat (Rassmann *et al*. 1991). Putative clones were picked, plated, and lifted again for secondary screening. The DNA insert and phagemid were then excised *in vivo* into a plasmid vector with an ExAssist helper phage (Stratagene). The insert-containing plasmid was grown and isolated using a Wizard Minipreps kit (Promega). The insert was then directly manually sequenced (Sanger *et al*. 1977) using the M13 T7 and BK reverse primers located on either side of the multiple cloning site, and the microsatellite repeat was identified using a Sequenase version 2.0 kit (US Biochemicals) labelling nucleotides with ^{35}S. The sequencing reaction products were separated by electrophoresis in a 6% polyacrylamide gel for 3 h at 55 W in a Stratagene Base Ace Sequencing apparatus. The insert DNA sequence was read into a Macintosh Quadra 950 computer using an IBI Gel Reader and the MACVECTOR 4.1.4 program (Eastman Kodak). The flanking regions of the repeat were used to design polymerase chain reaction (PCR) primers using the PRIMER program (Lincoln *et al*. 1991). Primers were then synthesized and used to amplify single loci from genomic DNA of 251 individuals from 21 populations of *S. ornatus* to assay the degree of polymorphism. Primers of 18–24 bp in length were designed to flank the microsatellites, giving PCR products that range in size from 110 to 280 bp in length. Amplification of microsatellites was carried out in a 15 μL volume containing 30 ng of DNA, 0.2 μM of each primer, 0.2 μM dNTP, 1× *Taq* buffer (1.5 μM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl) and 0.75 U of *Taq* polymerase (Boehringer Mannheim). Amplification conditions were 94 °C for 5 min, followed by 34 cycles at 94 °C for 45 s, T_a for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 5 min. Locus-specific annealing temperatures are given in Table 1. Amplifications were performed in a programmable thermal cycler (PerkinElmer-Cetus, Model 480). To assess allelic variation at each locus, DNA samples were amplified as above, but with one primer end-labelled with ^{32}P-ATP. The PCR products were optimized and analysed on 6% denaturing polyacrylamide sequencing gels (SequaGel-6, National Diagnostics). Radioactively labelled M13 mp18 sequence was used as a size standard on all gels.

Over 1000 clones were identified for further analysis when the library was screened with a CA_{15} probe. We isolated 100 clones that showed the strongest signal (1–2% of all clones). The Southern blots of DNAs from these clones were probed with a labelled CA_{15} probe, to confirm that the inserted sequences contained microsatellite repeats

Table 1 Primer sequences for microsatellite loci developed in this study together with annealing temperatures optimized for *Sorex ornatus relictus* genomic DNA. The number of alleles found in the ornate shrew and the observed percentage heterozygosity were also presented. Clone sequences have been submitted to GenBank

Locus	GenBank Accession no.	Allele no.	Size range (bp)	*n*	Repeat sequence	PCR primer sequence (5′–3′) Forward Reverse	Annealing temp. (°C)	H_O	H_E
A4-20	DQ204582	21	251–273	157	$(CA)_{13}$	5′-ACCATGCGTGTGACACAGACGTAC-3′ 5′-ACATGAGAAGGAAATCCCCAGTG-3′	55	0.50	0.53
A4-7	DQ204583	11	72–98	157	$(TG)_3(TA)(TG)_{10}$	5′-TCACTATTTTTTGGGGGGAG-3′ 5′-CAAAAAAAGACACACATT-3′	50	0.40	0.46
A3-5	DQ204580	16	267–293	157	$(CA)_{17}(CT)(CA)_5$	5′-GGATACTTTCTATGGGACTTCTGTG-3′ 5′-ATACCTGAGTTTGGGGAC -3′	60	0.66	0.70
A4-5	DQ204578	27	270–292	157	$(CA)_9(AA)(CA)_9$	5′-AACCCGTGTATCTGAGTTCAGGAG -3′ 5′-CCTAAATTGTCCAGTGTGAGTGAGG-3′	55	0.70	0.70
SH-22	DQ204584	17	337–365	157	$(GT)_{15}$	5′-AAGTACCTTGGGGCATAAAAC -3′ 5′-CAACCTGACAAACTCCAGTTC-3′	53	0.80	0.71
A3-26	DQ204587	5	155–171	157	$(GT)_3G(GT)_5(GC)(GT)_4$	5′-TTCTGAGCCCTTGCCCCCTC-3′ 5′-GCACAGAAGACACAGGAATG-3′	55	0.70*	0.42*
A4-1	DQ204581	6	275–281	157	$(TG)_6(TC)(TG)_3$	5′-TGTGTTGGGCACATTCCTG-3′ 5′-CGGTAGTCCAGAATCTCGTC-3′	55	0.60	0.66
SH-5	DQ204586	6	198–206	157	$(GT)_7C(GT)_4$	5′-GCTCTGTGCATTTGCTAC-3′ 5′-TAGTCCAGAATCTCGTCGCC-3′	55	0.50	0.61
SH-25	DQ204585	18	271–315	157	$(GT)_6C(GT)_{14}$	5′-CCAGGAATAAAATCTCTGG-3′ 5′-ATGGCATGGCTGGTGCATTC-3′	53	0.50	0.53
A3-35	DQ204579	17	132–160	157	$(TG)_{22}$	5′-GATTGGTTTGTTTGTGCCAG-3′ 5′-CCAGGAGAGAAGTGAAGGAGAC-3′	60	0.20	0.24

*Significant deviation from Hardy–Weinberg equilibrium.

and that the insert was within the size range of the DNA that was used to make the library (350–1000 bp). Of the positive clones, 77 were sequenced. A microsatellite region was identified in 44 of them; they contained 4–20 CA_n units. Primer pairs were designed for 10 microsatellite loci that contained 10 or more repeats (Table 1). A preliminary screening of these loci showed all of them to be polymorphic. Finding polymorphism in 100% of loci screened was unexpected, as rates of 50% have been reported in humans (Edwards *et al*. 1991). This may have been a consequence of selecting loci that contained 10 or more repeats. Alternatively, shrews may have a higher rate of mutation than other mammals.

Observed heterozygosity values ranged from 0.20 to 0.80; meanwhile, the expected values ranged from 0.24 to 0.71. We tested for deviation from Hardy–Weinberg equilibrium (HWE) at each locus as implemented in GENEPOP (Raymond & Rousset 2000). Most of the polymorphic loci were in HWE with the exception of locus A3-26, which yielded a deficiency of heterozygous genotypes. We tested for linkage disequilibrium (LD) using an extension of the Fisher's exact test as described by Slatkin (1994). Significant levels were generated based upon 10 000 permutations of Markov chain as implemented in ARLEQUIN (Schneider *et al*. 2000). We also used the Bonferroni technique to correct significant levels of this test. Results showed that none of our loci were in (LD).

All loci were tested in the nine subspecies of *S. ornatus* under the same PCR conditions (Table 2). Microsatellites amplified successfully in these subspecies, showing high

Table 2 Results of the cross-species amplifications of Buena Vista Lake shrew loci in eight subspecies of *Sorex ornatus*, seven species of shrews and one mole. For each taxa, the number of alleles are given except when no amplification of the homologous locus was detected

	A4-20 Allele no. Size range	A4-7 Allele no. Size range	A3-5 Allele no. Size range	A4-5 Allele no. Size range	SH-22 Allele no. Size range	A3-26 Allele no. Size range	A4-1 Allele no. Size range	SH-5 Allele no. Size range	SH-25 Allele no. Size range	A3-35 Allele no. Size range	*n*
S. o. californicus	6	6	9	19	8	4	4	4	8	10	19
	261–273	72–98	267–293	270–292	337–363	155–167	275–281	198–206	271–315	132–160	
S. o. juncensis	9	8	11	11	11	5	4	4	7	11	21
	251–273	72–98	265–291	276–288	339–365	155–171	275–281	198–204	271–311	136–158	
S. o. lagunae	13	8	10	14	10	6	4	3	8	9	17
	267–275	70–98	265–293	270–290	339–357	155–171	277–283	198–202	275–309	132–154	
S. o. ornatus	14	8	11	13	9	6	4	4	8	8	17
	257–273	76–96	271–293	278–292	337–365	157–169	275–283	198–206	271–311	132–160	
S. o. salarius	11	7	10	9	10	4	4	4	6	11	15
	257–271	72–98	265–291	270–280	337–365	155–169	275–281	198–204	275–307	130–158	
S. o. salicornicus	8	8	7	10	9	5	3	4	7	12	11
	263–273	72–98	267–289	274–290	345–365	155–169	275–281	200–206	275–303	132–158	
S. o. sinuosus	9	6	9	11	10	3	2	3	8	10	15
	261–273	80–98	271–291	274–294	339–359	155–163	275–277	198–204	271–311	132–160	
S. o. willetti	16	8	8	16	9	2	3	4	8	9	17
	241–273	74–96	275–289	270–288	337–365	155–159	275–281	200–206	269–311	144–160	
S. arizonae	1	0	0	3	0	0	1	0	2	2	13
	251	—	—	272–292	—	—	277	—	315–317	150–154	
S. monticolus	3	2	5	4	4	0	1	2	1	3	10
	241–261	74–80	255–267	282–306	349–361	–	275	200–202	271	140–144	
S. nanus	0	1	0	0	0	0	0	0	0	0	2
	—	72	—	—	—	—	—	—	—	—	
S. pacificus	5	3	7	5	9	0	2	1	5	5	8
	241–265	74–80	261–287	270–306	353–371	–	277–281	200	295–305	144–150	
S. tenellus	0	1	0	1	0	0	0	1	0	0	1
	—	74	—	270	—	—	—	204	—	—	
S. vagrans	3	1	3	5	3	0	2	0	1	4	4
	261–267	72	269–277	282–308	341–353	—	281–289	—	271	138–146	
Notiosorex	0	0	1	0	0	0	0	0	0	0	1
crawfordii	—	—	265	—	—	—	—	—	—	—	
Crocidura	0	0	0	0	0	0	1	0	0	0	1
viaria	—	—	—	—	—	—	275	—	—	—	
Neurotrichus	0	0	0	0	1	0	1	0	1	0	1
gibbsii	—	—	—	—	339	—	277	—	271	—	

levels of polymorphism. Species belonging to the genus *Sorex* showed high to moderate levels of amplification and polymorphism. *Sorex monticolus* presented the highest amplification values and polymorphisms among the different species in the genus *Sorex*. Test for amplification in distantly related shrew species of the genus *Notiosorex* and *Crocidura* and the shrew-mole was low and ranged from one to three successfully amplified loci, but polymorphism was not tested because we only had one sample for each of these species.

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