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A set of microsatellite loci for the hairy-nosed wombats (*Lasiorhinus krefftii* and *L. latifrons*)

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Introduction

Australia has three extant species of wombat, the northern and southern hairy-nosed (NHN and SHN) wombats (*Lasiorhinus krefftii* and *L. latifrons*, respectively), and the common wombat (*Vombatus ursinus*). These large herbivores spend a considerable amount of time underground, are hard to observe, are difficult to trap, and trapping is known to be disruptive (details in Taylor et al. 1998). This makes studies of wombats by conventional techniques relatively intractable, and has prompted the development of methods for molecular ecological analysis of wombat populations, such as censusing the highly endangered NHN wombat via remotely-collected hair samples. The NHN wombat is currently represented by only one known population consisting of probably less than 100 animals and has severely reduced genetic variation compared to the SHN wombat, which remains highly abundant in at least some parts of its South Australian range (Taylor et al. 1994). Microsatellite markers typically reveal high levels of polymorphism in natural populations (Bruford and Wayne 1993). We describe here the isolation and features of 12 novel wombat microsatellite loci and present details of an unpublished set of 16 loci already used extensively in the study of wombat populations (Taylor et al. 1994, 1997, 1998; Alpers et al. 1998). Details of the utility of these markers in the common wombat are also presented.

Methods

For the 12 new loci, NHN wombat genomic DNA previously isolated from blood samples (Taylor et al. 1994) was digested with *Alu*I, *Hae*III and *Eco*RV. Size-selected DNA (430–550bp) was used to construct a library of approximately 20,000 clones, which was screened for microsatellites according to Taylor et al. (1994), probing simultaneously with poly (AC/TG) and poly (TC/AG) (Pharmacia). Fifty-one putative positive clones were miniprepped and rescreened, and 36 clones showing intense hybridization were selected for sequencing. Primer sets were designed to amplify 14 long repeated dinucleotide regions with suitable flanking sequences.

Twelve of the 14 loci were successfully amplified by the polymerase chain reaction (PCR) in a 10 μl isotopic reaction containing 100–200 ng of template DNA, 0.5 units of *Taq* polymerase (Promega), 12 pmol of each primer, 200 μM of dCTP, dGTP, and dTTP, 20 μM of dATP, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100 and 0.05 μl [α-^{33}P]dATP at 1000 Ci/mmol. Cycling was performed in a MJ Research PTC100 thermocycler, starting with 94 °C for 2 min, followed by a ‘touchdown’ PCR (30 cycles of 94 °C/15s, annealing/30s, 72 °C/45s) and a final step of 72 °C for 2 min. We used three different ‘touchdowns’: in ‘65→60’ the annealing temperature decreased one degree per cycle, in ‘55→47’ two degrees, and the ‘62→55’ profile was 62-61-59-57-55 °C. PCR products were separated by

Table 1. Characteristics of 28 polymorphic wombat microsatellite loci isolated from *L. krefftii* (Lkr or Lk prefix) and *L. latifrons* (Lla or Ll prefix) genomic libraries. Number of alleles, along with expected (H_E) and observed (H_O) heterozygosity found in *n* samples of (from top to bottom) *L. krefftii*, *L. latifrons* and *V. ursinus*, are shown. Where no data are given for *V. ursinus*, either no product, or product of insufficient quality for scoring was obtained in a single PCR attempt. In the allele sizes column, 'nd' indicates markers for which accurate sizing of products was not performed. T*a* is the annealing temperature used in the 'touchdown' PCRs (see text for details). Repeat type corresponds to sequence of cloned allele. However, those loci marked with a * have multiple and often complex repeat regions (consult GenBank sequences for full details)

Locus	Primer sequences (5′–3′)	Repeat	T*a* (°C)	No. of alleles	*n*	Allele sizes (bp)	H_E	H_O	GenBank Accession no.
Lk09	GGTCTACAACAAGGTCTAGG	$(GA)_{33}$	65→60	4	64	191-209	0.69	0.66	AF178638
	TTTGGGGTGAGGCAGTGGAG			3	8	159–165	0.60	0.67	
				17	27	195–241	0.89	0.88	
Lk13	GATCCCCCCATAACAATAAC	$(CA)_{16}$	55→47	1	60	152	0.00	0.00	AF178639
	AACTCCATTTTTTCCTACAC			4	10	142–158	0.55	0.40	
				5	5	nd	0.70	1.00	
Lk19	TTTCTTTATCCTTTGCTTGC	$(GA)_{32}$	62→55	4	62	143–175	0.64	0.66	AF178640
	AGGACCTGATGCTTGGGACA			9	10	137–185	0.88	1.00	
Lk21	TGTTTCCACTCTTTCCATCC	$(GA)_{39}$	55→47	2	8	183–209	0.13	0.13	AF178641
	CCTGTGTATGACTGAGGTAGAG			5	8	181–203	0.79	0.83	
Lk23	TCTGGCTCCCAAACTAGAAG	$(CA)_{25}$	62→55	2	65	134–142	0.48	0.65	AF178642
	TGATAGAGATTAGCACTGGA			7	12	118–156	0.85	0.67	
				2	3	nd	0.44	0.00	
Lk24	CAGTGTGAACAGAGGGTTTG	$(GA)_{45}$*	62→55	2	8	179–183	0.13	0.13	AF178643
	AGTTAGAACNGGGGGAGG			4	8	151–185	0.82	0.83	
				3	6	nd	0.48	0.67	
Lk26	ACTTTCCAGACCCCATAGCC	$(GA)_{43}$	55→47	2	47	205–209	0.49	0.64	AF178644
	GGCAGGAGGAGAATGAAGAG			3	6	189–209	0.71	0.67	
				15	29	163–211	0.88	0.78	
Lk27	TCCTTGCTTTTGTCACCACC	$(GA)_{49}$*	55→47	3	64	306–322	0.33	0.44	AF178645
	GACCTCCAAAGNCACCTAGTC			3	6	312–322	0.68	0.33	
				13	29	216–377	0.86	0.59	
Lk31	ACATTTCCAGAGCCTTATCC	$(GA)_{25}$	62→55	2	7	157–185	0.25	0.26	AF178646
	CTGTGACCNTGTGATAGTGTCG			4	7	131–175	0.29	0.40	
Lk32	ACTTAACCCCATTGCCTAGC	$(GA)_{36}$	62→55	5	64	187–197	0.73	0.81	AF178647
	CTTTTCCCCACCCAGATTAG			7	8	169–197	0.82	0.89	
				1	5	nd	0.00	0.00	
Lk34	CACAGTGGATGCTAATAGTTC	$(GA)_{20}$*	55→47	1	8	207	0.00	0.00	AF178648
	GCACTTCCTCAAAAGGCATA			5	5	213–229	0.64	0.60	
				6	29	191–207	0.54	0.48	
Lk37	ATTCCTGATTTTGGGGTCTC	$(CA)_{16}$	62→55	2	8	192–194	0.26	0.29	AF178649
	CCTGTGAGTGGTAAGGATGG			5	8	192–216	0.79	0.75	
				2	6	nd	0.44	0.33	
Lla3AT[a]	GCAAAATGAACTGCAGAAGG	$(AT)_{26}$*	55→47	1	32	179	0.00	0.00	AF185101
	CATAGATATGAAGTATATAC			17	88	175–213	0.88	0.58	
				2	2	179–191	0.38	0.50	
Lla14CA[a]	GACACAGAAAACAAGGGCAG	$(CA)_{16}$	65→60	1	27	220	0.00	0.00	AF185097
	GATCCCTGAGCACTCTCTTG			2	7	230–232	0.57	0.50	
				2	2	238–242	0.38	0.50	

Table 1. Continued

Locus	Primer sequences (5′–3′)	Repeat	T*a* (°C)	No. of alleles	*n*	Allele sizes (bp)	H_E	H_O	GenBank Accession no.
Lla16CA[a]	GACTTCTTCCATATCTGGTG	$(CA)_{18}$*	55→47	1	32	222	0.00	0.00	AF185098
	CCCACATAACCCTTAAAGTG			3	88	256–262	0.74	0.59	
				7	29	236–264	0.85	0.78	
Lla20CA[a]	ACGTCCCCACATAACAAGAC	$(CA)_{23}$	62→55	1	13	128	0.00	0.00	AF185099
	TGGGGGAACTCAGTAACACC			5	5	116–136	0.84	0.80	
Lla25CA[a]	TGGACAAGTCACTTAACCCCAATTAC	$(CA)_{22}$*	62→55	3	60	132–136	0.67	0.68	AF185100
	CTCACTACCCCCACATTCAGTTACTT			2	5	134–148	0.36	0.40	
				1	5	132	0.00	0.00	
Lla51CA[a]	GCACTCTAATACATAAGCC	$(CA)_{14}$*	62→55	2	60	277–281	0.20	0.16	AF185102
	AGCAGGAGCACACATTGAC			8	88	267–281	0.74	0.66	
Lla54CA[a]	AAATATACTCATACTCAACC	$(CA)_{21}$*	62→55	3	60	129–162	0.65	0.75	AF185103
	ATCTTGCTACTACTAATGAC			9	89	131–175	0.82	0.82	
				8	37	123–160	0.74	0.31	
Lla55A[a]	ACCTCTCCTGGTTGC	$(A)_{14}$	62→55	2	60	79–80	0.43	0.51	AF185104
	TCAAGCCCTGGAAGC			3	89	80–82	0.62	0.69	
				2	24	78–79	0.22	0.25	
Lla67CA[a]	AGGTAGGCAAATAAGTAAAG	$(CA)_{27}$*	55→47	3	60	151–165	0.50	0.65	AF185105
	TGCTCAGGAGATGCTTAGCC			9	90	141–157	0.83	0.86	
				7	36	nd	0.81	0.78	
Lla68CA[a]	TCCTTTCCCACTCCACCAGC	$(CA)_{20}$*	55→47	3	60	96–102	0.67	0.62	AF185106
	GAAGCATTACCATCGGAAAACC			10	90	90–118	0.80	0.83	
				6	27	92–98	0.59	0.64	
Lla71CA[a]	AATGAGAAGGTATCTCCAGG	$(CA)_{23}$	55→47	2	60	183–191	0.24	0.25	AF185107
	GGTATAGGCTATGTCATAGG			10	90	179–199	0.80	0.83	
				6	27	177–191	0.78	0.84	
Lkr102[a,c]	CTTCAAAGGTCTTTTCTGTC	$(GA)_{28}$*	55→47	2	60	300–306	0.39	0.46	AF185109
	CTTAAAATCTAGCAGAGTG			2	3	322–324	0.53	0.67	AF185110
				4	36	nd	0.68	0.75	
Lkr105[a]	CAAGGCTCCGACGGTTGG	$(CA)_{19}$*	62→55	2	23	228–242	0.49	0.43	AF185111
	CCGGGGCTCCGTGTGTG			3	4	220–248	0.71	1.00	
Lkr107[a,c]	ATGGCATAACGTTTGTGTATGC	$(CA)_{23}$*	62→55	1	32	274	0.00	0.00	AF185113
	CTGACTCCAGAATCAGTGAACC			10	89	238–282	0.76	0.70	AF185114
				4	5	260–278	0.66	0.60	
Lkr109[a]	TCAACAAGACGGAAGGCTG	$(CA)_{21}$	62→55	1	32	190	0.00	0.00	AF185108
	AAGCCTTTTGGGACACTGTG			5	88	158–178	0.63	0.58	
				10	26	180–204	0.79	0.62	
Ll2[b]	TGGTGCTCCTTTGATATCCC	$(CA)_{19}$	62→55	2	60	120–132	0.53	0.59	AF191296
	CCTGGGTCAGTGGCTTTG			8	83	122–148	0.76	0.79	
				6	37	122–132	0.73	0.81	

[a]developed as described in Taylor et al. (1994).
[b]developed as described in Alpers (1998).
[c]loci for which incomplete sequence data were obtained, requiring separate accession numbers for flanking sequences.

polyacrylamide gel electrophoresis and visualized by autoradiography following Taylor et al. (1994).

Results and discussion

The utility of the 12 new primer sets was tested by genotyping individuals representing the three species of wombats (Table 1). These primers cross-hybridise extensively and reveal large amounts of genetic variation in common wombats (up to 17 alleles and 90% expected heterozygosity per locus; Table 1), consistent with fossil and mitochondrial DNA sequence evidence for a relatively recent (within the last seven million years) radiation of the wombat family (Stephenson 1967; Taylor et al. 1994). All 12 new loci were polymorphic in SHN (2–9 alleles), and all but Lk13 and Lk34 in NHN wombats (2–5 alleles) (Table 1). All loci were examined for sex linkage with a minimum of three males and three females per locus: no sex linked loci were indicated. Locus 3AT shows homozygous excess in many wombat populations (Table 1 and unpublished), but no other loci show consistent deviations from Mendelian expectations (exact probability tests implemented in GENEPOP Version 3.1; Raymond and Rousset 1995).

Incorporation of the new markers into a comparative analysis of genetic variation in the hairy-nosed wombats reinforces the earlier finding of significantly lower levels in NHN than in SHN wombats (Taylor et al. 1994). The current panel of 28 microsatellite markers reveals an average of only 2.1 alleles per locus in the NHN wombat, compared to the significantly higher number of 5.9 in SHN wombat populations (Wilcoxon signed rank test; Z = –4.3, p = 0.0001). Expected heterozygosity at the 28 loci is also significantly lower in the NHN (H_E = 0.32) than the SHN (H_E = 0.71) wombat (Z = –4.2, p = 0.0001).

The 12 new markers have greatly enhanced our ability to identify individual NHN wombats from remotely collected hairs: using all variable loci, the commonest multiple locus genotype is expected to occur in one in every 5.3×10^7 individuals, while the comparable number for the old 16 loci alone is 1.7×10^3 (calculated as per Taberlet and Luikart (1999) using program Prob-ID3 (G. Luikart, unpublished).). The microsatellites presented here have resolved, and will continue to resolve a large range of questions concerning the genetic variation and molecular ecology of wombats, and are an especially valuable tool for management and conservation programs of the highly endangered NHN wombat.

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