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PRIMER NOTE

Isolation and characterization of tetramicrosatellite DNA markers in the Eurasian otter (*Lutra lutra*)

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Abstract

Eight new tetramicrosatellite loci for Eurasian otter (*Lutra lutra*) were designed. Polymorphism of these eight loci in 29 otter individuals was tested. The results indicated that the allele numbers of each of loci ranged from three to five and the observed heterozygosity from 0.483 to 0.828. These new loci can be useful for population genetic research on otters and help improve the resolution of individual identification using noninvasive method.

Keywords: enrichment, Eurasian otter, *Lutra lutra*, microsatellite

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Microsatellite DNA is a useful genetic tool that allows noninvasive study of elusive species, such as the Eurasian otters (*Lutra lutra*) (e.g. Dallas *et al*. 1999). In Asia, Hung *et al*. (2004) used seven tetranucleotide microsatellite markers (Dallas & Piertney 1998) to estimate the population density of Eurasian otters in Kinmen, an island off the coast of Fujian Province in China. To further facilitate the study of conservation genetics of this threatened species across its range, more polymorphic microsatellite loci are needed.

In this study, we basically followed the protocol developed by Hsu *et al*. (2003). We developed new otter-specific microsatellite loci from a partial genomic library enriched for tetranucleotide $(GAAA)_n$ repeats. We extracted genomic DNA (Sambrook *et al*. 1989) from the tissue sample of an otter from Kinmen. Ten milligrams of gross DNA was digested with restriction endonuclease *Hae*III, *Rsa*I, and *Alu*I. These fragments were ligated with SNX linker (Hamilton *et al*. 1999), followed by a polymerase chain reaction (PCR) using primers complementary to linker sequence. The PCR was set up in a 50 μL, containing 0.2 μM of each primer, 10 mM of Tris-HCl, 50 mM of KCl, 0.4 U *Taq* DNA polymearse (Amersham Biosciences), 0.5 mM of dNTP and 1.5 mM of $MgCl_2$. PCR condition was set at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 70 °C for 2 min, and a final step at 72 °C for 10 min. PCR was performed in an iCycler thermal cycler (Bio-Rad). A solid phase hybridization with 3′-biotin-labelled $(GAAA)_{10}$ probe was used to preferentially select DNA fragments which contained microsatellite motif. These biotin-labelled fragments were captured by the streptavidin-coated Dynalbead M280 (Dynal) according to the manufacturer's instructions. The captured DNA fragments were eluted by heating at 95 °C for 10 min in 100 μL of 1 X PCR buffer. Fifteen microlitres of eluted DNA were PCR-amplified by the same condition previously described. These PCR-amplified DNA fragments were ligated into pGEM-T Easy vector (Promega) and transformed to *Escherichia coli* DH5α. A total of 1200 clones were lifted to Hybond-N+ membranes (Amersham Pharmacia Biotech) and hybridized with [γ^{32}P] ATP end-labelled $(GAAA)_8$ oliginucleotides, then 142 hybridized clones were selected for sequencing using DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE (Amersham Bioscience) on a MegaBACE 1000 autosequencer (Amersham Bioscience). Sequences were proofread using SEQUENCER version 4.0.5 (Gene Codes Corporation). We found 98 clones with microsatellite motif, of which 16 loci containing more than 10 units of GAAA motif were randomly chosen to design the PCR primers. PCR products with expected length were amplified successfully in eight primer pairs. The forward primers of these loci were labelled with HEX, FAM or TAMRA fluorescent dyes. DNA extracted form tissue samples of four otters and from faecal samples of 25 otters with unknown relationship were used to characterize these eight loci. PCRs were set up in 10-μL reaction volumes containing about 30 ng genomic DNA, 0.2 μM of each

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Table 1 Characterization details of the eight microsatellite loci in the Eurasian otter (*Lutra lutra*)

Locus	Repeat motif	Primer sequences	Fluorescent dye labelled	H_O	H_E	Size of cloned allele	No. of allele	$MgCl_2$ (mM)	Genebank Accession no.
04OT02	$(GAAA)_{16}$	F: 5′-AGGTCCTGAACCAAGACATTTAAT R: 5′-TCACAGTAACCCAGATGATTTTG	HEX	0.655	0.537	145–193	3	2.5	AY786983
04OT04	$(GAAA)_{16}$	F: 5′-AACTCTGACTCTGGGTGGAGGTGTT R: 5′-GCCTGGGAGGCAGCATGATTAGT	FAM	0.586	0.753	178–210	5	1.5	AY786984
04OT05	$(GAAA)_{14}$	F: 5′-TGGAGAAAAGCATTATCTTACTG R: 5′-ATTCAGGGAGGCAGGAGAGC	HEX	0.828	0.718	165–191	4	2.5	AY786985
04OT07	$(GAAA)_{12}$ $GAAGG\,(GAAA)_9$	F: 5′-CACAGTGAAGGGTGACCAGATCACC R: 5′-CCACCTCATCCCAAATGATCCTCT	TAMRA	0.621	0.621	182–200	4	2.5	AY786986
04OT14	$(GAAA)_{13}$	F: 5′-GGTCCAAGTCCAAGCCTGCCT R: 5′-TTCATATTCTTCAGGTGAATCCCAT	TAMRA	0.621	0.648	123–139	5	1.5	AY786987
04OT17	$(GAAA)_{13}$	F: 5′-ATCAGGTATGAGGATACATTTACCT R: 5′-TGCAACCTACTTCTATATGAATTT	HEX	0.483	0.593	153–169	5	1.5	AY786988
04OT19	$(GAAA)_{12}$	F: 5′-ATAGGTCTCTCAGCACGGTGTCT R: 5′-TTAAATCCACATCTGTGACTCTGCA	FAM	0.379	0.627	197–219	4	2.5	AY786989
04OT22	$(GAAA)_{16}$	F: 5′-CTATCTGACCATTGTCCCATGA R: 5′-ACCCATGTAGGGTGCCATGCT	TAMRA	0.586	0.678	149–157	3	2.5	AY786990
Overall				0.595	0.647				

Table 2 *P* value for the test of gametic disequilibrium among eight pairs of microsatellite loci

	Microsatellite loci							
	04OT02	*04OT04*	*04OT05*	*04OT07*	*04OT14*	*04OT17*	*04OT19*	*04OT22*
04OT02	—	0.03*	0.56	0.33	0.02*	0.38	0.12	0.10
04OT04		—	0.11	0.003*	0.11	0.08	0.34	0.12
04OT05			—	0.50	0.29	0.02*	0.10	0.01*
04OT07				—	0.30	0.05	0.25	0.11
04OT14					—	0.01*	0.12	0.09
04OT17						—	0.35	0.03*
04OT19							—	0.42
04OT22								—

*significantly departed from gametic equilibrium.

primer, 1X PCR buffer (0.5 mM dNTP, 10 mM of Tris-HCl, 50 mM of KCl, pH = 9.0), 0.25 U *Taq* DNA polymerase (Amersham Biosciences) and 1.5–2.5 mM of $MgCl_2$ (Table 1). We use a touchdown PCR protocol to increase the specificity and the amount of PCR product. PCR profile was at 95 °C for 2 min, followed by 20 cycles at 95 °C for 30 s, 60 °C–0.5 °C per cycle for 20 s, and 20 cycles at 95 °C for 30 s, 50 °C for 30 s, and a final extension at 72 °C for 10 min. The PCR products were electrophoresed in MegaBACE 1000 autosequencer (Amersham Biosciences). Sizes of alleles were scored using GENETIC PROFILER version 2.0 (Amersham Biosciences).

Data were analysed using CERVUS version 2.0 (Marshall *et al*. 1998) to calculate the observed and expected heterozygosities, and for tests of Hardy–Weinberg equilibrium (HWE). The test of gametic disequilibrium was calculated using GENEPOP version 3.3 (Raymond & Rousset 1995). Overall, the mean number of alleles per locus was 4.125 (ranged from 3 to 5) and the mean observed heterozygosity was 0.595 (Table 1). No significant deviation from HWE was detected in each locus. Furthermore, seven of 28 possible pairwise comparisons between loci showed significant ($P < 0.05$) gametic disequilibrium (Table 2). This might be partially caused by random genetic drift in small island population (Ohta 1984). In conclusion, we believe that these new microsatellite loci should provide a new set of molecular makers to studying the ecology of this elusive carnivore.

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