Conserv Genet (2009) 10:1845–1847
DOI 10.1007/s10592-009-9830-3

TECHNICAL NOTE

Ten novel tetranucleotide microsatellite DNA markers from Asiatic black bear, *Ursus thibetanus*

Chih-Chin Shih · Chuan-Chin Huang · Shou-Hsien Li · Mei-Hsiu Hwang · Ling-Ling Lee

Received: 14 January 2009 / Accepted: 19 January 2009 / Published online: 13 February 2009

Abstract Ten polymorphic microsatellite markers were developed for the endangered Formosan black bear (*Ursus thibetanus formosanus*) from a partial genomic library enriched for GAAA repeat. Polymorphism of these loci was evaluated in 27 Formosan black bear specimens of unknown relationship. The number of alleles per locus ranged from 5 to 15 and the observed heterozygosity of each locus ranged from 0.556 to 0.889. These loci should provide useful molecular tools to study conservation genetics of the Formosan black bear and other Asiatic black bears.

Keywords Tetranucleotide microsatellite · Tailed primers · *Ursus thibetanus* · Formosan black bear

The Formosan black bear (*Ursus thibetanus formosanus*) is an endemic subspecies of Asiatic black bear inhabiting Taiwan (Wozencraft 2005). Similar to all other Asiatic black bears, degradation and fragmentation of habitat as well as poaching have caused a decrease in population and distribution of the Formosan black bear (Wang 1999; Hwang and Wang 2006). To formulate proper conservation strategies, it is important to understand the genetic diversity and genetic structure within and among populations of this subspecies.

In this study, we reported ten novel easy-scored polymorphic tetranucleotide repeat (GAAA) microsatellite loci from the Formosan black bear. We followed the protocol developed by Hsu et al. (2003) to enrich microsatellite-contained fragment in a partial genomic library. The library was constructed from genomic DNA which extracted from tissue sample of a Formosan black bear individual using the proteinase *K*-chloroform method (Sambrook et al. 1989). Microsatellite-enriched PCR (polymerase chain reaction) library was ligated into pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* DH5α. A total of 880 clones were lifted to Hybond-N + membranes (Amersham Pharmacia Biotech) and hybridized with [γ^{32}P] ATP end-labelled $(GAAA)_{10}$ oligonucleotides, then 56 hybridized clones were sequenced using DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE (Amersham Bioscience) on a MegaBACE 1000 autosequencer (Amersham Bioscience). Sequences were proofread using software SEQUENCER 4.2 (Gene Codes). We found 47 clones with microsatellite motif, of which 33 loci containing more than 10 units of GAAA motif were chosen to design the PCR primers.

All forward primers were 5′-tailed with an M13-tail (5′-GGAAACAGCTATGACCAT-3′) or a CAG-tag (5′-CAG TCGGGCGTCATCA-3′) (Schuelke 2000; Boutin-Ganache et al. 2001). DNA extracted from tissue samples of 17 Formosan black bears and from faecal samples of ten Formosan black bears with unknown relationship were used to characterize these 33 loci. PCRs were set up in

C.-C. Shih · C.-C. Huang · L.-L. Lee (✉)
Institute of Ecology and Evolutionary Biology, National Taiwan University, Taipei 106, Taiwan, ROC
e-mail: leell@ntu.edu.tw

C.-C. Shih
Conservation and Research Center, Taipei Zoo, Taipei 116, Taiwan, ROC

S.-H. Li
Department of Life Science, National Taiwan Normal University, Taipei 116, Taiwan, ROC

M.-H. Hwang
Institute of Wildlife Conservation, National Pingtung University of Science and Technology, Pingtung 912, Taiwan, ROC

Table 1 Characterization of the ten microsatellite loci of Formosan black bear (*Ursus thibetanus formosanus*)

Locus	Core motif	Primer sequence (5′–3′)	*N*	T_a (°C)	$MgCl_2$ (mM)	No. of alleles	Allele size (bp)[a]	H_O	H_E	*P*-values (HWE)	GenBank Accession no
UT1	$(GAAA)_9GGGA(GAAA)_{10}$	F: CAG-AGCAACTCTTCTCAGATGTTCACAAA	27	64	2.5	5	176–192	0.556	0.584	0.461	FJ640076
		R: CCCAGGTCAGCACTTGGCATAC									
UT3	$(GAAA)_{18}$	F: CAG-AAGACATACAGAAGCCAAGACTAG	25	56	2.5	7	256–282	0.640	0.776	0.186	FJ640077
		R: TACTCAATTACAAAGGATAACTATA									
UT4	$(GAAA)_6GAGA(GAAA)_{11}$	F: M13-GAGTTATTGGCACTAAAATCTAATG	27	56	2.5	7	157–182	0.704	0.814	0.107	FJ640078
		R: CTGCAAATCCCTGCTCAACTTTC									
UT23	$(GAAA)_{10}GA(GAAA)_{22}$	F: M13-GCTGGATACATCATCCTGGCTC	27	62	2.5	12	349–382	0.778	0.881	0.040*	FJ640079
		R: GGAATCAAGTTCGGCATCGGG									
UT25	$(GAAA)_2(GA)_{12}$ $(GAAA)_{16}$	F: M13-GCTCAGGGCGTGATCCCAGAG	27	62	2.5	6	314–333	0.704	0.720	0.011*	FJ640080
		R: GGCTCCCCTGCACTAGAGATTTAAC									
UT29	$(GAAA)_2AA(GAAA)_{17}$	F: CAG-GACATTGCCTTTTACAGAGCAG	27	64	2.5	8	204–236	0.889	0.788	0.058	FJ640081
		R: GGGCAGATCTCAACCACCATAAGC									
UT31	$(GAAA)_{17}GG(GAAA)_3$	F: CAG-AATAAACTGATGCAGCCATACTAG	26	64	2.5	15	315–369	0.846	0.909	0.560	FJ640082
		R: CTGCCACTGAATCTTCTGATCTTAG									
UT35	$(GAAA)_{15}$	F: CAG-ACTCCCTAGTAAGTAGAAAGCACAC	27	64	2.5	7	218–247	0.630	0.825	0.022*	FJ640083
		R: CCCACAGGATGGGCTCAAGAA									
UT36	$(GAAA)_{16}$	F: CAG-AGACTCAGGAAGTCTGGAGTGGGA	27	62	2.5	7	276–309	0.630	0.727	0.154	FJ640084
		R: CTTTCGGCTCAGGGATCGAGC									
UT38	$(GAAA)_{24}$	F: M13-ATTATTGATGAGCAGGGACAG	27	56	2.5	10	196–232	0.778	0.839	0.039*	FJ640085
		R: CTAAAGCAACAACATGTGAATG									

N, number of individuals genotyped; T_a, PCR annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, Hardy–Weinberg equilibrium; *, $P < 0.05$

[a] Allele size includes the additional size of tails added to forward primer

10 μl reaction volumes containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.05 μM of tailed forward primer, 0.12 μM of reverse primer, 0.18 μM of fluorescent-labelled M13 or CAG-tag primer that were labeled with HEX, FAM or TAMRA fluorescent dyes, 0.2 U *Taq* DNA polymerase (Biotech), and around 30 ng genomic DNA. The PCR condition was 95°C for 4 min, then 40 cycles at 95°C for 30 s, 30 s at the optimal annealing temperature of each primer pair (Table 1) and 72°C for 20 s, followed by a final extension at 72°C for 7 min. The PCR products were electrophoresed in a MegaBACE 1000 autosequencer (Amersham Biosciences). Sizes of alleles were scored with software GENETIC PROFILER 2.0 (Amersham Biosciences).

Twenty-three loci that appeared difficult to score or monomorphic were excluded from subsequent analyses. Genotype frequencies of ten loci were analysed using CERVUS 2.0 (Marshall et al. 1998) to calculate the observed and expected heterozygosities. Tests for departure from Hardy–Weinberg equilibrium and linkage equilibrium between pairs of loci were performed using GENEPOP 3.4 (Raymond and Rousset 1995). Polymorphism assessment at these ten microsatellite loci is summarized in Table 1. The number of alleles per locus ranged from 5 to 15 and the observed heterozygosities ranged from 0.556 to 0.889. There was no evidence for large allele dropout and null alleles detecting by MICRO-CHECKER (van Oosterhout et al. 2004) in all ten loci. Four loci (UT23, UT25, UT35 and UT38) represented significant differences between the observed and expected heterozygosities ($P < 0.05$), which are probably due to genetic drift driven by Formosan black bear's small population size. No significant deviation from linkage equilibrium was detected after Bonferroni correction.

With microsatellites that isolated from Japanese black bear (*U. thibetanus japonicus*) (Kitahara et al. 2000) and other Ursids (Paetkau et al. 1995; Taberlet et al. 1997), the tetranucleotide microsatellites we isolated should provide an ideal genetic tool kit to study the population genetics of the endangered Formosan black bear and other Asiatic black bears that are also under threat.

Acknowledgments We acknowledge Mr. Chieh-Chung Yang in Taiwan Endemic Species Research Institute for providing bear samples. We also thank Ms. Rong-Chien Lin in the Genetic Diversity Laboratory of National Taiwan Normal University for her help with the lab work. This study was funded by the Taiwan Forest Bureau, Council of Agriculture, ROC (95AS-11.1.3-FB-e1) to L-L Lee and supported by Taipei Zoo to C–C Shih.

References

Boutin-Ganache I, Raposo M, Raymond M, Deschepper CF (2001) M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. Biotechniques 31:24–28

Hsu YC, Severinghaus LL, Lin YS, Li SH (2003) Isolation and characterization of microsatellite DNA markers from the Lanyu scops owl (*Otus elegans botelensis*). Mol Ecol Notes 3:595–597. doi:10.1046/j.1471-8286.2003.00523.x

Hwang MH, Wang Y (2006) The status and management of Asiatic black bears in Taiwan. In: Japan Bear Network (ed) Understanding Asian bears to secure their future. Japan Bear Network, Japan, pp 107–110

Kitahara E, Isagi Y, Ishibashi Y, Saitoh T (2000) Polymorphic microsatellite DNA markers in the Asiatic black bear *Ursus thibetanus*. Mol Ecol 9:1661–1662. doi:10.1046/j.1365-294x.2000.01030.x

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol 7:639–655. doi:10.1046/j.1365-294x.1998.00374.x

Paetkau D, Calvert W, Stirling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian polar bears. Mol Ecol 4:347–354. doi:10.1111/j.1365-294X.1995.tb00227.x

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Hered 86:248–249

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18(2):233–234. doi:10.1038/72708

Taberlet P, Camarra JJ, Griffin S et al (1997) Noninvasive genetic tracking of the endangered Pyrenean brown bear population. Mol Ecol 6:869–876. doi:10.1111/j.1365-294X.1997.tb00141.x

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538. doi:10.1111/j.1471-8286.2004.00684.x

Wang Y (1999) Status and management of the Formosan black bear in Taiwan. In: Servheen C, Herrero C, Peyton B (eds) Bears: status survey and conservation action plan. IUCN, Gland, Switzerland, pp 213–215

Wozencraft WC (2005) Order Carnivora. In: Wilson DE, Reeder DM (eds) Mammal species of the world: a taxonomic and geographic reference, 3rd edn. John Hopkins University Press, Baltimore, pp 532–628