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TECHNICAL NOTE

Isolation and characterization of eight microsatellite loci for the vulnerable Hainan Eld's deer (*Cervus eldi hainanus*) in China

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Abstract We report on the isolation and characterization of eight microsatellite markers in the Hainan Eld's deer (*Cervus eldi hainanus*) from genomic DNA-enriched libraries. Thirty-three microsatellites were screened from the libraries, and 8 of the screened microsatellites were polymorphic. The number of observed alleles for each locus in 47 individuals ranged from 2 to 9, and the expected and observed heterozygosity was 0.141–0.792 and 0.128–0.957, respectively. Three loci (CEH-2, CEH-6 and CEH-8) of eight deviated from Hardy-Weinberg expectation and no significant linkage association was found among all these loci. These microsatellite markers provide useful tool for population genetic studies of the Eld's deer.

Keywords *Cervus eldi hainanus* · Cervidae · Population bottleneck · Microsatellite DNA · Polymorphic · Hainan Eld's deer

The Hainan Eld's deer (*Cervus eldi hainanus*) is a subspecies of the Eld's deer (*Cervus eldi*) and only occurs in Hainan Island of China (Zeng et al. 2005). In the 1970s, the Hainan Eld's deer was once reduced to 26 individuals due to poaching and the degradation of habitats (Wang 1979), thus has experienced a severe population bottleneck. At present, the species is listed in Appendix I of the Convention on International Trade in Endangered Species (CITES) and is considered endangered by the World Conservation Union (IUCN). The overall population size has now increased to more than 1000 individuals (Yuan et al. 2001), largely due to restoration and captive-breeding programs implemented over the last 30 years. However, earlier conservation efforts have largely focused on increasing population size, and not enough attention was paid to breeding management. There is practically no information on genetic relatedness of the 26 founder individuals. This will undoubtedly affect further effective management of the deer population and place them under potential risk of serious inbreeding. Therefore, genetic analysis of Hainan Eld's deer populations will play an increasing role in their conservation, and these microsatellite loci presented in this work and those screened from published bovine microsatellites in previous study (Zhang et al. 2005) will be an important tool in these genetic analyses (Zhang and Hewitt 2003).

A genomic library was produced according to an enrichment method similar to that described by Hammond et al. (1998). The blood samples of *C. e. hainanus* were collected and stored in DNA preservation buffer [0.1 M Tris-HCl (pH 8.0):0.1 M EDTA (pH 8.3):1% SDS, volume ratio of blood and buffer 1:1] and preserved at –20°C. Total DNA was extracted from a blood sample using a standard phenol–chloroform method (Sambrook et al. 1989) and digested with MboI restriction enzyme (Promega). Then a 300–1000 bp DNA fragments was isolated from total genomic DNA and ligated to MboI linkers made by annealing equimolar amounts of MboI1 (5′-GATCGCAGAATTCGCACGAGTACTAC-3′) and MboI2 (5′-CGTCTTAAGCGTGCTCATGATGC-3′). The ligated fragments were enriched and made double-stranded by polymerase chain reaction (PCR) (5 min 72°C, then 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 67°C and 2 min extension at 72°C) using MboI1 as the PCR primer. The whole PCR products were

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Table 1 Characteristics of eight polymorphic microsatellite loci in Hainan Eld's deer (*Cervus eldi hainanus*)

Locus	Primer sequence (5′-3′) (F, forward; R, reverse)	Repeat motif	Labelling dye	T_a (°C)	*A*	Size range (bp)	H_O	H_E	GenBank Accession no.
CEH-1	F:TAGGTGGACATGCTTGCC[a] R:GAGTACTACGATCGAACCTGC	$(GT)_5GC(GT)_4AT(GT)_3$	HEX	55	2	132–146	0.234	0.209	EU016518
CEH-2	F:TTCCCTTCTGGCGGTTGA[a] R:AAGAGGATATGCGCGTGTGTA	$(GT)_{25}$	FAM	55	9	216–242	0.830	0.792	EU016519
CEH-3	F:CTGTGAGGTGCTTGATTGAA[a] R:TACTGTAGGTGGACATGCTTG	$(AC)_3AT(AC)_9$	FAM	52	3	91–97	0.191	0.197	EU016520
CEH-4	F:ACGTGTGATGAGCGGTGT[a] R:CACTATTACCTGAACCCTTCG	$(GT)_6$	FAM	54	2	143–147	0.277	0.259	EU016521
CEH-5	F:GAGCTGGTCCTCTGCGTGAT[a] R:CAGGCAGATTCTTTACCGTTG	$(AC)_3AA(AC)_{11}$	FAM	55	5	190–198	0.660	0.682	EU016522
CEH-6	F:CTACGATCTGTTTGTGAGCAT[a] R:TATAAGCACGTGCACC	$(GT)_5GCA(GT)_2$	FAM	52	2	93–95	0.851	0.506	EU016523
CEH-7	F:AGTACTACGATCGAACCTGC[a] R:ACTGTAGGTGGACATGCTTGC	$(AC)_{12}$	FAM	54	2	128–132	0.128	0.141	EU016524
CEH-8	F:TGGTCACAGGTGTGGACG[a] R:CAGAGGAGCCTGGTGGGTTAT	$(TG)_{13}$	FAM	57	2	128–206	0.957	0.504	EU016525

[a] Labeled primer; T_a, annealing temperature; *A*, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity

hybridizated to biotin-labelled probe [biotin-ATAGAATAT $(CA)_{20}$] (Kandpal et al. 1994) and subsequently isolated with the streptavidin-coated magnetic beads (Promega). The enriched microsatellite fragments were ligated into pGEM-T easy vector plasmid (Promega) and transformed into competent *Escherichia coli* DH5α cells, then plated up on Luria Bertani (LB) agar containing ampicillin, isopropyl-beta-D-thiogalactopyranoside (IPTG) and X-gal. Colonies were screened following the polymerase chain reaction based screening method of Lunt et al. (1999). Of the 392 colonies screened, 117 were positive and were sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI 377 automated DNA sequencer. 90 colonies had repeat sequences. Thirty-three primer pairs were designed using the software program oligo ™ 6.0 (Molecular Biology Insights).

PCR amplifications were performed in 10 μl reaction mixture, consisting of approximately 30 ng of template DNA, 0.2 mM of each dNTP, 1 × PCR buffer (TaKaRa Biomedical), 1.5 mM Mg^{2+}, 0.15 U of *Taq* DNA polymerase (TaKaRa Biomedical) and 0.15 μM of each primer. The reaction mixture was amplified using an MBS Satellite thermal cycler (Thermo Electron Corp., USA), using an initial denaturing step of 5 min at 94°C and 35 cycles of 94°C for 30 s, 50–58°C for 30 s, and 72°C for 30 s, followed by 7 min at 72°C. For genotyping, fluorescently labelled PCR products (Table 1) were electrophoresed along with GeneScan ROX 400 internal size standard on an ABI PRISM 3700 Genetic Analyser (Applied Biosystems). Allele sizes were assigned against the internal size standard and individuals were genotyped using Genescan version 2.0 (Applied Biosystems).

We observed that eight microsatellite loci showed polymorphic in 47 individuals. Population genetic parameters were estimated with Genepop version 3.4 (Raymond and Rousset 1995; available: http://wbiomed.cutin.edu.au/genepop/), all the effects of multiple tests were adjusted by Bonferroni method (Rice 1989). The number of observed alleles per locus ranged from 2 to 9. Observed heterozygosity (H_O) ranged from 0.128 to 0.957 and expected heterozygosity (H_E) ranged from 0.141 to 0.792. After sequential Bonferroni corrections, three loci (CEH-2, CEH-6 and CEH-8) of eight showed deviation from Hardy-Weinberg predictions (exact probability test). The departure from HWE is probably caused by random sampling from different populations (Datian, Bangxi, Ganshiling, Fengmu, Jinniuling and Wenchang populations) and we have no information about the relationship among individuals. No significant linkage association was found among all these loci. The result showed that the eight microsatellite markers will provide a powerful tool for the further investigation of the genetic diversity of Hainan Eld's deer. Furthermore, these microsatellite markers can be used in genetic studies of .other 3 subspecies of Eld's deer, *C. e. eldi*, *C. e. siamensis* and *C. e. thamin* to develop conservation measures for this species.

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References

Hammond RL, Saccheri IJ, Ciofi C, Coote T, Funk SM, McMillan O, Bayes MK, Taylor E, Bruford MW (1998) Isolation of microsatellite markers in animals. In: Karp A, Isaac PG, Ingram DS (eds) Molecular tools for screening biodiversity, Chapman & Hall, London, pp 279–287

Kandpal RP, Kandpal G, Weissman SM (1994) Construction of libraries enriched for sequence repeats and jumping clones, and hybridisation selection for region-specific markers. Proc Natl Acad Sci U S A 91:88–92

Lunt DH, Hutchinson WF, Carvalho GR (1999) An efficient method for PCR-based isolation of microsatellite arrays (PIMA). Mol Ecol 8:891–894

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

Rice WR (1989) Analysing tables of statistical tests. Evolution 43:223–225

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

Wang Q (1979) The precious animals-Hainan Eld's deer. Wildlife 1:29–31 (In Chinese)

Yuan XC, Xue C, Yun DX, Wang CX, Li SY (2001) Insite and Offsite Conservation of Hainan Eld's Deer. Chin Wildlife 21: 6–8 (In Chinese)

Zeng ZG, Song YL, Li JS, Teng LW, Zhang Q, Guo F (2005) Distribution, status and conservation of Hainan Eld's deer (*Cervus eldi hainanus*) in China. Folia Zool 54:249–257

Zhang DX, Hewitt GM (2003) Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. Mol Ecol 12:563–584

Zhang Q, Ji YJ, Zeng ZG, Song YL, Zhang DX (2005) Polymorphic microsatellite DNA markers for the vulnerable Hainan Eld's deer (*Cervus eldi hainanus*) in China. Acta Zool Sinica 51: 530–534