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TECHNICAL NOTE

Characterization of novel microsatellite loci in the great leaf-nosed bat, *Hipposideros armiger* and cross-amplification in other related species

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Abstract Eight microsatellite loci were isolated from an enriched genomic library of the great leaf-nosed bat, *Hipposideros armiger*. The polymorphism of these loci was tested on a population of 48 individuals from Anhui Province, China. All loci revealed the polymorphism ranging from three to 12 alleles. The observed heterozygosity values were from 0.213 to 0.875 and expected heterozygosity from 0.232 to 0.820. No significant linkage disequilibrium was detected. Two loci significantly deviated from Hardy–Weinberg equilibrium after Bonferroni correction. In addition, successful cross-amplification also suggested that these microsatellite markers will facilitate research on the population genetics and gene flow of *H. armiger* and other related species.

Keywords Microsatellite loci · *Hipposideros armiger* · Gene flow · Polymorphism · Cross-species amplification

The great leaf-nosed bat *Hipposideros armiger* (Hipposideridae, Chiroptera) roosts in caves and feeds in open spaces in woodlands, gardens, and around trees (Bates and Harrison 1997). It has a wide Asian geographical distribution that includes India, Nepal, Myanmar, Vietnam, Laos, Cambodia, Thailand, S and SE of China and Malay Peninsula (Simmons 2005). Currently, the bat populations are in decline because of habitat destruction. Studies on this species have been carried out on the echolocation calls (Bogdanowicz et al. 1999; Zhao et al. 2003), karyology analysis (Gu 2002; Wu et al. 2004) and some mitochondrial DNA sequences (Li et al. 2006*)*. However, little is known about the molecular biology and genetic diversity of this species. In this paper, we reported eight polymorphic microsatellite loci isolated from *H. armiger* using the microsatellite enriched method. These molecular markers are needed for ongoing population research of *H. armiger*.

The genomic DNA was extracted from the wing membrane of *H. armiger* collected from Chizhou (30°20.497′ N, 117°50.104′ E) in Anhui Province, South China with a standard phenol-chloroform extraction method (Sambrook and Russell 2002). The enriched library protocol for developing CA repeats was similar to that previously described by Karp et al. (1998) and Hua et al. (2006). Genomic DNA was digested with restriction enzyme *Mbo*I (Takara) overnight and the fragments (0.4–0.8kb) were selected and purified with a DNA purification kit (Takara). Then the DNA fragments were ligated to adapters (SAULA/SAULB) with T_4 DNA ligase (Takara) and the ligation products were amplified with the adapter sequence SAULA as primers. After being recovered and denatured, the PCR products were hybridized with a biotin-labelled dinucleotide repeat $(CA)_{15}$ probe in sodium phosphate buffer (0.5 M sodium phosphate, 0.5% SDS, PH7.4) at 50°C overnight. The hybridization products were mixed with VECTREX Avidin D (Vector Laboratories) and incubated at 37°C for 30 min. The mixture was washed for four times by TBS (150 mM NaCl, 100 mM Tris, PH7.5) at different temperatures to remove the unbound sequences (50, 50, 65, 65°C). The bound single-strand fragments were eluted with 80 μl ddH_2O at 65°C for 30 min. After recovered into double-strand by PCR with primer

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Table 1 Isolation and characterization of polymorphic microsatellite loci from the great leaf-nosed bat (*Hipposideros armiger*)

Locus	Primer sequence (5′–3′)	Repeat motif	*Ta* (°C)	N_A	Size range (bp)	H_E	H_O	GenBank accession no
CHANG242	F:TGGCTGCTGGGAAGTGCTG R:CGAGTGCACCGATGGACGT	$(CA)_5N_{10}(CA)_4TG(CA)_4$	67	4	218–228	0.232	0.213	EU165327
BAM09	F:CGCCTCGACAACTTGTTCC R:TCCGAGTGAATGCCAAGTGT	$(AC)_{14}$	56	9	252–274	0.510	0.419	EU165328
TT18*	F:GCACGCACATAAACACCCTC R:TTGCCAGCGTGATAAAGACC	$(AC)_{16}$	59	10	245–271	0.752	0.364	EU165329
CHANG260*	F:CCTCACTACCTCCTCCCA R:ATACACCAGGCAGTTTCTTAC	$(AC)_{19}$	56	12	134–158	0.820	0.585	EU165330
TE2	F:GCAAGTGGCTATGCTTCAGG R:GTGCTACAGTCCCCATCTCC	$(GT)_{14}$	55	5	341–351	0.710	0.875	EU165331
NEAM15	F:GATCACCCTTCCCTTTCCCC R:GCGTCCCAAGCCAGTTCTTA	$(CA)_{22}$	60	11	176–214	0.805	0.714	EU165332
T121	F:CCAAGCCAACTCTTAACCAAC R:GTCTAGAAAACGGGACCATGT	$(CA)_{12}N_{14}(CA)_5N_{14}(CA)_5$	56	9	184–208	0.762	0.778	EU165333
HAM15	F:AGGACCAGTGCAGGGAAAGG R:GATGCTCCAGCCTCACCACA	$(TG)_4CA(TG)_4N_8(TG)_5$	66	3	102–114	0.262	0.300	EU165334

Ta, Annealing temperatures; N_A, Number of alleles; H_O, Observed heterozygosity; H_E, Expected heterozygosity

*Significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction ($P < 0.006$)

SAULA, the fragments were ligated with pMD19-T vector (Takara) and transformed to *E. coli* TOP10 competent cells (Tiangen). Out of 212 recombinant clones, 78 positive clones were picked up through M13 primers amplication. These clones were selected to sequence using M13 universal primers. (Invitrogen). To find that 22 had dinucleotide repeat regions. The primers were designed flanking the repeat regions using PRIMER PREMIER 5.0 (Premier Biosoft International). 13 primer pairs were amplified successfully through PCR and used to test the polymorphism of the loci.

All 48 specimens of *H. armiger* captured from Chizhou of Anhui, China, were genotyped. Genomic DNA was extracted from the wing membrane biopsies with the Dneasy Tissue Kit (Qiagen). PCRs were conducted in a total of 15 μl volume containing 50–100 ng genomic DNA, 0.25 mM of each primer (the 5-end of the forward primers were modified with FAM, HEX or TAMRA fluorolabels), 0.2 mM of each dNTP, 0.2 U hot-start Taq DNA polymerase (QIAGEN) and 1.5 mM $MgCl_2$. The reactions were carried out on a PTC-220 thermalcycler (Biorad) as follows: denaturation at 95°C for 15 min, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at specific temperatures (see Table 1) and 30 s at 72°C, and a final extension for 20 min at 72°C. PCR products were tested using an ABI377 sequencer (Applied Biosystems) and analyzed with Genescan v3.7 and Genotyper v3.6 (Applied Biosystems). Observed and expected heterozygosity values, tests for deviations from Hardy–Weinberg equilibrium and linkage equilibrium were assessed with Genepop v3.4 software (Raymond and Rousset 1995).

Table 2 Cross-species amplification using primers developed from *Hipposideros armiger*

Locus	Species						
	H. larvatus	*H. pomona*	*H. pratti*	*R. pusillus*	*R. pearsonii*	*R. macrotis*	*R. affinis*
CHANG242	+	–	+	–	–	–	–
BAM09	+	+	+	+	+	+	+
TT18	+	+	+	+	+	+	+
CHANG260	+	+	+	–	+	–	–
TE2	+	+	+	–	–	–	–
NEAM15	+	+	–	+	–	–	–
T121	+	+	+	+	–	–	–
HAM15	+	+	+	–	–	–	–

+, PCR amplification; –, No PCR amplification; Sample size = 3

Eight loci were polymorphic (see Table 1). The average number of alleles per locus was 7.9, ranged from 3 (HAM15) to 12 (CHANG260). The results showed the observed heterozygosity ranged from 0.213 to 0.875, and two loci (CHANG260 and TT18) deviated from Hardy–Weinberg equilibrium after Bonferroni correction for multiple testing (adjusted $\alpha = 0.006$, $k = 8$). The analysis with Micro-checker v2.2.3 (Van Oosterhout et al. 2004) showed both had null alleles. No loci pairs revealed significant linkage disequilibrium. These eight polymorphic microsatellite loci will be effective molecular tools for further research on population genetic structure and gene flow in *H. armiger*. Moreover, we performed cross-amplification in related species of Hipposideros and Rhinolophus (Table 2). The result showed that most of the loci amplified some PCR products in Hipposideros. Especially, the two loci BAM09 and TT18 showed highly successful amplifications in all these species, also suggesting that these markers may be used for similar applications in other related species.

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