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Short Communication

Isolation and Characterization of Microsatellite Markers for the Sable, *Martes zibellina* (Mammalia: Mustelidae)

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WZ, HZ and OW conceived the study, designed the experiments. WZ, TL and SX performed the experiments. WZ analyzed the data and wrote the article.

Key words
***Martes zibellina*, Sable, FIASCO, Microsatellite, Genetic diversity.**

ABSTRACT

The number of wild sables has declined dramatically in recent years due to the high value and increasing demand of their fur. In order to evaluate the genetic diversity and population structure for the conservation and management of sables (*Martes zibellina*), we developed six microsatellite markers using a method of fast isolation by AFLP of sequences containing repeats (FIASCO) from them in this study. The primers designed by Primer Premier 5.0 based on conservative regions were tested on 22 individuals. Our results demonstrated that the number of alleles per locus ranged from two to five and the mean number of alleles was 3.67. The observed and expected heterozygosities ranged from 0.227 to 0.545 and 0.201 to 0.630, respectively. The polymorphic information content value ranged from 0.181 to 0.573. Null allele frequency varied from -0.0548 to 0.0563. The sets of loci proved to correctly discriminate populations using Principal Coordinate analysis. These polymorphic markers will be useful tools for investigating genetic diversity and population structure of this species.

The geographic range of sable (*Martes zibellina*), extends in Russia (Siberia and the Far East), Kazakhstan, Mongolia, China, Korea and Japan (Geptner, 1967). There are four subspecies of sable (*M. zibellina princeps, M. zibellina linkouensis*, *M. zibellina altaica* and *M. zibellina hamgyenensis*) in China. They inhibit vast territories of the northeast and northwest China. Unfortunately, the demand for their highly valued fur has resulted in intense hunting, which led to a substantial population depression of the sable in China (Kashtanov *et al*., 2011). An assessment of their genetic diversity is necessary, since the genetic diversity is an important indicator for populations. It is all the more important since sable populations are evolving in response to environmental changes (Reed and Frankham, 2003). Several studies based on the cytochrome b (Cyt-b) gene or control region (D-loop) polymorphism data focused on sable have been carried out (Hosoda *et al*., 1999; Kurose *et al*., 1999; Murakami *et al*., 2004; Inoue *et al*., 2010). However, mtDNA as molecular marker has some unreliability because of molecular structure diversity, interspecific transfer and the nuclear copy (insert) of mtDNA (Ferris *et al*., 1983; Zullo *et al*., 1991; Niu *et al*., 2001). Therefore, some more reliable inherited molecular markers are necessary for studying the genetic diversity and population structure of sable.

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), are highly polymorphic and codominant molecular markers that are based on simple repeated and frequent sequences common in eukaryotic genomes, which have proven to be an effective tool in genetic diversity and molecular ecology studies (Goldstein and Schlöterer, 1999). In the present study, we chose tetranucleotide repeat stretches that are less compound and easier to type to develop some reliable microsatellites for *M. zibellina* and test their validity using genetic analyze (Vandevliet *et al*., 2009).

Material and methods

Twenty two *M. zibellina* individuals, including specimens, bodies of natural death and road killing, were collected from two populations in Northeast China. Among them, 12 individuals of *M. zibellina princeps* were collected in Daxinganling (50°10′- 53°33′ N, 121°12′-127°00′ E), and other 10 individuals (*M. zibellina*

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linkouensis) were collected in Xiaoxinganling (48°52′-49°11′ N, 126°45′-127°25′ E). All the muscle tissues were stored at -80°C. Total genomic DNA was extracted using DNeasy® Blood & Tissue Kit (QIAGEN GmbH) following the manufacturer's guidelines. All DNAs showed qualified after the detection of 1% agarose gel electrophoresis.

In accordance with the FIASCO protocol (Zane *et al*., 2002), we constructed a partial genomic library and used 5'-biotinylated $(AAAC)_8$ oligonucleptide probe for enrichment selection from it. Isolated DNA was digested by *MseI* and ligated to double strand AFLP linkers (*MseI* F: 5'-TACTCAGGACTCAT-3' and *MseI* R: 5'-GACGATGAGTCCTGAG-3'). The hybrids strands were captured with Dynabeads® M-280 Streptavidin (Invitrogen) and were amplified to recover the dsDNA. The enriched fragments were ligated into pMD18-T simple cloning vector (TaKaRa) and transferred into Trans1-T1 chemically competent cells (TransGen Biotech). Recombinants were screened by PCR with three primers: vector primers (M13F and M13R) and $(AAAC)_8$ oligonucleotide. Then, the PCR products of clones were examined by 1% agarose gel electrophoresis, and lanes showing multiple bands were considered as positive clones. The positive clones were sequenced by Sangon Biotech (Shanghai) with ABI3730XL Genetic Analyser (Applied Biosystems, Carlsbad, CA, USA).

A total of 196 positive clones were identified from 432 plasmids. Then 100 selected positive clones were sequenced and 79% of them had tandem repeats.

Thirty specific-primers were designed based on conserved regions using the software Primer Premier 5.0 (Lalitha, 2000) and eight primers were successfully yielded clear bands. PCRs were performed in a 25-µl reaction volume containing 7.5 µl 2×Easy Taq PCR Supermix (TransGen Biotech), 100–200 ng genomic DNA and 2.0 pM of each primer pair (forward primer fluorescently labeled with FAM, HEX). PCR-amplifications were carried out in an Applied Biosystems (ABI) 9700 thermal cycler under the following conditions: a first denaturation at 95°C for 5 min, 35 cycles at 95°C for 30 s, 30 s at the annealing temperature (Table I), and 72°C for 30 s, followed by a last cycle at 72°C for 10 min, and a hold step at 4°C. PCR products were analysed on an ABI 3500 genetic analyser (Applied Biosystems) with a LIZ600 size standard, and the allele sizes were estimated using the software GeneMapper (version 5.0, ABI).

The detection of potential scoring errors (*e.g.* stutter bands, large allele dropout and null alleles) for the data was analysed using Micro-Checker 2.2.1 (van Oosterhout *et al*., 2004). The number of alleles (N_A), observed heterozygosities (H_O), expected heterozygosities (H_E) and Principal Coordinate analysis (*PCoA*) were estimated using GenAlEx 6.5 (Peakall and Smouse, 2012). Linkage disequilibrium (*LD*) between pairs of microsatellite loci and deviations from the Hardy-Weinberg equilibrium (*HWE*) were calculated by GENEPOP v4.2 (Rousset, 2008). Cervus 3.0 was used to assess the polymorphism information content (PIC) and the frequency of null alleles (Kalinowski *et al*., 2007). Inbreeding coefficients (*Fis*) were determined by FSTAT v2.9.3.2 (Goudet, 1995).

Table I.- Characterization for six microsatellite loci isolated from *Martes zibellina*.

Locus	Primer sequence	Repeat motif	Ta (ºC)	Allele size (bp)	N_A	H_E	H_O	P_{HW}	*PIC*	*F* (null)	Accession no.
Mzf49	F: CACCTTATTGCCATTCAG	$(TGAA)_7$	52	163-171	3	0.408	0.364	0.380	0.341	0.0521	KX712105
	R: AAAGTGGGTATCATTGTC										
Mzf51	F: TTGTGGGTGCTTGGTAGTA	$(GTTT)_8$	54	149-161	5	0.508	0.500	0.519	0.458	0.0221	KX712106
	R: CCTCATTTGGGTTGGTAG										
Mzf56	F: ATTGTGAGGGTAGGTTCG	$(CCAT)_8$	54	292-296	2	0.449	0.500	0.816	0.348	-0.0533	KX712107
	R: ATTTATGGGCTTGCTGTC										
Mzf57	F: AAACTCACTTTGCTGTAT	$(TTGT)_7$	50	243-275	5	0.519	0.545	0.595	0.477	-0.0371	KX712108
	R: GAATGAAATCGCTTAGGT										
Mzf58	F: GAATAGCCAAAGGAATGT	$(AAAC)_7$	52	172-192	2	0.201	0.227	1.000	0.181	-0.0548	KX712109
	R: GTCCATAAACTTGAGGGT										
Mzf61	F: TGTAGCCCCATAGGTTTG	$(TTTG)_7$	52	172-188	5	0.630	0.545	0.069	0.573	0.0563	KX712110
	R: TGCTTTGAATCGCATCTT										

Ta, annealing temperature; N_A, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; *PIC,* polymorphic information content value; *F,* null allele frequency.

Table II.- Genetic diversity over all loci for the two populations of *Martes zibellina*.

Locus	Daxinganling (N=12)					Xiaoxinganling (N=10)				
	N_A	N_E	H_O	H_E	*Fis*	N_A	N_E	H_O	H_E	*Fis*
Mzf49	3	1.291	0.250	0.226	-0.065	2	1.980	0.500	0.495	0.043
Mzf51	5	1.895	0.500	0.472	-0.015	3	2.062	0.500	0.515	0.082
Mzf56	2	1.800	0.500	0.444	-0.082	2	1.835	0.500	0.455	-0.047
Mzf57	4	2.165	0.667	0.538	-0.197	4	1.905	0.400	0.475	0.209
Mzf58	2	1.180	0.167	0.153	-0.048	2	1.342	0.300	0.255	-0.125
Mzf61	5	2.824	0.500	0.646	0.267	3	2.469	0.600	0.595	0.044

N, number of individuals tested; N_A, number of alleles; N_E, number of effective alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; *Fis*, inbreeding coefficient.

Results and discussion

Although a high proportion of sequences contained repeats, the majority of them was unusable for primer design for several reasons such as the higher or lower annealing temperature (Zhi *et al*., 2014). Thirty primer pairs were designed, of which eight successfully yielded clear bands. Futhermore, only six markers yielded polymorphic amplification products in the test for polymorphisms across 22 individuals from two natural populations of *M. zibellina*. DNA sequences of the six microsatellites were deposited into Genbank (accessions KX712105- KX712110).

N_A per locus varied from two to five, with an average of 3.67 (Table I), which showed a lower level than previous study (Kashtanov *et al*., 2011). At the species level, H_O and H_E ranged from 0.227 to 0.545 and from 0.201 to 0.630, with an average of 0.447 and 0.453, respectively (Table I).

None of these loci were found to deviate from the *HWE* (Table I), and significant *LD* was detected between the locus Mzf49 and Mzf56 after Bonferroni correction ($p<0.05$), which indicated significant allelic association between the two markers. The *PIC* value ranged from 0.181 to 0.573. Locus Mzf58 showed low polymorphism (*PIC*<0.25) and Mzf61 showed high polymorphism (*PIC*>0.5), while the rest were moderately polymorphic (0.25<*PIC*<0.5). The frequency of null alleles varied from -0.0548 to 0.0563 and none of these loci showed evidence of null alleles. None of the loci showed evidence for large allele dropout or stutter bands. Genetic diversity parameters in each population are presented in Table II. Numbers of effective alleles (N_E) were all less than 3, and ranged from 1.180 to 2.824 in the two populations. The locus Mzf61 showed the highest inbreeding coefficient (Fis=0.267) in Daxinganling population. Furthermore, using the 6 microsatellites, *PCoA* permitted the discrimination of the populations of Daxinganling and Xiaoxinganling, although any overlap was observed. It suggested that the sets of loci proved to correctly discriminate populations (Fig. 1).

In conclusion, microsatellite DNA is an effective molecular marker for studying the genetic diversity of sables. The FIASCO protocol is fast and simple, and its utility is better than traditional enrichment methods. The six microsatellite markers show polymorphism in the two populations, which is helpful for the research and conservation of *M. zibellina* in the future.

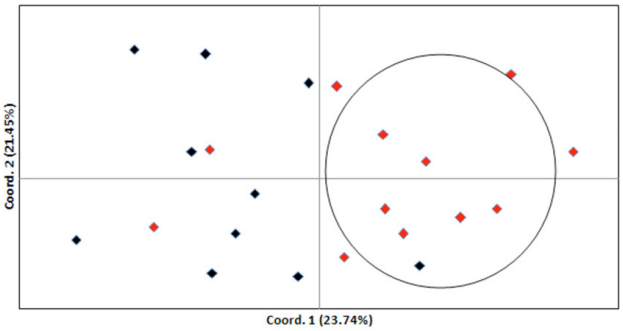

Fig. 1. Principal coordinates analysis (PCoA) for the two regional populations of sable based on the six microsatellite loci (Red, Daxinganling; Black, Xiaoxinganling).

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Statement of conflict of interest

Authors have declared no conflict of interest.

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