



# Isolation and characterization of polymorphic microsatellite loci of the Chinese muntjac (*Muntiacus reevesi*)

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**ABSTRACT.** Eight polymorphic microsatellite markers for *Muntiacus reevesi* were identified and characterized in this study. The number of alleles per locus ranged from 3 to 10 across 24-48 samples. The loci showed expected and observed heterozygosities of 0.577-0.876 and 0.387-0.933, respectively, with an average polymorphic information content value of 0.682. These markers should be a useful tool for further population and conservation genetic studies of *Muntiacus reevesi*.

**Key words:** Chinese muntjac; *Muntiacus reevesi*; Microsatellite; Polymorphism; Population genetics

## INTRODUCTION

The Chinese muntjac (*Muntiacus reevesi*) is a small deer species with a native distribution centered in South China and parts of Southeast Asia (Zhang et al., 2004). It was introduced to England in the 19th century and has expanded its range broadly in the last 30 years (Harris et al., 1995). In some areas, *M. reevesi* density became so high that it was considered to be exerting a negative impact on timber production and on the biodiversity and conservation interests of forests (Putman and Moore, 1998; Fuller and Gill, 2001; Joys et al., 2004; Rooney et al., 2004). It has also been suggested that the roe deer (*Capreolus capreolus*) may suffer from competition with introduced muntjac populations (Chapman et al., 1993; Staines et al., 1998; Hemami et al., 2005). In China, however, the *M. reevesi* population has declined sharply due to habitat degradation and overhunting, and it is in urgent need of protection (Ye et al., 2008). Unfortunately, little attention has been paid to its conservation, apart from a few ecological and phylogeographical studies (Shi et al., 2010). In this investigation, we developed eight polymorphic microsatellite markers that can be used to study the population and conservation genetics of *M. reevesi*, and help to determine and identify effective conservation measures for this species.

## MATERIAL AND METHODS

Microsatellite library enrichment was performed following the fast isolation by amplified-fragment length polymorphism (AFLP) of sequences containing repeats (FIASCO) protocol (Zane et al., 2002). Genomic DNA from the muscle tissue of one individual was extracted and purified using the EasyPure Genomic DNA kit (TransGen Biotech) following manufacturer guidelines. Then, it was digested by the *MseI* restriction enzyme (New England Biolabs) and ligated to a double-stranded *MseI* AFLP adaptor (*MseI* F: 5'-TACTCAGGACTCAT-3' and *MseI* R: 5'-GACGATGAGTCCTGAG-3') at 37°C for 3 h. The digested and ligated product was then amplified using an *MseI*-N primer (5'-GATGAGTCCTGAGTAAN-3') for 26 cycles. After denaturation, DNA was hybridized with 5'-biotinylated (AC)<sub>12</sub> oligonucleotide probes. Hybridization products were captured by Streptavidin MagneSphere Paramagnetic Particles (Promega) and unhybridized DNA was washed away. Enriched fragments were recovered from the denaturation steps by polymerase chain reaction (PCR) amplification with the *MseI*-N primer.

Agarose gel visualization of amplified fragments should appear in PCRs as a smear above 200 bp (Zane et al., 2002). PCR products were then ligated into the pEASY-T1 Simple Cloning Vector (TransGen Biotech) and transferred to Trans5α Chemically Competent Cells (TransGen Biotech). Recombinants were screened by PCR using three primers: vector primers (M13F and M13R) and the (AC)<sub>12</sub> oligonucleotide. The lanes showing multiple bands on 2% agarose gel electrophoresis were considered putative microsatellite repeats. Plasmids from positive clones were sequenced and primers were designed by the Primer Premier 5 software (Lalitha, 2000).

PCRs were performed in 20-μL volumes containing 100-200 ng genomic DNA, 10 μL 2X EasyTaq PCR Supermix (TransGen Biotech), and 2.5 pmol of each primer pair (the forward primer was fluorescently labeled with FAM, HEX, or TAMRA). Amplification was performed on an Applied Biosystems (ABI) 2720 Thermal Cycler under the following conditions: initial denaturation at 95°C for 5 min; followed by 32 cycles of 30 s at 94°C, 30 s at the annealing temperature (Table 1), 40 s at 72°C; and a final extension at 72°C for 10 min. All

PCR products were separated on an ABI PRISM 3730 genetic analyzer (Applied Biosystems) with a GS500 size standard, and analyzed using GENEMARKER (version 1.3, SoftGenetics LLC). Allele size ranges, numbers of alleles, observed heterozygosity, expected heterozygosity, and polymorphic information content values were calculated using GENETIX version 4.0 (Belkhir et al., 2001). Deviations from Hardy-Weinberg equilibrium and linkage disequilibrium at each locus were measured using GENEPOP version 4.0 (Rousset, 2008).

**Table 1.** Characterization of 8 *Muntiacus reevesi* microsatellite loci.

Locus	Primer sequences (5'-3')	Repeat motif	Ta (°C)	Size range (bp)	No. of alleles	$H_o$	$H_e$	PIC	Accession No.
Mre35	F: GTCCTGGTAAATGGGGTTCA R: GCCGATGCTTTGTTCTCC	(GT) <sub>31</sub>	57	194-202	3	0.533	0.577	0.496	KC166223
Mre39	F: AATTGGGAGACTGGGACTGAGA R: TGAATGAATGAAGCTGCTTGTA	(AC) <sub>17</sub>	57	237-255	6	0.714	0.820	0.761	KC166224
Mre49	F: CGGCCAATTATCTGCGAGTGTC R: CTAGGCAGTGCTTGGTTGAGAA	(AC) <sub>30</sub>	57	276-282	4	0.625	0.597	0.510	KC166225
Mre61	F: AAGGGGGACGTTGGTTTGAC R: GATGCCTGTGTGGACAGAGTTTGA	(AC) <sub>18</sub>	57	187-211	9	0.933	0.876	0.83	KC166226
Mre04*	F: GTCACAAGCCGCAITCAGAG R: ATGCCCTTTGTTCTTCTTATTG	(AC) <sub>20</sub>	57	329-339	6	0.387	0.644*	0.568	KC166227
Mre22	F: CAAACATGACTGAGCGACTGA R: TGCATCTCTTTCTAACTCCACATT	(AC) <sub>16</sub>	57	132-144	6	0.625	0.717	0.658	KC166228
Mre383	F: AGACGGTCACCACTTCTATTAC R: AGTAGAGTTTGTGTGTGAGG	(AC) <sub>21</sub>	57	172-190	9	0.857	0.838	0.808	KC166229
Mre448*	F: GAGACAGCAGGATGAGATGT R: ACAGTAGCCTCTCCTTCTC	(TG) <sub>12</sub>	57	188-214	10	0.438	0.855*	0.453	KC166230

\*Indicates significant deviations from Hardy-Weinberg equilibrium. Ta = annealing temperature;  $H_o$  = observed heterozygosity;  $H_e$  = expected heterozygosity; PIC = polymorphic information content value.

## RESULTS AND DISCUSSION

Plasmids from 70 positive clones were sequenced and 20 primers were designed. Among the 20 primer pairs, 12 microsatellite loci produced consistent amplifications and were chosen for further analysis by screening 24-48 individuals. The analysis results showed that the number of alleles ranged from 3 to 10; the expected heterozygosity ranged from 0.577 to 0.876 and observed heterozygosity ranged from 0.387 to 0.933 (Table 1). The average polymorphic information content value was 0.682. Together, these results indicated that these loci were polymorphic. No deviation from Hardy-Weinberg equilibrium was observed, except for loci Mre04 and Mre448. We did not detect any linkage disequilibrium between these loci.

These polymorphic microsatellite loci may be useful for investigating population structure, levels of gene flow, mating systems, and conservation genetics of *M. reevesi*.

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