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Zoological Research

Isolation and Characterization of Microsatellite DNA Markers from Forest Musk Deer (*Moschus berezovskii*)

ZHANG Shu-chao, YUE Bi-song, ZOU Fang-dong*

(Key Laboratory of Bio-resources and Eco-environment, Ministry of Education, College of Life Sciences, Sichuan University, Chengdu, Sichuan 610064, China)

Abstract: In this report, we describe the isolation and characterization of microsatellite loci for forest musk deer (*Moschus berezovskii*) through an improved enrichment protocol. Five new markers were isolated from the genomic DNA of forest musk deer and showed high polymorphism with 4 – 13 alleles in 24 sampled individuals from the population of Jinfeng Mountain, Sichuan Province, China. The observed and expected heterozygosities were from 0.429 – 0.957 and 0.587 – 0.902, respectively. The average polymorphism information content (PIC) value in these five loci was 0.730. This suggests that the five microsatellite loci are a valuable tool for further studies about forest musk deer.

Key words: *Moschus berezovskii*; Microsatellites; Polymorphism

林麝微卫星座位的分离和鉴定

张述超, 岳碧松, 邹方东*

(四川大学 生命科学学院, 生物资源与生态环境教育部重点实验室, 四川 成都 610064)

摘要: 利用改进的磁珠富集法从林麝(*Moschus berezovskii*)的基因组中分离到 10 个新的(AC)n 重复微卫星座位, 并分析其在 24 个林麝个体(取样于中国四川金凤山群体)中的多态性。其中, 5 个位点在 24 个林麝个体中具有 4—13 个等位基因, 观察杂合度和期望杂合度分别是 0.429—0.957 和 0.587—0.902, 平均多态信息含量是 0.730。表明这 5 个微卫星位点具有高度的多态性, 可以用于林麝遗传多样性研究, 对林麝的保护具有很重要的意义。

关键词: 林麝; 微卫星; 多态性
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Forest musk deer (*Moschus berezovskii*), one of the five reported musk deer species in the world, is distributed in the southwest part of China and the adjacent areas (Zou, 2005; Xia, 2005). Due to habitat destruction and over-exploitation of the musk, the population of wild musk deer sharply decreased from more than 3 000 000 in the 1950s to no more than 100 000 in the 1990s, according to a new report released by, the world's leading wildlife trade monitoring network (TRIFFIC), and the World Wildlife Fund (WWF) (Zou, 2005). As a result, all musk deer species have been listed in the Appendices of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1979. In 2002, they were cited in the first class of "key" species of wildlife as protected by Chinese legislation. In China some 1900 musk deer are kept in farms (Yang, 2003). However, current studies on the level of genetic variability in wild

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* Corresponding author(通讯作者), Tel: +86 – 28 – 85412488; Fax: +86 – 28 – 85414886; E-mail: fundzou@scu.edu.cn
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第一作者简介: 张述超(1981 –), 男, 硕士研究生, 研究方向为分子细胞生物学。E-mail: chaoring@163.com

forest musk deer is scant. It is difficult to avoid inbreeding due to the lack of accurate pedigree notes in musk deer farms. These problems can be settled by using microsatellites.

Microsatellites are generally considered to be the most powerful Mendelian markers currently available for population genetic studies (Jarne, 1996). The information provided by these markers can address biological questions ranging from the fine scale determination of individual identity and relatedness to broader scale questions, including the determination of population genetic structure and evolutionary relationships. At present only a few microsatellite loci have been isolated from the forest musk deer distributed in Miyaluo and Maerkang of Sichuan Province (Zou, 2005; Xia, 2005). There is a limited number of these loci and it is not clear whether they are applicable to the forest musk deer population of Jinfeng Mountain, Sichuan Province. In this study, five new highly polymorphic microsatellite loci were isolated and characterized in 24 musk deer from the population on Jinfeng Mountain. These new loci should be beneficial to the study of population and conservation genetics of forest musk deer.

1 Materials and Methods

1.1 Materials

Twenty-four forest musk deer were sampled under the standard care procedures from the population on Jinfeng Mountain, Sichuan Province, China.

1.2 Isolation and characterization of microsatellite loci for forest musk deer

Microsatellites were isolated based on the enrichment technique described by Zou et al (2005). Male musk deer genomic DNA was obtained from muscle tissue by the standard proteinase K/phenol extraction procedure. The genomic DNA was digested with *Sau*3AI at 37℃ overnight and ligated to *Sau*3AI adaptors (oligo A: 5′-GGCCAGAGACCCCAAGCTTCG-3′ and oligo B: 5′-pGATCCGAAGCTTGGGGTCTCTGGC-3′) using T4 DNA ligase at 4℃ overnight. Successful ligation of the adaptors to the digested DNA was tested by polymerase chain reaction (PCR) with oligo A as the primer. After denaturing at 95℃, the ligated fragments were hybridized to a biotin-labelled probe $(AC)_{12}$, which were attached to Streptavidin-coated magnetic beads, at 57℃ for 30 min. The fragments containing $(AC)_n$ were enriched and amplified by PCR with the primer oligo A. Then 300 – 1 000 bp PCR products were recovered and ligated into pMD18-T vector (TaKaRa, Japan) and transfected into JM109 competent cells. PCR with oligo A and $(AC)_{12}$ as primers were carried out to identify the clone carrying $(AC)_n$ repeats. After sequencing, primers were designed according to flank sequences of the microsatellites using the software PRIMER 3.0 (Rozen, 2000). The PCR conditions were optimized by changing the concentration of $MgCl_2$, the annealing temperature, and by applying a touchdown PCR program in an iCycler PCR (Bio-Rad). In the first 22 cycles of touchdown PCR, the initial annealing temperature was decreased by 1℃ every two cycles, then the annealing temperature of the remaining 15 cycles was increased by 3℃. 5.0 μL of PCR products were used for electrophoresis in a 1.0% agarose gel with Ethidium bromide (EB) to determine if there had stable products.

Ten microsatellites with specific and repeated PCR amplifications were chosen for further polymorphism analysis in 24 forest musk deer. 2 – 4 μL of PCR products of those 10 microsatellites were separated on a 6% urea-polyacrylamide gel with a sequencing electrophoresis cell (Bio-Rad) along with pUC19DNA/*Msp* Ⅰ (*Hap* Ⅱ) (MBI Fermentas) and visualized with an improved silver staining protocol as described by Zou et al (2005).

1.3 Statistical analysis

The gels with clear bands were scanned using GeneGenius (Synoptics Ltd.) and the allele length of each microsatellite was determined by GENETOOLS software (Synoptics Ltd.). Allele frequencies and heterozygosities were calculated with the CERVUS 2.0 program (Marshall, 1998). Deviations from Hardy-Weinberg equilibrium (HWE) were analyzed and linkage disequilibrium among all loci were tested with GENEPOP3.3.

2 Results

2.1 Isolation of microsatellites from forest musk deer

After PCR screening with primers oligo A and $(AC)_{12}$, 16 microsatellite loci with AC/TG repeat motifs were isolated from the enrichment library of forest musk deer.

2.2 PCR optimization of microsatellite loci

The PCR conditions of each microsatellite loci from forest musk deer were optimized as listed in Tab. 1. In this study, gradient PCR was chosen to obtain the optimal annealing temperature and concentration of $MgCl_2$. When optimal PCR conditions could not be ascertained, touchdown PCR was used to solve the problem. Some microsatellite loci, for example locus Mber118H,

did not generate the expected PCR products until the concentration of the reaction system had been changed. Exceptunder PCR conditions, some microsatellite loci could not be amplified successfully due to some inherent qualities about the loci or poor primer design. After optimization, 10 loci from forest musk deer had specific and stable PCR products (Tab. 1).

2.3 Characterization of microsatellites for forest musk deer

Of the 10 microsatellite loci, only five loci were highly polymorphic with 4 – 13 alleles in 24 individuals. The observed and expected heterozygosities ranged from 0.429 to 0.957 and 0.587 to 0.902, respectively. The mean observed heterozygosity (Ho) was 0.779. The two loci (Mber76C and Mber116H) deviated significantly from HWE ($P < 0.05$). The five loci were tested for linkage disequilibrium to ensure marker independence using Genepop version 3.3, and no linkage associations were evident from pairwise comparisons of loci ($P > 0.001$ for all comparisons). The average polymorphism information content (PIC) value was 0.730, indicating that the five microsatellite loci are highly polymorphic (Tab. 2).

Tab. 1 The PCR condition of 10 microsatellite loci

Loci	Primer sequences(5′-3′)	Mg^{2+} (mm/L)	Annealing temperature(℃)	Touchdown PCR	Size (bp)
Mber118H	TGTCAAGCACCAACCTCC	1.5	54	NO	159
	GTGCGTATTGAAGTGATGAGA				
Mber116H	TGCGTATTGAAGTGATGAGA	1.5	59 – 49	YES	160
	GCTGTCAAGCACCAACCT				
Mber76C	GATGAGAATCAGGACGGGA	1.0	54	NO	158
	CCCTTACTGCTGCTGTCAA				
Mber102C	TGACTGATACTCTGAAGGGTGT	1.0	59 – 49	YES	264
	GCTCCTCTCATTACTGGCTC				
Mber112A	ATGGCGAACTGCTGAATC	1.0	60 – 50	YES	229
	CCCCACTACAAGCATCTC				
Mber1110G	GAACATCAACCCACTCCAG	1.0	58 – 48	YES	178
	AAGAAAGGGACCTGAAATGA				
Mber102H	TCCACGGAGTCACCAAGA	1.0	53	NO	193
	ACTTTGACTCTGCCTTTGC				
Mber111E	ATCCAACCAGCCTCAATC	0.83	60 – 50	YES	249
	AGTCTGCCACTCAGTCAAG				
Mber912D	GGTAACCCACTCCAGTATTC	1.0	57 – 47	YES	282
	GGAAGCAGGTTCTTTACCACTA				
Mber912F	ATACACTGCGGAGAAAGG	0.67	58 – 48	YES	327
	TGGAGAAGGGAATGGCTAC				

Tab. 2 Characteristics of five polymorphic microsatellite loci in 24 forest musk deer individuals

Locus	Repeat motif	Size (bp)	No. of alleles	H_O	H_E	PIC	GenBank Accession No.
Mber76C	$(gt)_{23}$	126 – 172	11	0.864	0.817	0.771	DQ852332
Mber112A	$(gt)_4...(gt)_7...(gt)_4$	222 – 348	8	0.952	0.799	0.748	DQ852333
Mber116H	$(gt)_{23}$	126 – 182	13	0.870	0.902	0.872	DQ852334
Mber118H	$(ac)_{23}$	128 – 182	8	0.957	0.791	0.738	DQ852335
Mber102C	$(gt)_{10}...(gt)_8...(gt)_9$	220 – 348	4	0.429	0.587	0.519	DQ852336

Points (...) indicating non-repeated nucleotides.

3 Discussion

Thus far, few microsatellite loci have been reported (Zou, 2005; Xia, 2005), which are not enough to analyze the genetic diversity of forest musk deer populations. Therefore, five new microsatellite loci were isolated and characterized in this research. Each locus had a high polymorphic information content value (PIC ⩾ 0.519), and only two loci (Mber76C and Mber116H) deviated significantly from expected heterozygosities, which could be due to the presence of null alleles or the small sample size used in this study. This suggested

that those five microsatellite loci could be a valuable tool for further studies about population structures, gene map construction, gene diversity and paternity identification in wild or captive forest musk deer populations.

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(中国科学院昆明动物研究所　计财处，650223)