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Research paper

Isolation of novel microsatellite markers and their application for genetic diversity and parentage analyses in sika deer

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ABSTRACT

Every part of the sika deer (*Cervus nippon*) body is valuable traditional Chinese medicine. And sika deer is the most important semi-domestic medicinal animal that is widely bred in Jilin province northeast of China. But few studies had been conducted to characterize the microsatellite markers derived from sika deer. We firstly used IlluminaHiSeq™2500 sequencing technology obtained 125 Mbp genomic data of sika deer. Using microsatellite identification tool (MISA), 22,479 microsatellites were identified. From these data, 100 potential primers were selected for further polymorphic validation, finally, 76 primer pairs were successfully amplified and 29 primer pairs were found to be obvious polymorphic in 8 different individuals. Using those polymorphic microsatellite markers, we analyzed the genetic diversity of Jilin sika deer population. The mean number of alleles of the 29 loci is 9.31 based on genotyping blood DNA from 96 Jilin sika deer; The mean expected heterozygosity and polymorphic information content (PIC) value of the 29 loci is 0.72 and 0.68 respectively, and among which 26 loci are highly polymorphic (PIC > 0.50). According to the electrophoretic results and PIC value of these 29 loci, 10 loci with combined paternity exclusion probabilities > 99.99% were selected to use in parentage verification for 16 sika deer. All the offspring of a family could be successfully assigned to their biological father. These microsatellite markers generated in this study could greatly facilitate future studies of molecular breeding in sika deer.

1. Introduction

Microsatellites are short tandem repeating sequences (STRs) of 1–6 genetic elements distributed throughout vertebrate genomes (Toth et al., 2000). Compared with other molecular markers such as randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), microsatellite markers have many unparalleled advantages such as codominant inheritance, high level of polymorphism, easy to be detected and so on. Especially because of his codominant inheritance, heterozygotes can quickly distinguished with homozygous alleles, which allow it becomes the marker of choice for studies of kinship and has been widely used in humans and animals parentage analysis (Jin et al., 2016; Kang et al., 2009). Except for parentage analysis, microsatellite markers can also be applied in other animal genetic researches such as genetic diversity analysis (Tian et al., 2014), Quantitative Trait Locus (QTL) (Lu et al., 2013), construction of genetic maps (Wells et al., 2011) and so on.

Traditional methods of developing microsatellite markers need to establish genomic library to get the fragmented sequence, then use probes to hybridization in situ which are usually time-consuming and labor-intensive (Li et al., 2015). With the rapid development of high throughput sequencing technology, the research methods of microsatellite markers have been greatly improved. Automated sequence analysis instruments make the detection of microsatellite DNA more rapid and accurate (Vukosavljev et al., 2015). At the same time, the application of microsatellite markers has become more and more widely.

Sika deer (*Cervus nippon*) is an important economic animal used for producing antlers and meat. According to the Chinese pharmacology monograph, antler is a valuable tonic which can be used to treat osteoporosis (Szuwart et al., 2002). The venison has long been favored by consumers because it has high protein and low fat content. Nowadays,

Abbreviations: STRs, short tandem repeating sequences; PE, probability of exclusion; CPE, combined probability of exclusion; PIC, polymorphism information content; Ho, observed heterozygosities; He, expected heterozygosities; Tm, annealing temperatures; K, numbers of alleles; N, numbers of individuals genotyped; MISA, microsatellite identification tool

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sika deer have been farmed in many countries such as China, New Zealand, Japan, Russia, Korea and so on (Dvořák and Palyzová, 2016). But the deer breeding technology is relatively behind other livestock. For example, in northeast China, people often used mixed semen for artificial insemination or let female deer repeated mate with different males to improve the conception rate, so it is impossible to maintain an accurate pedigree record. Accurate paternity records and genetic selection are very important for continued genetic improvement of the breed (Theunissen, 2014). Well, lacking sufficient molecular markers has hampered the molecular research for sika deer. The number of existing deer microsatellite markers is only 200 +, of which about 170 microsatellites are "borrowed" from cattle and their polymorphism is relatively low, can't meet the needs of genetic research for sika deer(Xu et al., 2001). To solve this problem, we firstly using the high throughput sequencing technology isolated 29 high polymorphic microsatellites markers for sika deer and 10 of them were discovered can be using in parentage analyses in sika deer. These microsatellite markers would provide great help for future studies of population genetic analysis and molecular breeding in sika deer.

2. Experimental section

2.1. SSR isolation and primer design

In this study, we firstly using IlluminaHiSeq™2500 sequencing technology obtained 125 Mbp genomic data of sika deer which is included 302,642 fragments sequences. According to previous studies (Li et al., 2004; Subramanian et al., 2003), microsatellite requires a single base 10 minimum repeats, two base 6 minimum repeats, the other types of base 5 minimum repeats. Based on this criterion, the assembled contigs and singletons were screened by the MISA software (Thiel et al., 2003) and 22,479 microsatellites were isolated. The primers of those microsatellites and their predicted PCR products were obtained by QDD software package (Meglécz et al., 2010). Primers were designed with the following parameters: The length of the generated sequences were 150–350 bp, primer length ranging from 18 to 24 bp, annealing temperature ranging from 55 °C to 65 °C, and GC content ranging from 40% to 60%.

2.2. Sample collection

According to the published data, there was about 1.1 million sika deer in China and approximately half of them distributed in Jilin province (Wang and Song, 2014). Our samples were come from four different farms of Jilin province (northeast China). About one hundred different sika deer blood samples were collected. All of the samples were blooded from jugular vein of healthy sika deer and avoided asking farmers about animal relatedness. Blood samples were collected using anticoagulant tubes then stored in − 20 °C until DNA extraction.

2.3. Potential polymorphism microsatellite selection

In order to isolate polymorphism microsatellites, we selected 100 potential polymorphic microsatellites from the isolated 22,479 microsatellites according to the criteria which are as follows (1) The core of the repeat should not be mono-nucleotide; (2) According to previous studies (Wierdl et al., 1997), microsatellites with a high number of repeats often have a high mutate rate, so we try to choose those microsatellites whose repeat number were relatively high: the number of di- nucleotide repeats should be > 18, and the tri-nucleotide repeats should be > 10, the other type of microsatellite repeats should be > 8; (3) In addition, we would like to choose the microsatellite which core sequence was "GNC" and "GAA" repeats, because "GNC" repeats may result in a hairpin structure due to the base pairing of the GC (Moore et al., 1999), whereas GAA repeats easily produce a special triple helix structure (Gacy et al., 1995), these structures are very stable and difficult to be repaired by system, so their polymorphism is generally higher than others. (3) The flanking sequences of microsatellites should be long enough to design primers (> 50 bp); (4) In order to avoid the same sequence, we compared those selected microsatellites using the software DNAMAN version 4.0, their similarity of each other should be lower than 65%.

2.4. Polymorphism microsatellite verification

DNA was extracted from 300 μl deer blood using the Blood genomic DNA extraction kit (Bioteke). PCR amplifications were carried out in 15 μl reaction mixtures, comprising approximately 20 ng of template DNA, 6 pmol of each primer, 1.5 μl of dNTP, 1.5 μl of PCR buffer (10 ×), 0.15 μl of TE Taq polymerase (TaKaRa, Japan). Amplifications were performed using the following PCR procedure: an initial denaturation step for 5 min at 95 °C, followed by 35 cycles of 94 °C for 30 s, 30 s at 60 °C uniform annealing temperature and 30 s at 72 °C, and a final elongation for 10 min at 72 °C. The PCR products were separated on 3% agarose electrophoresis. The primers with a single band of expected size in the amplification were selected to further polymorphic test. We performed polyacrylamide gel electrophoresis to test the polymorphisms of the selected loci in 8 individuals, and only those loci which showed obvious polymorphism were selected for the next experiment.

2.5. Genetic diversity analysis

In order to analyze the genetic diversity of the Jilin captive sika deer population and further research the polymorphisms of these polymorphic loci, we extracted 96 blood DNA of Jilin captive sika deer. All of the forward primers of the 29 loci were labeled with one of three fluorescent dyes (FAM, TAMRA or HEX) and their PCR amplification programs were as the same as the above. Their PCR products were analyzed by Applied Biosystems 3730 Genetic Analyzers using the Gene Marker V2.2.0 software package of Applied Biosystems.

2.6. Parentage analysis

Previous studies have demonstrated the ability of microsatellite markers to determine parentage and pedigree analysis (Li et al., 2010). Whereas, the genotyping errors may cause an inaccurate results when conduct paternity test with microsatellites (Marshall et al., 1998). However, the ease of distinguish genotypes can vary dramatically across different microsatellite loci. According to previous studies (Webster and Reichart, 2005), those microsatellites which with single large "peak" characters can easily be genotyped when running fluorescently labeled PCR products on the ABI 3730 Genetic Analyzer. Whereas, those complex profiles and lower amplification microsatellites are more easily to cause genotyping error. According peak characters and PIC value, 10 loci were selected from above 29 loci to use in parentage verification for sika deer (Table 3). We used 16 sika deer which from four families to verify the efficacy of paternity test of the 10 microsatellite markers.

2.7. Statistical and genetic data analysis

To test the level of polymorphism of those loci, the number of alleles (A), observed heterozygosities (HO), expected heterozygosities (HE) and polymorphism information content (PIC) values were calculated using CERVUS (Version 3.0.3) software. For parentage analyses, non-exclusion probability and non-exclusion probability for identity were calculated by CERVUS software; probability of exclusion (PE) and combined probability of exclusion (CPE) were calculated by Gen AIEX (Version 6.503) software.

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