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Individual and sexual identification for the wild black muntjac (*Muntiacus crinifrons*) based on fecal DNA

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ABSTRACT

The black muntjac (*Muntiacus crinifrons*), endemic to China, was categorized as a Grade I National Key Protected Animal by the Ministry of Agriculture of China and classified as Vulnerable (VU) by IUCN. Recent years, studies had been conducted on this species mainly focusing on habitat selection, food habit, gene flow etc., only with a few reports on the population dynamics. Individual identification of wild animals is one of the most important subjects in the population dynamic research. Of various molecular markers, microsatellite DNA fingerprinting has been used most frequently and successfully on many kinds of animals. Here, we constructed identification system for the black muntjac using 8 microsatellite loci. 31 black muntjacs were identified from 141 fecal samples, whereas 43 samples could be used for PCR after repeated trials. Further, the sequencing for Cyt*b* gene was also conducted for convincing us the identity of fecal samples. The results, highly consistent between sequencing consequence and sequence data from Genebank, implied that those experienced local people are of the convincing knowledge about wild animals, especially at the respects of identification to black muntjac' feces pellets. Moreover, we detected the specificity of identification system to black muntjac. BM1225 was the only one locus that unsuccessful PCR for the muscle samples of *Muntiacus reevesi* was observed, which suggested that our identification system could be used for excluding the non-researched objects in some cases. Analyses using softwares CERVUS 3.0 and POPGENE 1.21 showed that the present identification system had strong discrimination power: 0.938 per loci (*DP*) or 0.999 in total (*CDP*). The mean observed number of alleles (*Na*), mean effective number of alleles (*Ne*), and mean excepted heterogosity (*He*) were 8.875, 6.375, and 0.829 respectively. Considering that the change of sex ratio in population could exert significant impact on population growth, density to some extent, we also analyzed the sex ratio of those individuals that had been identified based on fecal samples from filed using *SRY* (Sex-determining region Y) gene amplification, which identified 19 males and 12 females.

0. Introduction

Small mammals are easy to be captured and marked, so the use of traditional ecological methods can achieve the purpose of individual identification for the further studies of population ecology and behavior [1]. At present, the applications of radio tracking technology seem to have general attention for some bigger mammals [2–4]. However, it is difficult even invasive to utilize this method for individual identification of some large, vulnerable and strong concealment wildlife. With the development of molecular biology techniques, DNA molecular markers, non-invasive methods, are gradually used to solve the problem of individual identification of larger wildlife. Recently, 42 individuals of giant pandas (*Ailuropoda melanoleuca*) have been successfully identified using 10 microsatellite loci from 201 fecal samples collected at Tangjiahe Nature Reserve [5]. Now, it is generally believed that microsatellite (Simple sequence repeats, SSR) fingerprinting system is of a certain feasibility for individual identification. A battery of studies about individual identification for wildlife have been reported using the SSR fingerprinting, such as mountain lions (*Puma concolor*) [6], Chinese alligators (*Alligator sinensi*) [7], white-headed langurs (*Trachypithecus leucocephalus*) [8], Yangtze finless porpoises (*Neophocaena phocaenoides asiaeori-entali*) [9], and so on. The black muntjac (*Muntiacus crinifrons*), endemic to China, was categorized as a Grade I National Key Protected Animal by the Ministry of Agriculture of China and classified as Vulnerable (VU) by IUCN. Previous studies of this species mainly focus on a survey of population density [10], habitat selection [11–13], food habit [14], gene flow [15,16] and population genetic status quo [17–19], while with few reports on the population growth, dispersal, namely the population dynamics, which are important

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to better protection of this species. Individual identification is the foundation in research of the population dynamics.

In addition, sex ratio of the population is closely related to its dynamics. Wang et al. [20] had finished clone of the sex-determining gene (Sex-determining region Y, SRY) using the muscle tissue of black muntjacs, but the sex determination and individual identification based on fecal samples were not reported. In the present study, we will construct DNA fingerprinting system using 8 microsatellite markers for the individual identification of wild black muntjacs at first place and identify their sex based on the fecal samples. It is of great significance in application of non-invasive methods on sexual and individual identification. The use of microsatellites based on fecal samples of black muntjacs was mainly to reveal the genetic diversity of its population in past years [21]. For better protection of it, we conducted this research in individual and sexual identification for this species. This research could lay a good foundation for researches of black muntjacs population ecology.

1. Study area

Jiulongshan National Nature Reserve which is the junction of Zhejiang, Fujian and Jiangxi provinces with the area of 55.25 km^2, the highest elevation of 1724 m locates in southwest of Suichang county (118°49′–118°55′E°19′–28°24′N), Zhejiang Province in China. It belongs to the main branch of the Xianxialing mountains of Wuyishan department, which is rich in water resources as well as vegetations. The zonal vegetation type is evergreen broadleaf forest, and glauca (*Cyclobalanopsis glauca*) is the dominant species in the community. The fauna is Oriental realm and sub-realm of the eastern hills and plains of central China. This reserve influenced by the summer monsoon is of significant seasonal variations: the spring (March to May) with average temperature of 14.1–8.1 °C; the summer (June to August) with the average temperature of 25.0–18.0 °C; the fall (September to November) with an average temperature of 13.5–7.0 °C; and the winter (December to next February) with the average temperature of 3.8–0 °C.

2. Materials and methods

2.1. Collection and preservation of samples

A total of 141 black muntjac fecal samples were collected from Jiulongshan Nature Reserve in November 2009, March 2011 and June. All the samples were stored in individual screw-cap vials that contained anhydrous ethanol. Samples were transported to the laboratory and kept at −20 °C. For species identification and collection of fecal samples, our recent work have shown some detailed description [22], i.e., fecal pellets of the black muntjac are oval-shaped with the shape of large peanuts, less than 40 mm in length and a width of 25 mm around. They sometimes are of different sizes, different shades of 'dimples' and often stacked on a tree foot, the open space on the advancing line. We collected fecal samples as fresh as possible (light green, intact particles, adhesion). Otherwise, muscle samples of a black muntjac and four reeve's muntjacs (*Muntiacus reevesi*) were also used as a comparison control group, which were kept at −20 °C too.

2.2. Reagents and primers

The main reagents such as proteinase K, Taq DNA polymerase, dNTPs, were purchased from TaKaRa cooperation, Bovine serum albumin (BSA), DNA Makers and primers were purchased from Shanghai Sangon Limited. In this study, 8 loci (BM1225RT1, RT7, BN203, CSSM41, BM720, BM1706, INRA121) were used ultimately according to the experimental amplification of 10 loci (BM1225, RT1, RT7, BN203, CSSM41, BM720, BM1706, INRA121, OARVH110, BOVIRBP). Demonstration of these SSR primers could be found in the studies of Zhou [18] and Wu [21]. Otherwise, the primers of SRY gene have been reported by Wang et al. [20], and here we have presented the primers (i.e., Primer L 5′-gctggggtatgagtggaaaa-3′, Primer R 5′-ctgtatgtgaagggctgcaa-3′).

2.3. DNA extraction and polymerase chain reaction (PCR)

A slight improvement has been made for fecal DNA extraction compared to conventional extraction methods [22–23]. 1 mL of 2% cetyltrimethylammonium bromide (CTAB) lysis buffer was added in each centrifugal tube and mixed with the fecal sample in a 56 °C water bath for 2 h. Here we propose to turn tubes upside down with a 30 min interval, and then add 7 μL proteinase K in each tube. Heat the samples with proteinase K for another 30 min before centrifugation. In order to have a better result of amplification, we purified DNA templates using AXYGEN genomic DNA purification kit (Axygen Biotechnology, Hangzhou, China). DNA extracted were detected by agarose gel electrophoresis and stored at −20 °C. For muscle tissue DNA extraction, the classical method of phenol chloroform was used [17].

Amplifications were performed in a reaction volume of 50 μL, which contained 5 μL 10 × PCR buffer, 4 μL of 2.5 mmoL/L each dNTPs, 2.5 or 3.0 μL of 25 mM $MgCl_2$ (optimized for specific primer pairs), and 0.4 μL of 5U/mL Taq DNA polymerase, 0.4–0.8 μL genomic DNA, 0.6–1.5 μL each primer. The following amplification conditions were used: 95 °C for 5 min, followed by 45 cycles of 40 s at 94 °C, 40 s at the optimized annealing temperature (determined for each primer pair), 40 s at 72 °C, and a final extension at 72 °C for 10 min. The PCR products were stored at 4 °C. The amplification of SRY was performed according to the reference of Wang et al. [20].

2.4. Polyacrylamide gel electrophoresis

The SSR typing was performed on a 6% polyacrylamide gel [24] using a rapid silver staining protocol, an opening patent from Cao et al. [25]. Gel imaging system, Gel DocTM XR+(BIO-RAD), was used for making records of pictures. For the purpose of accuracy and stability in SSR typing, we strictly controlled the length of time (250 min) and voltage (250 V).

2.5. Data analysis

Image analysis and fragment size determination were performed using Gel-Pro32 Software. The population genetic analysis software POPGENE 1.21 [26] was used to calculate the number of alleles (*Na*), effective number of alleles (*Ne*), excepted heterogosity (*He*), and to assume Hardy–Weinberg equilibrium (HWE). The genetics software CERVUS 3.0 [27] was used to evaluate discrimination power (*DP*) for each loci and cumulative discrimination probability (*CDP*).

3. Results

3.1. Fecal DNA extraction and PCR

In 141 samples collected in the field, only 64 of which can be used for stable DNA extraction (the number of repetitions is greater than or equal to 2 times). From the results of agarose gel electrophoresis, the strips of total DNA were greater than 2000 bps with regular shape (Fig. 1a). The amplification of 8 SSR loci for these 64 DNA templates was successful as well as the DNA templates