Sex Determination of the Japanese Serow (*Capricornis crispus*) by Fecal DNA Analysis

Takashi NISHIMURA¹, Kiyoshi YAMAUCHI², Yasushi SAITO³, Yoshitaka DEGUCHI¹, Toshiki AOI¹, Tsunenori TSUJIMOTO⁴ and Kazuei MATSUBARA¹

1) The United Graduate School of Agricultural Sciences, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan
2) Research Institute for Environmental Sciences and Public Health of Iwate Prefecture, 1-36-1 Iiokashinden, Morioka, Iwate 020-0852, Japan
3) Morioka Zoological Park, 60-18 Shimoayagita, Shinjou, Morioka, Iwate 020-0803, Japan

(2009 年 7 月 28 日受領、2010 年 5 月 18 日採択)

ニホンカモシカ (*Capricornis crispus*) の糞中 DNA 解析による雌雄判別

西村貴志¹, 山内貴義², 斎藤靖史¹, 出口善隆¹, 青井俊樹¹, 辻本恒穂³, 松原和雄¹

1) 岩手大学大学院農学研究科 〒 020-8550 盛岡市上田 3-18-8
2) 岩手県環境保健研究センター 〒 020-0852 盛岡市版野新田１地割 36-1
3) 盛岡市動物公園 〒 020-0803 盛岡市新庄字下八木田 60-18

**ABSTRACT.** In this study we extracted DNA from feces of the Japanese serow and developed a PCR-based sex identification method using the amelogenin gene. Fecal DNA was extracted by the phenol chloroform method with purification by the CTAB method. Analysis of PCR products showed clear female and male banding patterns with one homozygous band (*AMEL XX*) and 2 or 3 heterozygous bands (*AMEL XY*), respectively, with lengths of approximately 250 bp and 200-300 bp, respectively, by electrophoresis on 2% agarose gel. Determination of sex was possible for fecal samples collected from the rectum of Japanese serows in 38 of 40 individuals (95%), and the designations were completely consistent with determination by physical examination. Determination of sex was successful for 5 of 6 fecal masses (83.3%) collected in the Takizawa Forest, with 3 judged to be from female origin and 2 of male origin. As the sexual dimorphism of the Japanese serow is not remarkable, the sex determination method developed in this study can be used to estimate the sex based on field feces analysis.

Key words : amelogenin, feces, Japanese serow, non-invasive sampling, sex determination


**INTRODUCTION**

The Japanese serow (*Capricornis crispus*) is endemic to Japan, and indiscriminate hunting and deforestation have considerably reduced the population size in the past. It is supposed that the present genetic diversity of the Japanese serow is limited due to the rapid decrease in population, or so-called bottleneck event [1, 2]. Following designation as a special national natural monument in 1955, the population size has recovered. And in recent years, Japanese serow has caused agricultural and forestry damage. In areas where damage is particularly serious, specific wildlife management plans are put in place for population control. Scientific information is necessary for the conservation and management of the Japanese serow. Generally, direct observations or biotelemetry tracking have been used for ecological research of Japanese serows. And quadrat method [3] and the driving-out method have been used to investigate their habitat and population size. Recently, genetic analysis from the non-invasive samples such as feces is used for the ecological research of the wild animal. Methods for analyzing fecal DNA have been developed and applied to some wild animals that are difficult to capture [4-6]. In Japanese serow, sexual dimorphism is not remarkable, making it difficult to determine the sex by appearance. We developed a fecal DNA analysis for determining sex for the Japanese serow based on the amelogenin gene (*AMEL*). *AMEL* is widely used for sex determination of the mammals, including livestock and wild animals, because it has a locus on both sex chromosomes (X and Y) and respective base sequences [6-9].

We extracted DNA from tissues and from feces collected from
the rectum each individual and conducted sex determination independently. On the occasion of sex determination from fecal DNA, we performed replicate analysis according to the “multiple tubes approach” reported by Navidi et al. [10] and Taberlet et al. [11]. Furthermore, we performed an experimental analysis of feces of Japanese serows collected in the field.

MATERIALS AND METHODS

Collection of tissue samples and fecal samples from the Japanese serows

Tissue and fecal samples were collected over a period of 2 years and 6 months from the summer of 2001 to the winter of 2004 from 51 Japanese serows collected in the suburbs of Morioka City. A total of 8 individuals were alive (♀ 5, ♂ 3) and 43 were dead (♀ 18, ♂ 25) at the time of collection. Of the live individuals, 6 were bred or kept temporarily at the Morioka Zoological Park (N39° 41’ E141° 11’), the Akita Omoriyama Zoo (N39° 40’ E140° 4’), or the Wildlife Rescue and Rehabilitation Center of Iwate Prefecture (N39° 48’ E141° 7’), and 2 were captured in the wild. Samples of blood and tens of whole hair strands including the root were collected from live individuals, and skin and muscle samples were collected from dead individuals. In addition we collected feces from their rectum, but the amount and consistency was insufficient in some individuals, and samples from 40 individuals were judged suitable for DNA analysis. We wore new latex gloves to make all collections and renewed them each time in order to avoid contamination. Samples were transported to our laboratory at a low temperature (about 4°C) on ice and cryopreserved at −20°C until use in experiments.

Collection of fecal samples in the field

Field surveys were conducted in the Takizawa Forest (N39° 47’ E141° 8’; total area, 280.58 ha; elevation, 170 to 274 m), Field Science Center, Faculty of Agriculture, Iwate University in the autumn (from November to December) of 2002, the autumn (from November to December) of 2003, and the spring (May) of 2004 to collect and record the locations of feces of the Japanese serow. Fecal masses that were collected from the accumulated feces, and transported to our laboratory at a low temperature (about 4°C) on ice after collecting them. A total of 6 fecal samples were analyzed, which could be obtained in large quantities especially. Although it is difficult to distinguish between the feces of the Japanese serow and sika deer (Cervus nippon), sika deer were not observed in the Takizawa Forest at the time that samples were collected.

Extraction and purification of DNA

Genomic DNA extraction was performed by the phenol chloroform (PC) method [12] for the tissue samples following preparation. Blood samples were centrifuged at 3,000 rpm, and leucocytes were collected for DNA extraction. Skin and muscle samples were cut into slices 5-7 mm² for DNA extraction. Tens of hair strands in each individual were cut and these roots were prepared for DNA extraction.

DNA extraction from feces was carried out following the method reported by Yamauchi et al. [6]. Briefly, the fecal surfaces were gently scraped with a sterilized cotton swab to collect residual epithelial cells from the intestinal mucosa. The tip of the cotton swab was cut off and placed in a 2-mL microtube for DNA extraction. Next, two kinds of DNA purification methods were performed: use of QIAamp DNA Mini Kit (QIAGEN) (kit method, K) and the cetyl trimethylammonium bromide (CTAB) method [13]. The CTAB method, which is generally used for DNA purification in plants to remove pigments and the polysaccharides of plant origin that inhibit PCR, is also applied to DNA extraction from damaged crops with bite marks of Asiatic black bears (Ursus thibetanus) [14]. Following PC extraction, it was most often impossible to obtain PCR products, as described below. We analyzed DNA extracted from feces collected from the rectum that was purified by each of these 2 purification methods and compared the determination rate between 2 different methods. Briefly, K was only used for samples that could not be amplified by PCR following PC extraction. The CTAB purification was applied on all samples. The analyses (DNA extraction, PCR and electrophoresis) for determining sex were replicated 3 times in each individual in order to confirm the accuracy of the results and monitor PCR errors (especially, allelic dropout). For feces collected in the field, extraction and purification by PC/CTAB was performed in triplicate for each fecal mass.

PCR and electrophoresis for sex determination

The AMEL was amplified in 25 µl reactions containing 10 × PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, SIGMA), 2.5 mM of each dNTP, 1.5 mM MgCl₂ (SIGMA), 1.25 units of Taq DNA polymerase (SIGMA), 50-100 ng of DNA, and 0.1 mM of each of the following primers: SE47, 5’-AGGAGAACT CCCTCTCTGC-3’ and SE48, 5’-CCGCCGTTTGTCTGCTGTNTGC- 3’ [7]. Amplification was carried out in the MyCycler thermal cycler (BIO-RAD) as follows: an initial hot start at 97°C for 3 min, 30-45 cycles (for tissue samples) or 55 cycles (for fecal samples) each consisting of denaturation at 95°C for 1 min, annealing at 66°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min.

The PCR products were electrophoresed on 2% agarose gel with a 0.5 × TBE buffer (44.5 mM Tris-borate (pH 8.0), 1 mM EDTA). The gel was stained by ethidium bromide and visualized under UV illumination to determine the sex based on the band patterns of the PCR products. In addition from these PCR products, the AMEL gene of Japanese serow was partially sequenced.

For feces samples, analyses were conducted in triplicate, and DNA bands confirmed in at least 2 out of 3 replicate samples were used.

RESULTS

PCR products of the target fragment of the AMEL were obtained from all tissue samples. Clear banding patterns
of one homozygous band (AMEL XX) in female and 2 or 3 heterozygous bands (AMEL XY) in male were detected at approximately 250 bp and 200-300 bp, respectively (Fig. 1). Banding patterns were consistent with the sex of the individual for all tissue samples (51/51 individuals). Additionally, results on DNA sequencing from PCR products, it was confirmed that this primer pair also amplified part of AMEL gene for Japanese serow (Note: Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers ’AB555648’ and ’AB555649’).

Band patterns of PCR products amplified using DNA isolated from feces were consistent with patterns obtained from body tissues (Fig. 2). However, band pattern analysis was possible for PC extraction alone only in 8 out of 40 individuals (20%) (Table1). Purification by K allowed detection in 26 of the 32 individuals that could not be detected by PC extraction alone. Therefore, detection of PCR product banding patterns and determination of sex was possible in 34 of 40 individuals (85%) (Table 1). Using the CTAB purification after PC extraction resulted in determination produced identifications in 38 of 40 individuals (95%) (Table 2). Moreover, results of sex determination by DNA analysis were consistent for replicates and were identical with those by physical observation.

Based on results for tissue samples, we performed DNA purification by PC/CTAB from feces collected in the Takizawa Forest and analyzed them as described above. Sex determination was possible in 5 out of 6 fecal masses (83.3%) (Table 3), and 3 were judged to be of female origin and 2 were of male origin. All replications produced the same results.

**DISCUSSION**

In this study, we demonstrated that amplification of a fragment of AMEL from body tissues and feces was suitable for correctly determining the sex Japanese serow. Although the detection rate of PCR products from fecal DNA was low for PC extraction alone, further purification by K and CTAB methods improved the detection rate to 85% and 95%, respectively. Compared to bands from amplifications with body tissues, those with fecal samples were faint, but this did not pose any problem in determining the sex of the individual. The male
banding pattern comprised 3 heterozygous bands, including 1 minor band of non-specific amplification (at approximately 300 bp). On the other hand, the female banding pattern was only one homozygous band without non-specific amplification. This result agrees with the report for sika deer by Yamauchi et al. [6], and sheep (*Ovis aries*) and European red deer (*Cervus elaphus*) by Pfeiffer et al. [9]. In addition, no difference in detection rates between male and female was confirmed.

Because there is very little quantity of DNA but a large quantity of potential contaminants in non-invasive samples, such as feces or hair, these are generally considered to be low-quality sources as samples for PCR analysis [11]. The decreased success rate of PCR in feces in this study was thought to be due to soluble contaminants that could not be removed by post-extraction purification. Application of CTAB purification drastically improved the amplification success, probably due to the effective removal of polysaccharides and humic acids present in soil [15]. Compared to PC extraction alone, the quantity of DNA obtained following K and CTAB purification was significantly decreased (data not shown) but was sufficient for determining the sex by PCR amplification. Judging from the determination rate, costs and known content of reagents, it was suggested that the CTAB method could be superior to the K method for the purification of DNA extracted from feces.

To test this method, we analyzed the CTAB purified DNA from feces in the field. Sex was determined in 5 of 6 fecal masses. Based on the complete confirmation of sex for corresponding tissue and fecal samples for individual samplings (alive and dead), we consider that PCR analysis test is accurate and reliable for determination of sex and that the sex determined in these field samples has some measure of confidence, too. The sex could not be determined for 1 fecal mass sample because of the extent of DNA deterioration and adhesion of PCR inhibitors due various environmental factors of the field, such as weather. Due to the small sample size for feces collected in the field, we cannot compare the determination rate with feces collected from the rectum feces. Further research using more field feces

---

**Fig. 2** Band profile of amelogenin gene amplified from fecal samples of Japanese serow
Sex determination by fecal DNA

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>Number of fecal samples</th>
<th>Number and rate (%) of PCR products detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PC</td>
</tr>
<tr>
<td>Captive serows</td>
<td>6</td>
<td>2 (33.3)</td>
</tr>
<tr>
<td>Wild-captured serows</td>
<td>2</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Dead serows</td>
<td>32</td>
<td>5 (15.6)</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>8 (20)</td>
</tr>
</tbody>
</table>

1) Values indicate the number of fecal samples.
2) Detection rates are shown in parenthesis. PC and K denotes phenol-chloroform and kit method, respectively.

Table 2 Detection rate of PCR products for fecal DNA purified by the CTAB method

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>Number of fecal samples</th>
<th>Number and rate (%) of PCR products detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PC</td>
</tr>
<tr>
<td>Captive serows</td>
<td>6</td>
<td>6 (100)</td>
</tr>
<tr>
<td>Wild-captured serows</td>
<td>2</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Dead serows</td>
<td>32</td>
<td>30 (93.8)</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>38 (95)</td>
</tr>
</tbody>
</table>

1) Values indicate the number of fecal samples.
2) Detection rates are shown in parenthesis.

Table 3 Detection rate of PCR products for fecal DNA from field samples purified by the CTAB method

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>Number of fecal masses</th>
<th>Number and rate (%) of PCR products detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 (83.3)</td>
</tr>
</tbody>
</table>

1) Values indicate the number of fecal samples.
2) Detection rates are shown in parenthesis.

and matched tissue samples will be required to analyze the reliability and reproducibility for field samples.

DNA analysis is known to be affected by errors that are the results of low sample quality, which is common for sample types such as feces [10, 11, 16-18]. Fernando et al. [16] review the analytic reliability of fecal DNA analysis in Asian elephants. They found false heterozygote or false homozygote, allelic dropout, and multiple alleles—errors that may result in the misassignment of allelic homozygotes and heterozygotes. In this study, determination of sex was not possible in some samples because PCR products were not detected by electrophoresis. However, we cannot determine whether this is simply due to errors in amplification due to the presence of PCR inhibitors, a lack of DNA quantity, or some failure in PCR. Further investigation will be necessary to examine these factors, including the use of replicate sampling from the same fecal mass or performing PCR multiple times as in Morin et al. [17].

Sexual dimorphism is not remarkable in the Japanese serow, making it difficult to determine sex based on appearance. However, by using methods, as developed in this study, we can estimate the sex of a wild individual from the field feces. In addition, it is considered that this method will be applicable to calculation of sex ratio of wild population in a given area with using individual identification method and be of some help to investigate the structure or number of local population in the future. Therefore, it is expected that the findings from this study can be used as a new tool to develop ecological study of wild Japanese serow.

要約

ニホンカモシカ（Capricornis crispus, カモシカ）は日本固有種で、特別天然記念物に指定されている。本研究では、カモシカの巣中DNAのPCR法によるアメロゲニ遺伝子（AMEL）を指標にした雌雄判別を行った。フェノール・クロロホルム法による抽出とCTAB法による精製を行い、糞中DNAを回収した。2%アガロースゲル電気泳動でPCR産物を解析した結果、250bp付近に雌では1本のホモバンド（AMEL XX）、雄では200～300bp付近に2本のヘテロバンド（AMEL XY）がそれぞれ検出された。直腸糞から抽出したDNAを解析した結果、40個体中38個体（95%）で雌雄判別が可能であり、判定された性別は主に既知の性別と一致していた。同様に、渋沢河原で採取した野外のため雌からの解析を行った結果、6巣塊のうち5巣塊で雌雄判別が可能であり（83.3%）、3巣塊が雄、2巣塊が雌のものが判定された。カモシカは性二型が顕著でないため、直接観察で雌雄を判別できない場合には、本研究で開発した雌雄判別法を利用し野外の糞から個体の性別を推定することが可能になる。
REFERENCES


