Sex Determination of Sika Deer (Cervus nippon yesoensis) Using Nested PCR from Feces Collected in the Field

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ABSTRACT. We describe a method for determining the sex of sika deer (Cervus nippon yesoensis) from feces collected in the field. Using a nested polymerase chain reaction (nested PCR), partial sequences of the sex determination region of the Y chromosome (SRY) gene and X zinc finger protein (ZFX) gene were amplified. In 19 individuals with sex information, the correct sex was successfully detected and sequences of target amplicons were completely matched between muscle and feces from the rectum. Among 75 fecal samples collected noninvasively in the field, 68–71 samples (90.7–94.7%) yielded successful sex determinations. Using this technique, feces collected in the field would enhance the utility of genetic analysis. As a result, instead of biomaterials, these samples can serve as new or alternative materials. Finally, it can be expected that this technique will contribute to reveal in advanced detail of the population dynamics and genetic diversity that needed to carry out effective population control and to reduce the extinction risk of sika deer.

KEY WORDS: feces, nested PCR, noninvasive, sex determination, sika deer.

Currently, because of the over population of sika deer (Cervus nippon) in Japan, problems such as damage to agriculture and natural vegetation have markedly increased. One subspecies of sika deer, Cervus nippon yesoensis, is distributed widely in Hokkaido, the northernmost major island of Japan. The Hokkaido Government has established the conservation and management plan for sika deer in eastern Hokkaido [10], and has promoted aggressive population control [14].

From the standpoint of the conservation genetics, genetic analyses can contribute to carrying out effective population control by revealing population dynamics, especially seasonal migration, and to reducing the extinction risk by revealing genetic diversity [8, 9]. So far, several studies have been conducted on sika deer using biomaterials such as blood, muscles and liver tissue or bone [18–21, 28].

Although the DNA of biomaterials is easy to extract, it is sometimes difficult to collect these samples due to the cost and effort involved in capturing and to capture limitations, especially in wildlife protection areas. In this situation, fecal samples that can be collected noninvasively are simply counted and sequences of target amplicons were completely matched between muscle and feces from the rectum. Among 75 fecal samples collected noninvasively in the field, 68–71 samples (90.7–94.7%) yielded successful sex determinations. Using this technique, feces collected in the field would enhance the utility of genetic analysis. As a result, instead of biomaterials, these samples can serve as new or alternative materials. Finally, it can be expected that this technique will contribute to reveal in advanced detail of the population dynamics and genetic diversity that needed to carry out effective population control and to reduce the extinction risk of sika deer.

When applying fecal DNA to PCR, there are several important factors to keep in mind to achieve reliable genotyping. First, low DNA quality and quantity affected by degradation and the presence of PCR inhibitors in the extract may produce poor results [27]. Second, the unavailability of target or primer DNA by nonspecific blocking or sequestration may result in misleading band variations [31]. For instance, in a study using feces for the sex determination of red deer (Cervus elaphus) reported by Huber et al. (2002), it was suggested that a faint band may produce unresolved results. Therefore, not only a high sensitivity but also a high specificity method of genotyping will be required. To achieve this requirement, nested PCR often used for a pathogen diagnosis from fecal sample [7, 13] may improve genotyping reliability.

The aim of this study is to develop a new method for the sex determination of sika deer from feces collected in the field. With that in mind, partial sequences of SRY gene on the Y chromosome and ZFX gene on the X chromosome were amplified using nested PCR. In addition, to simplify the procedure, a commercial kit was used for DNA extraction because of its rapidity and simplicity of use.

MATERIALS AND METHODS

Sample collection: Sampling was conducted on the Shiretoko Peninsula, Hokkaido, Japan in the winter seasons of 2009 and 2010. From 19 wild sika deer, including 4 males and 15 females, sets of muscle and feces from the rectum were collected. In addition, 75 fecal samples on the snow which were suspected to have been defecated within a few days were collected noninvasively in the field. All samples were stored at –30°C until DNA extraction.
DNA extraction: For DNA extraction, commercial kits, the Qiagen Blood and Tissue Kit (Qiagen, Tokyo, Japan) and the QIAamp DNA Stool Mini Kit (Qiagen) were used for muscle samples and for fecal samples from both the rectum and the field, respectively. For fecal samples, some procedures were modified. First, one fecal pellet was generally used. In case the feces were suspected to be from a fawn, two pellets were used. Almost all the outer pellet layer of each fecal pellet scraped with a surgical blade was applied as fecal material. Since the weight of feces changes depending on its moisture content and, as has been suggested by Wehausen et al. (2004), the outer pellet layer contains a significant amount of high quality template DNA. Second, 1.5 ml of Buffer ASL was added to each fecal sample to accumulate enough supernatant following centrifugation, while a pestle (LMS, Tokyo, Japan) was used to crush fecal material to obtain the maximum DNA concentration. Finally, 100 µl of Buffer AE was used for the final elution.

Designing of primers: The SRY gene on Y chromosome and the ZFX gene on X chromosome were used for sex determination. To prevent sex misidentification, it was necessary to provide a higher level of PCR sensitivity to the SRY gene than that to the ZFX gene. Therefore, primers were designed to induce an amplicon sequence of the SRY gene shorter than that of the ZFX gene. As external primers for a primary reaction and internal primers for a nested reaction against both genes, a previously reported primer was used or new primers were designed using PRIMER3 computer software [26]. In the SRY gene, the sequence data of sika deer (Cervus nippon yesoensis) available from the DDBJ (DNA Database of Japan; accession no. AB247627). MT1 reported by Takahashi et al. (1998) and, designed primer, DSRY1-H were used as external primers amplifying a 290-bp fragment. DSRY2-L and DSRY2-H were designed as internal primers amplifying a 113-bp fragment (Fig. 1). In the ZFX gene, due to a lack of sequence data for sika deer in the DDBJ, new primers were designed using the Big Dye Terminator cycle sequencing kit (Applied Biosystems), and was analyzed on an ABI 3100 DNA Sequencer (Applied Biosystems).

Amplification test for muscle samples: To check whether target genes were amplified, the muscle DNAs of 19 individuals were applied to nested PCR, and PCR products were visualized after both reactions. Sex was determined based on band patterns, and sequences of the target amplicons from both reactions were detected.

Sequence analysis: The target amplicon was purified from the gel using a Nucleo Spin Extract II kit (Macherey-Nagel, Tokyo, Japan). A direct sequence was performed using the Big Dye Terminator cycle sequencing kit (Applied Biosystems), and was analyzed on an ABI 3100 DNA Sequencer (Applied Biosystems).

Amplification test for fecal samples from the rectum: Fecal DNAs from the rectum of 19 individuals were applied to nested PCR, and PCR products were visualized after the nested reaction. Sex was determined based on band patterns, and sequences of the target amplicons from a nested reaction were detected. In addition, to compare PCR sensitivity between the target SRY and ZFX genes, template

Fig. 1. Sequence of the SRY gene fragment on Y chromosome and 2 sets of primers for nested PCR in sika deer.
DNAs of 4 males with different concentration were applied to nested PCR, and amplification of the target band was confirmed. Template DNA was prepared at a concentration of 10–0.01 ng/µl by a serial 10-fold dilution, and each 1 µl was applied to nested PCR.

**Application to fecal samples collected in the field:** The DNAs of 75 fecal samples collected in the field without sex information were applied to nested PCR, and PCR products were visualized after the nested reaction. Sex was determined based on band patterns. The trials of sex determination were conducted two times to ensure the PCR results and check the PCR error. Furthermore, in the 4 samples including the 2 males and 2 females randomly selected, sequences of the target amplicons from the nested reaction were detected.

**RESULTS**

**Amplification test for muscle samples:** In both primary and nested reactions, the bands for target molecular weights were detected without any extra band, and sequences were successfully determined in all samples. The results of sex determination based on band patterns were matched with the actual sex (Fig. 3). In the SRY gene, the amplicon sequences of all males were matched with the reported sequence (DDBJ; accession no. AB247627). In the ZFX gene, there was an over 97% homology between the amplicon sequences and the reported sequence of red deer (DDBJ; accession no. DQ415950), with a single repeated sequence of 8 bases detected in 1 sample of male (Fig. 2).

**Amplification test for fecal samples from the rectum:** The bands for target molecular weights were detected without any extra band, and the sequences were successfully determined in all samples. The results of sex determination and amplicon sequences were completely matched with the results of muscle samples (Fig. 3). In a comparison of PCR sensitivity between the target SRY and ZFX genes, following dilution of template DNA, the band for SRY gene existed at a lower concentration than that for ZFX gene in 2 samples, and both bands became negative at the same con-
Application to fecal samples collected in the field: The bands for target molecular weights were detected without any extra band (Fig. 4). Sex determination was applicable in 71 of 75 samples (94.7%; 19 males and 52 females). In two trials of sex determination, 68 samples (90.7%) showed the same results, and 3 samples (4%) showed the band in either trial. Among 19 fecal samples determined as male, 1 sample only had a band for the SRY gene. There was no amplification in 4 samples (5.3%). The amplicon sequences of the 4 samples matched the sequences identified in this study.

DISCUSSION

In muscle samples, the availability of primers was demonstrated by the result that target amplicons were detected without any extra band after both reactions, while sex determination results were matched with the actual sex. Though a repeated sequence of 8 bases was detected on the ZFX gene in 1 sample of male (Fig. 2), there was no effect on sex determination, because an amplicon sequence for the ZFX gene was designed to be sufficiently longer than that of the SRY gene. There was no amplification in 4 samples (5.3%). The amplicon sequences of the 4 samples matched the sequences identified in this study.

Table 1. Results of PCR sensitivity between the target ZFX (X) and SRY (Y) genes using fecal DNA from the rectum of 4 males

<table>
<thead>
<tr>
<th>Template DNA (ng/µl)</th>
<th>Male 1</th>
<th>Male 2</th>
<th>Male 3</th>
<th>Male 4</th>
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<tbody>
<tr>
<td></td>
<td>X</td>
<td>Y</td>
<td>X</td>
<td>Y</td>
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<td>10³</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>1.0</td>
<td>–</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>0.1</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<td>0.01</td>
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</table>

| a) Each 1 µl of DNA was applied to nested PCR. b) Positive with band (+) or negative without band (−). c) General concentration of nested PCR in this study.

As for fecal samples collected in the field, the success rate of sex determination was 90.7% (68/75), even when 3 samples (4%) with a band in either trial were excluded. This success rate was higher than that of the 85% demonstrated in red deer previously reported by Huber et al. (2002). In 8 samples (10.7%) including 3 samples with a band in either trial, 1 sample with only a band for the SRY gene and 4 others without an amplicon, those results might have been caused by the low amount of DNA, DNA degradation or PCR inhibitors [27]. In the present nested PCR, it was easy to determine the sex because the bands appeared quite obvious in almost all samples and there was no extra band. These results supported the utility of the present nested PCR for sex determination from fecal samples collected in the field.

When applying this method to a different location or season without modification, it is important to collect fresh feces containing a high quality and quantity of DNA for maximizing the efficiency of genotyping. In such a case,
several points should be kept in mind. As for factors related to the DNA condition, climate including temperature, humidity and rainfall, as well as diet or elapsed time from defecation are suggested [3, 17, 24]. For feces in a low DNA condition, repeating fecal DNA extraction or PCR amplification will be needed due to allelic dropout and false alleles resulting from DNA degradation [2, 11, 25]. Therefore, a pilot study for determining a suitable strategy was recommended.

With such a method for sex determination, feces collected noninvasively in the field would enhance the utility of genetic analysis. As a result, those samples can consist of new or alternative materials instead of biomaterials. From a population demographic perspective, estimating sex ratios and monitoring trends in abundance can be available [16, 29]. By combining them with other genetic analyses using mitochondrial DNA or microsatellite DNA [18–22], advanced details of population dynamics and genetic diversity can be revealed [4]. Finally, it can be expected that these combined analyses would contribute to carry out effective population control and to reduce the extinction risk of sika deer.

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REFERENCES


