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APPLICATION ESSAYS

Species identification based on the faecal DNA samples of the Japanese serow (*Capricornis crispus*)

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Abstract The Japanese serow (*Capricornis crispus*) is endemic to mountain regions in Japan. Due to the difficulty of directly observing Japanese serows, traces such as faeces have been utilized to study their distribution and estimate their population size. However, the recent population increase of sika deer (*Cervus nippon*) often leads misidentification of sika deer faecal pellets as those of Japanese serows. Therefore, we developed species identification methods to differentiate between the Japanese serow and sika deer based on mtDNA cytchrome *b* gene sequences, and applied the method to faecal samples collected for a survey program on the Japanese serow. In 67 field-collected faecal samples examined, we could amplify mtDNA at relatively high PCR succession rates (86.6%). Species identification based on PCR–RFLPs using the cytochrome *b* gene revealed that 41.4% of faecal pellets of sika deer were misidentified as being from Japanese serow. This suggests that the present population size of the Japanese serow may be overestimated. This species identification method eliminates such misidentifications and should become a powerful tool for studying Japanese serow, including their distribution, precise population size, and estimations of density.

Keywords Faecal DNA · Japanese serow · Species identification

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Introduction

Genetic analysis using non-invasive samples, such as faeces, has provided a breakthrough for the conservation of elusive or endangered animal species. However, PCR amplification of target DNA regions from faecal samples is difficult due to the poor quality and low quantity of DNA resulting from degradation and the presence of PCR inhibitors. Recently, several methods were developed for DNA extraction from faeces, including a collection method, sample preservation, and PCR amplification (Brinkman et al. 2009; Goossens et al. 2000; Nsubuga et al. 2004; Piggott et al. 2004; Zhang et al. 2006). Although problems with non-amplification, false alleles, and allelic dropout in microsatellites remain (Brinkman et al. 2009; Murphy et al. 2007), faecal DNA analysis has become popular for inferring phylogeny or intraspecific hybridization and for analyzing population genetics (Adams et al. 2003; Ernest et al. 2000; Matsui et al. 2007).

Species identification based on faecal DNA is effective in regions where several animals with similar faeces are present. Faecal DNA-based species identification has been primarily applied to carnivores such as bears, martens, lynxes, tigers, and cats, as well as primates such as macaques and gorillas (Dalèn et al. 2004; Chu et al. 2006; Kurose et al. 2005; Livia et al. 2007; Sugimoto et al. 2006). It has been possible to successfully analyze multiple faecal samples collected in the field. In addition to these animals, faecal DNA-based methods for identifying species, sex, and individuals have been developed for several ungulate species (Kim et al. 2008; Miyazaki et al. 2001; Yamauchi et al. 2000), however, few empirical studies involving large numbers of faecal samples have been conducted because tissue samples are readily available through hunting (Brinkman et al. 2009). Furthermore, superior techniques in terms of cost, time, and precision exist for estimating the population size and density of ungulates (Brinkman et al. 2009).

The Japanese serow (*Capricornis crispus*) is endemic to mountain regions of Honshu, Shikoku, and Kyushu in Japan. Hunting in the early twentieth century severely reduced its numbers. Therefore, it has been protected as a "national monument" under the Japanese Law for Protection of Cultural Properties (LPCP) since 1934 and was promoted to "special national monument" status in 1955 (Tokida and Miura 1988). At present, some controlled hunting is permitted in Gifu, Nagano, and Shizuoka Prefectures in central Honshu of Japan; however, no predictions regarding population recovery have been made for the prefectures of Shikoku or Kyushu (Tokida 2008).

The Japanese Agency of Cultural Affairs has monitored population trends and habitat conditions of the Japanese serow since 1985. For each protected area, a primary survey program is conducted by specialists every 6 or 7 years to monitor population indices (i.e., habitat change, damage to habitat, and an estimate of population size based on field traces), a supplemental survey program is conducted annually by local volunteers (Tokida 2008). Because most field traces (such as feeding marks) of the Japanese serow are similar to those of the sika deer (*Cervus nippon*), information from faecal pellets is the most reliable sign in the field. However, the faecal pellets of Japanese serow and sika deer are sometimes difficult to be distinguished in the field.

Faecal dropping behavior does differ between the two species, with the Japanese serow depositing more than twice the number of pellets than the sika deer (Takatsuki et al. 1981). The habitat of the sika deer population has been increasing conspicuously throughout Japan in recent years, and it overlaps that of the Japanese serow (Tokida 2008). This makes it more difficult to distinguish between faeces of the sika deer from those of the Japanese serow. Because current estimates of population size are generated by counting faecal pellet groups, proper differentiation of faeces between Japanese serow and sika deer is crucial for the Japanese serow conservation program.

We developed an identification technique to differentiate between the Japanese serow and the sika deer based on mitochondrial cytocrome *b* gene (Cyt *b* gene) sequences, and applied the technique to field-collected faecal samples. The method eliminates problems with false positive samples because the enzyme-digestion patterns of the two species are sufficiently different.

Materials and methods

Samples and DNA extraction

We used tissue samples from four Japanese serow and four sika deer collected from individuals killed in traffic accidents or by hunting in Oita and Shizuoka Prefectures. Faecal samples from two Japanese serow (one male and one female) were obtained from the Tokushima Zoo. In addition, faecal samples were collected from the rectums of two sika deer (one male and one female) killed in a pest-control program in Tokushima Prefecture. We also used 67 faecal samples morphologically estimated as faeces of the Japanese serow by volunteer researchers during the supplemental survey program in 2008 in Tokushima Prefecture.

Tissue samples and approximately 20 g of each faecal sample were preserved in 100% ethanol at room temperature until DNA extraction. Total genomic DNA was extracted from tissue samples by the phenol/proteinase K/sodium dodecyl sulfate methods of Sambrook et al. (1989). For faecal samples, DNA was extracted following the methods of Zhang et al. (2006).

PCR restriction fragment length polymorphism (RFLP) analysis for species identification

To amplify the mitochondrial Cyt *b* gene of Japanese serow and sika deer, we designed primers on the conserved region for both species based on sequence information from the DDBJ/EMBL/GenBank databases (Accession nos: Japanese serow, AB097256-60, Min et al. 2004; sika deer, AB021093, Kuwayama and Ozawa 2000). For all tissue and faecal samples, we conducted PCR using the primer Cb-GS2 (5′-TGA GGA CAA ATA TCA TTC TGA GG-3′) and Cb-GSR2 (5′-ATT GAT CGT AGG ATT GCG TAT GC-3′). PCR reaction mixtures contained 1.5 mM $MgCl_2$, Taq buffer, 0.2 mM of each dNTP, 0.25 μM of each primer, 0.5 U/μl Ex Taq polymerase (Takara Bio), and distilled water to a total volume of 25 μl. The reaction conditions were 94°C for 2 min, 30 cycles of 94°C for

30 s, 55°C for 30 s, and 72°C for 2 min, followed by a final extension for 10 min at 72°C. When we could not amplify sufficient target fragments, we employed nested PCR using outer primers Cb-GS1 (5′-ATT CAT ACA CGT AGG ACG AGG C-3′) and Cb-GSR1 (5′-TGT CCT CCA ATT CAT GTG AG T-3′) and inner primers GS2 and GSR2. The reaction mixture of the first PCR amplification was the same as described above, except use of Cb-GS1 and Cb-GSR1 (0.05 M each) as outer primers. The reaction conditions were 94°C for 2 min, 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, followed by a final extension for 10 min at 72°C. In the following PCR amplification with inner primers (GS2 and GSR2), the reaction mixture and conditions were the same as described above, except used of the first PCR product (1 μl) was used as the template.

PCR products were purified with a 2% low-melting-point agarose gel and digested by a restriction enzyme, *Bsm*AI (GTCTC/), following the manufacturer's instructions (Promega).

Results and discussion

We amplified the Cyt *b* genes in 58 of 67 (86.6%) faecal samples. Several previous studies have reported similarly high rates (65–96%) of successful PCR amplification of mtDNA from faecal samples of wild carnivores and primates (Chu et al. 2006; Kurose et al. 2005; Murphy et al. 2007; Palomares et al. 2002; Sugimoto et al. 2006).

The molecular size of the PCR products for the Cyt *b* gene by using the primers Cb-GS2F and Cb-GS2R were 404 bp both for the Japanese serow and sika deer. The restriction enzyme *Bsm*AI produced fragments that were 327 bp and 77 bp for the Japanese serow and 225 bp and 179 bp for the sika deer (Fig. 1). Fifty-four of the 58 PCR products from field-collected faecal samples were successfully digested with BsmAI (Fig. 2). Of the 54 amplified field-collected faecal samples (which had been identified by morphology as Japanese serow samples), 55.2% were found to be Japanese serow, and 41.4% were sika deer, based on banding patterns after BsmAI digestion. This indicates that many sika deer faecal pellets were misidentified as being from Japanese serow by volunteer researchers.

Counts of faecal pellet groups are commonly used to indirectly estimate population size and density of ungulates (Mayle et al. 1999). However, in habitats where several ungulate species coexist, density estimates are often in error due to the misidentification of faeces (Theuerkauf et al. 2008). At present, distribution studies and population size estimations for Japanese serow also involve counting faecal pellet groups. The results of the present study and the compiled data on the distribution of Japanese serow as determined by supplemental survey programs from 1996 to 2001 in Tokushima Prefecture are shown in Fig. 3. Our results revealed that the range of Japanese serow and sika deer overlapped in several areas. Moreover, we identified

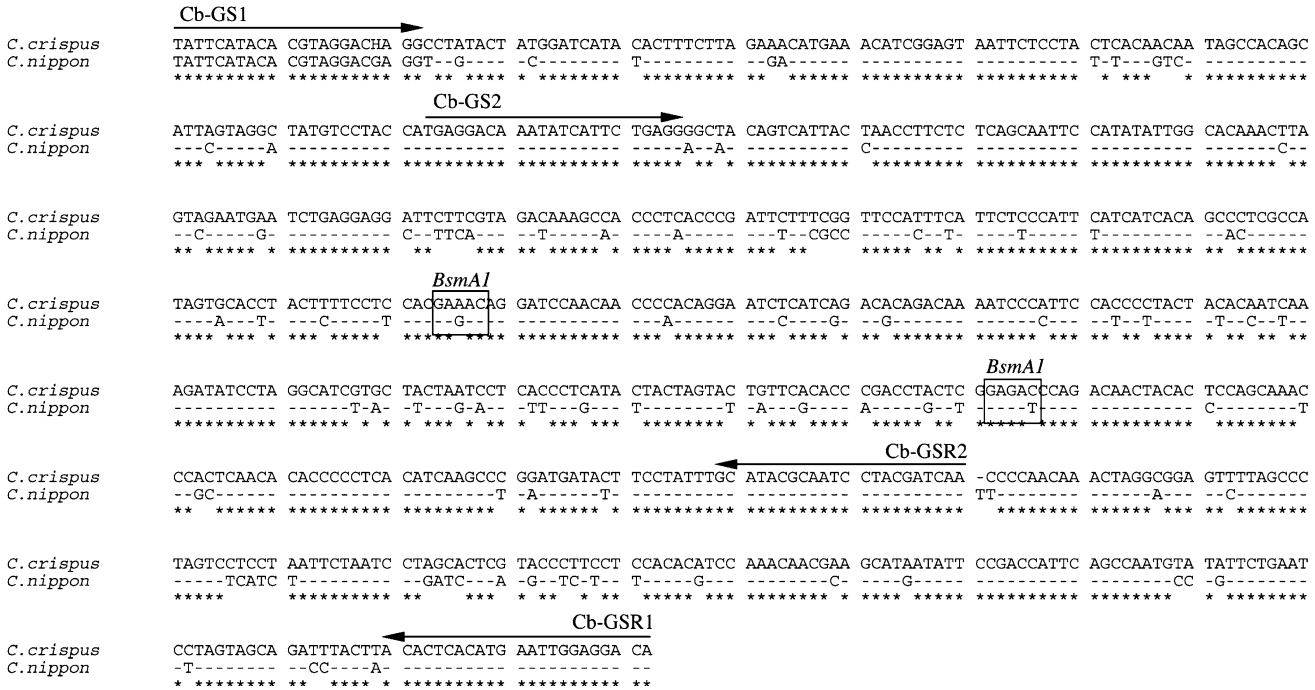

Fig. 1 Partial mitochondrial cytochrome *b* sequences and *Bsm*AI restriction sites of the Japanese serow and sika deer

several locations with no previous distribution record. PCR–RFLP based species identification revealed that the misidentification of faecal samples between the Japanese serow and sika deer was lowest in the west and south mountain regions (i.e., the Turugi mountain range and Kaifu mountainous region, respectively), which has been considered the central distribution area of Japanese serow in Tokushima Prefecture for the past few decades. In contrast, misidentified samples were primarily found in the other areas. The Tokushima and Kochi Prefecture Board of Education (2004) reported that the distribution of Japanese serow has expanded gradually from its central habitat, and its population increased until reaching approximately 2,700 individuals in Shikoku. However, our results indicate that the current distribution of the Japanese serow is probably overestimated.

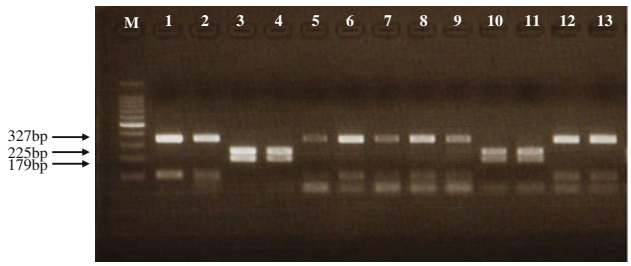

Fig. 2 Species identification by PCR-RFLP using tissue and faecal samples from Japanese serows, sika deer, and field-collected faecal samples. tissue sample of Japanese serow *1*, faecal sample of Japanese serow *2*, tissue sample of sika deer *3*, faecal sample of sika deer *4*, field-collected faecal samples *5–13, M* Molecular size marker, 100 bp ladder

The misidentification of faecal pellets between Japanese serow and sika deer stems from two primary causes, namely, the pellets' high morphological resemblance and recent increases in distribution overlap between the two species. A few decades ago, the Japanese serow was restricted to temperate forests in higher mountains and its range did not overlap with that of the sika deer (Tokida 2008). Recently, however, populations of sika deer have increased conspicuously, and the range of the species has expanded. In Shikoku, the sika deer's distribution has spread toward high-altitude areas, and currently overlaps with that of the Japanese serow. The population density of the sika deer in overlapping areas has been estimated at 2.1–52.2 deer/km^2 (Tokushima and Kochi Prefecture Board of Education 2004). The faecal piles and the amount of faeces per defecation of Japanese serow (200–360 pellets/defecation, 810–980 pellets/day) are larger than those of sika deer (81–95 pellets/defecation, 880–1,200 pellets/day) (Takatsuki et al. 1981), which should allow for

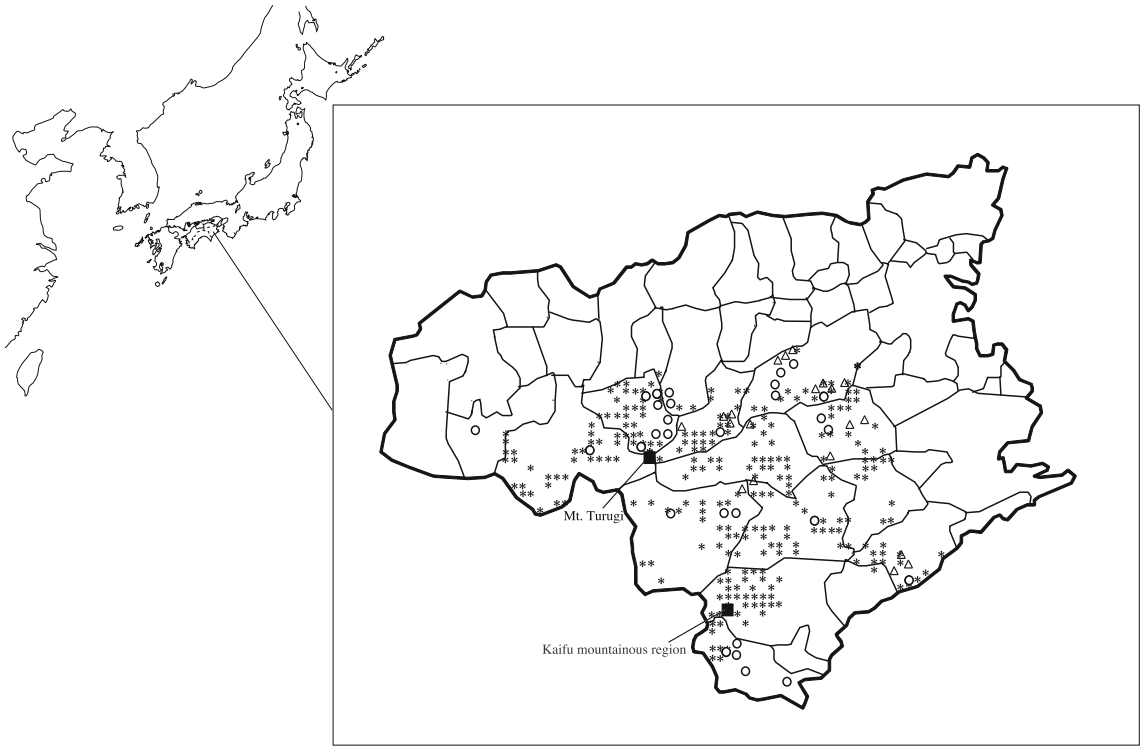

Fig. 3 Distribution map of the Japanese serow in Tokushima Prefecture. *Circle* indicates faecal samples of Japenese serow and *triangle* indicates those of sika deer (based on faecal DNA samples). *Asterisk* indicates the compiled data on the distribution of Japanese serow as determined by supplement survey programs from1996 to 2001 in Tokushima Prefecture (Tokushima and Kochi Prefectures Board of Education 2004)

reliable discrimination between the two species. However, in places where the density of the sika deer is high, faecal deposits from several individuals may become piled, producing samples that look like the faeces deposits of Japanese serow.

Volunteers also sometimes make mistakes and misidentify faecal piles. The supplemental survey program for Japanese serow has been conducted annually by local volunteers. Engel and Voshell (2002) pointed out several benefits of using volunteers for biological monitoring, i.e., groups of volunteers can sample multiple locations at one time, and local volunteers can monitor changing conditions and report in a timely fashion because they live in the region being monitored. The supplemental survey program for Japanese serow can monitor a wider area than the primary survey program. Moreover, the results of the supplemental survey reveal annual changes in habitat conditions for Japanese serow. However, there are limits to each volunteer's ability to collect precise data. Therefore, the misidentification of some faecal samples is unavoidable using the methods employed to date. The species identification method developed here can eliminate the misidentification of faeces and will become a powerful tool for precise estimation of population size or density for Japanese serow by overcoming the weakness of the faecal pellet-group counting method. Applying this method to the supplemental survey program will not only contribute to the precise estimation of Japanese serow distribution and population density, but also improve the monitoring of long-term population dynamics.

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