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Fecal DNA Analysis for Identifying Species and Sex of Sympatric Carnivores: A Noninvasive Method for Conservation on the Tsushima Islands, Japan

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Abstract

Fecal analysis is a useful tool for the investigation of food habits and species identity in mammals. However, it is generally difficult to identify the species based on the morphological features and contents of feces deposited by mammals of similar body size. Therefore we developed noninvasive DNA analysis methods using fecal samples for identification of the species and sex of four small sympatric carnivores living on the Tsushima Islands of Japan: the leopard cat (*Felis bengalensis*), Japanese marten (*Martes melampus*), Siberian weasel (*Mustela sibirica*), and feral cat (*Felis catus*). Based on DNA sequence data from previous phylogenetic studies, we designed species-specific primers for polymerase chain reaction (PCR) amplification of the partial mitochondrial cytochrome *b* gene (112–347 bp) to identify the species and primers for the partial SRY gene (135 bp) to determine the sex. Due to the adjustment of PCR conditions, those specific DNA fragments were successfully amplified and then applied for species and sex identification. Nucleotide sequences obtained from the PCR products corresponded with cytochrome *b* sequences of the carnivore species expected. The protocol developed could be a valuable tool in the management and conservation of the four carnivore species occurring on the Tsushima Islands.

On the Tsushima Islands of Japan, which are located between Kyushu and the Korean Peninsula, five carnivore species are sympatrically distributed: the Tsushima leopard cat (*Felis bengalensis euptilura*), Tsushima marten (*Martes melampus tsuensis*), Siberian weasel (*Mustela sibirica coreana*), domestic cat (*Felis catus*), and domestic dog (*Canis familiaris*) (Abe and Ishii 1987). The Tsushima Islands have an area of approximately 708 km^2, with a length (north–south) of approximately 82 km and a width (east–west) of approximately 18 km. The forests, where these animals reside, are mostly secondary forests and occupy approximately 89% of the islands, while agricultural fields comprise approximately 3% of the islands. Although the leopard cat is distributed widely in south and east Asia, in the Japanese islands the Tsushima leopard cat occurs only on the Tsushima Islands. It is considered an endangered species because of its reduced (estimated at less than 100 individuals) population size (Izawa and Doi 1991; Izawa et al. 1991). Recently in the southern areas, which have been developed more by human activities than the northern areas, no traces of the leopard cat have been found. The Tsushima marten is an isolated population and is a subspecies of the Japanese marten, which is an indigenous species to Japan; their population is also thought to be reduced (Tatara 1994; Tatara and Doi 1991). These two endangered carnivores are designated as natural monument species in Japan. In addition, although the Siberian weasel is distributed widely on the eastern Eurasian continent, on the Japanese islands the native population lives only on the Tsushima Islands. This weasel is distributed over the islands and, to date, a reduction in their population size has not been reported. Thus the Tsushima Islands have both Japan-endemic and continental species of carnivores because of their migration via land bridges which were formed in the Pleistocene between the islands, the Japanese main islands, and the Korean Peninsula (Abe and Ishii 1987).

The domestic cat was introduced to the Tsushima Islands via human activity, resulting in transmission of feline immunodeficiency virus (FIV) to the Tsushima leopard cat (Doi and Izawa 1997; Nishimura et al. 1999). In addition, feral dogs are seen on the Tsushima Islands. There is an urgent need to clarify the precise distribution of the five carnivores and the biological relationships between them to promote an effective conservation plan.

Generally, analysis of feces has been used to identify the species and food habits of animals. The information obtained from feces is important to elucidate behavioral and ecological features of target animals. However, it is often difficult to identify the species among sympatric animals with similar body sizes and food habits (Tatara and Doi 1994). In such cases, fecal DNA analyses can provide species identification. For instance, in order to investigate the population structure of brown bears (*Ursus arctos*), Hoss et al. (1992) developed the technique of examining the species, individual, and variability using fecal samples. They amplified two kinds of mitochondrial DNA (mtDNA) fragments of brown bears and their food plants, and evaluated the extent of genetic variation among brown bear populations. Gerloff et al. (1995) analyzed microsatellite loci using feces of wild bonobos (*Pan paniscus*) and discussed the kinship within the population. Paxinos et al. (1997) distinguished canid species using fecal DNA digested with some restriction enzymes. In Japanese mustelids such as *Martes melampus*, *Martes zibellina*, and *Mustela itatsi*, Murakami (2002) identified the species using fecal DNA and reported that the success rate of species identification was only 2.7%.

Meanwhile, it is also important to identify the sex of animals in wildlife studies in order to examine ecological and social features of animal populations. Sex determination on mammals has been performed by detection of genes on the Y chromosome, such as the SRY gene (the sex-determining region on the Y chromosome) (Abe et al. 2001), amelogenin gene (Ennis and Gallagher 1994; Yamauchi et al. 2000), and ZFX and ZFY genes (Aasen and Medrano 1990).

In the present study, we developed DNA markers and analytical techniques using fecal samples of the four sympatric carnivores from the Tsushima Islands for identification of the species and sex. We then discuss their usefulness and applications in the conservation and management of these mammals.

Materials and Methods

Samples and DNA Extraction

Muscle and fecal samples from four sympatric carnivore species (Tsushima leopard cats, Tsushima martens, Siberian weasels, and domestic cats) were obtained from field sites, zoos, and an animal clinic. Relatively larger feces in the field, which were considered to be from feral dogs, were eliminated from the sample collection. Muscles (Figure 1) and feces (Figure 2), whose original species were known, were used in order to confirm the species specificity of polymerase chain reaction (PCR) amplification using primers designed in the present study. Muscle tissues were frozen at −80°C or preserved in 100% ethanol at 4°C until use. Thirty fecal samples were collected from field sites on the Tsushima Islands and preserved in 100% ethanol at 4°C at the Tsushima Wildlife Conservation Center. The original species and sexes of these fecal samples from the field sites were unknown. Total DNAs were extracted from muscles by the phenol/proteinase K/sodium dodecyl sulfate method of Sambrook et al. (1989) with some simplified modifications as indicated by Masuda and Yoshida (1994). Total DNAs from the feces samples were extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Venlo, The Netherlands).

PCR Amplification and Sequencing for Species Identification

In order to establish a method for species identification using fecal DNA, species-specific PCR primers (Table 1) were newly designed based on mtDNA cytochrome *b* gene sequences of the four carnivore species reported in the previous studies: the leopard cat and domestic cat (Masuda and Yoshida 1995), and the Japanese marten and Siberian weasel (Kurose et al. 2000). The PCR amplification was performed in a 50 μl reaction volume including 1 μl of DNA extract, 1.25 U of r*Taq* DNA polymerase (Takara Co., Tokyo, Japan), 5 μl of 10× buffer (Takara), 4 μl of dNTPs (2.5 mM of each dNTP; Takara), and 0.5 μl of two primers (25 pmol/μl), using the conditions shown as results in Table 2. For inactivation of PCR inhibitors present in fecal DNA extracts, 20 μg of bovine serum albumin (Boehringer Ingelheim, Ingelheim, Germany) was added to the reaction mixture. Thirty-five cycles were performed with the following programs using a DNA thermal cycler (GeneAmp 9600 PCR system or 9700 PCR cycler; PerkinElmer, Wellesley, MA): denaturing at 94°C for 1 min, annealing at 50°C–67°C for 1 min, and extension at 72°C for 1 min, and the reaction was completed at 72°C for 10 min. Primer sets and annealing temperatures are summarized in Table 2. To check PCR amplification, an aliquot (10 μl) of the PCR product was electrophoresed on a 3% agarose gel.

As a control for the fecal DNA extraction procedure, we confirmed that dog mtDNA control region primers mitL57 and mitH52 (Okumura et al. 1999) amplified the expected 258 bp product from DNA extracted from dog feces. The remaining PCR products (40 μl) from fecal DNA, whose original species were unknown, were purified using a centrifugal dialysis kit (QIAquick; Qiagen). Cycle sequencing was performed using the Thermo Sequence Pre-Mixed Cycle Sequencing kit (Amersham, Piscataway, NJ). Primers were labeled with Texas red at the 5′ end position. All products were sequenced using an automated DNA sequencer (Hitachi SQ-5500; Hitachi, Tokyo, Japan) to check the reliability of the species identification by the developed PCR method.

PCR Amplification for Sex Identification

For PCR amplification of partial SRY gene regions from feces whose original species had been known or determined

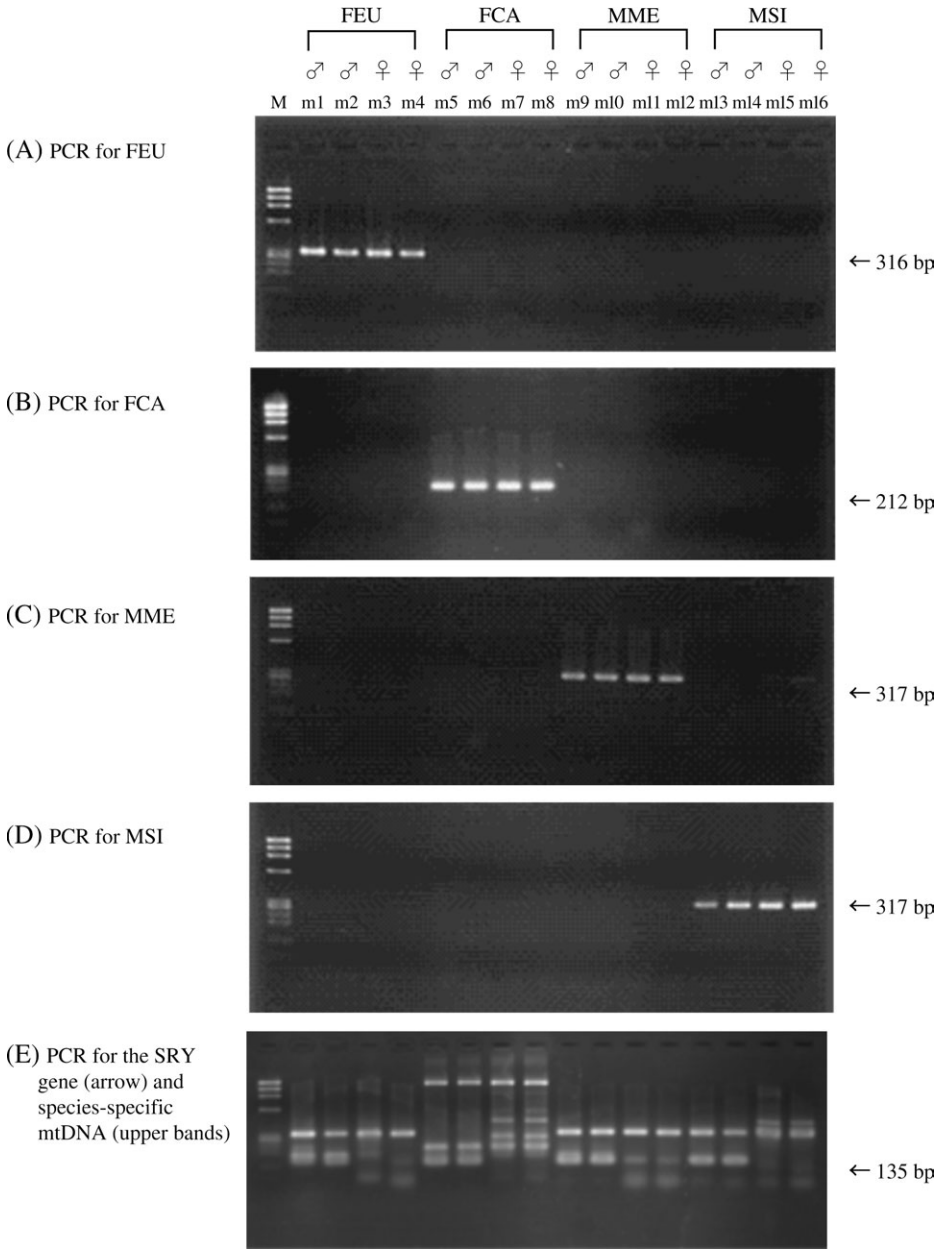

Figure 1. PCR amplification of partial fragments of mtDNA cytochrome *b* and SRY genes in 3% agarose gels using DNA extracted from species-known muscle samples. M, size marker (ϕX174/HaeIII digest). Samples m1–m16. FEU, leopard cats; FCA, domestic cats; MME, Japanese martens; MSI, Siberian weasels. (A) PCR amplification using primers Cb-FE1 and Cb-FER2, specific to leopard cats (FEU). (B) PCR amplification using primers FC-3 and FC-5R, specific to domestic cats (FCA). (C) PCR amplification using primers Cb-MM1 and Cb-MMR2, specific to Japanese martens (MME). (D) PCR amplification using primers Cb-MS1 and Cb-MSR2, specific to Siberian weasels (MSI). (E) PCR amplification using primers for the SRY gene for sex determination and those that are species specific as internal controls.

by the above mtDNA amplification method, we performed the first and nested PCRs using the following primers: RG4 and RG7 (Griffiths and Tiwari 1993) for the first PCR, and two new primers (Carni-SRY2: 5′-GTGGCTCTA-GAGAATCCCCAA-3′, and SRY-CR1: 5′-CTCTCGGTG-CATGGCCTGTAG-3′) for the nested PCR, which were modified in the present study using the sequences reported by Griffiths and Tiwari (1993) (Table 3). In the first PCR, the partial SRY gene region was amplified using the RG4/RG7 primer set with the following program: 35 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, then the reaction was completed

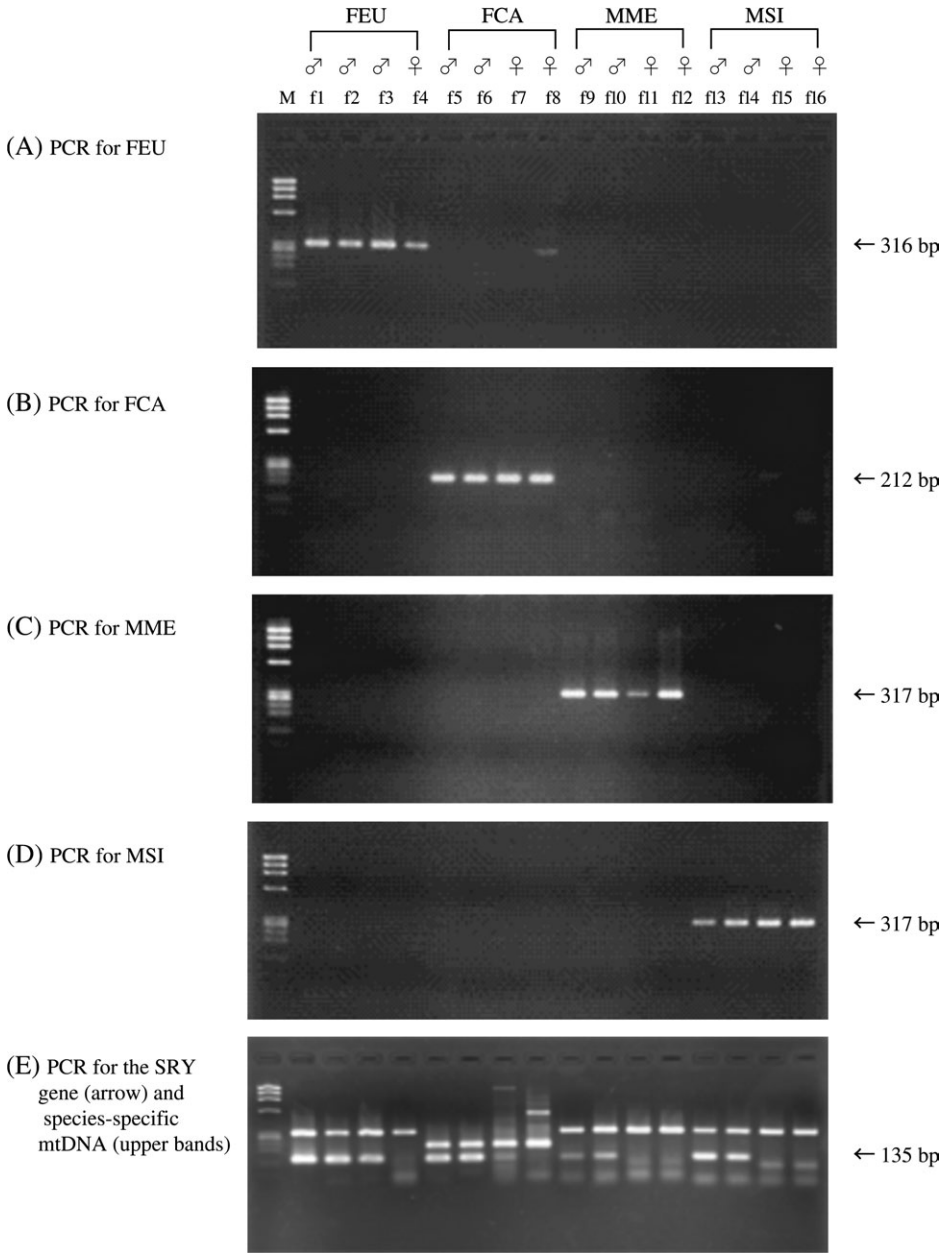

Figure 2. PCR amplification of partial fragments of mtDNA cytochrome *b* and SRY genes in 3% agarose gels, using DNA extracted from species-known fecal samples. M, size marker (ϕX174/HaeIII digest). Samples f1–f16. FEU, leopard cats; FCA, domestic cats; MME, Japanese martens; MSI, Siberian weasels. (A) PCR amplification using primers Cb-FE1 and Cb-FER2, specific to leopard cats (FEU). (B) PCR amplification using primers FC-3 and FC-5R, specific to domestic cats (FCA). (C) PCR amplification using primers Cb-MM1 and Cb-MMR2, specific to Japanese martens (MME). (D) PCR amplification using primers Cb-MS1 and Cb-MSR2, specific to Siberian weasels (MSI). (E) PCR amplification using primers for the SRY gene for sex determination and those that are species specific as internal controls.

at 72°C for 10 min. At this point the PCR products were too weak to be visualized on agarose gels. The second PCR amplification was performed using the first PCR product (5 μl), the nested primers (0.5 μl of 25 pmol/μl), and an additional DNA extract sample (5 μl). Simultaneously one of the primer sets (Table 1) for species identification was added to the reaction mixture. The partial SRY fragment and the cytochrome *b* fragment with a different molecular size from the SRY fragment were amplified using the following PCR program: 45 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, then the reaction was completed at 72°C for 10 min.

Table 1. PCR primers for identification of species which were newly designed in the present study

Targeted species	Primer name	Strand[a]	Sequence
Felis bengalensis	Cb-FE1	F	5′-ATGACCAACATTCGAAAATCA-3′
	Cb-FE2	F	5′-ATGATGGAACTTCGGCTCCCTA-3′
	Cb-FER2	R	5′-GAGCCATAATATATTCCTCGA-3′
	FE-3	F	5′-GATGGAACTTCGGCTCCCTA-3′
	FE-5	F	5′-TATCTGTCTATACATGCACGTAGGT-3′
	FE-5R	R	5′-TCGACCTACGTGCATGTATAGA-3′
	FE-6R	R	5′-GAAAGCTGTGGCTATGACTGCG-3′
Felis catus	FC-3	F	5′-GATGAAACTTCGGCTCCCTT-3′
	FC-4	F	5′-TTATGGCTGAATCATCCGATATT-3′
	FC-5	F	5′-TATCTGCCTGTACATACATGTGGGA-3′
	FC-4R	R	5′-AACTGATGAAAAGGCGGTTATTGTG-3′
	FC-5R	R	5′-CCGTCCCACATGTATGTACAGG-3′
	FC-6R	R	5′-AAAAGCTGTGGCTATGACTGTA-3′
Martes melampus	Cb-MM1	F	5′-ATGACCAACATTCGTAAAACT-3′
	Cb-MM2	F	5′-ATGATGAAACTTCGGCTCCCTC-3′
	Cb-MMR2	R	5′-GATCCATAGTATAGGCCCCGT-3′
	Cb-MMR3	R	5′-GGTATATATAAGATCCATAGTATAGG-3′
	Cb-MMR4	R	5′-CCAATGTTCCATGTTTCGGG-3′
Mustela sibirica	Cb-MS1	F	5′-ATGACCAACATTCGCAAAACC-3′
	Cb-MS2	F	5′-ATGATGAAACTTCGGCTCCCTT-3′
	Cb-MSR2	R	5′-GATCCGTAATATAAACCTCGC-3′
	Cb-MSR3	R	5′-TGAATATATAAGATCCGTAATATAAA-3′
	Cb-MSR4	R	5′-CCGATGTTTCATGTTTCGGT-3′

[a] F, forward; R, reverse.

Results

Species Identification

To design cytochrome *b*-specific PCR primers for each of the four carnivore species (leopard cat, domestic cat, Japanese marten, and Siberian weasel) of the Tsushima Islands, the previously reported sequences (Kurose et al. 2000; Masuda and Yoshida 1995) were aligned and then primer sequences were selected such that nucleotides of the 3′ ends differed among the species. Table 1 shows PCR primer sequences that successfully amplified the expected DNA fragments. For each species, two to four primers per strand were synthesized to amplify partial cytochrome *b* fragments whose molecular sizes were less than 350 bp using any combination of primers. Table 2 shows successful primer sets, the annealing temperatures of PCR amplification, and the size of the PCR products.

The PCR amplification was performed using muscle DNA in order to confirm that the expected PCR products were amplified with newly designed primers. The molecular sizes of PCR products were between 109 and 347 bp (Table 2). Figure 1A–D shows an example for PCR amplification

Table 2. PCR primer sets and program data for species identification

Targeted species	Primer set	Annealing temperature (°C)	Fragment length (bp)
Felis bengalensis	Cb-FE1; Cb-FER2	50	316
	Cb-FE2; Cb-FER2	50	230
	FE-3; FE-5R	50	212
	FE-3; FE-6R	50	296
	FE-5; FE-6R	50	109
Felis catus	FC-3; FC-4R	50	110
	FC-3; FC-5R	50	212
	FC-4; FC-6R	50	163
	FC-5; FC-6R	50	112
Martes melampus	Cb-MM1; Cb-MMR2	63	317
	Cb-MM1; Cb-MMR3	60	328
	Cb-MM1; Cb-MMR4	60	347
	Cb-MM2; Cb-MMR3	61	242
	Cb-MM2; Cb-MMR4	67	261
Mustela sibirica	Cb-MS1; Cb-MSR2	50	317
	Cb-MS1; Cb-MSR3	50	328
	Cb-MS1; Cb-MSR4	60	347
	Cb-MS2; Cb-MSR3	50	242

from muscle DNA of the four carnivore species (except for the dog) using species-specific primers for cytochrome *b*.

We then performed PCR amplification using DNA extracts from fecal samples whose original species were known. The PCR products with expected molecular sizes as to species-known fecal DNAs were amplified using those primers (Figure 2A–D). For cases in which any faint band was observed in a different species-specific PCR, in addition to a strong band in one species-specific PCR, other primer sets were selected from Table 2 and applied again for PCR of that fecal sample. For example, sample f8 had a strong band for the domestic cat with primers FC-3/FC-5R (Figure 2B) and a faint band for the leopard cat with primers Cb-FE1/Cb-FER2 (Figure 2A). In this case, the primer set FC-3/FC-4R for the domestic cat and CB-FE2/Cb-FER2 for the leopard cat (Table 2) yielded a strong band and no band, respectively (data not shown), demonstrating that sample f8 was a domestic cat. Similarly species of other samples with two or more positive bands could be identified by PCR using additional primer sets of Table 2. Thus an addition of PCR using other primers guaranteed the species identification.

Using the PCR conditions mentioned above, we identified the species and sex from feces collected in the field (Figures 3 and 4). Figure 3 shows the results of PCR amplification of fecal samples collected from the northern areas of the Tsushima Islands. Samples 3, 4, 6–10, 13, and 14 were identified as leopard cats (FEU) (Figure 3A), sample 2 was identified as the domestic cat (FCA) (Figure 3B), and sample 1 was identified as the Japanese marten (MME) (Figure 3C). Nucleotide sequences determined from these PCR products were compared with sequences of the carnivores (Kurose et al. 2000; Masuda and Yoshida 1995). As a result, samples identified as the leopard cat by the PCR method shared a partial sequence of the leopard cat cytochrome *b*. Samples identified as the domestic cat and marten by PCR showed partial sequences of cytochrome *b* for the respective species. No PCR products were obtained from samples 5, 11, and 12. Figure 4 shows the results of PCR amplification of fecal samples collected from the southern areas of the Tsushima Islands. Samples 1, 2, 6, and 8–16 were identified as the domestic cat (FCA) (Figure 4B) and sample 7 was from the Japanese marten (MME) (Figure 4C). No PCR products were obtained from samples 3–5. In addition, no products were PCR amplified from these fecal DNAs using the dog mtDNA-specific primers. No amplification with any primer sets indicates DNA fragmentation in the samples or feces from other mammalian species which are unknown. Feces from Siberian weasels was not identified among samples collected for the present study from either northern or southern Tsushima.

Sex Identification

Sex identification was performed by PCR amplification using an initial primer set RG4/RG7, followed by the nested primer set Carni-SRY-2/SRY-CR1 and the cytochrome *b* primer set as internal control. Consequently, partial SRY fragments with a 135 bp molecular size were amplified from both the muscles and feces of males, while no amplification was observed from the muscles and feces of females (Figures 1E and 2E). Nonspecific PCR products observed did not impede the sex identification. Figures 3E and 4E show the results of the sex identification using fecal samples collected from fields in the northern and southern areas of the Tsushima Islands.

Discussion

Although biological data is indispensable for wildlife and habitat conservation for the carnivores of the Tsushima Islands, basic data such as reliable distributional areas and population sizes have not been investigated in detail. Difficulties with species and sex identification of feces found in the habitat are one of the reasons. In the present study we established techniques to identify the species and sex of four carnivores using fecal samples collected in the field.

The investigative techniques applied to endangered wildlife such as the Tsushima leopard cat should be carefully considered to avoid any detrimental effects on individuals. Investigation using traditional ecological methods, such as capture traps, is generally stressful. In contrast, this procedure, which makes use of readily found fecal samples, is a noninvasive method that does not involve catching wildlife. However, it is sometimes difficult to analyze long DNA fragments when fecal samples are used, because fecal DNA is often fragmented due to the time process and environmental conditions. First, it is necessary to make PCR amplification practicable using fecal DNA. Lathuilliere et al. (2001) performed a study that compared methods of DNA extraction using fecal samples. As a result, the cetyltrimethylammonium bromide (CTAB) DNA extraction method obtained a higher success rate (70%) for PCR amplification than the silica DNA extraction method. On the other hand, in the present study, the rate of successful PCR amplification was 80% (24/30 samples; Figures 3 and 4) using the QIAamp DNA Stool Mini Kit, although the condition of the fecal samples affects the success rate of PCR amplification. Frantzen et al. (1998) evaluated the relative effectiveness on preservation methods of fecal samples for DNA analysis and suggested that storage in a DMSO/EDTA/Tris/salt solution (DETS) was most effective in preserving DNA. Moreover, they showed that preservation in 70% ethanol is good for mtDNA and short (less than 200 bp) nuclear DNA fragments. In the present study, fecal samples were stored in 100% ethanol and the PCR amplification of short DNA fragments (135–347 bp) succeeded at a high rate (Figures 2–4). On the other hand, Melanie et al. (2000) reported that fecal drying as one of the preservation methods was also available.

Using the method established in the present study, we identified the species origin of feces among the four sympatric carnivores. The partial mtDNA fragments were successfully amplified as species-specific bands (Figures 1 and 2). In one sample, even when a faint PCR band appears in a different species-specific PCR in addition to a strong band in one species-specific PCR, other PCR primer sets (Table 2)

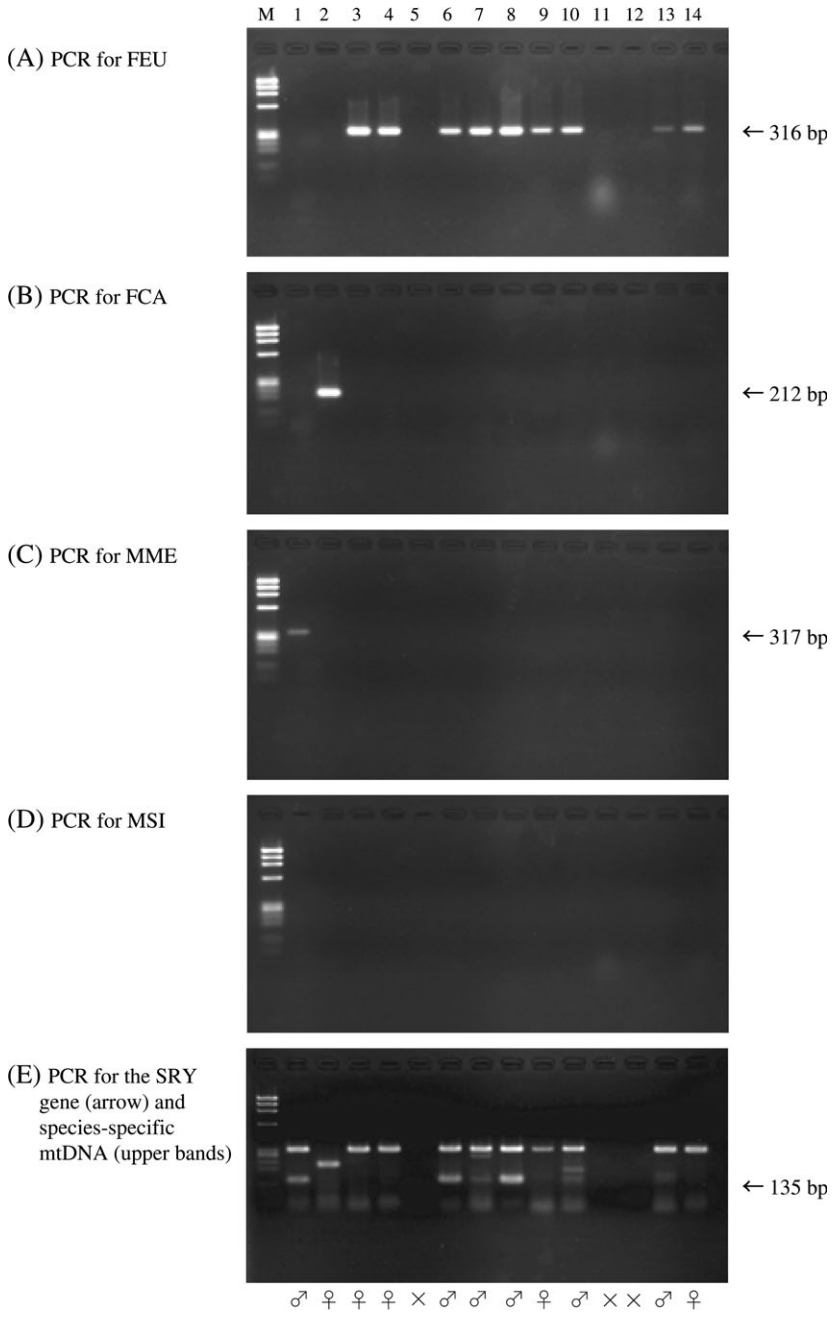

Figure 3. PCR amplification of partial fragments of mtDNA cytochrome *b* and SRY genes in 3% agarose gels using DNA extracted from species-unknown fecal samples collected from the northern Tsushima Islands. M, size marker (ϕX174/HaeIII digest). Fourteen samples were examined. FEU, leopard cats; FCA, domestic cats; MME, Japanese martens; MSI, Siberian weasels. (A) PCR amplification using primers Cb-FE1 and Cb-FER2, specific to leopard cats (FEU). (B) PCR amplification using primers FC-3 and FC-5R, specific to domestic cats (FCA). (C) PCR amplification using primers Cb-MM1 and Cb-MMR2, specific to Japanese martens (MME). (D) PCR amplification using primers Cb-MS1 and Cb-MSR2, specific to Siberian weasels (MSI). (E) PCR amplification using primers for the SRY gene for sex determination and those that are species specific as internal controls. Consequently sample 1 was judged a male marten and sample 2 was a female domestic cat. No PCR products were obtained from samples 5, 11, and 12. The other nine feces samples were from leopard cats with clear sexing.

specific to each species are able to confirm the species identification. In addition, nucleotide sequences supported the species identified by the PCR method developed. In the present study, no feces positive to the dog were found, probably because relatively larger feces estimated as those from the dog were not collected from the Tsushima Islands. Such

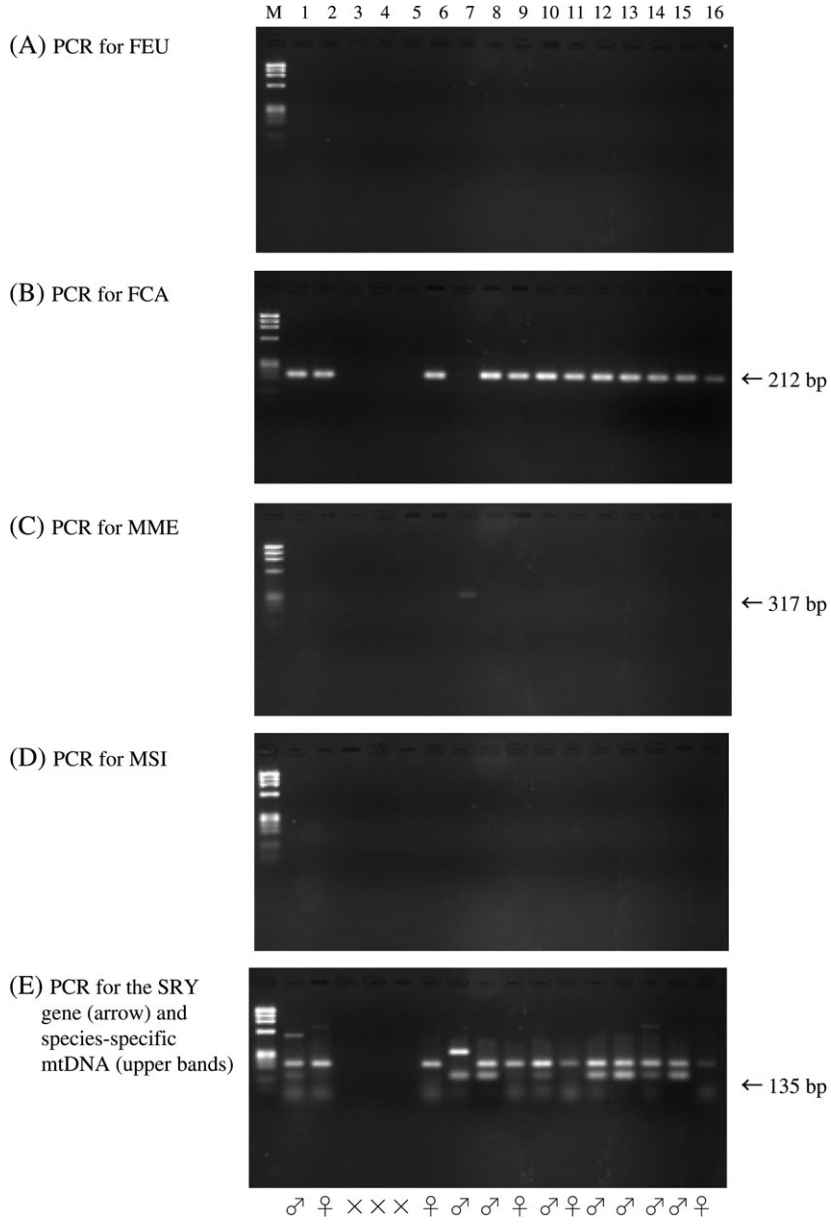

Figure 4. PCR amplification of partial fragments of mtDNA cytochrome *b* and SRY genes in 3% agarose gels using DNA extracted from species-unknown fecal samples from the southern Tsushima Islands. M, size marker (ϕX174/HaeIII digest). Sixteen samples were examined. FEU, leopard cats; FCA, domestic cats; MME, Japanese martens; MSI, Siberian weasels. (A) PCR amplification using primers Cb-FE1 and Cb-FER2, specific to leopard cats (FEU). (B) PCR amplification using primers FC-3 and FC-5R, specific to domestic cats (FCA). (C) PCR amplification using primers Cb-MM1 and Cb-MMR2, specific to Japanese martens (MME). (D) PCR amplification using primers Cb-MS1 and Cb-MSR2, specific to Siberian weasels (MSI). (E) PCR amplification using primers for the SRY gene for sex determination and those that are species specific as internal controls. The results show that sample 7 was a male marten and samples 3–5 had no amplification. The other 12 feces samples were judged to be domestic cats showing clear sexing.

an outcome helps investigations of detailed distribution areas and the relationships among the carnivores. Likewise, some studies to identify the species using fecal DNA have been reported.

To investigate endangered San Joaquin kit foxes (*Vulpes macrotis mutica*), Paxinos et al. (1997) developed mtDNA markers to be amplified using fecal DNA, and then DNA was digested using three restriction enzymes. The resultant restriction profiles discriminated among five sympatric canid species (kit fox, red fox [*Vulpes vulpes*], gray fox [*Urocyon cinereoargenteus*], coyote [*Canis latrans*], and domestic dog). In contrast with their method, the method developed in

the present study has an advantage that species identification is possible only by PCR amplification. Fecal DNA has also been used to study dietary separation and to identify the species among four sympatric carnivores (the puma [*Felis concolor*], jaguar [*Panthera onca*], ocelot [*Felis pardalis*], and crab-eating fox [*Cerdocyon thous*]) Farrell et al. 2000). In the present study, the PCR primers were designed specifically to the cytochrome *b* fragment of each species, therefore no products from the prey were contained in the feces. This was confirmed by DNA sequencing. Palomares et al. (2002) reported their analytical method using fecal samples in order to investigate the presence and distribution patterns of an elusive carnivore (the Iberian lynx [*Lynx pardinus*]). Species identification using fecal DNA is very effective and efficient because fecal samples are collected noninvasively.

For sex determination in the present study, a second (nested) PCR was needed, because SRY is a single-copy gene, while there are numerous copies of the mtDNA cytochrome *b* gene per cell. Consequently some nonspecific PCR products in addition to SRY fragments were found (see Figures 1E, 2E, 3E, and 4E). Since the primers were developed to amplify the SRY fragment across several species, there is a concern that they might amplify some pseudogenes or other homologues from the female genome. In some cases, such as samples m11 and m12 of Figure 1E, weak bands from females appeared to comigrate with the Y-specific band. By comparing the band intensity to that of the internal control mtDNA cytochrome *b*, these discrepancies could be resolved. In the human and cattle, the X-Y homologous amelogenin gene is used to determine the sex. As for the amelogenin X (on the X chromosome) and amelogenin Y (on the Y chromosome), the molecular size is different (Ennis and Gallagher 1994; Gibson et al. 1992; Salido et al. 1992). The assay using this marker was available for the sex determination of wildlife such as the sika deer (*Cervus nippon*) (Yamauchi et al. 2000) and the Japanese black bear (*Ursus thibetanus japonicus*) (Yamamoto et al. 2002). However, in the present study, the sex determination of the four carnivores of Tsushima was not successful using amelogenin markers because the fragment size of the amelogenin X and Y of the four species was almost the same (data not shown). On the other hand, the ZFX/Y genes have been used for sex identification in the hyena (*Crocuta crocuta*) (Schwerin and Pitra 1994) and the sea otter (*Enhydra lutris*) (Hattori et al. 2003).

For wild carnivores such as the leopard cat, marten, and weasel, capture in traps is extremely stressful and may result in death. A noninvasive method using fecal DNA markers can resolve this problem and greatly contribute to a wildlife distribution survey. Furthermore, population genetic analysis using fecal samples has previously been reported for the mountain lion (puma) in the Yosemite Valley using microsatellite DNA markers (Ernest et al. 2000), which suggests that fecal samples are useful for the investigation of population genetics in wildlife.

In the present study, to obtain detailed distribution patterns of wild cats, the species of origin were identified for the southern areas of the Tsushima Islands using fecal samples. No feces from leopard cats was obtained and most of the animals identified were domestic cats (Figure 4). On the other hand, feces from the northern island was identified as leopard cat, as expected (Figure 3). The population of Tsushima leopard cats has been reduced to fewer than 100 individuals (Izawa and Doi 1991; Izawa et al. 1991). It has been reported that FIV originating from the domestic cat was detected in the Tsushima leopard cat (Doi and Izawa 1997; Nishimura et al. 1999). Therefore conservation of the leopard cats as well as their habitat is urgently needed. These DNA markers and methods using fecal samples could be useful to further track the presence of leopard cats in the southern Tsushima Islands. Collection of fecal samples from all over the Tsushima Islands and utilization of highly polymorphic DNA markers such as microsatellites to allow genetic individualization of samples will help us to better understand the genetic status of these endangered species.

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