Identification of tamaraw (Bubalus mindorensis) from natural habitat-derived fecal samples by PCR-RFLP analysis of cytochrome b gene

Shinya ISHIHARA,1 Rommel J. HERRELA,2 Daichi IJIRI,1 Hisashi MATSUBAYASHI,3 Miho HIRABAYASHI,1 Arnel N. DEL BARRIO,2 Rodel M. BOYLES,4 Medardo M. EDUARTE,5 Ronilo L. SALAC,6 Libertado C. CRUZ7 and Yukio KANAI1

1Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, 2Philippine Carabao Center – University of the Philippines at Los Baños, Laguna, 3Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia, 4Tamaraw Conservation Program, Department of Environment and Natural Resources (DENR), 5Protected Areas and Wildlife Bureau (PAWB), DENR, Quezon City, 6Provincial Environment and Natural Resources Office Region IV, DENR, Occidental Mindoro and 7Philippine Carabao Center, National Headquarters, Munoz, Nueva Eciia, Philippines

ABSTRACT
Fecal DNA analysis is a useful tool for the investigation of endangered species. Tamaraw (Bubalus mindorensis) is endemic to the Philippine island of Mindoro but knowledge of its genetic and ecological information is limited. In this study, we developed a species identification method for tamaraw by fecal DNA analysis. Eighteen feces presumed to be from tamaraw were collected in Mount Iglit-Baco National Park and species-known feces from domestic buffaloes and cattle were obtained from a farm. Additionally, one species-unknown fecal sample was obtained in Mount Aruyan Preserve, where the sighting of tamaraw has not been reported in recent years. Based on DNA sequence data previously reported, the genus Bubalus- and tamaraw-specific primers for PCR of cytochrome b gene were newly designed. The Bubalus-specific primer yielded a 976 bp fragment of cytochrome b for all fecal samples from tamaraw and domestic buffaloes, but not for cattle, whereas the tamaraw-specific primer yielded a 582 bp fragment for all tamaraw fecal samples and for one of the four domestic buffalo samples. PCR-RFLP (restriction fragment length polymorphism) analysis of the 976 bp PCR fragment with AvrII or BsaXI provided distinct differences between tamaraw and domestic buffalo. PCR-RFLP analysis also showed that the species-unknown sample obtained in Mount Aruyan Preserve, originates from tamaraw.

Key words: cytochrome b, fecal DNA, species identification, tamaraw.

INTRODUCTION
Tamaraw (Bubalus mindorensis) is a wild dwarf buffalo endemic to the Philippine island of Mindoro. It was formerly abundant and well-distributed across the island, but its current range is restricted to three small protected areas: Mount Iglit-Baco National Park (MIBNP), Mount Aruyan and Mount Calavite Tamaraw Preserve in Occidental Mindoro (Cox & Woodford 1990). International Union for Conservation of Nature and Natural Resources (IUCN) first categorized tamaraw as an endangered species in 1986, and then as a critically endangered species in 2000. Recent estimation of tamaraw populations by IUCN indicates that the number of mature individuals is less than 250 with more than 90% of individuals assigned to the MIBNP subpopulation, giving an estimated continuing decline of 25% over the next three generations or the next 30 years (Hedges et al. 2008).

The population of tamaraw in the early 1900s was estimated to be around 10 000 individuals across the whole of Mindoro Island, but over-hunting and exploitation of their habitat with an increasing human population resulted in a drastic decline of the tamaraw population to less than 1000 in 1949 (Harrison 1969). To save the tamaraw from extinction, the Philippine Government prohibited by law the killing, wounding
or taking away of tamaraw from their habitat as early as 1936, and also initiated official conservation measures called the Tamaraw Conservation Program (TCP) in 1979, which consists of three major components, namely, conservation of habitat, captive breeding and education of the populace in cooperation with various non-government organizations (NGOs) and Department of Environment and Natural Resources of the Philippines, or DENR (Caringal-Panga et al. 1994). Despite such conservation efforts, the tamaraw population still remains low and up to now only limited information has become available regarding ecological behavior of tamaraw and tamaraw–human conflict (Maala 2001). One reason for these constraints could be the difficulty in accessing their habitat and in sighting tamaraw in the field. The tamaraw are known to be fierce, nocturnal and wary animals, and therefore it is difficult to directly observe them in the daytime (Kuehn 1977, 1986). At present, confirmed sightings are largely limited to MIBNP where the vegetation is dominated by grasses with small scattered forest areas (Ishihara et al. 2007). In contrast to MIBNP, little information is available for the other two protected areas of Mount Aruyan and Mount Calavite where human–tamaraw conflict or the dense secondary forest makes the sighting of tamaraw more difficult (Matsubayashi et al. 2009).

As the technique of molecular analysis has developed, DNA became a useful tool in conservation biology and wildlife management in terms of acquiring valuable information such as behavioral and ecological features. Mitochondrial DNA (mt-DNA) is known to evolve much faster than nuclear DNA and thus contains more sequence diversity compared to nuclear DNA, facilitating the identification of closely related species (Brown et al. 1979, 1982). Above all, the nucleotide sequence of the cytochrome b gene on mt-DNA which contains species-specific information has been used in forensic analysis in a number of studies (Irwin et al. 1991; Smith & Patton 1991; Cees pedes et al. 1998). Tanaka et al. (1996) successfully sequenced the whole cytochrome b gene (1140 bp) using blood samples from captured tamaraw and demonstrated that tamaraw is phylogenetically closer to swamp buffaloes than riverine buffaloes. Furthermore, it has been shown that non-invasive samples such as feces are a valuable source for mt-DNA analysis (Höss et al. 1992; Constable et al. 1995). Paxinos et al. (1997) discriminated fecal DNA of sympatric canid species using three restriction enzymes on cytochrome b gene. Meanwhile, Kurose et al. (2005) distinguished carnivore species using fecal DNA amplified with a species-specific primer on PCR.

The object of this study was to establish the method for identifying tamaraw from its close relative species by using cytochrome b gene extracted from outdoor-derived fecal samples. We present here that the newly designed genus *Bubalus*-specific or tamaraw-specific primer pair can amplify the tamaraw cytochrome b gene fragment, and the combination of PCR and restriction fragment length polymorphism (RFLP) analysis with restriction enzymes could successfully discriminate tamaraw from domestic buffalo.

**MATERIALS AND METHODS**

**Fecal sampling**

Fecal samples from putative tamaraw (*Bubalus mindorensis*), domestic buffalo (*Bubalus bubalis*) and cattle (*Bos taurus*) were used in the present study. Eighteen fecal samples presumed to be from the tamaraw by its size and shape were collected at 11 different observation sites within the tamaraw conservation area (approximately 16 000 ha) of MIBNP in April, 2007. Species-known fecal samples were collected from each two individuals of swamp buffaloes, crossbred buffaloes (swamp × river) and cattle (crossbred Brahman) in the Philippine Carabao Center, University of Phillippines at Los Banos (PCC-UPLB), immediately after sighting of defecation from each animal. Additionally, one species-unknown fecal sample obtained at the ridge area of Mount Aruyan Preserve in our previous study (Matsubayashi et al. 2009), was also included to determine the host species of this fecal sample at the DNA level. The collection site of this sample is the eastern part of Aruyan near the Buayan and Kapihan creeks (altitude range 210–350 m), where the sighting of tamaraw has not been reported in recent years.

Intestinal epithelial cells on the surface of feces were collected using a sterilized cotton swab, and then dipped into 1 mL of lysis buffer (White & Densmore 1992) in a 1.5 mL micro-test tube. The lysis buffer contained 0.5% sodium dodecyl sulfate (SDS), 100 mmol/L ethylenediaminetetra-acetic acid (EDTA: pH 8.0), 100 mmol/L Tris-HCl (pH 8.0), and 10 mmol/L NaCl. All tubes with samples were transferred to PCC-UPLB and kept in a regulator at 4°C until analyzed.

**DNA preparation**

DNA extraction was conducted using the standard method of proteinase K digestion, phenol/chloroform extraction, and precipitation with ethanol by Sambrook et al. (1989). Proteinase K and NaCl were added into the fecal sample tube to reach a final concentration of 250 μg/mL and 0.15 mol/L, respectively, and incubated for 1 h at 55°C and then for 16 h at 37°C. After removing the residue of the feces and centrifugation (25 000 × g, 5 min at 4°C), DNA was extracted and purified as described by Sambrook et al. (1989). The extracted DNA was dissolved in 50 μL Tris-EDTA buffer (each 1 mmol/L of Tris-HCl and EDTA, pH 8.0).

**Oligonucleotide primers**

In order to establish a method for species identification using fecal DNA, the genus *Bubalus*-specific primer pair and tamaraw-specific primer pair for PCR were designed based on the mtDNA cytochrome b gene. Sequences of whole cytochrome b gene regions (1140 bp) from different species were referred to the GenBank database and Tanaka et al. (1996) (GenBank accession; *Bos taurus*, D34635; *Bubalus bubalis*, D82894; *Bubalus mindorensis*, D82895). Primers for amplifying cytochrome b gene of the genus *Bubalus* and the species tamaraw were designed on the mutated regions between the
three species as indicated in Figure 1. The genus *Bubalus*-specific primer, PBb-CbF (5'-CATTCTAGGACCTCCGCTGCT-3')/PBb-CbR (5'-GGGTCTTCTCCAAATTGATG-3'), and the tamaraw-specific primer, PBm-CbF (5'-GGCACAACCTAGTTGAACTGA-3')/PBm-CbR (5'-CGATGTTAATATATGCGGGTTGTTCG-3'), were newly designed. Expected amplified products for PBb-CbF/PBb-CbR and PBm-CbF/PBm-CbR were 976 bp and 582 bp in length, respectively. Additionally, a universal primer pair for cytochrome b gene (Irwin et al., 1991), L14724B (5'-CGAAGCTTATATGAACACCTATGTTG-3')/H15915R (5'-GGAATTCCATATCTCCCCGTTACAAAGAC-3'), was also used as a positive control for PCR amplification.

**PCR**

The final PCR mixture contained 0.4 μL EX Taq polymerase (Takara Bio Inc., Tokyo, Japan), 2 μL of 10 × EX taq buffer (Takara), 1.6 μL of dioxynucleotide triphosphate (dNTP: 2.5 mmol/L of each dNTP; Takara), 1.2 μL of 10 μmol/L of each primer and 1.6 ng DNA, in a total volume of 20 μL. PCRs were performed in an automated DNA thermal cycler (Applied Biosystems), according to the manufacturer’s instructions.

**Direct sequencing**

PCR products from 18 putative tamaraw feces from MIBNP, a species-unknown feces from Mount Aruyan Preserve and four domestic buffalo feces were subjected to direct sequencing to confirm whether the products actually matched with cytochrome b gene for each species. A fragment of cytochrome b for sequencing was obtained by a PCR with PBb-CbF/PBb-CbR primer pair. Each PCR product was mixed with 1 μL dye terminator (Big Dye Terminator, Applied Biosystems, Tokyo, Japan), 1 μL primer (3 μmol/L, PBb-CbF/ PBb-CbR), 6.5 μL distilled purified water and 1.5 μL sequencing buffer. PCR conditions were: initial denaturation at 98°C for 3 min followed by 25 cycles of denaturation at 96°C for 10 s, primer annealing at 50°C for 5 s and extension at 72°C for 4 min. The PCR products were subjected to capillary gel electrophoresis in an ABI Prism 310 genetic analyzer (Applied Biosystems), according to the manufacturer’s instructions.

**PCR-RFLP analysis**

For PCR-RFLP, two restriction endonucleases, AvrII (Takara) and BsaXI (New England Biolabs Inc., Tokyo, Japan) were used. AvrII recognizes the 5’-CCTAG-3’ and BsaXI recognizes the 5’-CGAATTC-3’ (Fig. 1). The PCR products amplified with PBb-CbF/PBb-CbR were used for digestion. Digestions were carried out in 50 μL volumes using reaction conditions recommended by the manufacturer. The restriction products were separated on 2% agarose gel by electrophoresis, visualized with ethidium bromide staining, and sized with reference to a DNA size marker. Expected digested products by AvrII were two fragments of 589 and 387 bp for domestic buffaloes and one undigested fragment of 976 bp for tamaraw, while those by BsaXI were three fragments of 679, 267 and 30 bp for domestic buffalo and five fragments of 367, 282, 267, 30 and 30 bp for tamaraw.

**RESULTS**

** Sequencing of cytochrome b gene**

The 976 bp fragment of cytochrome b was successfully amplified for all fecal samples, from both the putative tamaraw and domestic buffalo, by the genus *Bubalus*-specific primer pair (PBb-CbF/PBb-CbR). However, due to sequence ambiguity at the end of some amplified fragments, only 854 bp (position 113 to 966) of 976 bp fragments of PCR products were used in the analysis. The nucleotide sequence of the species-unknown sample collected in Mount Aruyan Preserve showed complete homology to the sequence of tamaraw cytochrome b reported by Tanaka et al. (1996), while all of the putative tamaraw samples from MIBNP showed single nucleotide transition (T→C) at position 255 bp (registered in DDBJ with accession no. AB526220).

As for the sequence of cytochrome b in domestic buffalo, one sequence from crossbred buffalo had a single nucleotide mutation (T→C) at position 471 bp. This mutation (registered in DDBJ with accession no. AB529514) was not matched with any haplotype previously reported in the GenBank database. The other three domestic buffaloes showed entirely the same sequence in the database (GenBank accession D34637 for one buffalo, and FJ556563 for the other two buffaloes).

**PCR with the genus *Bubalus* or tamaraw-specific primer**

The PCR amplification of a fragment of the cytochrome b gene by different primer pairs in representative samples are shown in Figure 2. The universal primer pair (L14724B/H15915R) yielded an approximate 1200 bp fragment in all fecal samples irrespective of the species (Fig. 2a), whereas the genus *Bubalus*-specific primer pair (PBb-CbF/PBb-CbR) allowed PCR amplification of the 976 bp fragment of cytochrome b only for the samples from domestic buffaloes and putative tamaraw (Fig. 2b). On the other hand, the tamaraw-specific primer pair (PBm-CbF/PBm-CbR) yielded the 582 bp fragment for all 18 fecal samples from putative tamaraw and one out of four fecal samples from crossbred buffaloes (Fig. 2c).

**RFLP of PCR product**

The restriction endonuclease digestion of the PCR products with AvrII yielded two distinct fragments (589 and 387 bp) for domestic buffalo feces, but its restriction site was not present for putative tamaraw feces (Fig. 3, upper panel). Another restriction endonuclease BsaXI resulted in profiles that distinguished all of the putative tamaraw from domestic buffaloes, visualizing two bands each with different sizes as shown in the lower panel of Figure 3 (679 and 267 bp fragments for domestic buffaloes and 367 and 282/
Figure 1  Whole cytochrome b (1140 bp) sequence from three bovidae species referred to Genbank; tamaraw (Bubalus mindorensis), D82895; domestic buffalo (Bubalus bubalis), D82894; and cattle (Bos taurus), D34635. The sites of each primer are surrounded by a square. The genus Bubalus-specific primer, PBb-CbF/PBb-CbR, and the tamaraw-specific primer, PBm-CbF/PBm-CbR, were newly designed. Endonuclease restriction sites of AvrII and BsaXI are underlined and the cutting locus is shown by a vertical line. Arrows indicate a mutation of tamaraw at 255 bp and domestic buffalo at 471 bp.
267 bp fragments for tamaraw). The PCR product from the species-unknown fecal sample from Mount Aruyan Preserve showed the same band patterns as the putative tamaraw feces from MIBNP after treatment with AvrII or BsaXI.

**DISCUSSION**

Results of the present study demonstrate that both the genus *Bubalus*-specific and tamaraw-specific primer pair could successfully amplify the tamaraw cytochrome *b* gene fragment from outdoor-derived fecal samples, and that RFLP analysis of the PCR products with restriction enzymes, AvrII or BsaXI, could accurately discriminate tamaraw from domestic buffalo and cattle.

To design tamaraw-specific PCR primers for the cytochrome *b* gene, two sites were selected where the nucleotides sequence of tamaraw differed from both domestic buffalo and cattle. The newly designed primer set successfully amplified the expected DNA fragments in all 18 fecal samples of putative tamaraw, but also reacted to one of the four domestic buffalo feces. Sequences with a nucleotide mutation at the 3’ end are often chosen for species-specific primer designing; however, in the present study there was a lack of nucleotide difference at the 3’ end between the two species, which could have reduced the specificity of the tamaraw-specific primers. This should be confirmed by further analysis. Degeneration may have also affected the specificity of the fecal DNA PCR amplification, as it has been shown that damaged DNA reduces the fidelity of Taq DNA polymerase (Sikorsky et al. 2007). Kurose et al. (2005) successfully discriminated the four carnivore species of the leopard cat (*Felis bengalensis*), Japanese marten (*Martes melampus*), Siberian weasel (*Mustela sibirica*), and feral cat (*Felis catus*) from fecal samples, using a species-specific primer on PCR to amplify cytochrome *b* fragments. They also found that in a small number of the samples, a PCR band appeared in a different species-specific primer pair in addition to a band in one species-specific primer pair, and they recommended using other PCR primers simultaneously to guarantee high accuracy. Nevertheless, PCR for cytochrome *b* fragment with a tamaraw-specific primer pair used in the present study can be a useful tool for the species identification of tamaraw, since it is rare for tamaraw to share their habitat with domestic buffaloes. This is supported by a recent report that indigenous peoples of Mangyan are living within the vicinity of tamaraw conservation areas and they still conduct slash-and-burn farming with no domestic animals (Matsubayashi et al. 2009).

The method to identify a species from fecal samples requires high accuracy. Restriction enzyme digestion of PCR-amplified species-specific mtDNA has been widely used for species identification in a variety of animals such as canid species (Paxinos et al. 1997), flatfish species (Cespedes et al. 1998) and Chinese alligators (Yan et al. 2005). In the present study, the genus *Bubalus*-specific PCR primer pair for a 976 bp fragment of cytochrome *b* was able to discriminate domestic buffalo and tamaraw from cattle, and restriction enzyme digestion of the PCR products with AvrII or BsaXI could identify tamaraw in all fecal samples which were presumed to be from tamaraw. Thus, the methods developed in the present study enabled the
species identification of tamaraw from outdoor-derived fecal samples with high precision. The rate of successful PCR amplification for outdoor-derived fecal samples in the present study (100%, 18/18 samples) is higher than that (80%, 24/30 samples) reported in sympatric carnivore species by Kurose et al. (2005). Since fecal DNA is often damaged by ultraviolet rays, the time process and environmental conditions before fecal sampling might affect the success rate of PCR amplification. In the present study, relatively fresh feces were sampled to minimize possible DNA damage. Further study is recommended to determine how time and environmental factors affect the success rate of PCR amplification from fecal samples.

Fecal DNA analysis enables us to acquire valuable information for the conservation of endangered animals, namely, phylogeny (Irwin et al. 1991; Smith & Patton 1991; Hammond et al. 2001), population genetics (Constable et al. 1995; Nielsen et al. 2008), distribution (Kurose et al. 2005) and feeding behavior (Höss et al. 1992). Currently, the habitat range of tamaraw is isolated into three regions in the Occidental Mindoro, and the largest population has been found in the tamaraw conservation area of MIBNP where annual population surveys by simultaneous multiple vantage-point counts has been undertaken as a part of TCP operation under the assignment of DENR since 1999 (Ishihara et al. 2007). However, only limited information is available in the other two tamaraw habitats, due to the difficulty in accessing these locations and in sighting tamaraw (Hedges et al. 2008). Recently, Matsubayashi et al. (2009) succeeded in identifying one male tamaraw by camera trap in Mount Aruyan Preserve. They also observed some signs of tamaraw presence such as footprints, foraging prints and fresh feces, but failed to obtain actual sightings. Fecal DNA analysis to identify tamaraw established in the present study makes it possible to screen large areas of habitat for the presence of tamaraw by collecting feces. In fact, the present study confirmed an unidentified fecal sample collected in Aruyan area as originating from tamaraw. Furthermore, we revealed that nucleotide sequence of amplified cytochrome b fragment in this sample completely matched that reported by Tanaka et al. (1996), while all fecal samples collected in MIBNP had a single transition (T→C) at position 255 bp. Since Tanaka et al. (1996) used blood samples from tamaraw captured in Mount Aruyan, these results indicate the possibility that tamaraw populations in MIBNP and Mount Aruyan have a different haplotype for cytochrome b gene. This finding should be verified with sufficient fecal samples in further studies.

Genetic diversity analysis by fecal DNA has been previously reported for the black rhinoceros (Diceros bicornis) and white rhinoceros (Ceratotherium simum) in Africa using microsatellite marker (Nielsen et al. 2008). Tamaraw may have already been affected by the bottleneck effect or by inbreeding because of their small population size. If we can collect putative tamaraw feces on a large scale, it may be possible to investigate any inbreeding effects, after the species identification of fecal samples and amplification of target DNA markers such as 12sRNA, D-loop and microsatellites which can be used for genetic diversity analysis.

Poaching by outsiders, not by indigenous people, is still a serious threat to tamaraw, and it has been presumed that tamaraw meat can be traded on the black market at a high price, two to three times the price of buffalo meat (Matsubayashi et al. 2009). The method of species identification developed in this study can be theoretically applied to any tissues of tamaraw. Therefore, it may also be a useful tool to intensify governmental regulation against the poaching of tamaraw and smuggling of tamaraw meat, as has been applied in other endangered animal species (Fang & Won 2002; Yan et al. 2005).

ACKNOWLEDGMENTS

We sincerely thank Ms. Josefina L. de Leon in DENR-PAWB for her dedicated support to the conclusion of the Memorandum of Agreements (MOA) between PCC and DENR, and the technical staff of the PCC/DENR and the tamaraw rangers in MIBNP/Mount Aruyan Preserve for their technical support to our field work. The study was reviewed and approved under the MOA, and partly supported by a Grant-In-Aid for Scientific Research (No. 17405004 and no. 21274) from the Japan Society for the Promotion of Science to YK.

REFERENCES


