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Non-Invasive Sex Determination of Asiatic Black Bear (*Ursus thibetanus*) via Sex-Specific Amplification of the Amelogenin Gene

Baek-Jun Kim*

National Institute of Ecology, Seocheon, Korea

ABSTRACT

The Asiatic black bear, *Ursus thibetanus*, is among the most threatened or endangered species in Asia. For its conservation and management, sex identification of *U. thibetanus* using non-invasive samples (e.g., hair and/or feces) is potentially valuable. In this study, a non-invasive molecular method for sex identification of *U. thibetanus* samples collected from various countries was first utilized, and it was based on polymerase chain reaction (PCR) amplification of the amelogenin gene via PCRs. Thirty-three bear DNA samples, extracted not only from blood (n=9) but also from hair (n=18) and feces (n=6), were used. We performed sex-specific PCR amplifications of the amelogenin gene using a primer set, SE47 and SE48. The primer set could successfully amplify a single X-specific band for females and both X- and Y-specific bands for males from all blood (100%) and hair (100%) samples. In addition, the primer set could distinguish the sex of bears in four out of a total of six fecal samples (approximately 67%). This study's findings suggest that this molecular method can be applied to sex identification of Asiatic black bears from various Asian regions using non-invasive samples, such as hair and feces.

Keywords: Amelogenin, Black bear, Feces, Hair, Sex-specific PCR, Sex-specific primer

Introduction

The Asiatic black bear, *Ursus thibetanus*, is among the most wide-spreading wildlife species in Asia. Unfortunately, many of these bears have been threatened or endangered in Asian countries by the impact of human activities, such as poaching and habitat destruction. The International Union for Conservation of Nature (IUCN, 1996) classified this bear in the vulnerable category, and the Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora listed it in Appendix 1 as an endangered species (Hutton & Dickson, 2000). Compared with those on the brown bear, studies that have been conducted on the Asiatic black bear are limited. Even fundamental sex identification of the species has only been subject to a single, previous study (Yamamoto *et al*., 2002).

Molecular methods of sex determination for several species have been based on detecting a sex-specific DNA sequence, such as Sex-determining Region Y (*SRY)* (Bellemain & Taberlet, 2004; Han *et al*., 2007; Murata & Masuda, 1996) and Zinc Finger protein, X-lined (*ZFX)*/ Zinc Finger protein, Y-linked (*ZFY)* (Aasen & Medrano, 1990; Hattori *et al*., 2003), using polymerase chain reaction (PCR) amplification. The former technique requires two primer

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***Corresponding author:** Baek-Jun Kim
e-mail naturalist71@nie.re.kr
https://orcid.org/0000-0002-8207-1931

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sets: one for the *SRY* gene and the other for an internal positive control. The latter technique necessitates an extra step, that is, restriction fragment length polymorphism after PCR (PCR-RFLP). In contrast, sex identification via PCR based on the amelogenin gene presents no such problems (Yamamoto *et al*., 2002).

The amelogenin gene encodes an important protein in the development of mammalian tooth enamel matrix (Lagerström *et al*., 1990; Termine *et al*., 1980) and is conserved in vertebrates (Lyngstadaas *et al*., 1990). In human (Salido *et al*., 1992; Sullivan *et al*., 1993) and bovine (Ennis *et al*., 1999; Gibson *et al*., 1992) cases, the amelogenin gene was detected on the X and Y chromosomes and assayed. Different-sized fragments were observed between the X- and Y-specific genes (only the X-specific gene for females, but both X- and Y-specific genes for males). Yamamoto *et al*. (2002) suggested that PCR amplification of the amelogenin gene using two primers, SE47 and SE48, is potentially applicable to DNA analysis of blood and hair samples of only Japanese black bears. However, no further sexing study was subsequently conducted using fecal samples (with which sex identification is more difficult owing to the low quantity and quality of extracted fecal DNA) from various Asiatic black bear subspecies.

In this study, we extensively identified the sexes of diverse Asiatic black bear subspecies (e.g., North Korean, Chinese, Russian, and Japanese black bears) from blood and hair samples using a primer set. We also sought to determine whether the primer set was useful for sex identification based on fecal samples. This study potentially provides a highly valuable tool for future forensic, phylogenic, and population-based studies.

Materials and Methods

Samples

Nine blood, 18 hair, and six fecal samples were obtained from the Conservation Genome Resource Bank (CGRB) for Korean Wildlife. The samples had been stored in −70°C deep freezers at the CGRB. The blood (n=9) and hair (n=18) samples originated from different Asian countries, namely, North Korea (three blood and eight hair samples), Tibet, China (one hair sample), Japan (one hair sample), Russia (six blood and two hair samples), and unknown origins (six hair samples). However, all fecal samples were collected from a bear farm in Heilungjiang, China (n=6). The detailed sample information is shown in Table 1.

DNA extraction

DNA was extracted from blood and hair samples using the commercial DNeasy Tissue Kit (Qiagen Inc., Germantown, MD, USA) and Chelex method (Walsh *et al*., 1991), respectively. DNA extraction from fecal samples was performed using the method described by Gerloff *et al*. (1995), with some modifications.

DNA amplification and analysis

One or two amelogenin regions were amplified by PCR for each bear sample. Two primers, SE47 (5'-CAG CCA AAC CTC CCT CTG C-3') and SE48 (5'-CCC GCT TGG TCT TGT CTG TTG C-3'), were used for PCR amplification (Yamamoto *et al*., 2002). The PCR was conducted in a 20-μL reaction volume containing 5 μL of DNA template, 2 mM $MgCl_2$, 1X buffer (iNtRON Inc., Seongnam, Korea), 0.2 mM of deoxyribonucleotide triphosphate, 0.25 μM of each primer, 5 μg of bovine serum albumin (Promega, Inc., Madison, WI, USA), and 1 U of *i-Star Taq* polymerase (iNtRON Inc.,). The PCR amplifications were performed in a Takara PCR Thermal Cycler (Takara Bio Inc., Kusatsu, Japan) with the following conditions: initial denaturation for 3 minutes at 95°C, followed by 50 cycles (94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute) and a final 3-minute extension at 72°C. PCR products were resolved using electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized under an ultraviolet illuminator.

Table 1. The samples and results of sex determination of the bears (n=33)

Sample size	NK (n=11)		CH (n=5/7)				JA (n=1)		RU (n=8)		UN (n=6)		Total no. success (n=33)	
			TB (n=1)		HL (n=4/6)									
Sex	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Blood (n=9)	2	1	-	-	-	-	-	-	3	3	-	-	5	4
Hair (n=18)	3	5	1	0	-	-	0	1	2	0	5	1	11	7
Feces (n=6)	-	-	-	-	4	0	-	-	-	-	-	-	4	0
Total (n=33)	5	6	1	0	4	0	0	1	5	3	5	1	20	11

NK, North Korea; CH, China; TB, Tibet, China; HL, Heilungjiang, China; JA, Japan; RU, Russia; UN, unknown source; -, not applicable.

Results

Blood, hair, and fecal samples were analyzed to ascertain the sexes of different Asiatic black bear subspecies using SE47 and SE48 primer sets. The sexing success rate was 100% in both blood and hair samples and approximately 67% in fecal samples (Fig. 1).

The sexing results from the bears' blood and hair samples exhibited one and two bands for female and male samples, respectively (Fig. 2). A fragment of approximately 245 bp was amplified from female samples, while both 245-bp and 191-bp fragments were amplified from male samples (Fig. 2). The sex determination of 27 samples was entirely successful (nine blood and 18 hair samples), with 16 males and 11 females detected from the total samples. In the fecal samples, the primer s*et al*so revealed PCR bands similar to those in the blood and hair samples; however, only four males were detected from six fecal samples, but no females (Table 1).

Table 1 shows the sex determination results of various Asiatic black bear subspecies. All the blood and hair samples, except those from Tibetan (only one male) and Japanese (only one female) black bears, yielded both male and female outcomes. However, male outcomes were exclusively observed in four fecal samples, with no sex outcomes in the other two.

Discussion

We confirmed the utility of a non-invasive molecular method for sex identification of several Asiatic black bear subspecies, including Korean, Chinese, Japanese, and Russian black bears. Yamamoto *et al*. (2002) found that the amelogenin gene of the Japanese black bear is located on the X- and Y- chromosomes and that 54 nucleotide deletions exist on the Y-specific gene in this region. In our study, all subspecies exhibited only the X-specific (approximately 245 bp) amelogenin gene for females and both X- and Y-specific (approximately 191 bp) amelogenin genes for males. In blood and hair samples, this method perfectly identified sex (100%; Fig. 1). In fecal samples, it distinguished the sex of approximately 67% of the bears (Fig. 1). This finding suggests that the two primers, SE47 and SE48, are considerably useful in determining the sex of different Asiatic black bear subspecies from feces as well as blood and hair. However, we could not decipher whether the lengths of the amplified products in this study were similar to those yielded by previous studies (Yamamoto *et al*., 2002).

PCR based on the amelogenin gene was more practical than that using the *SRY* and *ZFX/ZFY* genes. Murata and Masuda (1996) determined the sex of sea otters using the *SRY* region, which is exclusively located on the Y chromosome. When amplified, females yielded no band, whereas males generated one band. Bellemain and Taberlet (2004) also used the *SRY* region to identify the sex of brown bears. However, this technique requires two different primer sets: one for the *SRY* gene and the other for an internal positive control (Bellemain & Taberlet, 2004; Han *et al*., 2007; Murata & Masuda, 1996). In addition, Aasen and Medrano (1990) and Palsbø ll *et al*. (1992) suggested that distinguishing *ZFX/ZFY* using the RFLP assay in certain species is potentially challenging owing to a substantial degree of similarity between these sequences. Hattori *et al*. (2003) also developed an efficient method for sexing sea otters using the *ZFX/ZFY* regions. However, these techniques require an extra step, that is, restriction enzyme digestion after PCR (Aasen & Medrano, 1990; Hattori *et al*., 2003; Palsbøll *et al*., 1992). Although Kim *et al*. (2009) successfully utilized *ZFX/ZFY* introns for

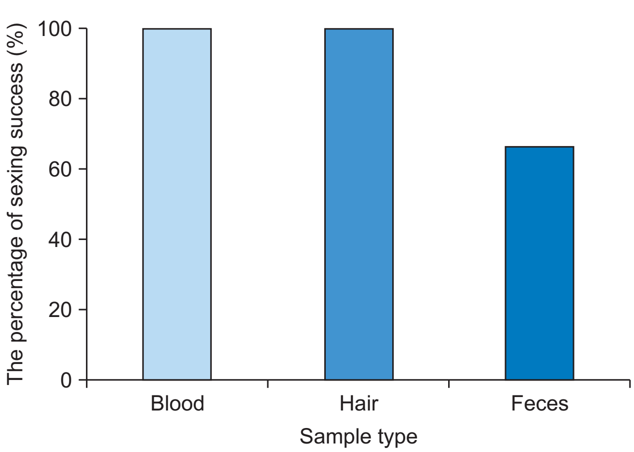

Fig. 1. Percentages of sexing success of Asiatic black bears from various types of samples (n=33).

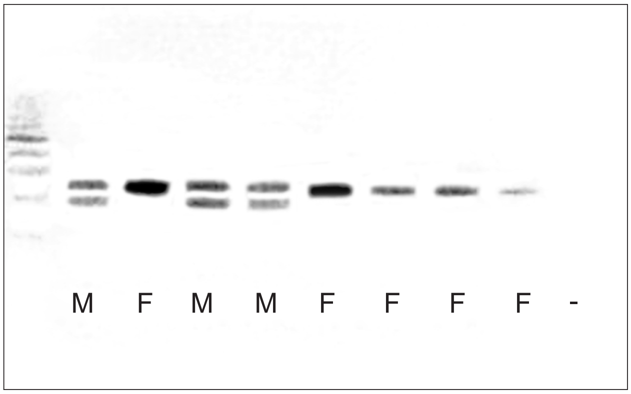

Fig. 2. Band patterns of sex identification using hair samples from Asiatic black bears in North Korea (n=8). 100 bp size marker was used to compare with PCR products. M, male; F, female; -, a negative control; PCR, polymerase chain reaction.

multiple PCR amplifications to identify the sex of five ungulate species, this technique has rarely been applied to bears.

In contrast, applying the amelogenin gene-based approach to humans (Salido *et al*., 1992; Sullivan *et al*., 1993), cattle (Ennis *et al*., 1999), horses (Fukushima *et al*., 1999), bears (Yamamoto *et al*., 2002), and ungulates (Kim *et al*., 2008) has the advantage of requiring only PCR amplification with one primer set, thus proving more convenient than other methods. However, the amelogenin gene-based method is not applicable to certain species, such as mice, monotremes, and marsupials (Chapman *et al*., 1991; Watson *et al*., 1992).

Our results revealed that only male sex could be detected from fecal samples; nonetheless, this bias may be explained by the lack of samples. Therefore, increasing the number of fecal samples during sex identification of bears may facilitate the detection of both males and females, just as in blood and hair samples. The present study's findings may be significantly useful in future forensic, phylogenic, and population studies of Asiatic black bears. In addition, the sequence data of amelogenin genes in Asiatic black bears may be a valuable index for identifying each subspecies, although we did not obtain amelogenin sequences in this study.

Conflict of Interest

The author declares that (s)he has no competing interests.

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