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Wildlife Science

# Sex identification using ZFX and ZFY genes in leopard cats (*Prionailurus bengalensis euptilurus*) in Korea

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**ABSTRACT.** In this study, we used multiplex polymerase chain reaction (PCR) to determine the sex of leopard cats (*Prionailurus bengalensis euptilurus*) in Korea. When we applied PCR using primers based on the intronic region between ZFX and ZFY, only one DNA band of 143 bp was detected in females, compared with two specific bands of 143 and 310 bp in males, indicating that the band patterns were clearly different between females and males. Since the set of primers also proved compatible with tissue and fecal samples, the results of the present study demonstrate that the present PCR could be a convenient tool for identifying the sex of leopard cats.

**KEY WORDS:** leopard cat, RFLP sex identification, ZFX, ZFY

The Asian leopard cat (Felidae: *Prionailurus bengalensis* Kerr, 1792) is found mainly in forests in the far east of Asia to western Indonesia and the Philippines [8]. *Prionailurus bengalensis euptilurus*, a subspecies of *P. bengalensis*, is distributed across Korea, the Russian Far East, and Northeast China [12]. However, this subspecies has been classified as endangered by the Korean Ministry of Environment [9].

Leopard cat has been intermittently rescued and referred to wildlife centers because of traffic accidents and starvation. It is so fierce that it is difficult for veterinarians and medical staffs to access it for capturing, restraint for treatment, and handling for care and rehabilitation. It is generally not accessible enough to distinguish the sex without anesthesia. However, anesthesia can put a stress on patients, especially those with severe wound and starvation. Also, the distinction between males and females after recovery is very important in terms of management, rehabilitation, and restoration research. This study was conducted to avoid risk factors for anesthesia and using noninvasive method with feces for sex differentiation in very fierce leopard cats. Therefore, we attempted to evaluate leopard cats' specific primers for PCR to identify the sex from fecal samples collected from the field.

## MATERIAL AND METHODS

### *Samples*

Four muscle ($n$=4) and five fecal ($n$=5) samples were collected from leopard cats in Gyeongnam Wildlife Center (GNWLC) in Korea. All samples were collected in sterilized plastic vials with approximately 30 m*l* of absolute alcohol. The samples were then stored in absolute ethanol at 4°C until DNA extraction.

### *DNA extraction*

Genomic DNA of leopard cats was extracted from fecal and muscle samples using the QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA, U.S.A.). DNA was stored at −20°C until use. In order to analyze the integrity of genomic DNA, all samples were subjected to 3% agarose gel electrophoresis. The quantity and quality of the DNA were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.).

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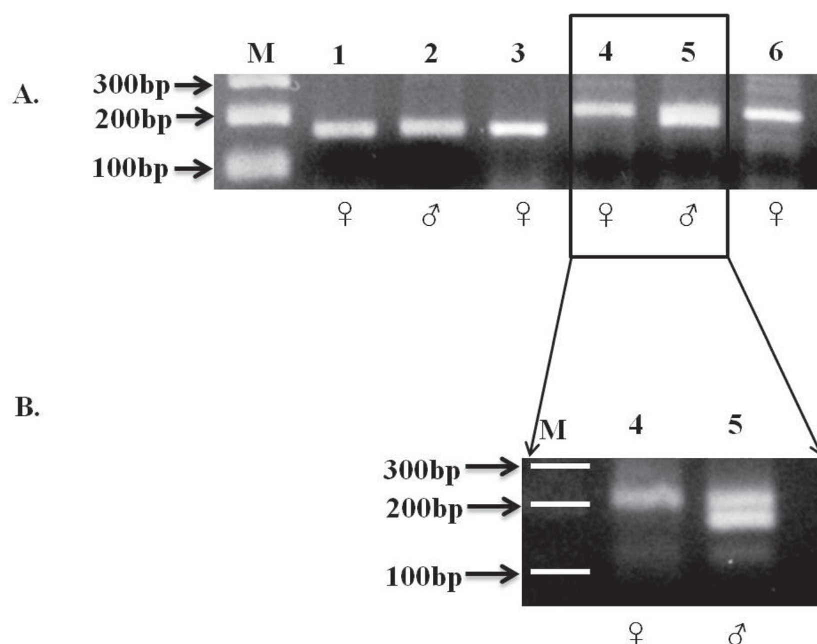


**Fig. 1.** Sex identification of leopard cats by analyzing the fragments of ZFX and ZFY with a zinc finger primer set and an amelogenin primer set, respectively [11]. (A) M: 100 bp markers, Lanes 1–3: zinc finger primer set. Lanes 4–6: amelogenin primer set. Lanes 1, 3, 4, and 6: female leopard cat fragments. Lanes 2 and 5: male leopard cat fragments. (B) A close-up of Lanes 4 and 5 in (A). Females showed a single band, whereas males revealed double bands. Electrophoresis was performed on a 2% agarose gel and visualized with ethidium bromide.

*Primer design*

Two male and two female reference leopard cats were used to confirm the PCR product size (males 194, 214 bp; females 214 bp), as described previously [10]. Following individual identification, their sexes were ascertained using amelogenin gene primers (Fig. 1). A check was also performed in order to determine whether the sex assignment was consistent for each individual that was recaptured multiple times in this study (Fig. 2). We developed a new primer set for ZFX and ZFY, as shown in Table 1. In addition, the SRY gene was analyzed using a primer set designed on the basis of the leopard cats' (*P. bengalensis*) SRY gene (GenBank accession no. DQ095184.1) [5]. Amplification of the ZFX and ZFY genes was performed on the basis of the leopard cats' ZFX and ZFY genes (GenBank accession nos. AB211420.1 and AB211428.1, respectively) [15]. By comparing the sequence information, primer sets of ZFX-LF/ZFX-LR for the X-chromosome and ZFY-LF/ZFY-LR for the Y-chromosome were developed, and their fragment sizes were 308, 143, and 551 bp, respectively. Multiplex PCR using these two primer pairs was expected to amplify two bands (143 and 308 bp) for males and a single band (143 bp) for females, as indicated in Table 1.

*DNA amplification*

PCR was performed in a total volume of 20 $\mu l$ containing 100 *ng* of genomic DNA, four 10 pM primers, 10x PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, and one unit of Taq Polymerase (Takara Inc., Otsu, Japan). The PCR reaction included an initial denaturation cycle at 94°C for 5 min; 29 cycles with a denaturation step at 94°C for 1 min, an annealing step at 59.5°C for 30 sec, and an extension step at 72°C for 30 sec; and a final extension step at 72°C for 10 min in a thermocycler (Bio-Rad, Foster City, CA, U.S.A.). The PCR products were then extracted for sequencing using the QIAquick Gel Extraction Kit (Qiagen Inc.).

## RESULTS

Initially, PCR products were produced using muscle DNA with newly designed primers. When the new primer set for the leopard SRY gene was tested, a PCR product of the expected size (551 bp) was successfully amplified for males, but not for females, as demonstrated in Figs. 3 and 4. However, since it is practically difficult to determine the sex of females without any signal, a reference male sample is required as a positive control along with test samples in order to confirm the validity of PCR in this experiment. In this perspective, it is necessary to detect the X-chromosome gene(s) specific for females. Therefore, we attempted to establish novel genotyping methods using the ZFX and ZFY genes because they have been successfully used to discriminate sexes in mammals [1, 4, 7]. With primers based on the sequence information of leopard cats' ZFX and ZFY, the production of the ZFX gene was 143 bp and the production of the ZFY gene was 308 bp, as shown in Fig. 4. Sequence alignment with amplified products also supported the specificity of the present PCR in this study. Moreover, we conducted the PCR using DNA extracted from fecal samples, for which the original species were known. Figure 5 shows the results of PCR amplification of fecal samples collected from western parts of Gyeongnam. In this experiment, the fragment length of the ZFX gene was 143 bp and that of the

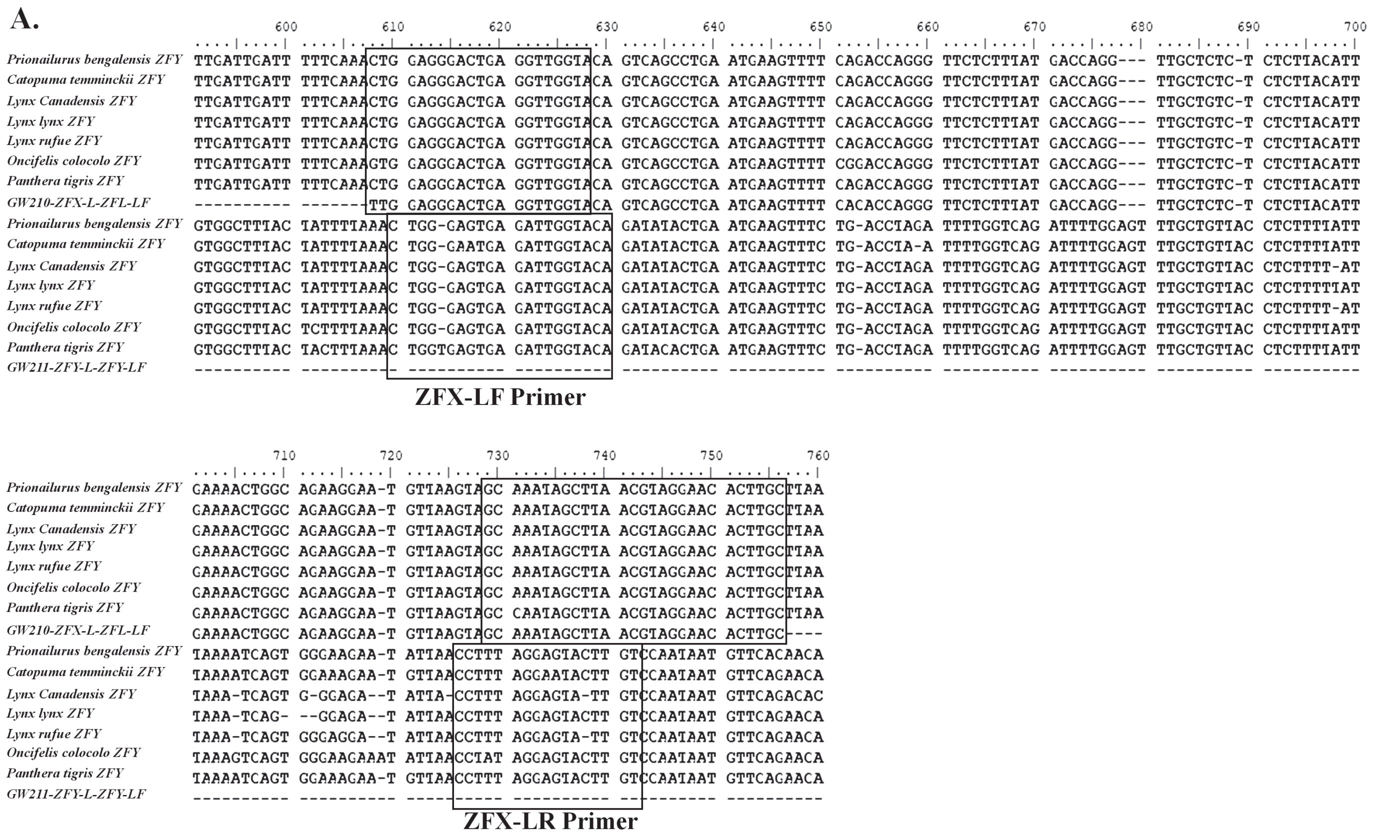


**Fig. 2.** Sequence alignments of ZFX (A) and ZFY (B) genes. The accession numbers of ZFX and ZFY in each species are as follows: ZFX-LF and ZFY-LF primers are each boxed. *Prionailurus bengalensis* AB211420.1, AB211428.1; *Catopuma temminckii* DQ086454.1, AY518666.1; *Lynx canadensis* DQ086447.1, AY518645.1; *Lynx rufus* DQ086448.1, AY518.646.1; *Lynx lynx* DQ6446.1, AY518644.1; *Oncifelis colocolo* DQ086428.1, AY518654.1; *Panthera tigris* AB211419.1, AY18650.1.

ZFY gene was 308 bp, as indicated in Fig. 5. As expected, the sequence from the fecal samples obtained through PCR revealed partial sequence identity of the leopard cats' ZFX and ZFY. The specificity of the Y-chromosome from the PCR was also confirmed by the presence of the expected product in males, but not in females. Taking the obtained findings together, the difference in length between ZFX and ZFY PCR products using the ZFX-LF/R primer was 165 bp. Notably, these eight species of leopard cats belong to the same order of Carnivora, family Felidae, subfamily Felidae, but they are within different genera.

## DISCUSSION

In this study, we used polymerase chain reaction (PCR) for sex identification of leopard cats on the basis of the sex-determining Y (SRY) gene located on the Y-chromosome. Actually, the fact that sex is determined by the SRY gene has been used in sex identification using PCR in various mammals [2, 3, 11, 13, 14, 18]. Therefore, genetic sex identification can be induced by investigating the SRY gene. However, the analysis of the SRY gene can be problematic if low-quality DNA samples are used; this analysis can be confirmed by failure in PCR amplification in female samples [10]. In addition, King *et al.* reported that the SRY gene was inconsistent in some species [5]. To overcome this problem, co-amplification of orthologous sexual chromosome genes, such as zinc finger proteins ZFX and ZFY, has been suggested to improve the precision and quality of sex identification [1]. PCR restriction fragment length polymorphism (RFLP) in various species has also been used to determine the sex with ZFX and ZFY on the X- and Y-chromosomes, respectively [1, 4, 7].

Generally, the size difference between ZFX and ZFY was too small to be used to differentiate the sexes in many mammals [10]. Although Sugimoto *et al.* reported the use of sex-specific primers (ZFX-PF/PR and DBY7-PF/R) for the leopard cats to generate large differences in PCR, this method required two primer pairs and RFLP analysis [15]. Since the zinc finger gene and the amelogenin gene resulted in similar sized PCR products between males and females, with sizes within 4 and 10 bp, respectively, it was practically difficult to differentiate them during gel electrophoresis. In this study, it was revealed that multiplex PCR based on ZFX-LF/R and ZFY-LF/R could conveniently identify two genes specific for the sex in leopard cats. In fact, the sequence alignment from the PCR products also confirmed the specificity of the primers, indicating that this protocol would presumably be feasible for other Felidae species. Moreover, it was also revealed that the DNA obtained from the fecal samples could be employed

**B.**

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                                      10         20         30         40         50         60         70         80         90        100
                              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Prionailurus bengalensis ZFY  GTGAGGGCAC ATGAGTTCCA CAGCGCAGCG TGCTTTGCGA GCTCTCAGAT GAAACCCTAG CGTGTGTCCA CGGAGGTGTT GTGATGGCAA TCTAGCTGCT
Catopuma temminckii ZFY       GTGAGGGCAC ATGAGTTCCA CAGCGCAGCG TGCTTTGCGA GCTCTCAGAT GAAACCCTAG CGTGTGTCCA CGGAGGTGTT GTGATGGCAT TCTAGCTGCT
Lynx Canadensis ZFY           GTGAGGGCAC ATGAGTTCCA CAGCGCAGCG TACTTTGCGA GCTCTCAGAT GAAACCCTAG CGTGTGTCCA CGGAGGTGTT GTGATGGCAT TCTAGCTGCT
Lynx lynx ZFY                 GTGAGGGCAC ATGAGTTCCA CAGCGCAGCG TGCTTTGCGA GCTCTCAGAT GAAACCCTAG CGTGTGTCCA CGGAGGTGTT GTGATGGCAT TCTAGCTGCT
Lynx rufue ZFY                GTGAGGGCAC ATGAGTTCCA CAGCGCAGCG TGCTTTGCGA GCTCTCAGAT GAAACCCTAG CGTGTGTCCA CGGAGGTGTT GTGATGGCAT TCTAGCTGCT
Oncifelis colocolo ZFY        GTGAGGGCAC ATGAGTTCCA CAGCGCAGCG TGCTTTGCGA GCTCTCAGAT GAAACCCTAG CGTGTGTCCA CGGAGGTGTT GTGATGGCAT TCTAGCTGCT
Panthera tigris ZFY           GTGAGGGCAC ATGAGTTCCA CAGCGCAGCG TGCTTTGCGA GCTCTCAGAT GAAACCCTAG CGTGTGTCCA CGGAGGTGTT GTGATGGCAT TCTAGCTGCT
GW210-ZFX-L-ZFL-LF            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Prionailurus bengalensis ZFY  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----GTGAGG GTGCACAAGT TCCACAGTGC
Catopuma temminckii ZFY       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Lynx Canadensis ZFY           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Lynx lynx ZFY                 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Lynx rufue ZFY                ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Oncifelis colocolo ZFY        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Panthera tigris ZFY           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
GW211-ZFY-L-ZFY-LF            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- GTGCACAAGT TCCACAGTGC
                                                                                                                      ZFY-LF Primer

                                     110        120        130        140        150        160        170        180        190        200
                              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Prionailurus bengalensis ZFY  AGACCACATT GTAGCTTCTG TGTACTGAAT TTGAAAATAT AATTTTAAGA ATTCAGTGAT ATTCATGAAT GATTTCCTGG GATAAAAAGA CACAAGGAAT
Catopuma temminckii ZFY       AGACCACATT GTAGCTTCTG TGTACTGAAT TTGAAAATAT AATTTTAAGA ATTCAGTGAT ATTCATGAAT GATTTCCTGG GATAAAAAGA AACAAGGAAT
Lynx Canadensis ZFY           AGACCACATT GTAGCTTCTG TGTACTGAAT TTGAAAATAT AATTTTAAGA ATTCAGTGAT ATTCATGAAT GATTTCCTGG GATAAAAAGA CACAAGGAAT
Lynx lynx ZFY                 AGACCACATT GTAGCTTCTG TGTACTGAAT TTGAAAATAT AATTTTAAGA ATTCAGTGAT ATTCATGAAT GATTTCCTGG GATAAAAAGA CACAAGGAAT
Lynx rufue ZFY                AGACCACATT GTAGCTTCTG TGTACTGAAT TTGAAAATAT AATTTTAAGA ATTCAGTGAT ATTCATGAAT GATTTCCTGG GATAAAAAGA CACAAGGAAT
Oncifelis colocolo ZFY        AGACCACATT GTAGCTTCTG TGTACTGAAT TTGAAAATAT AATTTTAAGA ATTCAGTGAT ATACATGAGT GATTTCCTGG GATAAAAAGA AACAAGGAAT
Panthera tigris ZFY           AGACCACATT GTAGCTTCTG TGTACTGAAT TTGAAAATAT AATTTTAAGA ATTCAGTGAT ATTCATGAAT GATTTCCTGG GAGAAAAAGA AACAAGGAAT
GW210-ZFX-L-ZFL-LF            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Prionailurus bengalensis ZFY  AGTGTGCT-C TGTGAGCTCT CAAATGAAAC TAGTATGTAT CCACAGAAGT GTTAATGATA GCAATTTAGC TGCTAGATCA CATGTAGCTT TTCTTTATTA
Catopuma temminckii ZFY       ---GTGCT-C TGTGAGCTCT CAAATGAAAC TAGTATGTAT CCACAGAAGT GTTAATGATA GCAATTTAGC TGCTAGATCA CATGTAGCTT TTCTTTATTA
Lynx Canadensis ZFY           ---GTGCT-C TGTGAGCTCT CAAATGAAAC TAGTATGTAT CCACAGAAGT GTTAATGATA GCAATTTAGC TGCTAGATCA CATGTAGCTT TTCTTTATTA
Lynx lynx ZFY                 ---GTGCC-C TGTGAGCTCT CAAATGAAAC TAGTATGTAT CCACAGAAGT GTTAATGATA GCAATTTAGC TGCTAGATCA CATGTAGCTT TTCTTTATTA
Lynx rufue ZFY                ---GTGCT-C TGTGAGCTCT CAAATGAAAC TAGTATGTAT CCACAGAAGT GTTAATGATA GCAATTTAGC TGCTAGATCA CATGTAGCTT TTCTTTATTA
Oncifelis colocolo ZFY        ---GTGCT-C TGTGAGCTCT CAAATGAAAC TAGTATGTAT CCACAGAAGG TTTAATGATA GCAATTTAGT TGCTAGATCA CATGTAGCTT TTCTTTATTA
Panthera tigris ZFY           ---GTGCTGC TGTGAGCTCT CAAATGAAAC TAGTATGTAT CCACAGAAGT GTTAATGATA GCAATTTAGC TGCTAGATCA CATGTAGCTT TTCTTTATTA
GW211-ZFY-L-ZFY-LF            AGTGTGCT-C TGTGAGCTCT CAAATGAAAC TAGTATGTAT CCACAGAAGT GTTAATGATA GCAATTTAGC TGCTAGATCA CATGTAGCTT TTCTTTATTA

                                     210        220        230        240        250        260        270        280        290        300
                              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Prionailurus bengalensis ZFY  GGATCGAACA CGGATGGGAA AAAAATTCAA AACGTGGGTG ATTTTTATGT GGAAATGAGA AGAAATCCCT CAAATATGTC GCAGGCATTA ACTCTTTTAA
Catopuma temminckii ZFY       GGATCGAACA CGGATGGGAA AAAAATTCAA AACGTGGGTG ATTTTTATGT GGAAGTGAGA AGAAATCCCT CAAATATGTC GCAGGCATTA ACTCTTTTAA
Lynx Canadensis ZFY           GGATCGAACA CGGATGGGAA AAAAATTCAA AACGTGGGTG ATTTTTATGT GGAAATGAGA AGAAATCCCT CAAATATGTC GCAGGCATTA ACTCTTTTAA
Lynx lynx ZFY                 GGATCGAACA CGGATGGGAA AAAAATTCAA AACGTGGGTG ATTTTTATGT GGAAATGAGA AGAAATCCCT CAAATATGTC GCAGGCATTA ACTCTTTTAA
Lynx rufue ZFY                GGATCGAACA CGGATGGGAA AAAAATTCAA AACGTGGGTG ATTTTTATGT GGAAATGAGA AGAAATCCCT CAAATATGTC GCAGGCATTA ACTCTTTTAA
Oncifelis colocolo ZFY        GGATCGAACA CGGATGGGAA AAAAATTCAA AACGTGGGTG ATTTTTATGT GGAAATGAGA AGAAATCCCT CAAATATGTC GCAGGCATTA ACTCTTTTAA
Panthera tigris ZFY           GGATCGAACA TGGATGGGAA AAAAATTCAA AACGTGGGTG ATTTTTATGT GGAAATGAGA AGAAATCCCT CAAATATGTC GCAGGCATTA ACTCTTTTAA
GW210-ZFX-L-ZFL-LF            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Prionailurus bengalensis ZFY  AATTTGAGAA TATAATTTTA AGAATTCAGT GATATTCATG AATGATTTCC TAGAATAAAA ACAACAGATT TT-GAGTCAG AATAAAATCT CAGGTTATTT
Catopuma temminckii ZFY       AATTTGAAAA TATAATTTTA AGAATTCAGT GATATTCATG AATGATTTCC TAGAATAAAA AGAACAGATT TTTGAGTCAG AATAAAATCT CAGGTTATTT
Lynx Canadensis ZFY           AATTTGAAAA TATAATTTTA AGAATTCAGT GATATTCATG AATGATTTCC TAGAATAAAA ACAACAGATT TT-GAGTCAG AATAAAATCT CAGGTTATTT
Lynx lynx ZFY                 AATTTGAAAA TATAATTTTA AGAATTCAGT GATATTCATG AATGATTTCC TAGAATAAAA ACAACAGATT TT-GAGTCAG AATAAAATCT CAGGTTATTT
Lynx rufue ZFY                AATTTGAAAA TATAATTTTA AGAATTCAGT GATATTCATG AATGATTTCC TAGAATAAAA ACAACAGATT TT-GAGTCAG AATAAAATCT CAGGTTATTT
Oncifelis colocolo ZFY        AATTTGAAAA TATAATTTTA AGAATTCAGT GATATTCATG AATGATTTCC TAGAATAAAA ACAACAGATT TT-GAATCAG AATAAAATCT CAGGTTATTT
Panthera tigris ZFY           AATTTGAAAA TATAATTTTA AGAATTCAGT GATATTCATG AATGATTTCC TAGAATAAAA AGAACAGATT TT-GAGTCAG AATAAAATCT CAGGTTATTT
GW211-ZFY-L-ZFY-LF            AATTTGAGAA TATAATTTTA AGAATTCAGT GATATTCATG AATGATTTCC TAGAATAAAA ACAACAGATT TT-GAGTCAG AATAAAATCT CAGGTTATTT

                                     310        320        330        340        350        360        370        380        390        400
                              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Prionailurus bengalensis ZFY  AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT
Catopuma temminckii ZFY       AAACTCAATG ACTACAAAGA ATCTGATTCT GTTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT
Lynx Canadensis ZFY           AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT
Lynx lynx ZFY                 AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT
Lynx rufue ZFY                AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT
Oncifelis colocolo ZFY        AAACTCAATG ACTACAAAGA ATCTGATTCC GTTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT
Panthera tigris ZFY           AAACTCAATG ACTACAAAGA ATCTGATTCT GTTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT
GW210-ZFX-L-ZFL-LF            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Prionailurus bengalensis ZFY  TAGGAGAAAT TAAAATAAAT CCCTAAAATA TTTTCTTTAA CTTTAAATGA ACAAATTTAA AGATTATTTC TGTCAGCATA AGCAGGGCTT CTTTACAGAG
Catopuma temminckii ZFY       TAGGAGAAAT TAAAATAAAT CCCTAAAATA TTTTCTTTAA CTTTAAATGA ACAAATTTAA AGATTATTTC TGTCAGCATA AGCAGGGCTT CTTTACAGAG
Lynx Canadensis ZFY           TAGGAGAAAT TAAAATAAAT CCCTAAAATA TTTTCTTTAA AT------GA ACAAATTTAA AGATTATTTC TGTCAGCATA AGCAGG-CTT CTTTACAGAG
Lynx lynx ZFY                 TAGGAGAAAT TAAAATAAAT CCCTAAAATA TTTTCTTTAA AT------GA ACAAATTTAA AGATTATTTC TGTCAGCATA AGCAGGGCTT CTTTACAGAG
Lynx rufue ZFY                TAGGAGAAAT TAAAATAAAT CCCTAAAATA TTTTCTTTAA AT------GA ACAAATTTAA AGATTATTTC TGTCAGCATA AGCAGGGCTT CTTTACAGAG
Oncifelis colocolo ZFY        TAGGAGAAAT TAAAATAAAT CCCTAAAATA TTTTCTTTAA CTTTAAA-GA ACAAATTTAA AGATTATTTC TGTCAGCATA AGCAGGGCTT CTTTACAGAG
Panthera tigris ZFY           TAGGAGAAAT TAAAATAAAT CCCTAAAATA TTTTCTTTAA CTTTAAATGA ACAAATTTAA AGATTATTTC TGTCAGCATA AGCAGG-CTT CTTTACAGAG
GW211-ZFY-L-ZFY-LF            TAGGAGAAAT TAAAATAAAT CCCTAAAATA TTTTCTTTAA CTTTAAATGA ACAAATTTAA AGATTATTTC TGTCAGCA-A AGCAGGGCTT A---------
                                                                                                                ZFY-LR Primer
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**Fig. 2.** Continued.

**Table 1.** Primer set and sequence information in the study

| Name | Primer sequence (5–3′) | Amplicon length (bp) | Annealing temperature | References |
|---|---|---|---|---|
| Zn-finger | F: CAAGTTTACACAACCACCTGG<br>R: CACAGAATTTACACTTGTGCA | Male: 163, 166<br>Female: 166 | 54 | [11] |
| Amelogenin | F: CGAGGTAATTTTTCTGTTTACT<br>R: GAAGCTGAGTCAGAGAGGC | Male: 194, 214<br>Female: 214 | 54 | [11] |
| ZFX-L1 | F: TGGAGGGACTGAGGTTGGTA<br>R: GCAAGTGTTCCTACGTTAATC | Male, Female: 143 | 59.5 | This study |
| ZFX-L2 | F: CGGCAAAGCAGGAATTGTGA<br>R: AAGCAAGTGTTCCTACGTTAAGC | Male, Female 263 | 59.5 | This study |
| ZFY-L | F: GTGCACAAGTTCCACAGTGC<br>R: AAGCCCTGCTTATGCTGACA | Male: 308 | 59.5 | This study |
| SRY | F: TATCCTCGCTGTGGAGGGAA<br>R: GTTGTGGAGGTCCTGTGAGG | Male: 551 | 59.5 | This study |

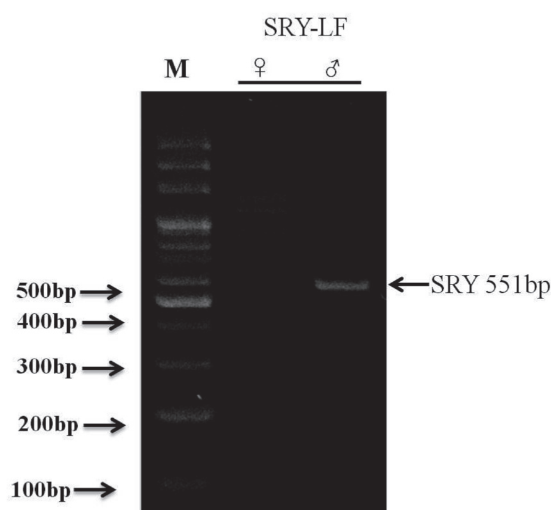


**Fig. 3.** PCR of the SRY gene. Lane 1: female leopard cat DNA. Lane 2: male leopard cat DNA. The male PCR products show amplification of a 551 bp SRY fragment. M: 100 bp molecular weight markers.

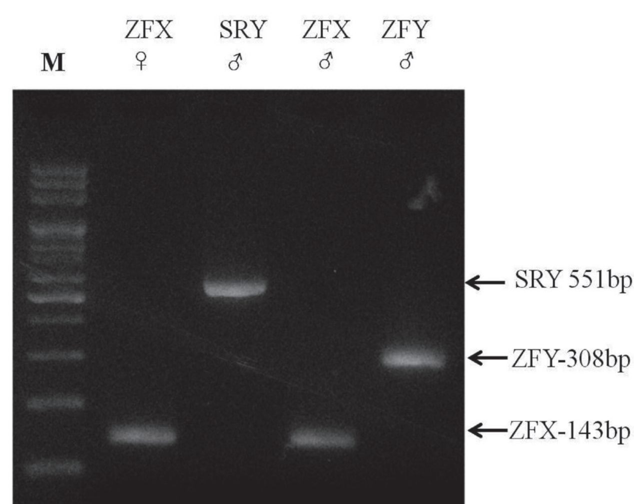


**Fig. 4.** PCR products of the SRY, ZFX, and ZFY genes. The SRY (551 bp) and ZFY (308 bp) bands were only present for males, but not for females. The ZFX band was positive in both males and females. M: 100 bp molecular weight markers.

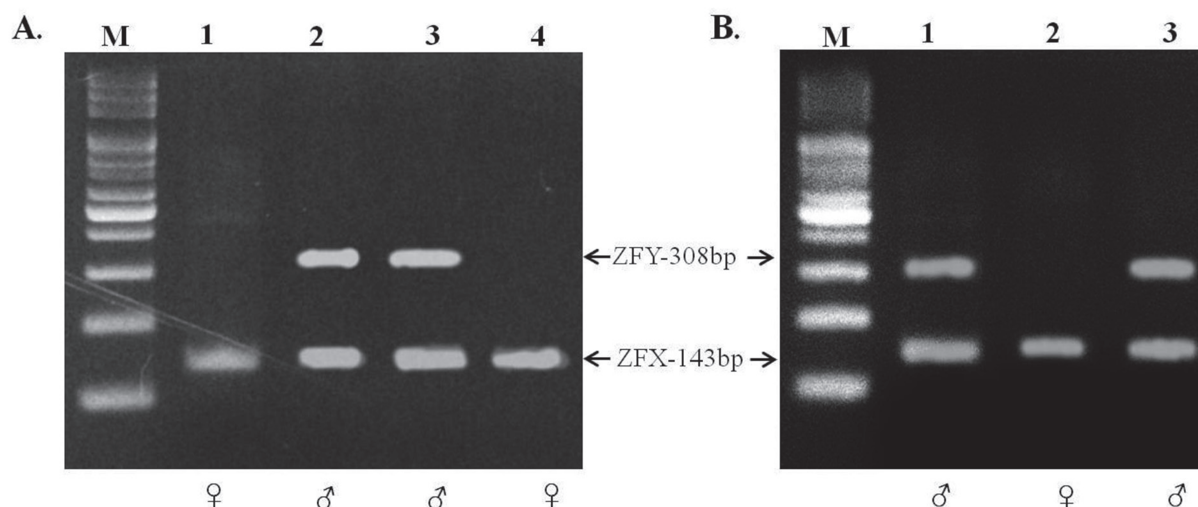


**Fig. 5.** PCR amplification of partial fragments of ZFX and ZFY genes using DNA extracted from leopard cats (reference muscles) and unknown fecal samples. (A) Lanes 1 and 2: leopard cats' reference muscles; Lanes 3 and 4: leopard cats' unknown fecal samples. (B) Unknown fecal samples.

as a suitable specimen for sex identification along with muscle or blood samples in the field. Although the DNA extracted from feces showed drawbacks in terms of quality, the utilization of fecal samples as a DNA resource has several advantages, such as there being no risk of injury or death associated with capturing or handling endangered species and the fact that one can obtain various types of important information on a target population [6, 16, 17]. Also, obtaining a good fecal sample from the specimens can help preserve the quality of the DNA and produce accurate results. Therefore, the results of this study demonstrate that PCR for sex identification can provide a convenient approach for breeding-related distribution and relationship studies for endangered leopard cats in Korea.

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