



FULL PAPER

Wildlife Science

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

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Received: 22 November 2018 Accepted: 23 March 2019 Published online in J-STAGE: 2 April 2019 **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 ml 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<sub>2</sub>, 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

<b>A.</b>	600	610	620	63	0 640	650	660	670	680	690	) 70
		· · · ·   · · <u>· · ·  </u>		<u></u> .							
Prionailurus bengalensis ZFY	TTGATTGATT	TTTCAAACTG	GAGGGACTGA	GGTTGGIACA	GTCAGCCTGA	ATGAAGTTTT	CAGACCAGGG	TTCTCTTIAT	GACCAGG	TTGCTCTC-T	CTCTIACATT
Catopuma temminckii ZFY	TTGATTGATT	TTTCAAACTG	GAGGGACTGA	GGTTGGIACA	GTCAGCCTGA	ATGAAGTTTT	CAGACCAGGG	TTCTCTTIAT	GACCAGG	TTGCTCTC-T	CTCTIACATT
Lynx Canadensis ZFY	TTGATTGATT	TTTCAAACTG	GAGGGACTGA	GGTTGGIACA	GTCAGCCTGA	ATGAAGTTTT	CAGACCAGGG	TTCTCTTIAT	GACCAGG	TTGCTGTC-T	CTCTIACATT
Lynx lynx ZFY	TTGATTGATT	TTTCAAACTG	GAGGGACTGA	GGTTGGTACA	GTCAGCCTGA	ATGAAGTTTT	CAGACCAGGG	TTCTCTTIAT	GACCAGG	TTGCTGTC-T	CTCTIACATT
Lynx rufue ZFY	TTGATTGATT	TTTCAAACTG	GAGGGACTGA	GGTTGGIACA	GTCAGCCTGA	ATGAAGTTTT	CAGACCAGGG	TTCTCTTTAT	GACCAGG	TTGCTGTC-T	CTCTIACATT
Oncifelis colocolo ZFY	TTGATTGATT	TTTCAAAGTG	GAGGGACTGA	GGTTGGIACA	GTCAGCCTGA	ATGAAGTTTT	CGGACCAGGG	TTCTCTTTAT	GACCAGG	TTGCTCTC-T	CTCTIACATT
Panthera tigris ZFY	TTGATTGATT	TTTCAAACTG	GAGGGACTGA	GGTTGGIACA	GTCAGCCTGA	ATGAAGTTTT	CAGACCAGGG	TTCTCTTIAT	GACCAGG	TTGCTGTC-T	CTCTIACATT
GW210-ZFX-L-ZFL-LF		TTG	GAGGGACTGA	GGTTGGIACA	GTCAGCCTGA	ATGAAGTTTT	CACACCAGGG	TTCTCTTTAT	GACCAGG	TTGCTCTC-T	CTCTIACATT
Prionailurus bengalensis ZFY	GTGGCTTIAC	TATTTIAAAC	TGG-GAGTGA	GATTGGIACA	GATATACTGA	ATGAAGTTTC	TG-ACCIAGA	TTTTGGTCAG	ATTTTGGAGT	TTGCTGTTAC	CTCTTTIATT
Catopuma temminckii ZFY	GTGGCTTIAC	TATTTIAAAC	TGG-GAATGA	GATTGGIACA	GATATACTGA	ATGAAGTTTC	TG-ACCIA-A	TTTTGGTCAG	ATTTTGGAGT	TTGCTGTTAC	CTCTTTIATT
Lynx Canadensis ZFY	GTGGCTTTAC	TATTTIAAAC	TGG-GAGTGA	GATTGGIACA	GATATACTGA	ATGAAGTTTC	TG-ACCIAGA	TTTTGGTCAG	ATTTTGGAGT	TTGCTGTTAC	CTCTTTT-AT
Lynx lynx ZFY	GTGGCTTIAC	TATTTIAAAC	TGG-GAGTGA	GATTGGIACA	GATATACTGA	ATGAAGTTTC	TG-ACCIAGA	TTTTGGTCAG	ATTTTGGAGT	TTGCTGTTAC	CTCTTTTIAT
Lynx rufue ZFY	GTGGCTTTAC	TATTTIAAAC	TGG-GAGTGA	GATTGGIACA	GATATACTGA	ATGAAGTTTC	TG-ACCIAGA	TTTTGGTCAG	ATTTTGGAGT	TTGCTGTTAC	CTCTTTT-AT
Oncifelis colocolo ZFY	GTGGCTTIAC	TCTTTIAAAC	TGG-GAGTGA	GATTGGIACA	GATATACTGA	ATGAAGTTTC	TG-ACCIAGA	TTTTGGTCAG	ATTTTGGAGT	TTGCTGTTAC	CTCTTTIATT
Panthera tigris ZFY	GTGGCTTIAC	IACTTIAAAC	TGGTGAGTGA	GATTGGIACA	GATACACTGA	ATGAAGTTTC	TG-ACCIAGA	TTTTGGTCAG	ATTTTGGAGT	TTGCTGTTAC	CTCTTTIATT
GW211-ZFY-L-ZFY-LF											
			ZEX-LE	Primer							
				I I IIIICI							
	710	) 720	730	740	) 750	760	)				
Prionailurus hengalensis ZFV	 	 ACAACCAA-T	CTTAACTACC	33373CCTT3	ACCTACCAAC	ACTTCCTTAA					
Catonuma temminckii ZFY	CANACTOCC		CTTAACTACC	AAATACCTTA	ACCEACCAAC	ACTIGCTIAN					
Lyny Canadansis ZEV	CANACTOGC	ACAAGGAA-I	CULTANCINCC	AAAIAGCIIA	ACCEACCAAC	ACTIGCTIAN					
Lynx Cumutensis 21 1 Lynx lynx ZFY	CANACTOCC	ACAACCAA-T	CULTANCINCC	AAATAGCIIA	ACCEACCAAC	ACTIGCTIAN					
Lynx rufue ZFY	CANACTOGC	ACAAGGAA-I	CULTANCINCC	AMAIAGCIIA	ACGIAGGAAC	ACTIGCTIAN					
Oncifelis colocolo ZFY	CARACTUGC	ACAAGGAA-T	GTIAAGIAGC	AAATAGCTTA	ACGIAGGAAC	ACTTGCTTAA					
Panthera tigris ZFY	CANANCIGGC	ACAAGGAA-T	GTIMAGIAGC	CANTAGOTIA	ACGIAGGAAC	ACTIGCTIAA					
GW210_ZFY_I_ZFI_I F	GAAAACTGGC	AGAAGGAA-T	GTTAAGTAGC	CAATAGCTTA	ACGIAGGAAC	ACTTGCITIAA					
Prionailurus hangalansis ZEV	GAAAACTGGC	ACAAGGAA-T	GTTAAGTAGC	AAATAGCTTA	ACGIAGGAAC	ACTTGC					
Catonuma tomninokii ZEV	TAAAATCAGT	GGGAAGAA-T	ATTAACCTTT	AGGAGIACTT	GTCCAATAAT	GTTCACAACA					
Calopuma temminicka ZF1	TAAAATCAGT	GGAAAGAA-T	GTIAACCTTT	AGGAATACTT	GTCCAATAAT	GTTCAGAACA					
Lynx Canaaensis ZFY	IAAA-TCAGT	G-GGAGAT	ATIA-CCTTT	AGGAGIA-TT	GTUCAATAAT	GTTCAGACAC					
Lynx tynx ZT I	'IAAA-TCAG-	GGAGAT	ATTAACCTTT	AGGAGIACTT	GTCCAATAAT	GTTCAGAACA					
Lynx rujue Z r Y Onoifalia aolooolo ZEV	IAAA-TCAGT	GGGAGGAT	ATIAACCTTT	AGGAGIA-TT	GTCCAATAAT	GTTCAGAACA					
Danih ma tionia ZEV	TAAAGTCAGT	GGGAAGAAAT	ATTAACCIAT	AGGAGIACTT	GTCCAATAAT	GTTCAGAACA					

IAAAATCAGT GGAAAGAA-T GTIAACCTTT AGGAGIACTT GTCCAAIAAT GTTCAGAACA



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

GW211-ZFY-L-ZFY-LF

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

В.	<u>10 20</u> 30 40 50 60 70 80 90 100
Prionailurus bengalensis ZFY	
Catopuma temminckii ZFY	GTEAGGEAC ATEACTTICAL CACCEGACG TECTTICEA CETTICAAT CHARACECTAG CETTETCAC CEGAEGETET GTEATEGCAT TETAGECTECT
Lynx Canadensis ZFY	GTCAGGGCAC ATCAGTTCCA CAGCGCAGCG IACTITCCCA GCTCTCACAT CAAACCCTAG CGTGTGTCCA CGCAGGTGTT GTCATGGCAT TCTAGCTGCT
Lynx lynx ZFY	GTCAGQGCAC ATCAGTTCCA CAGCGCACCG TECTTTECCA ECTCTCACAT CAAACCCIAG CETETECCA CEGAEGTETT ETCATEGCAT TCIAECTECT
Lynx rufue ZFY	GTGAGGGGAC ATGAGTTCCA CAGCGCACCG TGCTTTGCGA GCTCTCAGAT GAAACCCIAG CGTGTGTCCA CGGAGGTGTT GTGATGGCAT TCIAGCTGCT
Oncifelis colocolo ZFY	GTGAGGGGCAC ATGAGTTCCA CAGCGCA¢CG TGCTTTGCGA GCTCTCAGAT GAAACCCTAG CGTGTGTCCA CGGAGGTGTT GTGATGGCAT TCTAGCTGCT
Panthera tigris ZFY	GTEAGGECAE ATEAGTTECA CAGEGECAGEG TEETTTEEGA EETETLAEAT EAAACEETAG EGTGTETECA EGEAGGTETT ETEATGECAT TETAGETGET
GW210-ZFX-L-ZFL-LF	
Prionailurus bengalensis ZFY	GTEAGG GTGCACAAGT TCCACAGTGC
Catopuma temmineku ZT 1	
Lynx Canaaensis ZTT Lynx lynx ZFY	
Lynx rufue ZFY	
Oncifelis colocolo ZFY	
Panthera tigris ZFY	
GW211-ZFY-L-ZFY-LF	
	ZFY-LF Primer
	$^{\prime}$ 110 120 130 140 150 160 170 180 190 200 $^{\prime}$
Prionailurus bengalensis ZFY	ACACCACATT GIAGCTTCTG TGIACTGAAT TTCAAAAATAT AATTTIAAGA ATTCAGTGAT ATTCATGAAT GATTTCCTGG GATAAAAAAGA CACAAGGAAT
Catopuma temminckii ZFY	AGACCACATT GIAGCTTCTG TGIACTGAAT TTGAAAAATAT AATTTIAAGA ATTCAGTGAT ATTCATGAAT GATTTCCTGG GATAAAAAAGA AACAAGGAAT
Lynx Canadensis ZFY	AGACCACATT GIAGCTTCTG TGIACTGAAT TTGAAAAIAT AATTTIAAGA ATTCAGTGAT ATTCATGAAT GATTTCCTGG GAIAAAAAGA CACAAGGAAT
Lynx lynx ZFY	AGACCACATT GIAGCTTCTG TGIACTGAAT TTGAAAAIAT AATTTIAAGA ATTCAGTGAT ATTCATGAAT GATTTCCTGG GAIAAAAAGA CACAAGGAAT
Lynx rujue ZF Y Ousifulis solosolo ZEV	AGACCACATT GIAGCTTCTG TGIACTGAAT ITCAAAAIAT AATTTIAAGA ATTCAGTGAT ATTCATGAAT GATTTCCTGG GAIAAAAAGA CACAAGGAAT
Panthera tigris ZFY	ACACCACCAT GIACCTCTG TGIACTGAAT TTCAAAAAAT AATTTIAAGA ATTCAGTGAT AIACATCACT GATTTCCTGG GAIAAAAAGA AACAAGGAAT
GW210-ZFX-L-ZFL-LF	ACACCACAT GIACTICIG IGIACTEANT ITCAARATAT AATTITAAGA ATTCAGTCAT ATTCATCAT GATTCCIGG CACAAAAACA AACAACGAAT
Prionailurus bengalensis ZFY	ACTORNEOT-C TOTOACCTOT CAAATGAAAC TAGTATGTAT CCACAGAAGT GTTAATGATA CCAATTAGC TGCTAGATCA CATCTAGCTT TTCTTTATTA
Catopuma temminckii ZFY	GTGCT-C TGTCAGCTCT CAAATGAAAC IAGIATGIAT CCACAGAAGT GTIAATGATA GCAATTIAGC TGCTAGATCA CATGTAGCTT TTCTTTATIA
Lynx Canadensis ZFY	GTGCT-C TGTCAGCTCT CAAATGAAAC IAGIATGIAT CCACAGAAGT GTIAATGAIA GCAATTIAGC TGCIAGATCA CATGIAGCTT TTCTTTATIA
Lynx lynx ZFY	GTGCC-C TGTGAGCTCT CAAATGAAAC IAGTATGTAT CCACAGAAGT GTIAATGAIA GCAATTIAGC TGCIAGATCA CATGIAGCTT TTCTTIATIA
Lynx rufue ZFY	GTGCT-C TGTGAGGTCT CAAATGAAAC IAGIATGIAT CCACAGAAGT GTIAATGAIA GCAATTIAGC TGCIAGATCA CATGIAGCTT TTCTTIATIA
Oncifelis colocolo ZFY	GTGCT-C TGTCAGGTCT CAAATGAAAC IAGIATGIAT CCACAGAAGG TTIAATGAIA GCAATTIAGT TGCIAGATCA CATGIAGCTT TTCTTIATIA
Panthera tigris ZFY	GTGCTGC TGTGAGCTCT CANATGANAC IAGIATGIAT CCACAGAGGT GTIAATGAIA GCAATTIAGC TGCIAGATCA CATGAGCTT TTCTTIAIA
Gw211-27 1-L-27 1-L7	AGTGTGCT-C TGTCAGCTCT CAAATCAAAC IAGIATGIAT CCACAGAAGT GTIAATGAIA GCAATTIAGC TGCIAGATCA CATGIAGCTT TTCTTIATIA
	210 220 230 240 250 260 270 280 290 300
Prionailurus bengalensis ZFV	
Catopuma temminckii ZFY	GENEGRACA CEGARGEGAA ARABATICAA ANGINGGUTG ATTITITATET GEARTAGEA AGAATCCCT CARATATETE GEAGGAA AGUTTA ACCETTITA
Lynx Canadensis ZFY	GGATCGAACA CGCATCGCAA AAAAATTCAA AACGTCGGGTG ATTTTTATGT GCAAATCACA ACAAATCCCT CAAATATGTC GCAGCCATTA ACTCTTTTAA
Lynx lynx ZFY	GGATCGAACA CGGATGGGAA AAAAATTCAA AACGTGGGTG ATTTTIATGT GGAAATGAGA AGAAATCCCT CAAATATGTC GCAGGCATIA ACTCTTTIAA
Lynx rufue ZFY	GGATCGAACA CGGATGGGAA AAAAATTCAA AACGTGGGTG ATTTTIATGT GGAAATGAGA AGAAATCCCT CAAATATGTC GCAGGCATIA ACTCTTTIAA
Oncifelis colocolo ZFY	GGATCGAACA CGGATGGGAA AAAAATTCAA AACGTGGGTG ATTTTIATGT GGAAATGAGA AGAAATCCCT CAAAIATGTC GCAGGCATIA ACTCTTTIAA
Panthera tigris ZFY	GGATCGAACA TGGATGGGAA AAAAATTCAA AACGTGGGTG ATTTTIATGT GGAAATGAGA AGAAATCCCT CAAATATGTC GCAGGCATTA ACTCTTTIAA
GW210-ZFX-L-ZFL-LF	
Prionatturus bengatensis ZF 1 Catonuma tamminchii ZFV	AATTTGACAA TATAATTTA ACAATTCAGT CATATTCATG AATGATTTCC TACAATAAA ACAACAGATT TT-GACTCAG AATAAAATCT CAGGTTATTT
Lyny Canadensis ZFY	AATTIGAAAA IAIAATTITA AGAATIGAG GAIATTGATG AATGATTICE IAGAALAAAA AGAACAGATT TITGAGTCAG AALAAAATUT CAGGTLATTI Aammegaalaa galaanninga agaalaanninga ay
Lynx Cuntutensis 21 1 Lynx lynx ZFY	ANTIGRAMA TATAMITTA AGAMITGAGI GALATIGATIGA ANGALIGO TAGANTAGA AGAGAGAM UNGGONGA ANTAMAGU GAGGIATTI Dammagada galamuta dalamuta dalamugan dasangamuga dagamaga dalagan unggong dagamata agamaga dagamata
Lynx rufue ZFY	ВИТИСЛАВА ТАТАЛТИТИ ВОЛИТИСКИ СИЛИТИСКИ АЛГОГИ ПОЛИТИСКИ ПОЛИТИКИ И СЛОВОТИТИ И СОСТОВИТИ И СОСТОВИТИТИ Алгитесарая, татаятита, асаритески сатаятские артеситаетиски тасаратарая, асарсасит и по-сасиске алтараятся сосс
Oncifelis colocolo ZFY	ANTITICAANA TATAATITTA AGAATICAGT CATATICATE AATGATITCE TAGAATAANA ACAACAGATI IT-GAATCAG AATAAAATCI CAGGITATIT
Panthera tigris ZFY	AATTTGAAAA IAIAATTTIA AGAATTCAGT CAIATTCATG AATGATTTCC IAGAAIAAAA AGAACAGATT TT-GAGTCAG AAIAAAATCT CAGGTIATTT
GW211-ZFY-L-ZFY-LF	AATTTGAGAA IAIAATTTIA AGAATTCAGT GAIATTCATG AATGATTTCC IAGAAIAAAA ACAACAGATT TT-GAGTCAG AAIAAAATCT CAGGTIATTT
	310 320 330 340 350 360 370 380 390 400
Prionailurus bengalensis ZFY	
Catopuma temminckii ZFY	APACTCAATG ACTACAAAGA ATCTGATTCT GGTAGGATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCCAG TTCATGTGAC CTATTCCTGT
	AAACTCAATG ACTACAAAGA ATCTCATTCT GGTAGGATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT AAACTCAATG ACTACAAAGA ATCTGATTCT GTTAGGATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT
Lynx Canadensis ZFY	AAACTCAATG ACTACAAAGA ATCTCATTCT GGTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT AAACTCAATG ACTACAAAGA ATCTGATTCT GTTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT
Lynx Canadensis ZFY Lynx lynx ZFY	AAACTCAATG ACTACAAAGA ATCTCATTCT GGTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT AAACTCAATG ACTACAAAGA ATCTGATTCT GTTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGCATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGAC CTATTCCTGT AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGCATAA AGTAGGCATA
Lynx Canadensis ZFY Lynx lynx ZFY Lynx rufue ZFY Opsificia solosol - ZEV	AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGGATAA AGTAGGCATA AAACTCAATG ACTACAAAGA ATCTGATTCT GTTAGGATAA AGTAGGCATA AAACTCAATG ACTACAAAGA ATCTGATTCT GTTAGGATAA AGTAGGCATA AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGGATAA AGTAGGCATA AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGGATAA AGTAGGCATA AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGGATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGCAC CTATTCCTGT AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGGATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGCAC CTATTCCTGT AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGGATAA AGTAGGCATA CTTTATAGAG CAGGAGGACG GGTACTCGAG TTCATGTGCAC CTATTCCTGT AAACTCAATG ACTACAAAGA ATCTGATTCT GGTAGGATAA AGTAGGCATA
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Fig. 2. Continued.

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

Table 1. Primer set and sequence information in the 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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