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PRIMER NOTE

Development of primers to characterize the mitochondrial control region of the snow leopard (*Uncia uncia*)

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Abstract

The snow leopard (*Uncia uncia*) is a rare carnivore living above the snow line in central Asia. Using universal primers for the mitochondrial genome control region hypervariable region 1 (HVR1), we isolated a 411-bp fragment of HVR1 and then designed specific primers near each end of this sequence in the conserved regions. These primers were shown to yield good polymerase chain reaction products and to be species specific. Of the 12 snow leopards studied, there were 11 segregating sites and six haplotypes. An identification case of snow leopard carcass (confiscated by the police) proved the primers to be a useful tool for forensic diagnosis in field and population genetics studies.

Keywords: control region, Felidae, mitochondrial DNA, snow leopard, species-specific primers

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The snow leopard (*Uncia uncia*) is one of the most endangered big cats (Office of NABU 2001). It is categorized as 'Endangered' on the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List and is listed in the Appendix I of the Convention for the International Trade of Endangered Species of Wild Fauna and Flora (CITES) (Charudutt *et al*. 2003). Only about 4500–7500 (McCarthy & Chapron 2003) snow leopards survive in the wild. But the demand for snow leopard furs in international trade and other usage of snow leopard tissues threaten their survival. Extinction of the snow leopard will occur soon if no measures are taken. However, few data have been available on the population genetics of this species to provide information for targeted management. In this study, we developed species-specific primers for the mitochondrial control region hypervariable region 1 (HVR1) of the snow leopard, which will be useful in wildlife forensics, species identification and population genetics studies.

Skin and hair samples of snow leopard were collected and stored at –20 °C. Seven skin samples were collected from Qinghai Province, and the other two were collected in Yanchiwan Nature Reserve, Gansu Province; three hair samples were plucked from individual snow leopards in the Beijing Zoo. Hair samples of other eight species or subspecies of Felidae (*Panthera tigris tigris*, *Panthera leo*, *Panthera tigris altaica*, *Panthera tigris amoyensis*, *Lynx lynx*, *Acinonyx jubatus*, *Panthera pardus orientalis*, *Panthera pardus*) were plucked from the animals in the Beijing Zoo as well. Total genomic DNA was extracted using standard proteinase K, phenol–chloroform procedures (Sambrook *et al*. 1989). Polymerase chain reaction (PCR) was initially undertaken on eight of the DNA samples using universal primers L15926 and H16498 (Kocher *et al*. 1989; Shields & Kocher 1991). Amplification reactions were performed in a 50-μL volume, containing 10 ng of genomic DNA, 2.0 mM Mg^{2+}, 200 μM of each dNTP, 0.4 μM of each primer and 2 U of TaKaRa *Taq* DNA polymerase (TaKaRa). The optimal conditions in PCR were 94 °C initial for 5 min, 35 cycles of 94 °C denaturation for 45 s, 50 °C annealing for 30 s, 72 °C extension for 45 s, and a final 7-min extension at 72 °C. Because there is no snow leopard control region sequence data available in GenBank, we amplified cyt *b* gene from the same DNA samples using universal primers L14724 and H15149 to confirm that the PCR products were the mitochondrial DNA of snow leopard. Cyt *b* was amplified in similar conditions as control region except for annealing at 56 °C. Both PCR products of the control region and cyt *b* were purified using a QIAGEN PCR purification kit and subsequently sequenced for both forward and reverse strands using ABI BigDye chemistry on an ABI 377 Genetic

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Table 1 Segregating sites and their frequencies for each of the observed mitochondrial HVR1 haplotypes of the snow leopard. Observed frequencies of variable nucleotide and numbers of observed sequences in haplotypes are enclosed in parentheses

Positions of nucleotide of segregating sites											
Position	12	153*	355*	365*	368*	370	393*	399	400*	407	410
Variable nucleotide (frequency)	A (11) C (1)	G (6) A (6)	A (10) G (2)	G (8) A (4)	G (10) A (2)	T (11) C (1)	A (10) C (2)	C (10) G (1) T (1)	T (10) A (2)	T (11) C (1)	A (11) G (1)
Haplotypes											
Hap01 (n = 5)	A	G	A	G	G	T	A	C	T	T	A
Hap02 (n = 2)	A	A	A	G	G	T	A	C	T	T	A
Hap03 (n = 2)	A	A	G	A	G	T	A	C	T	T	A
Hap04 (n = 1)	C	G	A	G	G	T	A	C	T	T	A
Hap05 (n = 1)	A	A	A	A	A	T	C	G	A	C	G
Hap06 (n = 1)	A	A	A	A	A	C	C	T	A	T	A

*Parsimony informative sites.

Analyser according to the manufacturer's instructions. In a GenBank BLAST search, the 425 base sequences of cyt *b* aligned (98%) with a section of snow leopard mitochondrial DNA cyt *b* (GenBank Accession no. D28904).

The sequences representing control-region DNA from each of the eight snow leopards were aligned manually, and the sections of sequence towards each end of the generated sequences were observed to be fully conserved in these eight snow leopards. OLIGO 6.0 software (Molecular Biology Insights) was then used to determine an optimal primer pair anchored in the conserved regions at each end of the sequence. These primers were then synthesized by Biotech Company as sequencing quality. PCR was then undertaken on DNA samples from eight snow leopards and other species or subspecies of Felidae (mentioned in the sampling part). Amplification was then performed on the full 12 samples of snow leopard. A forensic identification of snow leopard in a poaching case provided a practical test of the applicability of the newly developed primers. PCR for HVR1 was performed using new primers in a 50-μL reaction mix as described previously. The protocol involved an initial 5 min at 94 °C followed by 35 cycles of 45 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C, followed by 7 min at 72 °C. Consistently, for verification, cyt *b* (425 bp) was also amplified separately on DNA from the confiscated suspect muscle in a 50-μL reaction mix using optimal conditions as described previously. Approximately 5 μL of each reaction mixture was electrophoresed in 1% agarose gel and sequences were analysed as described above.

The primers designed in this study are forward: GGAAGAAGCAACAGCCCC, and reverse: TCCATAGAAGAACATTGTTA. The amplicon length is about 500 bp as determined by sequencing. Results of the PCR and electrophoresis for the panel of felid species are shown in Fig. 1. Clear bands were evident in lanes 1–8 aligning approximately with the 500-bp marker band. These were the lanes generated from snow leopard DNA and were verified by sequencing for cyt *b* gene. In lanes 10–17, representing PCR on the DNA from the other felid species, no clear bands were seen at 500 bp, and only some light PCR/stain artefacts were observed in the gel. PCR using the specific primers on DNA from the suspected muscle of snow leopard gave similarly prominent single band; cyt *b* sequencing and phylogenetics verified that the sample was taken from an individual of snow leopard. In the phylogenetic trees of cyt *b*, the muscle has been clustered in snow leopard clade strongly (bootstrap is 100%, not shown). In the control region sequences from the overall 12 snow leopards, there were 11 segregating sites and six haplotypes (Table 1). Nucleotide positions at 153, 355, 365, 368, 393 and 400 were parsimony-informative sites.

It was concluded that these new primers provide a useful tool specifically for amplifying the control region of the mitochondrial genome in snow leopard. These primers may be used for population genetic studies and species identification in the field.

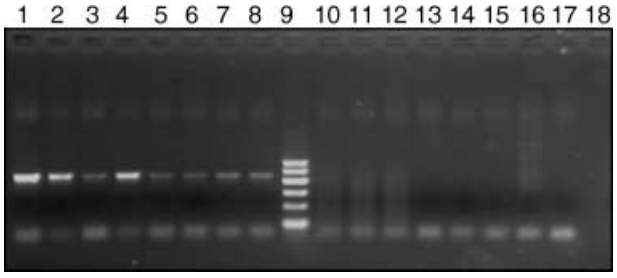

Fig. 1 Electrophoresis results of PCR products from DNA of eight snow leopards (lanes 1–8), other species or subspecies of Felidae (lanes 10–17, *Panthera tigris tigris*, *Panthera leo*, *Panthera tigris altaica*, *Panthera tigris amoyensis*, *Lynx lynx*, *Acinonyx jubatus*, *Panthera pardus orientalis*, *Panthera pardus*), blank control (lane 18) and size marker ladder (lane 9).

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