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Population monitoring of snow leopards using noninvasive collection of scat samples: a pilot study

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Abstract

The endangered snow leopard *Panthera uncia* occurs in rugged, high-altitude regions of Central Asia. However, information on the status of this felid is limited in many areas. We conducted a pilot study to optimize moecular markers for the analysis of snow leopard scat samples and to examine the feasibility of using noninvasive genetic methods for monitoring this felid. We designed snow leopard-specific primers for seven microsatellite loci that amplified shorter segments and avoided flanking sequences shared with repetitive elements. By redesigning primers we maximized genotyping success and minimized genotyping errors. In addition, we tested a Y chromosome-marker for sex identification and designed a panel of mitochondrial DNA primers for examining genetic diversity of snow leopards using scat samples. We collected scats believed to be from snow leopards in three separate geographic regions including north-western India, central China and southern Mongolia. We observed snow leopard scats in all three sites despite only brief 2-day surveys in each area. There was a high rate of species misidentification in the field with up to 54% of snow leopard scats misidentified as red fox. The high rate of field misidentification suggests sign surveys incorporating scat likely overestimate snow leopard abundance. The highest ratio of snow leopard scats was observed in Ladakh (India) and South Gobi (Mongolia), where four and five snow leopards were detected, respectively. Our findings describe a species-specific molecular panel for analysis of snow leopard scats, and highlight the efficacy of noninvasive genetic surveys for monitoring snow leopards. These methods enable large-scale noninvasive studies that will provide information critical for conservation of snow leopards.

Introduction

The endangered snow leopard *Panthera uncia* is primarily distributed in high-altitude mountain ranges in Central Asia (Fig. 1; Nowell & Jackson, 1996; Sunquist & Sunquist, 2002). It is believed to occur at low densities and has a patchy distribution as a result of natural habitat fragmentation, habitat degradation, declining prey populations, poaching and other anthropogenic threats (Nowell & Jackson, 1996). However, throughout much of its range, snow leopard distribution, status and population history remains poorly described as there have been few detailed studies (Jackson, 1996; Oli, 1997; McCarthy, Fuller & Munkhtsog, 2005). Additional information is needed for developing effective conservation plans that will enable the long-term persistence of this endangered felid.

The majority of snow leopard studies have consisted of surveys that relied upon sign (e.g. pugmarks, scrapes and scats) and interviews with local inhabitants, and provided only rudimentary information (Schaller, 1988; Schaller, Ren & Qiu, 1988; Jackson & Hunter, 1996; Hussain, 2003; McCarthy & Chapron, 2003; Ale, Yonzon & Thapa, 2007). Recently, Jackson *et al*. (2006) used camera trapping to obtain a quantitative estimate of population size of snow leopards in Hemis National Park, India (estimated densities of 4.5–8.5 snow leopards/100 km^2). However, this approach has several disadvantages including the need for extended time in the field ($\geq$40–50 days; Jackson *et al*., 2006), the

difficulty of setting camera traps in snow leopard habitat, and the high cost of field work in remote areas of Central Asia. Additional methods to supplement sign surveys and camera trapping are therefore needed for effective monitoring of snow leopards.

Population genetics has become widely used for research and conservation of both abundant and rare species (Schwartz, Luikart & Waples, 2007). Molecular methods incorporating noninvasive sampling via the collection of scats or hairs have become common for population monitoring of carnivores (Taberlet, Waits & Luikart, 1999; Piggott & Taylor, 2003; Waits & Paetkau, 2005; Schwartz *et al*., 2007). The successful application of noninvasive genetic techniques to different facets of carnivore biology suggests their usefulness for monitoring snow leopards. Snow leopards use predictable paths and mark frequently by scraping and depositing scat and urine in distinctive places (Ahlborn & Jackson, 1988), likely facilitating noninvasive sampling. However, the applicability of this technique to a new species must be explored before embarking on large-scale studies (Taberlet *et al*., 1999; Waits & Paetkau, 2005; Valiere *et al*., 2007).

One critical aspect of any noninvasive study involves the optimization of genetic techniques. Previous studies have observed error rates (i.e. amplification failure, allele dropout and false alleles) in microsatellite analyses that can significantly affect population size estimates (Taberlet *et al*., 1999; Mills *et al*., 2000; Waits, Luikart & Taberlet, 2001; Creel *et al*., 2003; McKelvey & Schwartz, 2004). Most errors have been attributed to the low quantity and quality of target DNA typically extracted from noninvasive samples (Taberlet *et al*., 1999; Pompano *et al*., 2005). However, inconsistencies across different microsatellite loci observed in many studies suggest errors may also result from other causes (Broquet & Petit, 2004). Particularly, genomic and evolutionary factors, such as repetitive elements and sequence divergence (Buchanan *et al*., 1993; Nadir *et al*., 1996; Lopez-Giraldez *et al*., 2006), have been previously underappreciated in noninvasive studies as a source of genotyping errors.

Many microsatellites were isolated before the distribution and behavior of repetitive elements, along with the formation of microsatellites, was understood. Microsatellites frequently occur in repetitive elements of vertebrate genomes, often found within or adjacent to short interspersed nuclear elements (SINEs), long interspersed nuclear elements (LINEs) and long terminal repeat (LTRs) transposons (Buchanan *et al*., 1993; Nadir *et al*., 1996; Lopez-Giraldez *et al*., 2006). Primers that are designed within a repetitive element adjacent to a microsatellite can cause weak amplification, background peaks and locus-specific dropout (Lopez-Giraldez *et al*., 2006). In addition, many studies use heterologous microsatellites. For example, primers that amplify domestic cat *Felis catus* microsatellites (Menotti-Raymond *et al*., 1999) have been recently used in snow leopards (Waits *et al*., 2007). As taxa diverge, mutations accumulate leading to primer mismatches that can increase the incidence of null alleles and cause weak PCR amplification (Callen *et al*., 1993; Menotti-Raymond *et al*., 1999; Culver, Menotti-Raymond & O'Brien, 2001; Menotti-Raymond *et al*., 2003). The impact of this potential source of error has not been thoroughly evaluated in previous noninvasive studies.

Here we describe the design and optimization of a snow leopard-specific mitochondrial, microsatellite and Y-chromosome panel and use it to conduct a preliminary noninvasive survey of the snow leopard in three diverse regions of Central Asia. We aim to provide robust molecular markers for facilitating large-scale in-depth surveys to determine the status of snow leopard populations, and illustrate the effectiveness of noninvasive sampling of snow leopard populations via scat collection.

Methods

Study area, sample collection and DNA extraction

Snow leopard populations were noninvasively sampled by collecting scats in three different geographic regions: (1) one site in Ladakh, India; (2) two sites in Dulan County, Qinghai, China; (3) three sites in South Gobi (Fig. 1). See Supplementary material for GPS locations and elevation. Local wildlife biologists and knowledgeable residents (e.g. herders and former hunters) guided field teams to areas inhabited by snow leopards.

Teams searched ridgelines and outcrops for snow leopard scrapes and tracks. A total of 5.4, 11.1 and 9.7 km were surveyed in Ladakh, Qinghai and South Gobi, respectively. Transects followed wildlife trails along features (i.e. saddles, ridgelines, and outcrops) frequently used by snow leopards (Ahlborn & Jackson, 1988; McCarthy & Munkhtsog, 1997). Putative species identification of scats was made in the field based on morphology and proximity to snow leopard sign (Jackson & Hunter, 1996). Primarily, snow leopard scats were collected in Ladakh and South Gobi, and all intact carnivore scats were collected in Qinghai. Samples were stored in 15 mL centrifuge tubes with *c*. 12 mL of silica desiccant covered by a clean Kimwipe® tissue (Kimberley-Clark, Irving, TX, USA) to separate the desiccant from the scat. DNA was extracted from scat using the Qiagen Stool DNA extraction kit (Qiagen, Valencia, CA, USA). Two snow leopard DNA samples (one male and one female) were obtained from the Zoological Society of San Diego and used as controls.

Species identification

A 148 bp region of the mitochondrial cytochrome *b* gene was amplified by PCR using carnivore-specific primers (Farrell, Roman & Sunquist, 2000), sequenced and used for species identification by comparing sequences to reference taxa (see Supplementary material for detailed methodology).

Redesigning microsatellite primers

We sequenced 12 microsatellite loci found to be variable in captive snow leopards (Waits *et al*., 2007) that were

Figure 1 Snow leopard *Panthera uncia* distribution (adapted from Fox 1994) along with the locations of the study areas.

originally isolated in the domestic cat (Menotti-Raymond *et al*., 1999). Primers were redesigned for seven loci to (1) precisely match the snow leopard sequence; (2) amplify segments <150 bp; (3) avoid designing primers in flanking sequences that contain SINEs, LINEs and LTRs. The specific details for methods used to redesign primers and optimize PCR conditions are described in the Supplementary material. These new primers were designated as 'PUN' with the same number as the respective FCA locus described by Menotti-Raymond *et al*. (1999). We compared genotypes between PUN primers and the original FCA primers for six loci using DNA extracted from 17 confirmed snow leopard scats. All amplifications of DNA extracted from scats were conducted in triplicate in a pre-PCR dedicated lab area.

Genotyping success, allele dropout and false alleles were compared between FCA and PUN primers for six loci. Genotyping success was defined as obtaining a consensus genotype from at least two unambiguous PCRs, observing each allele two or more times, and observing homozygous alleles three times. Allele dropout was defined as one allele in a heterozygote failing to amplify; only samples that had a consensus genotype for both FCA and PUN primer pairs were used for detecting this source of error. False alleles were defined as peaks that were misidentified as microsatellite alleles, but were not replicated in subsequent reactions. Nonmicrosatellite amplicons were defined as PCR products that did not have the characteristic 2 bp stutter of dinucleotide short tandem repeats (STRs) or were outside of the expected allele size. We also compared the quality index (Q) proposed by Miquel *et al*. (2006) for FCA and PUN primers. This is a standardized index of reliability of genotypes to enable more reasonable comparisons among studies. For the Q calculations, genotypes that were identical to the consensus genotypes were scored as 1 and those that were different were scored as zero (i.e. PCR failure, allele dropout and false alleles treated the same way). The total number of successful genotypes was added together and divided by PCR attempts, and summed across loci. The Student's t-test was used to compare Q for FCA and PUN primers.

Table 1 Sequences of seven snow leopard *Panthera uncia*-specific microsatellite primers

	Primer sequence	Allele size	Allele size with FCA primers
PUN82	F: CCGCTTAAGAAGAGGCTAAAAA	110–115	253–258
	R: TCAAAGAGTGTTGTTAGATAATATGGA		
PUN100	F: GATTTGAACCCAAAGAAAAAGA	88–96	107–115
	R: ATCCCTCTAGACATCTTAGTCACC		
PUN124	F: CCATTCCCTCCCTGTCTGTA	90–100	115–125
	R: TGTCCTCAAACCATAGACAGTTTC		
PUN132	F: CGAAATGCAGTAATGTTAGTTTTACA	113–123	165–175
	R: CACGGGTTCGTCTCTTTTG		
PUN225	F: CTGAGGCCAGCAAATCATTC	177–183	228–234[a]
	R: CCCACAGAAACTTCCTGCTT		
PUN229	F: AGACAAACTGACAAGCTTAGAGG	103–113	160–170
	R: TCATGTCTTTACATTCATTTCTTTTT		
PUN327	F: TTAATTTGGGGAATGTTAGGG	79–91	183–185
	R: TGCAACATCTATCAGTGAAATAACA		

Sizes of alleles are for those observed in snow leopards from Ladakh and South Gobi that were examined during this study.
[a]For the FCA/PUN225 locus, our study populations were not genotyped using the FCA primer so the allele sizes are those observed by Waits *et al.* (2007).

Individual identification using microsatellites

Seven loci were used to genotype 31 genetically identified snow leopard scats with the new PUN primers (Table 1). In a preliminary analysis of six snow leopard scats, six independent amplifications were conducted for all the seven loci. For three samples, increasing the number of amplifications to six did not affect our ability to produce reliable genotypes. For the other three samples, three amplifications were sufficient to obtain consistent genotypes. Therefore, we decided that three independent PCRs provided a reasonable compromise between minimizing error and maintaining cost effectiveness. To further reduce error, we required the following guidelines for a consensus genotype to be included in our sample set: heterozygous alleles must have been observed two or more times, and homozygous alleles three times. A similar filtering strategy (although only two detections of homozygous alleles were required) was used previously by Adams & Waits (2007) and Kay, Gompper & Ray (2008).

Microsatellite diversity and Hardy–Weinberg (HW) equilibrium were calculated in GENEALEX (Peakall & Smouse, 2006). The sequential Bonferroni technique was used to correct for multiple comparisons in the HW equilibrium tests (Rice, 1989). The probability of identity (P_{ID}) was estimated in GENEALEX for unrelated individuals and siblings ($P_{ID\text{-}sibs}$) in Ladakh and South Gobi based on the diversity observed in the respective areas (Paetkau & Strobeck, 1994; Waits *et al.*, 2001).

Mitochondrial DNA (mtDNA) panel for genetic structure

Mitochondrial primers were tested and designed for genetic structure analysis of snow leopard populations using portions of three protein-coding genes and the noncoding control region. Primers used for phylogeographic analysis of clouded leopards (241 bp of cytochrome *b*, 318 bp of *ND5* and 155 bp of *ATP8*; Buckley-Beason *et al.*, 2006) were tested by PCR in snow leopard scats. A published snow leopard control region sequence (Jae-Heup *et al.*, 2001) was used to design primers for the amplification of four segments (<185 bp each, total 631 bp). These mtDNA segments were sequenced in three individuals from Ladakh, three individuals from South Gobi and one individual from Qinghai. The number of variable sites, haplotype diversity, π (nucleotide diversity) and mean number of nucleotide differences were calculated in DNASP (Rozas *et al.*, 2003).

Sex identification of scats

Sex identification was performed with felid-specific primers (Murphy *et al.*, 1999) that amplified a 200 bp intronic segment of the *AMELY* gene. Amplifications were performed in triplicate along with one male positive control DNA, one female positive control DNA and one negative control (water only). The PCR products were run on a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. A sample was identified as male if at least two of three PCRs gave strong amplification of the Y-linked marker. If there was no amplification of the Y-linked marker in all three replicates the sample was considered to be female. Only scats that were successfully genotyped with the complete seven-microsatellite panel were positively identified as female, to avoid misidentifying male samples as female due to PCR amplification failure.

Results

Species identification

Phylogenetic analysis using a 100 bp segment of cytochrome *b* produced a tree consistent with more extensive

Table 2 Scats collected during field surveys in three study areas of Central Asia

	Ladakh	Qinghai	South Gobi
Total transect length	5.4 km	11.1 km	9.7 km
Scat collected	32	50	27
Genetic species ID	25 (78%)	36 (72%)	24 (89%)
Snow leopard scat	17	3	11
Lynx scat	0	10	0
Red fox scat	6	21	13
Wolf/dog scat	2	2	0
Field species ID error	35%	NA[a]	54%
Complete microsatellite genotypes	12 (71%)	0 (0%)	8 (73%)
Individual snow leopards detected	4 (2M, 2F)	1[b]	5 (3M, 2F)

Species identification is based on cytochrome *b* sequence. Individuals were identified using the seven-microsatellite panel described in this study and sex identification was made using felid-specific *AMELY* intron primers.

[a]All carnivore scats were sampled regardless of species field identification.

[b]The scats from Qinghai did not yield consensus genotypes and therefore it could not be determined that the Y-marker failed to amplify in the remaining two individuals as a result of PCR failure.

ID, identification.

Table 3 Repetitive elements detected in flanks of domestic cat *Felis catus* microsatellite loci

Microsatellite	5′ flank	3′ flank
FCA32	No repeats	No repeats
FCA75	No repeats	No repeats
FCA82	No repeats	No repeats
FCA88	SINE[a]	Uncharacterized repetitive element[b]
FCA96	SINE[c,b,a]	SINE[c]
FCA100	No repeats	LINE[a]
FCA124	No repeats	No repeats
FCA126	LTR[c]	LTR[c]
FCA132	No repeats	SINE[c]
FCA171	No repeats	Low complexity repeat[b]
FCA225	LINE[c,a]	LINE[a]
FCA229	No repeats	No repeats
FCA275	No repeats	SINE[c,b,a]
FCA327	No repeats	No repeats

The sequences were obtained from GENBANK and were those used by Menotti-Raymond *et al.* (1999) to design the original FCA primers. Searches for repetitive elements in flanking sequences were performed with three approaches: REPEATMASKER, BLAT using the domestic cat 1.9× genome sequence assembly and annotated repeats, and BLAST of trace archives.

[a]REPEATMASKER search for repetitive elements.

[b]BLAST of domestic cat trace archives.

[c]BLAT of assembled domestic cat genome.

LTR, long terminal repeat; LINE, long interspersed nuclear element; SINE, short interspersed nuclear element.

phylogenetic studies of carnivores (Fig. 2; Wayne *et al.*, 1997; Bardeleben, Moore & Wayne, 2005; Johnson *et al.*, 2006). The range of sequence divergence within species was 0–0.03 (e.g. tiger, wild cat) and the minimum sequence divergence between closely related felid taxa was 0.04 (Eurasian lynx *Lynx lynx* vs. Iberian lynx *Lynx pardinus*). This short segment was unable to differentiate between domestic dog and wolf, or between domestic cat and wildcat. The Tibetan and Corsac fox showed a low level of sequence divergence (0.03).

Positive species identification was made for 78% of the scat samples (Table 2). Of the 32 scats collected in Ladakh, the snow leopard was most common, with red fox and wolf/dog scats also being detected. In Qinghai, the most commonly observed species was red fox, with lynx also detected more often than snow leopard. The snow leopard and red fox were observed in South Gobi in approximately equal ratios. There was only one haplotype observed in the snow leopard (Fig. 2). In contrast, there were two lynx ($\pi = 0.030$), four red fox ($\pi = 0.017$) and two wolf/dog ($\pi = 0.010$) haplotypes observed among the different regions.

Microsatellite primers

Four microsatellite loci had flanks that were partially or entirely composed of known repetitive elements (FCA88, FCA96, FCA126 and FCA225), while four additional loci (FCA100, FCA132, FCA171, FCA275) had just one flank that contained repetitive sequences (Table 3). We were able to amplify only 12 of 14 loci (i.e. FCA32 and FCA75 failed) in the snow leopard using the sequencing primers redesigned to include additional flanking sequence from the domestic cat genome. Of these, two loci (FCA171 and FCA275) produced multiple amplicons of different sizes. These PCR products were cloned and sequenced, and did not contain any microsatellite repeats. However, large proportions of the sequences from these clones contained SINE or LINE elements.

For the remaining 10 microsatellite loci, we obtained 1287 bp of snow leopard sequence flanking the STRs. There were a total of 41 nucleotide substitutions and four indels between snow leopard and domestic cat flanking sequences, yielding a mean pair-wise mismatch of 3.2% for microsatellite flanks. The snow leopard sequence also had a 27 bp insertion in the 3′ flank of FCA132, and a 4 bp deletion in the 5′ flank of FCA327, relative to the orthologous domestic cat sequence. Five loci had at least one mutation distinguishing the snow leopard flank from the corresponding FCA primer (FCA88, FCA96, FCA100, FCA132, FCA225). We observed five point mutations in the STR repeats of four microsatellites (FCA82, FCA96, FCA100, FCA126) and a 1 bp indel in the FCA327 STR repeat.

Comparison between FCA versus PUN primers

We redesigned primer pairs for 10 microsatellite loci from the orthologous snow leopard sequences, and seven of these loci produced robust amplification in scat DNAs. The

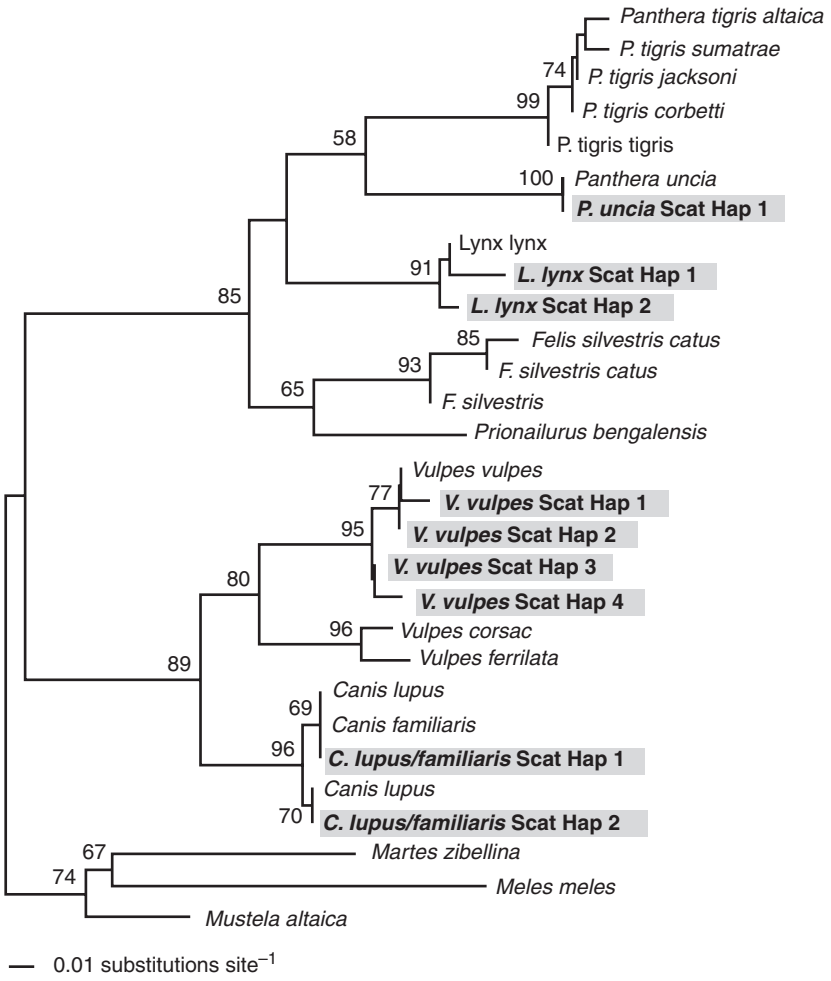

Figure 2 Neighbor joining tree reconstructed from 100 bp of cytochrome *b* for species identification of scats, using the Kimura two-parameter model of sequence evolution. Reference sequences are in regular italicized font and were obtained from GenBank or sequenced from DNA tissue extractions (see Supplementary material for accession numbers). Sequences obtained from scats collected in the field are shown in bold, highlighted in light gray and are denoted as scat haplotypes (Scat Hap). Species identification was made based on divergence from reference taxa (<0.03) and a $\geq 90\%$ bootstrap support clustering criterion. Bootstrap values are given for nodes with $>50\%$ support.

Table 4 Comparison between FCA primers and PUN primers for microsatellite genotyping of snow leopard *Panthera uncia* scats

	Genotyping success			Allele dropout			False alleles				N-M amplicons (10%)[a]				N-M Amplicons (30%)[a]			
Microsatellite locus	n[b]	FCA	PUN	n[c]	FCA	PUN	n[d]	FCA	n[d]	PUN	n[d]	FCA	n[d]	PUN	n[d]	FCA	n[d]	PUN
82	17	35.3%	70.6%	17	17.6%	0	19	10.5%	36	0	28	11%	45	0%	28	11%	45	0
100	17	58.8%	70.6%	21	19.0%	0	33	0.0%	33	0	45	7%	41	5%	45	0%	41	0
124	17	41.2%	70.6%	18	5.6%	5.6%	21	4.8%	41	2.4%	30	30%	50	0%	30	27%	50	0
132	17	23.5%	76.5%	6	0	0	14	0.0%	38	0	23	26%	47	0%	23	22%	47	0
229	17	58.8%	70.6%	10	30.0%	20.0%	32	3.1%	40	2.4%	41	2%	49	0%	41	0%	49	0
327	17	58.8%	76.5%	25	12.0%	0	32	0.0%	37	0	41	15%	46	0%	41	0%	46	0
Mean	17	46.1%	72.6%	16	14.0%	4.3%	25	3.1%	38	0.8%	35	15%	46	1%	35	10%	46	0

The percentage of genotyping success, allele dropout, false alleles and nonmicrosatellite (N-M) amplicons among samples genotyped at six microsatellite loci originally characterized in the domestic cat (Menotti-Raymond *et al.*, 1999). Loci were amplified using both the original FCA domestic cat primers and the snow leopard-specific PUN primers designed in this study.

[a]Electropherogram trace intensity of nonmicrosatellite amplicon relative to the minimum microsatellite allele intensity.

[b]The number of scat samples genotyped with both FCA and PUN primers.

[c]The number of heterozygous genotypes used to compare allele dropout between FCA and PUN primers. Only samples for which both FCA and PUN genotypes were obtained were used.

[d]The number of genotypes used in estimating the rate of false alleles and nonmicrosatellite amplicons.

overall genotyping success rate was higher for PUN (72.6%) primers than for the original FCA (46.1%) primers (Table 4). The PUN primers yielded consensus genotypes across six loci (composite genotype) for 11 scats, whereas only four scats had complete composite genotypes when FCA primers were used. Allele dropout was observed in five out of six FCA primers and only two out of six PUN primers, with a lower mean allele dropout rate among PUN

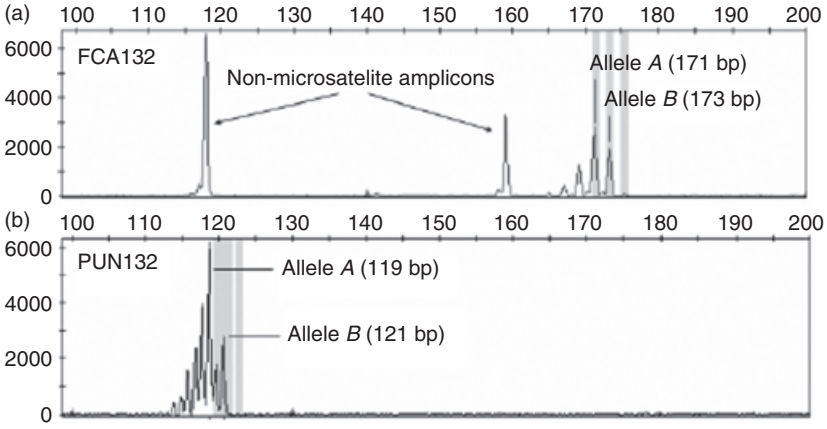

Figure 3 Electropherograms of PCR products amplified from a snow leopard *Panthera uncia* scat sample. (a) FCA132 amplified using the original domestic cat *Felis catus* primers (FCA132). (b) The bottom of the figure shows the amplified products for the same locus using the redesigned primers (PUN132) based on snow leopard sequence, positioned closer to the short tandem repeat (microsatellite alleles are 52 bp shorter) while avoiding repetitive elements.

Table 5 Levels of genetic diversity in seven microsatellite loci observed in snow leopards *Panthera uncia*

	Total number of alleles		Number of private alleles		Observed heterozygosity		Expected heterozygosity		Hardy–Weinberg equilibrium	
	Ladakh	S Gobi	Ladakh	S Gobi	Ladakh	S Gobi	Ladakh	S Gobi	Ladakh	S Gobi
PUN82	2	2	0	0	1.00	0.80	0.50	0.48	0.05	0.14
PUN100	3	3	0	0	0.75	0.40	0.53	0.56	0.70	0.14
PUN124	4	4	1	1	1.00	1.00	0.66	0.72	0.68	0.54
PUN132	3	2	2	1	0.50	0.20	0.59	0.18	0.70	0.80
PUN225	3	2	1	0	0.50	0.40	0.53	0.48	0.34	0.71
PUN229	3	3	2	2	0.50	0.60	0.59	0.46	0.70	0.82
PUN327	4	2	3	1	1.00	0.18	0.66	0.18	0.68	0.80
Mean	3.1	2.5	1.3	0.71	0.75	0.51	0.58	0.44	NA	NA

Data are based on unique genotypes among noninvasively sampled snow leopards in Ladakh ($n=4$) and South Gobi ($n=5$).
NA, not applicable.

primers. False alleles were observed in fewer samples with PUN primers. In addition, for three FCA primer pairs (FCA82, FCA124, FCA132; Fig. 3), some nonmicrosatellite amplicons had fluorescent signals $>30\%$ of the intensity of the microsatellite alleles, whereas the peak heights of nonmicrosatellite amplicons for the corresponding PUN primer pairs were $<10\%$ of the true microsatellite allele intensity. The global Q (index for all loci and samples combined) was 0.43 for PUN primers and 0.22 for FCA primers. The Q-value for genotypes generated with PUN primers was significantly higher than genotypes generated with FCA primers ($P = 0.0000064$).

Individual and sex identification, and microsatellite diversity

We genotyped 31 confirmed snow leopard scats with seven PUN microsatellites. The success rate for composite genotypes was 71 in Ladakh and 73% in South Gobi; however, we could not obtain composite genotypes for the three scats from Qinghai (Table 1). We identified two males and two females in Ladakh, one male in Qinghai and three males and two females in South Gobi.

All seven loci were polymorphic in both Ladakh and Qinghai with numbers of alleles ranging from two to four (Table 5). There were no available snow leopard samples to estimate allele frequencies *a priori* for the study populations, so we estimated allele frequencies and genetic parameters based on the unique genotypes observed. No loci were found to be out of HW equilibrium. The observed heterozygosity was lower in both areas compared with the expected heterozygosity. However, because of the low number of individuals available for allele frequency estimation statistical significance could not be tested. Overall, the number of alleles and levels of heterozygosity were lower in South Gobi (nine private alleles in Ladakh vs. five in South Gobi, Table 5). The probability of identity (P_{ID}) is the chance that two individuals in a population share the same genotype. The P_{ID} for each locus was higher than previously described by Waits *et al.* (2007); however, the estimates in the previous study were generated from snow leopards obtained from zoos, originating from different geographic regions. Therefore, these P_{ID} estimates are not specific to a population (see Supplementary material Table S1 for comparison). As there are no available reference samples from the snow leopard populations we sampled, we estimated P_{ID} from unique genotypes observed among the scat samples. In Ladakh,

P_{ID} was <0.01 for PUN124, PUN132, PUN327 and the $P_{ID\text{-}sib}$ was 0.01 with all seven loci (Fig. 4). The P_{ID} in South Gobi was <0.01 for PUN82, PUN100 and PUN124, and $P_{ID\text{-}sib}$ 0.035 with all seven loci (Fig. 4).

MtDNA genetic diversity

A total of 1445 bp of mtDNA was sequenced in eight individuals sampled noninvasively from the three populations, and the control DNA sample from CRES. No variation was observed within 814 bp of coding mtDNA sequence. In 631 bp of the control region, three variable sites were distributed among three haplotypes ($\pi = 0.0017$, $SD = 0.00039$), one of which was observed in the captive sample only. Both wild haplotypes were observed in Ladakh (haplotype diversity = 0.667). However, only one haplotype was observed in each of the other two regions, one in Qinghai and the other in South Gobi.

Discussion

Species identification of scats

The carnivore-specific cytochrome *b* primers designed for scat analysis of South American carnivores were useful for species identification of snow leopard, red fox, wolf/dog and lynx scats collected in Central Asia, with success comparable to other carnivore studies (Broquet, Menard & Petit, 2007). The snow leopard was the most common species identified among scats sampled in Ladakh, consistent with the availability of habitat and a more continuous population in the Himalayan region (McCarthy & Chapron, 2003). The red fox was most common in areas with higher human disturbance, such as Qinghai. Lynx were also observed in Qinghai more often than snow leopard, at elevations up to 3930 m. Red fox and snow leopard were observed with nearly equal frequency in South Gobi, whereas fox scats were regularly found on snow leopard scrape sites.

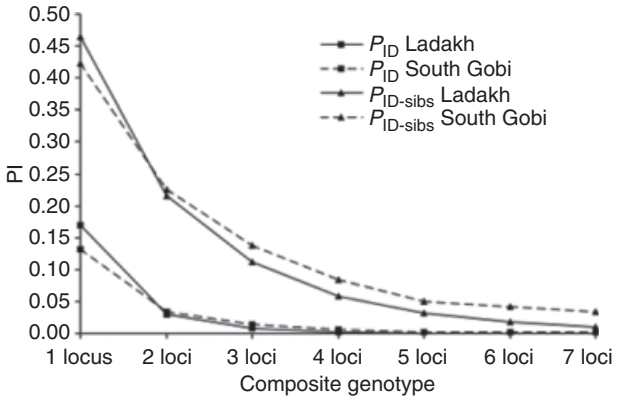

Figure 4 Probability of identity (P_{ID}) based on the allele frequencies observed in Ladakh and South Gobi. Probabilities were estimated from allele frequencies among unique snow leopard *Panthera uncia* genotypes sampled during this study. The locus combinations for composite genotypes are based on the variability observed in each population (Table 5), in order of decreasing H_E. Ladakh: PUN327, PUN124, PUN132, PUN229, PUN100, PUN225, PUN82. South Gobi: PUN124, PUN100, PUN82, PUN225, PUN229, PUN132, PUN327.

Because of the recent divergence and hybridization between the domestic dog and wolf (Leonard *et al.*, 2002) and the domestic cat and wildcat (Driscoll *et al.*, 2007), it was not possible to differentiate scat from these species with the 100 bp cytochrome *b* fragment. The Tibetan and Corsac fox had low levels of interspecies divergence, suggesting that this segment may be inappropriate for differentiating scats from these two species. Previous phylogenetic studies of Canidae have not included Tibetan fox and therefore the evolutionary relationship of this species to the other foxes is unknown (Wayne *et al.*, 1997; Bardeleben *et al.*, 2005). In order to develop species diagnostic markers for these two foxes, it will be necessary to identify the phylogenetic position of the Tibetan fox among *Vulpes* species.

Improving microsatellite primer design

As described in previous studies (Broquet & Petit, 2004; Valiere *et al.*, 2007), we found some evidence for a negative relationship between allele size and quality index for both PUN and FCA primers (see Supplementary material, Fig. S1); however, the correlation coefficients were low suggesting that a combination of factors contribute to genotyping success. In addition, previous studies describe locus-specific variation independent of allele size (Broquet & Petit, 2004). Over half of the domestic cat microsatellite loci we examined had repetitive sequences in one or both flanks. Primers designed from such sequences potentially anneal to many sites, in addition to the target locus flanking regions, during the PCR reaction leading to amplification of nonspecific products (Fig. 3), decreasing the yield of the target product, and increasing the potential for allele dropout (Lopez-Giraldez *et al.*, 2006). This is consistent with the lower genotyping success rate, higher rates of allele dropout and false alleles, and lower *Q*-values we observed using the FCA primers in snow leopard scat-derived DNAs. Although low quantities and quality of target DNA and PCR inhibitors (Taberlet *et al.*, 1999) contribute to genotyping errors, the location of microsatellites within repetitive elements (i.e. SINEs, LINEs and LTR retrotransposons) may explain genotyping errors in specific loci (Broquet *et al.*, 2007; Valiere *et al.*, 2007).

Many studies apply heterologous microsatellites designed from species closely related to the target species. Sequence divergence between the domestic cat and snow leopard has led to mutations in FCA primer annealing sites, which likely contribute to genotyping error when using these primers in other felids. Culver *et al.* (2001) reported 5% sequence divergence of microsatellite flanks between the domestic cat and mountain lion, *Puma concolor*. Our results suggest that it is beneficial to redesign primers that avoid repetitive sequences and are specific for the target species before embarking on noninvasive studies. Errors associated with analysis of scat-derived microsatellite genotypes can bias population size estimates and we were able to minimize

these errors by re-sequencing and designing species-specific primers (Creel *et al.*, 2003; Broquet & Petit, 2004; McKelvey & Schwartz, 2004). This initial investment in resources will reduce amplification errors and provide cleaner allele traces facilitating more accurate and rapid genotyping.

Application of techniques to snow leopard monitoring

Our brief expeditions to three geographically separated regions of snow leopard habitat illustrate the efficacy of scat surveys for monitoring snow leopards and other carnivores. In Qinghai, where snow leopard densities are believed to be low and fragmented (McCarthy & Chapron, 2003), we nonetheless were able to verify their presence despite a brief 2-day survey. In regions of India and Mongolia with higher known snow leopard densities, we were able to detect multiple cats in brief 2-day surveys. In Ladakh, Jackson *et al.* (2006) detected six individuals in a 135 km^2 area during a camera survey conducted 45 km southeast of our study site. Because, snow leopards have been found to have overlapping home ranges (Jackson, 1996), our detection of two males and two females on one transect seems consistent with the camera-trapping survey and ecology of the species. During a 2-month camera-trapping survey covering 145 km^2 (April–May 2007) in the Mongolian study site, four adult snow leopards and three cubs were detected (R. Jackson and B. Munkhstog, pers. obs.) compared with the three males and two females we detected via noninvasive genetics.

Comparable numbers of individuals were detected in Ladakh and Mongolia, despite a smaller number of snow leopard scrapes observed in Ladakh (2 vs. 23, respectively). Noninvasive genetic scat surveys are likely less prone to behavioral, environmental and seasonal effects than sign surveys (Ahlborn & Jackson, 1988; Jackson *et al.*, 2006). These techniques will be particularly useful for studying snow leopards in sites where it is not feasible to conduct in-depth 4–8-week camera-trapping surveys. In areas where camera trapping is practical, genetic analysis of scat can provide additional information on population connectivity and relatedness when this method is incorporated into monitoring programs.

The ability to rapidly obtain population samples by collecting scats will also facilitate population structure studies that to date have not been possible because of the inability of researchers to obtain tissue samples of wild snow leopards. There were no fixed haplotype differences between Ladakh and South Gobi or Qinghai, despite significant distance (>1000 km) and geographic barriers (e.g. Himalaya Mountains and nonmountainous regions of the Gobi Desert). However, we did observe low levels of variation in the control region ($\pi = 0.0021$). Coding regions are generally subject to purifying selection, noncoding regions (e.g. mtDNA control region) have higher substitution rates and higher levels of genetic diversity, consistent with the pattern we observed. Because the coding segments were invariant across a large geographic area, our data suggest that an extensive snow leopard phylogeographic study, along with more detailed population structure analyses will require more variable mitochondrial control region and nuclear microsatellite markers to define subspecies, examine patterns of differentiation among geographic regions and understand connectivity among snow leopard populations.

Species misidentification of scats and implications for snow leopard sign surveys

We observed a high level of scat species misidentification in the field, ranging from 35% in Ladakh to 54% in South Gobi. Many scats identified as putative snow leopard origin were found to be from red fox. Species misidentification in the field has been previously reported in studies that incorporate genetic identification of scats (Farrell *et al.*, 2000; Davison *et al.*, 2002). Scat is a common type of sign used for monitoring snow leopards (Schaller, 1988; Schaller *et al.*, 1988; Hussain, 2003; Ale *et al.*, 2007) and our results suggest that previous surveys incorporating scat data likely produced unreliable indices of snow leopard densities. As predator densities, diet and behavioral factors may influence the proportion of scat field-misidentification, we suggest that scat-based snow leopard surveys at a minimum incorporate genetic species identification. We tested the PUN-HVS1 control region primers and found they are specific to snow leopards using more stringent PCR conditions (i.e. 58 °C annealing temperature using a Hot-Start *Taq* polymerase). These primers therefore represent an inexpensive assay to identify snow leopard scat simply by PCR amplification and agarose gel electrophoresis.

Our main goal was to improve techniques for large-scale long-term monitoring of snow leopards and to examine the feasibility of sampling populations via scat collection. Our results demonstrate the advantages of redesigning microsatellite primers that avoid repetitive elements and are specific to the target taxon. As the snow leopard occupies regions that are difficult and expensive to access, it will be critical to coordinate a consistent sampling strategy and molecular marker panels so that results from different studies can be compared. One of the most difficult challenges to overcome is to design an effective sampling strategy that would yield biological realistic population estimates. As home ranges of snow leopards have been found to vary greatly, strategies may need to be flexible to take into account differing population densities. Our ability to detect multiple snow leopards on relatively short transects, surveyed over brief periods, suggests this method could be used to cover large areas. In addition, noninvasive survey schemes and the performance of rarefaction versus mark–recapture methods for population estimation for snow leopards need to be explored. The information obtained from across the species range will lead to effective conservation plans that ensure the persistence of this felid.

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Supplementary material

The following supplementary material for this article is available online:

Methods. Additional details on methology.

Table S1. Comparison of the number of alleles and the probability of identity (P_{ID}) observed for snow leopards by Waits *et al*. (2007) with estimates from snow leopard scat collected in Ladakh and South Gobi.

Fig. S1. A plot of the maximum allele size versus quality index (Q) for loci amplified with PUN and FCA primers from 17 snow leopard scats.

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