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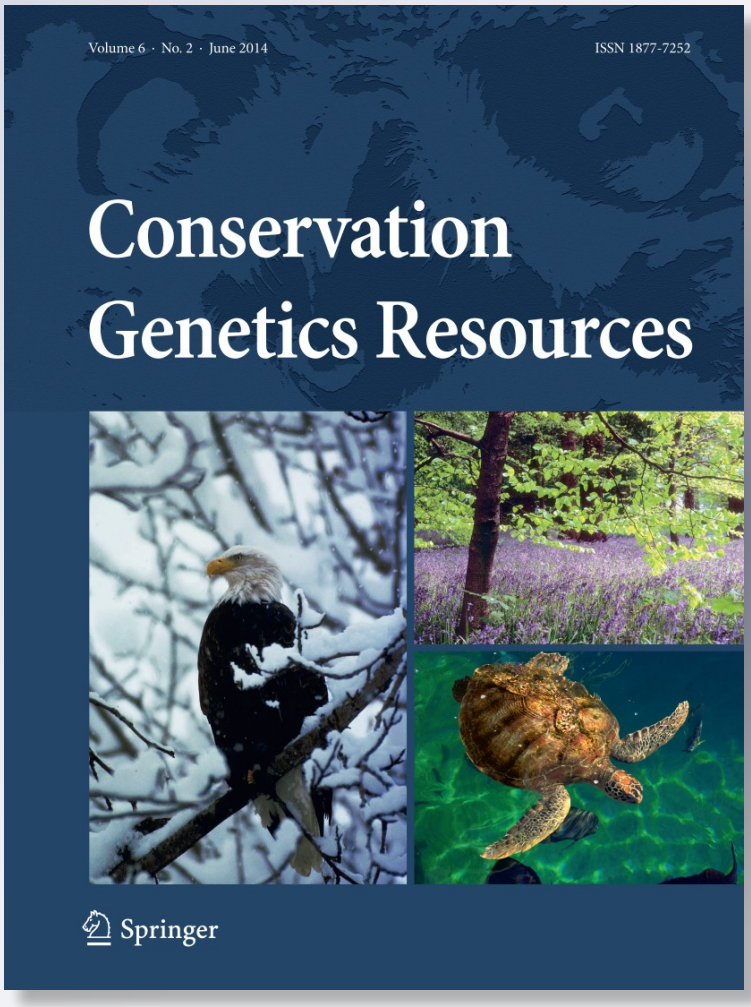

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MICROSATELLITE LETTERS

Characterization of 9 microsatellites and primers in snow leopards and a species-specific PCR assay for identifying noninvasive samples

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Abstract Molecular markers that can effectively identify noninvasively collected samples and provide genetic information are critical for understanding the distribution, status, and ecology of snow leopards (*Panthera uncia*). However, the low DNA quantity and quality in many noninvasive samples such as scats makes PCR amplification and genotyping challenging. We therefore designed primers for 9 microsatellites loci previously isolated in the domestic cat (*Felis catus*) specifically for snow leopard studies using noninvasive samples. The loci showed moderate levels of variation in two Mongolian snow leopard populations. Combined with seven other loci that we previously described, they have sufficient variation ($H_e = 0.504$, $A_n = 3.6$) for individual identification and population structure analysis. We designed a species-specific PCR assay using *cytochrome b* for identification of unknown snow leopard samples. These molecular markers facilitate in depth studies to assess distribution, abundance, population structure, and landscape connectivity of this endangered species.

Keywords Microsatellites · *Cytochrome b* · Snow leopard · Noninvasive genetics · Individual identification

The snow leopard (*Panthera uncia*) remains the most enigmatic big cat because it is highly elusive and occurs in very remote, mountainous regions of Central Asia (Sunquist and Sunquist 2002). Direct study of this species via live capture and telemetry is limited due to both logistical and ethical reasons. Camera-trapping has been used successfully to estimate distribution and abundance of snow leopards (Janecka et al. 2011). However, this approach does not yield physical samples, thus limiting its applications. Camera trapping is also not amenable to large-scale studies in mountainous habitat. Therefore, research on the status of this remarkable species, along with evolution, genetic diversity, and population structure, is necessarily limited to primarily noninvasive sampling.

Collecting scat on wildlife trails, saddles, and outcrops is an effective means of sampling snow leopard populations (Janecka et al. 2008, 2011). Despite noninvasive genetics being widely applied to felids, work on snow leopards has been more limited (Rodgers and Janecka 2013). Scat surveys are more cost and time efficient than camera trapping and they yield DNA samples that greatly increase the amount of information available on individuals and populations (Janecka et al. 2011). The low quantity of target DNA and its degradation leads to PCR failure, allele drop-out, and contamination making noninvasive genetic

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Table 1 Information on 9 microsatellite loci described in this study and seven loci previously described (Janecka et al. 2008)

Locus	Chr	LM[1] (cM)	LM[2] (cM)	RH[3] (cR)	Primers	Repeats	Differences with *Felis catus*	Pop	Alleles	A_N	H_O	H_E	*F*
PUN272	A3	123.3	n.a.	n.a.	CACCTTTGCATCCAATAAATTC	$(GT)_{19}$	5′ flank: 81 bp, 2.47 % (2 subs)	Gobi	125–129	2	0.286	0.245	−0.167
					AACTACCTTTACCTCCTTCCAAA	FCA + 5 rpts	3′ flank: 120 bp, 0.83 % (1 bp del)	Altai	123–129	4	1.000	0.684	−0.463
								All	123–129	4	0.643	0.543	−0.183
PUN834	B2	n.a.	n.a.	19.9	AAACACGTGCGCATGTAGAC	$(AC)_{17}$	5′ flank: 174 bp, 2.7 % (4 subs)	Gobi	109–113	3	0.250	0.539	0.536
					TGGAAGTGTGGTTTGACAGC	FCA + 0 rpts	3′ flank: 47 bp, 4.3 % (1 sub, 11-bp ins)	Altai	109–113	3	0.375	0.570	0.342
								All	109–113	3	0.313	0.580	0.461
PUN894	C2	n.a.	n.a.	132.0	CATGCCAGACTGCATTTGTT	$(GT)_{17}$	5′ flank: 41 bp, 2.4 % (1 sub)	Gobi	110–118	3	0.750	0.664	−0.129
					CCCACACATGACAATCCTGTT	FCA + 3 rpts	3′ flank: 155 bp, 1.3 % (2 sub)	Altai	110–118	3	0.500	0.398	−0.255
								All	110–118	3	0.625	0.604	−0.036
PUN1131	B1	n.a.	n.a.	33.4	TGGACAAATAGACCCAAGACTG	$(GT)_{23}$	5′ flank: 114 bp, 4.4 % (5 subs)	Gobi	118–124	3	0.375	0.398	0.059
					TAACCCCCATTGATGCTGTT	FCA − 5 rpts	3′ flank: 122 bp, 2.5 % (3 subs)	Altai	120	1	0	0	n.a.
							1 sub and two 1-bp del in STR	All	118–124	3	0.188	0.225	0.165
PUN1138	B1	n.a.	n.a.	753.3	TTGAAAAGCAAGACACTAATACATTTC	$(AC)_{17}$	5′ flank: 217 bp, 3.2 % (6 subs, 1-bp del)	Gobi	114–124	3	0.500	0.461	−0.085
					TGAGATATTCGTGATAGCTTGTTT	FCA + 0 rpts	3′ flank: 52 bp, 1.9 % (1 sub)	Altai	114–124	3	0.375	0.320	−0.171
								All	114–124	4	0.438	0.400	−0.093
PUN1157	B3	n.a.	n.a.	140.1	GAGAGTGCAGTCAGCCAGGT	$(AC)_{17}$	5′ flank: 251 bp, 3.2 % (7 subs, 7-bp del)	Gobi	101–109	3	0.875	0.594	−0.474
					TGAAATTCAGCTGCTTCAACTC	FCA + 3 rpts	3′ flank: 33 bp, 3.0 % (1 sub)	Altai	101–103	2	0.125	0.117	−0.067
								All	101–109	3	0.500	0.486	−0.028
PUN1262	D4	n.a.	n.a.	0.0	TCTGGAGAACTTGGGGACAC	$(AC)_{16}$	5′ flank: 98 bp, 1.0 % (1 sub)	Gobi	119–123	3	0.375	0.539	0.304
					TTCTGGGTCATGAGCCTTTC	FCA − 1 rpts	3′ flank: 163 bp, 3.1 % (5 sub)	Altai	121–125	3	0.250	0.227	−0.103

Table 1 continued

Locus	Chr	LM[1] (cM)	LM[2] (cM)	RH[3] (cR)	Primers	Repeats	Differences with *Felis catus*	Pop	Alleles	A_N	H_O	H_E	*F*
								All	119–125	4	0.313	0.412	0.242
PUN1283	E1	n.a.	139.1	333.4	TCATCACCAACCTTGCATTT	$(AC)_{14}$	5′ flank: 170 bp, 2.9 % (5 subs)	Gobi	125–129	2	0.000	0.490	1.000
					ACCAAGGATGTCTGCGTCTT	FCA + 0 rpts	3′ flank: 99 bp, 3.0 % (3 subs)	Altai	125–129	3	0.714	0.582	−0.228
								All	125–129	3	0.357	0.559	0.361
PUN1293	E3	n.a.	n.a.	425.4	GCACCCCTTCTTCTTGTAGTG	$(GT)_{18}$	5′ flank: 55 bp, 1.8 % (1 sub)	Gobi	116	1	0	0	n.a.
					AGAACTCACAGGGGCACAAT	FCA + 4 rpts	3′ flank: 144 bp, 2.1 % (3 subs)	Altai	116–118	2	0.125	0.117	−0.067
								All	116–118	2	0.063	0.061	−0.032
PUN082	E1	35.9	63.3	9.9	Janecka et al. (2008)	$(GT)_{26}$	5′ flank: 60 bp, 5.0 % (3 sub)	Gobi	109–113	2	0.875	0.492	−0.778
						FCA − 7 rpts	3′ flank: 70 bp, 0 %	Altai	109–113	3	0.875	0.648	−0.349
								All	109–113	3	0.875	0.643	−0.362
PUN100	A1	255.8	n.a.	n.a.	Janecka et al. (2008)	$(AC)_{18}$	5′ flank: 62 bp, 3.2 % (1 sub, 1 del)	Gobi	88–96	4	0.500	0.648	0.229
						FCA − 0 rpts	3′ flank: 45 bp, 6.7 % (3 subs)	Altai	90–96	3	0.875	0.648	−0.349
								All	88–94	5	0.688	0.721	0.046
PUN124	A2	126.2	n.a.	1,172.5	Janecka et al. (2008)	$(AC)_{22}$	5′ flank: 90 bp, 1.1 % (1 sub)	Gobi	90–100	4	1.000	0.680	−0.471
						FCA − 2 rpts	3′ flank: 89 bp, 0 %	Altai	96–100	3	0.625	0.602	−0.039
								All	90–100	4	0.813	0.701	−0.159
PUN132	D3	110.2	38.9	602.3	Janecka et al. (2008)	$(GT)_{19}$	5′ flank: 58 bp, 3.4 % (2 subs)	Gobi	121	1	0	0	n.a.
						FCA + 3 rpts	3′ flank: 105 bp, 7.6 % (5 subs, 3 indels)	Altai	117–123	4	0.750	0.711	−0.055
							2, 1-bp del and 29-bp ins in 3′ flank	All	117–123	4	0.375	0.521	0.281
PUN225	A1	202.8	231.1	n.a.	Janecka et al. (2008)	$(GT)_{15}$	5′ flank: 132 bp, 2.3 % (3 subs)	Gobi	176–178	2	0.375	0.492	0.238
						FCA + 5 rpts	3′ flank: 55 bp, 1.8 % (1 sub)	Altai	176–182	4	0.750	0.711	−0.055

Table 1 continued

Locus	Chr	LM[1] (cM)	LM[2] (cM)	RH[3] (cR)	Primers	Repeats	Differences with *Felis catus*	Pop	Alleles	A_N	H_O	H_E	*F*
								All	176–180	4	0.563	0.654	0.140
PUN229	A1	62.2	112.7	390.9	Janecka et al. (2008)	$(GT)_{23}$	5′ flank: 88 bp, 0 %	Gobi	104–112	4	0.375	0.539	0.304
						FCA + 1 rpt	3′ flank: no sequence	Altai	104–108	3	0.625	0.617	−0.013
								All	104–112	5	0.500	0.594	0.158
PUN327	A2	201.2	222.1	1,709.3	Janecka et al. (2008)	$(GT)_{14}$	5′ flank: 73 bp, 2.7 % (1 sub, 4-bp del)	Gobi	80–90	3	0.375	0.320	−0.171
						FCA + 5 rpts	3′ flank: 36 bp, 8.3 % (3 subs)	Altai	80–90	3	0.375	0.398	0.059
								All	80–90	3	0.375	0.361	−0.038
								Mean		*3.6*	*0.477*	*0.504*	*0.058*
								SE		*0.20*	*0.055*	*0.044*	*0.054*

Position in 1999 version of domestic cat linkage map

Position in 2009 version of the domestic cat linkage map

Position in domestic cat radiation hybrid map

LM linkage map, *RH* radiation hybrid map, *cM* centiMorgans, *cR* centiRads, *n. a.* not available, *rpt* short tandem nucleotide repeat, *sub* substitutions, *ins* insertion, *del* deletion, *indel* insertion/deletion, *Pop* population, A_N mean number of alleles, H_O observed heterozygosity, H_E expected heterozygosity, *F* fixation index = $(H_E–H_O)/H_E$

studies challenging. In addition, these problems are exacerbated when using primers designed originally for species other than the one being studied because of the potential for mutations in primer annealing sites and duplications or deletions of regions harboring these loci. The high rate of visual misidentification of scats in the field also necessitates an inexpensive means of screening fecal samples to minimize the cost of noninvasive genetics.

To facilitate population studies of snow leopards we sequenced flanking segments of 9 microsatellites described from the domestic cat (*Felis silvestris catus*) and designed primers from the snow leopard sequence. These primers yield amplicons smaller than 150 bp to facilitate PCR amplification and genotyping in degraded DNA preparations. They were designated with the same locus number as in the original microsatellites (Menotti-Raymond et al. 1999), but with the prefix "PUN" instead of "FCA". In addition, we aligned *cytochrome b* sequences of five species of *Panthera* and 3 other felids, designed PCR primers in sites that have snow leopard-specific substitutions (SCT-PUN-F: TGGCTGAATTATCCGATACC and SCT-PUN-R: AGCCATGACTGCGAGCAATA), and determined PCR conditions under which they are species specific. Please refer to Online Resource 1 for our detailed methodology and the sequences we generated.

Snow leopard samples obtained noninvasively were genotyped using the nine new and seven previously described microsatellites primers (Janecka et al. 2008). We estimated the number of alleles (A_N), observed heterozygosity (H_O), expected heterozygosity (H_E), and fixation index (F) in GenAlex v6.4. We genotyped 16 individuals from the western Altai and Gobi Desert regions of Mongolia (Janecka et al. 2011). The F_{ST} between populations was estimated using the AMOVA with 999 permutations in GenAlex to examine the level of differentiation. We then estimated the probability of identify for siblings (P_{IDsib}) to determine the number of loci required to achieve the $P_{IDsib} < 0.05$ criteria recommended for individual identification of noninvasive samples (Table 1).

When we combined our new microsatellite sequence data with loci previously sequenced in Janecka et al. (2008) we observed 2.86 % sequence divergence among 3,096 base pairs of STR-flanking sequence between the domestic cat and the snow leopard. We observed moderate levels of variation with $A_N = 3.6$, $H_O = 0.\ 477$, $H_E = 0.504$, and $F = 0.058$. These two sampled populations are ~1,200 km apart and therefore we would expect significant divergence if these microsatellites were informative for population structure. Based on AMOVA analysis we did indeed observe significant differentiation with an $F_{ST} = 0.122$ ($p < 0.001$). The 5 most variable loci were sufficient to achieve $P_{IDsib} < 0.05$ in both populations. The *cytochrome b* primers were snow leopard specific with the following conditions: 1.5 mM $MgCl_2$ and annealing temperature >60 °C. We validated this PCR assay by testing 30 scat samples that we previously identified as snow leopard by *cytochrome b* sequencing (Janecka et al. 2011). All 30 samples were confirmed to be of snow leopard origin using the new species-specific *cytochrome b* primers.

In summary, we have designed and validated 9 new snow leopard-specific microsatellite primers, bringing the total number of STR markers applicable for non-invasive snow leopard samples to 16. In addition, a new snow leopard-specific pair of *cytochrome b* primers provides a cost effective and efficient means for screening large numbers of scat samples with unknown species origin. These molecular tools will facilitate additional studies of snow leopards that will generate critical information for their conservation and management.

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