CENTER FOR MOLECULAR DYNAMICS - NEPAL

Genetic based population survey of snow leopard (*P. uncia*) in Annapurna Conservation and Rolwaling areas of Nepal.

FINAL REPORT

Submitted To

Snow Leopard Conservation Grants, Snow Leopard Network

Research Team

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1.0 EXECUTIVE SUMMARY

Snow leopard is an endangered species throughout its 12 range states in Asia including Nepal and is listed as 'endangered' in IUCN's Red List of Threatened Species. In Nepal, the endangered snow leopards are listed in Schedule 1 of the National Parks and Wildlife Conservation Act, 1973, thereby making it a priority species for conservation. While Nepal is a signatory to CITES since 1973, the species is further protected under the country's National Parks and Wildlife Conservation Act 1973. Before any effective conservation plan can be developed and implemented, it is very important to acquire reasonable estimates of existing abundance and distribution of snow leopard in the Nepal to formulate conservation strategy of the species. Conventionally applied field methods of population estimation of elusive species such as interviews with local inhabitants, sign and scat surveys etc. are cumbersome and laden with inaccuracies which often results in overestimation of population and misunderstanding of their behavioral observations. While other convincing field methods such as 'Camera trapping' is difficult, time consuming, expensive and even impractical in some cases. Non invasive genetic sampling and molecular scatology, on other hand, is an emerging scientific technique for applied conservation biology that can provide information, which when complimented with information garnered through conventionally applied methods, yields realistic population estimates and accurate behavioral patterns.

A total of 48 (n=48) scat samples of putative snow leopards, collected from two extreme habitats of Nepal- Mustang (n=32) of Annapurna Conservation area and Rolwaling region (n=16) near Mt. Everest, were analyzed at the molecular level. 54% (26/48) samples were found to be of snow leopard. 98% (47/48) samples were of carnivore species based on mt DNA Cytochrome b PCR amplification. Only 1 sample did not yield any amplifiable DNA. 20 snow leopard positive samples were successfully genotyped using six microsatellite markers; 14 individual snow leopards were found. We were able to identify 7 individual males and 3 females from Mustang area; 2 individual males and 2 females were identified from Rolwaling area. The snow leopard species negative and carnivore positive samples (n=21) were further sequenced; Red fox (*Vulpes Vulpes*, n=13), Leopard cat (*Prionailurus bengalensis*, n=4), Wolf (*Canis lupus*, n=1), Common leopard (*Panthera pardus*, n=1) and Lynx (*Lynx lynx*, n=1) were identified as samples belonging to carnivore species other than snow leopard, one sample did not yield any discernable sequencing data for identification.

2.0 OBJECTIVES

2.1 GENERAL OBJECTIVE

- To perform population survey study of snow leopards in Annapurna Conservation and Rolwaling areas of Nepal by non-invasive genetic analysis of scat samples.

2.2 SPECIFIC OBJECTIVES

- To collect potential snow leopard scat samples from Annapurna Conservation and Rolwaling areas of Nepal.
- To extract DNA from collected scat samples.
- To identify snow leopard scat samples by Species specific PCR assay.
- To identify sex of identified snow leopards by Sex identification PCR assay.
- To perform microsatellite genotyping analysis for individual characterization of positive snow leopard samples.
- To determine species of samples other than that of snow leopard.

3.0 MATERIALS & METHODS

3.1 SAMPLING

Putative snow leopard scat samples were opportunistically collected in 2011 from various areas of two different geographical regions of Nepal; Mustang in the west and Rolwaling in the East. The study sites were selected based on the suitable habitat for snow leopard and where their recent high activities were reported. There were two areas within Mustang where samples were collected from- Upper Mustang and Lower Mustang. Samples were obtained from ridgelines and outcrops in close proximity to visible snow leopard scrapes and tracks and the collection site were GPS marked.

Figure 1: Map of Nepal with highlighted study sites- Mustang of Annapurna Conservation Area and Rolwaling region near Mt. Everest.

3.2 SAMPLE COLLECTION

The scats were collected in tubes with silica desiccant. The tube was labeled with location initials, serial number, the date of collection and the initials of the sample collector. The GPS location of the area from where the scat was collected was noted along with other details of the scat. The scat collection area along with the tubes was photographed for available signs such as pugmarks and scrapes. A detailed field protocol of scat collection was developed and handed to the field researchers [Attached as Annex to this report].

3.3 SCAT DNA EXTRACTION

Scat DNA was extracted using the Qiagen QIAamp DNA Stool kit (Qiagen, Germany). Around 200mg of dried scat sample was scraped on the surface with sterile scissor and tweezers and was lysed in Lysis Buffer ASL and the supernatant was subjected to InhibitEX tablet treatment to adsorb PCR inhibitors. Proteinase K was added to the remaining supernatant and buffer AL was added and incubated for 10 minutes at 70°C. DNA was eluted to a total volume of 150ul.

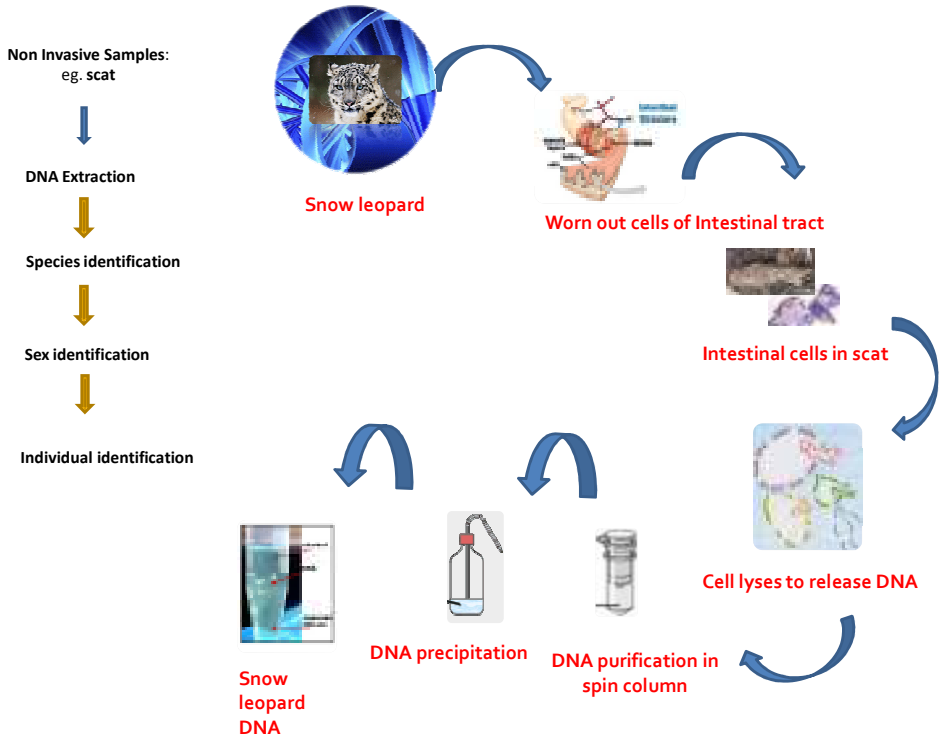


Figure 2: Schematic diagram showing DNA extraction technique and subsequent genetic analysis of snow leopard scats for the study.

3.4 SPECIES IDENTIFICATION

Snow leopard specific PCR primer set (CYTB-SCT-PUN F and CYTB-SCT-PUN R) was used for specific identification of Snow Leopards (Janecka et. al 2008).

CYTB-SCT-PUN F': 5' TGGCTGAATTATCCGATACC 3'

CYTB-SCT-PUN R': 5' AGCCATGACTGCGAGCAATA 3'

A 25ul PCR reaction was prepared containing 2.5 µl of 10XTaq Buffer, 2.5µl of 25mM MgCl2, 2µl of 25mM dNTPs, 0.25µl of 10µg/µl BSA, 0.1 ul of Taq Polymerase enzyme, 0.4µl of each primer and 14.65µl of distilled water to which 2µl of extracted undiluted DNA was added. The PCR reaction was carried out at the following thermo-cycling conditions: 94°C for 10 minutes followed by 50 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 1 minute. The PCR products, known snow Leopard positive sample incorporated as positive control, were visualized in 1.8% agarose gel.

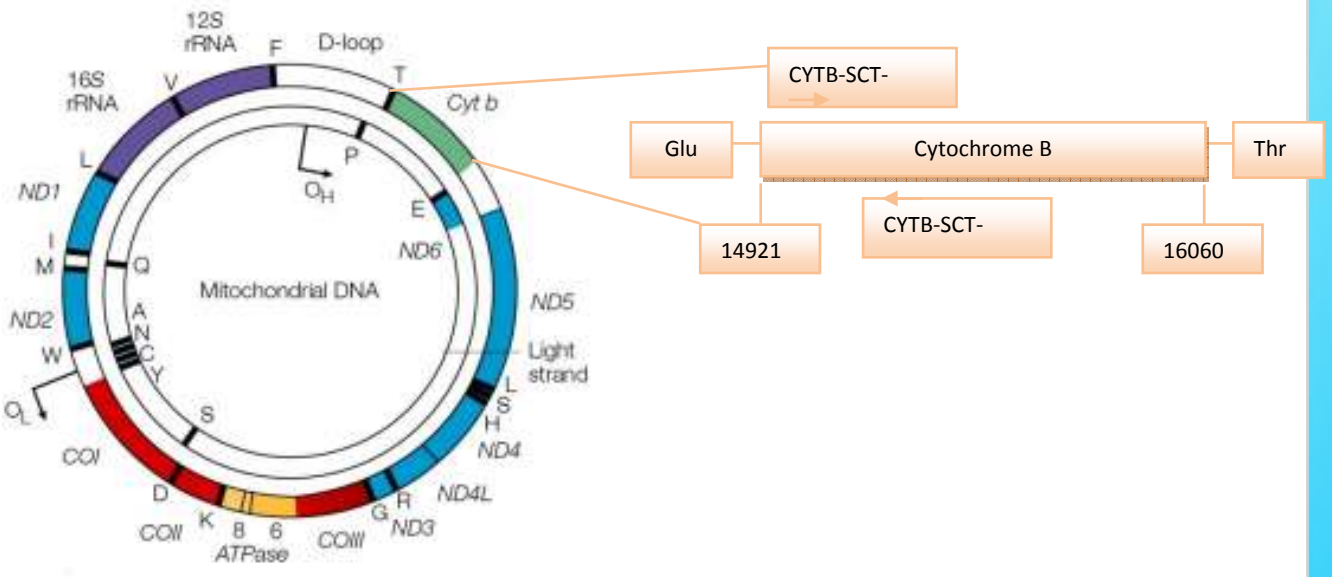


Figure 3: Schematic diagram of mitochondrial DNA of *Panthera uncia* depicting regions targeted for species identification.

3.5 GENERAL CARNIVORE IDENTIFICATION

Carnivore specific PCR primer set (CYTB-SCT F and CYTB-SCT R) was used for identification of general carnivores (Murphy et al 1999).

CYTB-SCT-F: 5' AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA 3'

CYTB-SCT-R: 5' TATTCTTTATCTGCCTATACATRCACG 3'

A 25ul PCR reaction was prepared containing 2.5 µl of 10X Amplitaq Buffer, 2.5 µl of 25mM MgCl2, 1.0 µl of 10mM dNTPs, 0.1µl of 10µg/µl BSA, 0.2ul of Amplitaq exzyme (ABI, USA), 1.0 µl of each primer and 15.7 µl of distilled water to which 1.0 ul of extracted undiluted DNA was added. The PCR thermo-cycling conditions: 95°C for 10 minutes followed by 50 cycles of each 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 1 minute with final extension step at 72°C for 5 minutes. The PCR products, along with incorporated carnivore positive control, were visualized in 2% agarose gel.

The carnivore positive PCR amplicons were sequenced using ABI 310 Genetic Analyzer using forward primer.

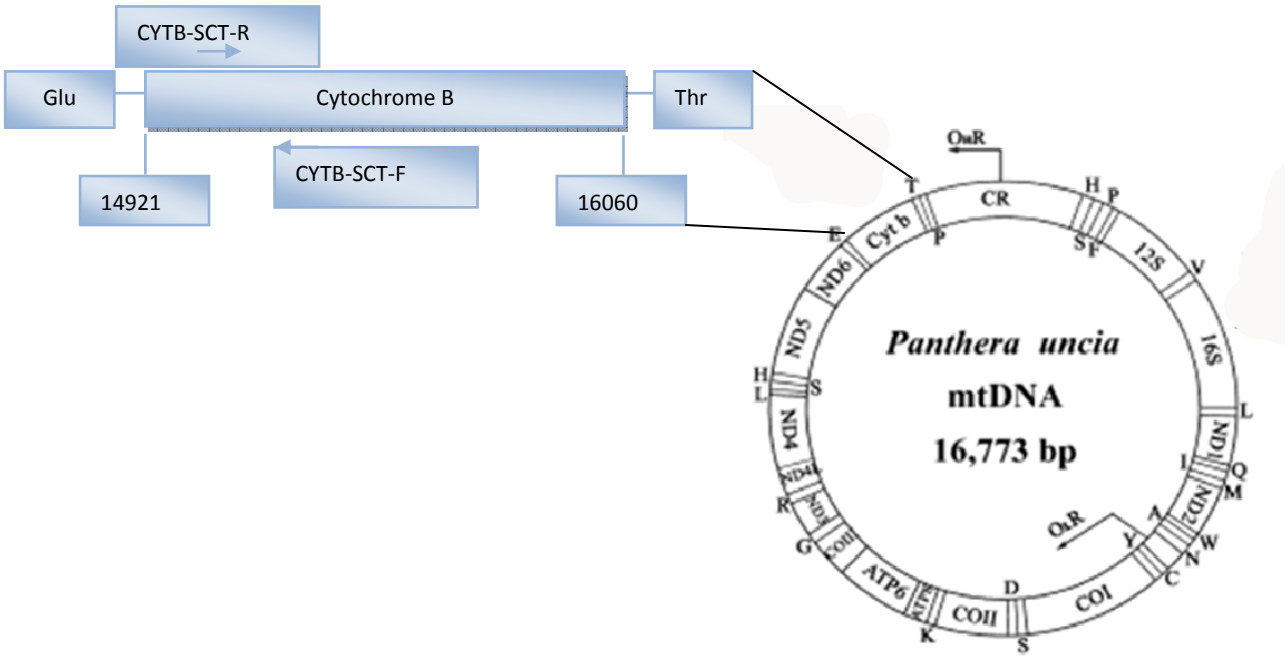


Figure 4: Schematic diagram of mitochondrial DNA of Panthera uncia depicting regions targeted for carnivore identification.

3.6 SEX IDENTIFICATION

Sex identification using PCR was done on snow Leopard species PCR positive samples with felid sex specific PCR primers (AMELY F and AMELY R) (Murphy et al, 1999).

AMELY F: 5' CCCAGCACACTCCTATTTGG 3'

AMELY R: 5' GGAATTTCAGCTGCAAAGGA 3'

A 10ul PCR reaction was prepared containing 1 µl of 10XPfu Buffer, 0.8µl of 25mM MgCl2, 0.08µl of 25mM dNTPs, 0.1 µl of 10µg/µl BSA, 0.05 ul of 5U/µl Pfu enzyme, 0.24 µl of each primer and 4.99 µl of distilled water to which 2.5 ul of extracted undiluted DNA was added. PCR thermo-cycling condition: 94°C for 2 minutes followed by 50 cycles of each 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 1 minutes. The PCR products, with incorporated male and female snow leopard positive controls, were visualized in 2% agarose gel.

3.7 INDIVIDUAL IDENTIFICATION

3.7.1. Individual identification PCR assay

6 microsatellite loci located at 6 different chromosomes of snow leopard were targeted using sets of following six fluorescent dye tagged primers. Six polymorphic microsatellite loci chosen were sufficient to give the value of P_{ID} (Probability of Identity) that was adequate to characterize individuals in a population sharing the same genotype (Janecka et al 2008).

PRIMERS	SEQUENCE
PUN124-F	NED- 5'-CCATTCCCTCCCTGTCTGTA-3'
PUN124-R	3'-TGTCCTCAAACCATAGACAGTTTC-5'
PUN132-F	NED-5'-CGAAATGCAGTAATGTTAGTTTTACA-3'
PUN132-R	3'-CACGGGTTCGTCTCTTTTG-5'
PUN229-F	VIC-5'-AGACAAACTGACAAGCTTAGAGG-3'
PUN229-R	3'-TCATGTCTTTACATTCATTTCTTTTT-5'
PUN894-F	VIC-5'-CATGCCAGACTGCATTTGTT3'
PUN894-R	3'-CCCACACATGACAATCCTGTT-5'
PUN935-F	FAM-5'-GCTGCTGTGACCTTCTGTGA 3'
PUN935-R	3'-CAGTGTTCCTGGTTTGCTCA-5'
PUN1157-F	FAM-5'-GAGAGTGCAGTCAGCCAGGT-3'
PUN1157-R	3'-TGAAATTCAGCTGCTTCAACTC-5'

Genotyping for individual identification was optimized for multiplex PCR. Six microsatellite primers were divided into two combinations each containing three of the primer pairs. One combination of primer pairs included tagged forward and untagged reverse primers for loci PUN124, PUN229 and PUN1157. Second combination consisted of primer pairs, tagged forward and untagged reverse primer, for loci PUN132, PUN894 AND PUN935. For each sample, multiplex PCR was performed for both combinations in a 7ul reaction volume. For first multiplex PCR combination, a total of 7ul reaction volume contained 3.5ul of Qiagen master mix (2x)(Qiagen,Germany), 0.7ul of Q-solution(5x)(Qiagen, Germany), primers for PUN124 at 0.5uM, primers for PUN229 at 0.4uM, primers for PUN1157 at 0.2uM and 1.5ul of DNA. Similarly for second multiplex PCR combination, a total of 7ul reaction volume contained 3.5ul of Qiagen mastermix (2x)(Qiagen, Germany), 0.7ul of Q-solution (5x)(Qiagen, Germany), primers for PUN132 at 0.5uM, primers for PUN894 at 0.4uM, primers for PUN935 at 0.2uM and 1.5ul of DNA.

The PCR reaction was carried out at the following thermo-cycling condition: 95°C for 15 minutes followed by 40 cycles of each 94°C for 30 seconds, 55°C for 90 seconds and 72°C for 90 seconds and a final extension of 72°C for 10 minutes. The PCR product was diluted to 1:60 by adding 118ul of distilled water to 2ul of the PCR product. 1ul of the diluted PCR product was then mixed with 9ul of Hi-Di formamide (Applied Biosystems, USA) and 0.25ul of GeneScan 500-Liz DNA size standard (Applied Biosystems, UK). The mixture was denatured at 94^0C for 5mins and immediately cooled. The samples were then injected into a capillary tube (36cm, POP4 polymer) by applying an injection voltage of 1kV for 5sec in ABI310 Genetic Analyzer (Applied Biosystems, USA). The capillary electrophoresis was performed at a voltage of 15kV at 60^0C.

3.7.2 Analysis

We determined the size of alleles using the software Genemapper 4.0. General descriptions of genetic diversity (i.e., percentage of polymorphic loci, heterozygosity, etc.) and deviations from Hardy Weinberg equilibrium were estimated in GenAlEx 6.41. In addition, because we did not have an independent sample available to estimate population allele frequencies, we used unique composite genotypes to estimate allele frequencies for calculating probability of identity for both unrelated and related individuals. Individuals were identified using unique composite genotypes and then we examined their spatial distribution.

4.0 RESULTS

A total of 48 (n=48) scat samples were collected in 2010 from Mustang region of Annapurna Conservation area (n=21) and Rolwaling region (n=16) of Nepal.

There were more samples collected opportunistically in Upper Mustang (sample collection area of 10 Km^2) than in the Lower Mustang (covering more than 2800 Km^2); meanwhile sample collection area for Rolwaling was around 7 Km^2.

Table 1: Summary of snow leopard genetic survey of Mustang and Rolwaling areas of Nepal.

Total No. of Samples	**48**
Total Snow leopard positives samples	**26**
Samples successfully Genotyped	20
No. of unique individuals	14
No. of unique individuals in Mustang	10
Male	7
Female	3
No. of Unique individuals in RW	4
Male	2
Female	2
No. of carnivores identified	20
No. of carnivores unidentified	1
Total Carnivores	**21**
No. of sample failed on PCR amplification	**1**

Figure 5: Area of Study for the prevalence of Snow Leopard.

4.1 SPECIES IDENTIFICATION

Out of 48 scat samples, 54% (26/48) samples were found to be of snow leopard in species identification based on PCR assay. Only 31% (5/ 16) of samples from Rolwaling were of snow leopard; 63% (20/32) of samples from Mustang were of snow leopard. 44% (21/48) of samples from Mustang and Rolwaling were not of snow leopard. One scat sample did not yield any PCR amplifiable DNA.

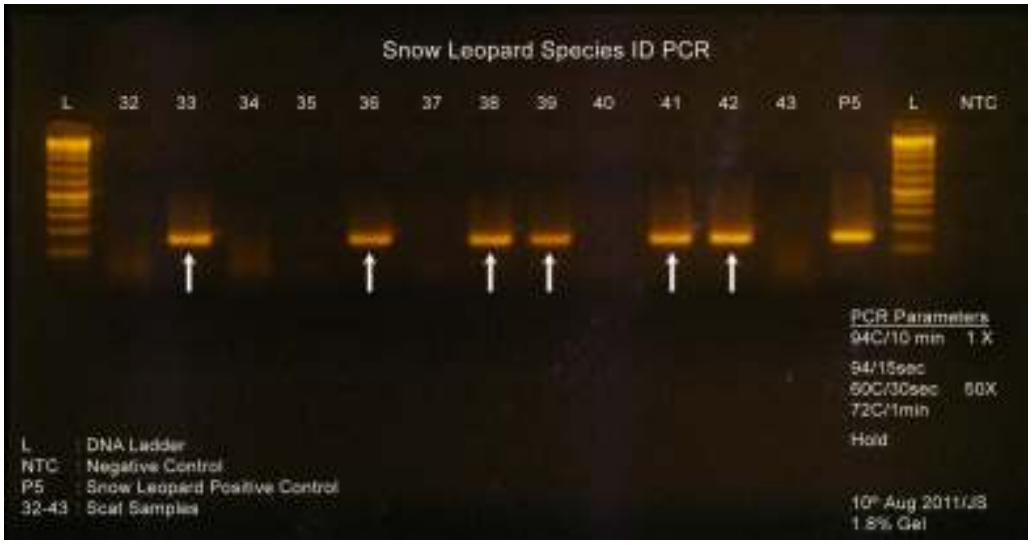


Figure 6: Snow leopard specific PCR assay

4.2 GENERAL CARNIVORE IDENTIFICATION

Out of 48 scat samples, 98% (47/48) of samples were positive on PCR assay targeting Carnivore specific Cytochrome b region. All samples identified as that of snow leopard were also positive on carnivore specific PCR assay. Of 21 samples (21/48, 44%) that were of carnivore species other than snow leopard, it was determined through DNA sequencing of carnivore PCR amplicon that they belonged to Red fox (*Vulpes Vulpes*, n=13), Leopard cat (*Prionailurus bengalensis*, n=4), Wolf (*Canis lupus*, n=1), Common leopard (*Panthera pardus*, n=1) and Lynx (Lynx lynx, n=1).

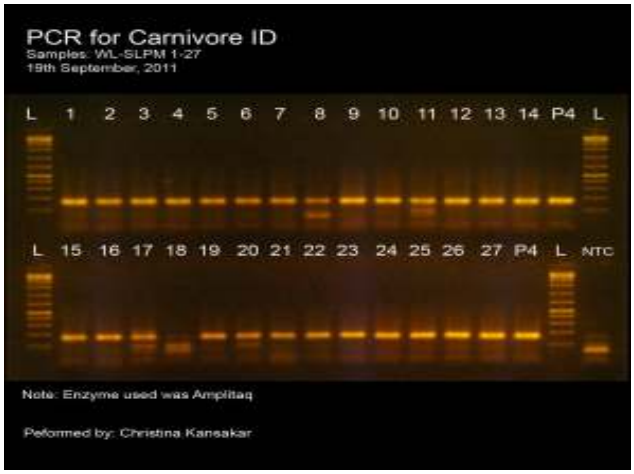


Figure 7: General Carnivore specific PCR assay.

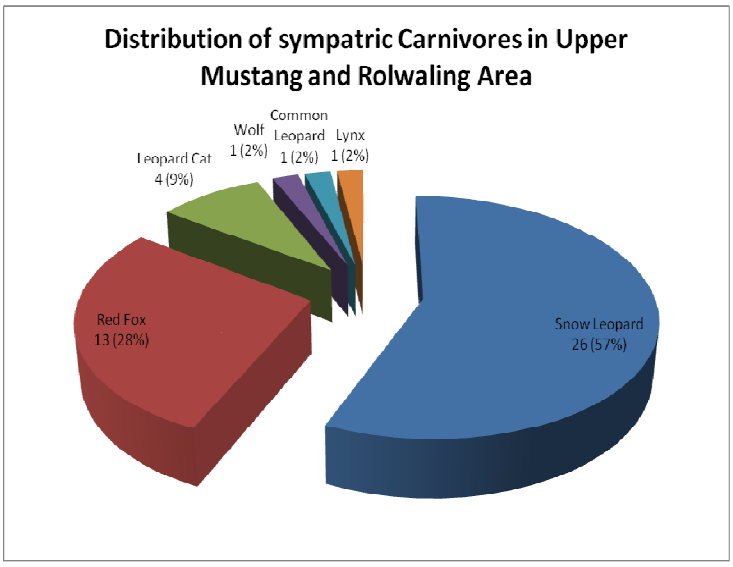


Figure 8: Carnivore species in the collected samples.

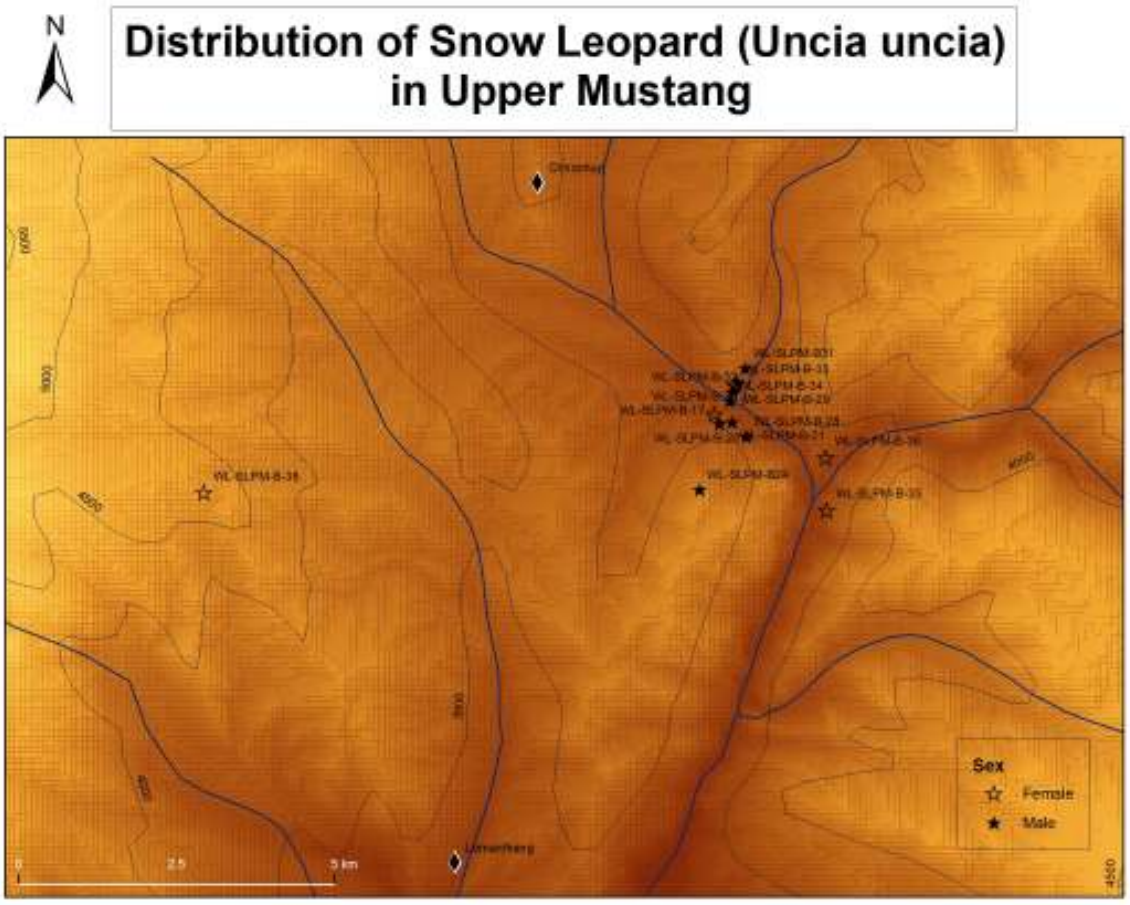
Table 2: BLAST results of carnivore positive and snow leopard negative samples; target amplification of Cytochrome b region of mtDNA.

Samples	Identified Species from Sequencing	Max. ID	E-value
1	Red fox (*Vulpes vulpes*)	92%	1.00E-43
2	Red fox (*Vulpes vulpes*)	94%	1.00E-47
3	Red fox (*Vulpes vulpes*)	98%	3.00E-55
4	Red fox (*Vulpes vulpes*)	98%	7.00E-55
5	Red fox (*Vulpes vulpes*)	94%	2.00E-54
6	Red fox (*Vulpes vulpes*)	94%	2.00E-52
7	Leopard cat (*Prionailrus bengalensis*)	98%	4.00E-50
8	Leopard cat (*Prionailrus bengalensis*)	98%	2.00E-51
9	Leopard cat (*Prionailrus bengalensis*)	97%	3.00E-54
10	Red fox (*Vulpes vulpes*)	96%	2.00E-50
11	No Sequencing Data		
12	Red fox (*Vulpes vulpes*)	98%	9.00E-54
13	Red fox (*Vulpes vulpes*)	94%	1.00E-44
14	Red fox (*Vulpes vulpes*)	98%	7.00E-55
15	Red fox (*Vulpes vulpes*)	98%	3.00E-55
16	Red fox (*Vulpes vulpes*)	98%	4.00E-55
17	Wolf (*Canis lupus laniger*)	96%	1.00E-53
18	Leopard cat (*Prionailrus bengalensis*)	97%	3.00E-54
19	Common leopard (*Panthera pardus*)	98%	1.00E-54
20	Red fox (*Vulpes vulpes*)	97%	3.00E-54
21	Lynx (*Lynx lynx*)	98%	8.00E-56

4.3 SEX IDENTIFICATION

Out of identified 26 snow leopard samples, 58% (15/26) were identified as male and 42% (11/26) as female. To avoid false negative results, each assay had both positive and negative controls and the samples were processed in triplicates.

Figure 9: Sex identification PCR assay.



Map 1: GIS map showing distribution of male and female snow leopards in Mustang.

4.4 INDIVIDUAL IDENTIFICATION

Out of 26 snow leopard positives PCR samples, 77% (20/26) were successfully genotyped using 6 microsatellite markers. 10 individuals (Males = 7, Females = 3) were identified in Mustang area and 4 (Males = 2, Females = 2) in Rolwaling region. There were 4 individual snow leopards in Mustang area having multiple samples determined by exact allele fragments across all 6 loci with calculated P_{ID} value of $1.7X\ 10^{-4}$ in Mustang and $2.8\ X\ 10^{-3}$ in Rolwaling populations. The calculated P_{ID} sibling was $2.5\ X\ 10^{-2}$ and $6.5\ X\ 10^{-2}$ in Mustang and Rolwaling populations respectively.

Table 3: Microsatellite Genotype Allele call for snow leopard samples. MUS=Mustang; RW=Rolwaling; F=Female; M=Male. Alleles at all loci could be scored for all the samples except in two samples where the data is missing at two loci and in one sample.

S.no.	Samples	Region	Sex	Genotypes											
				PUN124	PUN124	PUN229	PUN229	PUN1157	PUN1157	PUN132	PUN132	PUN894	PUN894	PUN935	PUN935
1	WL-SLPM-B-33	MUS	F	90	90	106	106	104	104	-	-	112	112	115	115
2	WL-SLPM-B31	MUS	M	90	100	102	108	100	104	112	116	112	112	115	119
3	WL-SLPM-B-17	MUS	F	90	100	-	-	103	103	-	-	112	112	115	115
4	WL-SLPM-B-32	MUS	M	90	100	106	110	104	104	118	118	112	112	115	115
5	WL-SLPM-B-30	MUS	M	**90**	**100**	**106**	**110**	**104**	**104**	**112**	**118**	**112**	**112**	**115**	115
6	WL-SLPM-B-34	MUS	M	**90**	**100**	**106**	**110**	**104**	**104**	**112**	**118**	**112**	**112**	**115**	115
7	WL-SLPM-B-43	MUS	M	90	100	106	108	100	104	118	120	112	112	115	115
8	WL-SLPM-B-42	MUS	M	**90**	**100**	**106**	**106**	**100**	**104**	**118**	**120**	**112**	**112**	**115**	**115**
9	WL-SLPM-B-48	MUS	M	**90**	**100**	**106**	**106**	**100**	**104**	**118**	**120**	**112**	**112**	**115**	**115**
10	WL-SLPM-B11	RW	M	92	100	104	110	104	108	116	116	112	124	115	115
11	WL-SLPM-B-15	RW	M	92	96	94	94	96	98	112	114	110	116	133	133
12	WL-SLPM-B-09	RW	F	96	98	102	106	104	104	116	120	112	112	115	119
13	WL-SLPM-B-29	MUS	F	**96**	**100**	**102**	**110**	**104**	**104**	**118**	**118**	**112**	**112**	**115**	**115**
14	WL-SLPM-B-35	MUS	F	**96**	**100**	**102**	**110**	**104**	**104**	**118**	**118**	**112**	**112**	**115**	**115**
15	WL-SLPM-B-46	MUS	M	98	100	106	106	104	104	112	118	112	112	115	119
16	WL-SLPM-B-20	MUS	M	**98**	**98**	**102**	**102**	**100**	**104**	**118**	**118**	**112**	**112**	**115**	**121**
17	WL-SLPM-B-21	MUS	M	**98**	**98**	**102**	**102**	**100**	**104**	**118**	**118**	**112**	**112**	**115**	**121**
18	WL-SLPM-B24	MUS	M	**98**	**98**	**102**	**102**	**100**	**104**	**118**	**118**	**112**	**112**	**115**	**121**
19	WL-SLPM-B-28	MUS	M	**98**	**98**	**102**	**102**	**100**	**104**	**118**	**118**	**112**	**112**	**115**	**121**
20	WL-SLPM-B-03	RW	F	90	100	-	-	104	104	116	120	-	-	107	119

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Table 4: Genetic diversity of snow leopard positive samples from two different populations (Mustang and Rolwaling) across selected 6 microsatellite markers.

Pop	Locus	N	Na	Ne	I	Ho	He	UHe	F
MUS	**PUN124**	10	5.000	4.255	1.522	0.800	0.765	0.805	-0.046
	PUN229	9	6.000	4.500	1.611	0.667	0.778	0.824	0.143
	PUN1157	10	6.000	2.222	1.190	0.400	0.550	0.579	0.273
	PUN132	8	5.000	3.368	1.369	0.500	0.703	0.750	0.289
	PUN894	10	4.000	1.370	0.588	0.200	0.270	0.284	0.259
	PUN935	10	4.000	1.709	0.826	0.300	0.415	0.437	0.277
RW	**PUN124**	4	3.000	2.462	0.974	1.000	0.594	0.679	-0.684
	PUN229	3	2.000	1.385	0.451	0.333	0.278	0.333	-0.200
	PUN1157	4	2.000	1.600	0.562	0.500	0.375	0.429	-0.333
	PUN132	4	4.000	3.200	1.255	1.000	0.688	0.786	-0.455
	PUN894	3	1.000	1.000	0.000	0.000	0.000	0.000	#N/A
	PUN935	4	3.000	2.133	0.900	0.500	0.531	0.607	0.059

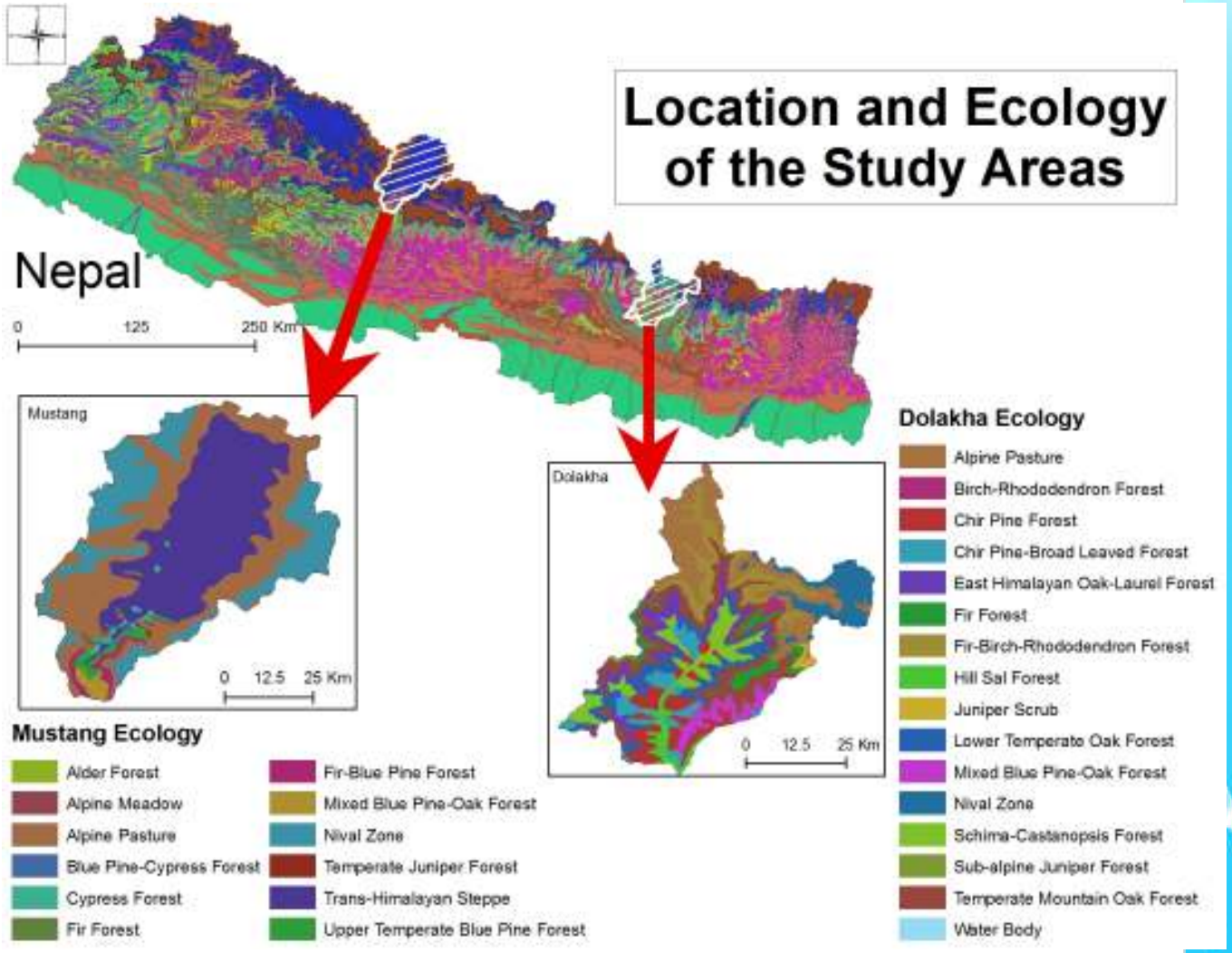
N=Total number of samples; Na=Number of observed alleles; Ne=Number of effective alleles; I=Shannon's Information Index; Ho=Observed heterozygosity; He=expected heterozygosity; UHe= Unbiased expected heterozygosity; F=Fixation Index; MUS=Mustang population; RW=Rolwaling population

Table 5: Genetic diversity of two separate snow leopard populations (Mustang and Rolwaling). SE=Standard Error of mean.

Mean and SE over Loci for each Pop									
Pop		**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**UHe**	**F**
MUS	**Mean**	9.500	5.000	2.904	1.184	0.478	0.580	0.613	0.199
	SE	0.342	0.365	0.543	0.165	0.092	0.084	0.090	0.054
RW	**Mean**	3.667	2.500	1.963	0.690	0.556	0.411	0.472	-0.323
	SE	0.211	0.428	0.327	0.182	0.159	0.102	0.116	0.113
Grand Mean and SE over Loci and Pops									
		N	**Na**	**Ne**	**I**	**Ho**	**He**	**UHe**	**F**
Total	**Mean**	6.583	3.750	2.434	0.937	0.517	0.496	0.543	-0.038
	SE	0.900	0.463	0.334	0.139	0.088	0.068	0.073	0.097

Table 6: Percentage of polymorphic loci in two separate snow leopard populations (Mustang and Rolwaling).

Population	%P
MUS	100.00%
RW	83.33%



Map 2: The study area and GIS generated ecology profile map based on GPS locations of genetically verified snow leopard positive samples.

5.0 DISCUSSION

A total of 26 scat samples were identified as that of snow leopard out of 48 samples screened. 44% (21/48) of scat that were presumed to be that of snow leopard were either misidentified in the field. Only 2% (1/48) of sample yielded DNA that was not suitable for genetic analysis. This has validated our previous hypothesis that the carnivore samples belonged to species other than snow leopard (Karmacharya et. al. 2012). There is significant amount of misidentification of scat samples in the field. High level of species field misidentification of scats has been documented in numerous studies that used genetic data to identify fecal material. (Farrell et al., 2000; Davison et al., 2002; Janecka et al. 2008). This suggests that proper training to field biologists is important for sample identification and collection. Scats that were collected for this study mostly yielded good DNA; our field protocol and training of field researchers on safe handling and preservation of scat prior to the field work might have contributed to good quality scat for genetic analysis.

Scat along with various signs (scrapes, pugmarks etc.) are common types of markers used for monitoring snow leopards (Schaller, 1988; Schaller et al., 1988; Hussain, 2003; Ale et al., 2007). However, various factors like predator densities, diet and behavioral factors may influence proportion of scat field misidentification while sign surveys may similarly be affected by seasonal, environmental and behavioral factors. Hence surveys based on scat and sign data are likely to produce unreliable overestimating indices of snow leopard densities as it might have happened in this study. Since non invasive genetic scat surveys are less likely to be affected by such effects, we suggest that scat and sign based Snow Leopard surveys should compliment genetic species identification.

Mitochondrial DNA (mtDNA) is found in hundreds to thousands of copies per cell compared to only two copies of nuclear DNA (nDNA) genome. This makes nDNA based genetic analyses e.g., Sex identification PCR (dependent on sex chromosome) and Individual identification PCR (based on multiple autosomal chromosomes) much sensitive than that based on mtDNA e.g., Species identification PCR (Waits et. al., 2005). This might explain 77% (20/26) genotype success across all 6 microsatellite loci.

The microsatellite markers used to identify individuals in Ladakh, India and Gobi Desert, Mongolia had sufficient variation to also identify individuals in Nepal. This enables us to expand these studies and conduct surveys that are designed to estimate the population abundance in different regions of Nepal. The mean He in our two study sites [0.49 is similar to those estimated in Mongolia (mean He = 0.51); Janecka et al. 2011]. We did not have a sufficient sample size, to test for significant differences in genetic variation between the populations. However, we are currently collecting additional samples from these areas and regions that are yet to be sampled, which will enable us to estimate population structure and landscape connectivity.

We were able to successfully enumerate the number of individuals among scats collected in our genetic lab in Kathmandu. This makes our laboratory the first local institution that has such capacity in Nepal and Bhutan. We observed more individuals in the Annapurna area than in Rolwaling. However, we currently

cannot make conclusions regarding the differences in abundance between these regions because our sample collection was not systematic. However, we are using this information to design a systematic survey that will enable us to estimate population densities in Annapurna, Rolwaling, and other regions of Nepal. This will be critical for prioritizing the conservation actions in our country.

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