



Conserving Natural Heritage through Genetic Assessment:

The case of Snow Leopards in Ladakh

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By

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CERTIFICATE

This is to certify that **Mr. Vishnuvardhan** has carried out an original piece of research in partial fulfilment of Master's Degree in Heritage Conservation and Management from Saurashtra University, Rajkot, Gujarat. The topic of his dissertation is "**Conserving Natural Heritage through Genetic Assessment: The case of Snow Leopards in Ladakh**". The study was carried out under our supervision from January 2021 to July 2021. We hereby certify that this work has not been submitted for any degree to any university.

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DECLARATION

I **Vishnuvardhan**, hereby declare that the research work entitled “**Conserving Natural Heritage through Genetic Assessment: The case of Snow Leopards in Ladakh**”, carried out in partial fulfillment of **M.Sc. degree in Heritage Conservation and Management** from Saurashtra University, Rajkot is an original work. This work was carried out under the supervision of Dr. Y.V. Jhala, Dean & Scientist-G, Wildlife Institute of India and co-supervision of Mr. Yellapu Srinivas, Ph.D. Scholar, Wildlife Institute of India from January to July. I hereby declare that this work has not been submitted in any form for any other degree or diploma at any university or other institutions.



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List of Abbreviations

SL	Snow Leopard
Km²	Square Kilometer
IUCN	International Union for Conservation of Nature
CMS	Convention on Migratory Species
CITIS	Convention on International Trade in Endangered Species of Wild Fauna and Flora
WWF	World Wide Fund
WII	Wildlife Institute of India
bp	Base pair
DNA	Deoxyribonucleic Acid
MtDNA	Mitochondrial Deoxyribonucleic Acid
Cyt b	Cytochrome B
Min	Minutes
Sec	Seconds
BSA	Bovine Serum Albumin
PCR	Polymerase Chain Reaction
N	Number of Alleles
HWE	Hardy-Weinberg Equilibrium
LD	Linkage Disequilibrium
PIC	Polymorphic Information Content

PID	Probability of Identity
PID sib	Probability of Identity among siblings
Ho	Observed Heterozygosity
He	Expected Heterozygosity
FIS	Fixative Statistics
ADO	Allelic dropout
AR	Allelic richness
MNA	Mean Allelic range
MCMC	Markov Chain Monte Carlo
DAPC	Discriminant Analysis of Principal Component
AMOVA	Analysis of molecular variance
FA	False Allele
LOCPRIOR=0	Without Location Prior Information
LOCPROR=1	With Location Prior Information
ARA	Allelic Range
P	Positive
%	Percentage

°C	Degree Celsius
u	Mu (Micro)
=	Equal to
~	Approximately
<	Less than
>	Greater than
≥	Greater than or equal to
©	Copyright
Δ	Delta
F (Null)	Frequency of Null Allele
G-W Index	Garza-Williamson Index
IBD	Isolation by Distance
SMM	Stepwise Mutation Model
TPM	Two Phase Mutation Model
SD	Standard Deviation
Ln	Logarithm

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SUMMARY

Natural world heritage sites are landscapes that sustain rare ecological processes, provide crucial habitats for the survival of many iconic species of significance and provide a wealth of ecosystem services. A part of India's natural heritage lies in its youngest landscape, the Himalayas. This natural heritage is an asset in itself due to its immense intrinsic values. Snow leopard, an elusive inhabitant of the cold desert represents an important cultural and ecological symbol of the Central and south Asian mountain systems and acts as a flagship as well as an umbrella species. This study has been designed to assess the genetic status of this vulnerable species distributed across the union territory of Ladakh using non-invasive genetic methods. The study site comprised of four distinct regions, Hemis-Leh, Kargil, Changthang and Nubra. Putative snow leopard scat samples (N=391) were collected from the study area. DNA was extracted from all the samples and a 148 bp Mt-DNA region was amplified with a carnivore specific primer. Successfully amplified samples were sequenced for species identification. From the 95 snow leopard positive samples 56 individuals were identified using a panel of 10 microsatellites. Sex identification was also done by using AMELY gene and results showed male biased sex ratio in Ladakh. PID_{sib} value was 3.8×10^{-4} was indicative of sufficient power of the microsatellite panel to discriminate between siblings in a population of 10,000 snow leopards.

Low genetic variation was observed in snow leopards ($H_o = 0.585/ H_e = 0.674$) and bottleneck analyses were indicative of genetic bottleneck events in the recent past. Genetic distance and geographic distance showed no significant correlation. Migration rates between the four regions were high showing maximum dispersal between

Hemis–Leh and Kargil. Population genetic structure revealed one single population with high admixture in the region. This study provides a baseline for future comparisons since increased anthropogenic development projects may fragment this contiguous population. Genetic integrity and large genepools are essential for long-term viability of species especially in the advent of climate change and anthropogenic modifications. This study using genetic markers on the snow leopard exemplifies the use of this important tool for natural heritage management and conservation.

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1 Introduction

Natural heritage encompasses natural features like geological and physiographical formations, delineated areas that constitute the habitat of threatened flora and fauna and natural sites of importance from the point of view of science, conservation or natural beauty (Titchen S.M 1995). In the Anthropocene era, humans are constantly shaping the environment and have emerged as the biggest drivers of environmental change (Steffen et al., 2007). Biological diversity of the earth is rapidly declining as a direct or indirect consequence of these human activities (Frankham et al., 2002). Many species are already extinct, and several others are on the verge of extinction owing to drastic decline in population sizes. Currently, many species require appropriate human interventions to aid in their conservation. According to Leakey and Lewin (1995), the current extinction problem has been termed as the 'sixth extinction', as its magnitude is as high as the other five mass extinctions (Frankham et al., 1999). In response to these situations, the International Union for Conservation of Nature (IUCN) recognizes the need of conservation at all the three diversity levels (i.e. species diversity, ecological diversity, and genetic diversity) (Hoban et al., 2020). An in-depth research is necessary to understand the relationship between, and impacts of, humans on the natural environment (Rust et al., 2017).

In order to protect an ecosystem from facing the consequences of species extinction some studies have shown that often conserving a top predator species could be beneficial for an entire ecosystem (Frankham 2002; Sergio, et al., 2006). Therefore, conservation plans based on apex predators could be implemented to deliver broader biodiversity benefits. Predators have manifold significance in sustaining an ecosystem. Apex predators often select sites with high topographic and habitat complexity (Sergio, et al., 2006, 2008), their diet is dominated by relatively few main prey species but a large number of minor prey species are also consumed thus maintaining a wide range of prey base; Furthermore, such prey species

depend on a complex suite of biotic and abiotic conditions. On one hand the presence of such predator implies the persistence of this diffuse complexity but on the other hand they can also be sensitive to ecosystem dysfunctions, such as chemical pollution, habitat alteration and other anthropogenic disturbances (Newton 1998; Thiollay 1989; Sergio et al. 2006). Lower trophic level predators cannot suffice the strategic and ecological importance of top predator species. Thus, predators coined as flagship, keystone or umbrella species are vital for maintaining the entirety in the biodiversity heritage of an ecosystem (Caro & O'Doherty 1999; Entwistle 2000; Bowen et al., 2002; Sergio et al., 2006; Carignan & Villard 2002).

A suitable representation of rich natural heritage lies in the youngest landscape of India, the Himalayas. Besides harboring a wealth of medicinal plants, globally important wildlife it also supports river systems vital for the nation's food security and a vast rangeland system to sustain important traditional pastoral economies and lifestyles. A significant proportion of these values provided by high altitude areas located above the tree line. Snow leopard (*Panthera uncia*), the largest Himalayan Felid serves the role of the apex predator in this ecosystem, and is one among the array of mystic inhabitants of the Himalayas. On the IUCN Red List, snow leopards are categorized as Vulnerable C1, meaning the observed, estimated or projected decline is expected to be 10% in 10 years or three generations and their population is above 2,500 but fewer than 10,000 (McCarthy et al., 2017). It is also listed in Appendix I of the Convention on Migratory Species (CMS) and Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The snow leopard has been accorded highest level of legal protection under Schedule I of the Indian Wildlife (Protection) Act, 1972 (McCarthy et al., 2017) due to its vulnerability for illegal trade of its coveted pelt. India perhaps holds 10 % of the

global population in less than 5% of its global range, and indeed the entire Himalayan landscape and wildlife, forms part of India's unique natural heritage.

The snow leopard habitats are not restricted to the protected areas but also spread across almost the entire trans-Himalaya landscape. However, due to natural barriers like permafrost at higher altitudes, large water bodies as well as the large geographical distances between the snow leopard populations inhibit their dispersal patterns (Farrington and Li 2016). Apart from these, such low-density wildlife populations are also facing increasing threats posed by competition with livestock, degradation, poaching, and some of them as extreme as to cause local extinctions (Beale and Botezatu 2016; Li, Juan, et al., 2020; McCarty et al., 2016).

India's high altitude landscape is enriched with tremendous biodiversity values and the resident communities respected, understood and coexisted with wildlife for centuries. People inhabiting the region mostly above the cultivable zone, are primarily pastoral with limited alternatives for survival. The coexistence of people and wildlife in the region is threatened due to pressures of escalating human and livestock populations. Conservation efforts in the region, therefore, need to be innovative, ensuring wildlife conservation not only within protected areas, but also in the larger landscape. The conservation of apex predator would enable the conservation of the landscape and our natural heritage in a holistic manner.

1.1 Introduction to the species

A fascinating large carnivore, snow leopard distributed over Trans-Himalayas and Greater Himalaya is an Umbrella species, as well as a flagship species. These solitary cats are distributed as fragmented populations over less than 1.8 million km² across their global range. Snow leopards being ubiquitous species, in India ranges over approximately 130,000 km² but only 200-600 individuals are present in India (Bhatnagar et al., 2016). Hence, they are included in the IUCN red list as a vulnerable species.

Snow leopard is smallest among the big cats of genus *Panthera* with head and body length is 1-1.3m, Tail length is 0.8-1.1m, weighing between 20-50 kg. Annually each individual snow leopard requires approximately 20-30 adult Bharals, or around 730 kg of meat to feed this felid. Its prey primarily includes Blue sheep (bharal), Ibex, Marmots, Lagomorphs, and small birds (Kitcher et al., 2016). Adaptation to harsh climatic conditions like low temperature and high altitude makes snow leopards distinct from other big cats in phylogeny and taxonomy. The decline of prey leads to an adverse effect leading to depredation of livestock and conflict with residents. The low resource habitats and isolated small populations of the species increases the threat of extinction inferred from estimated population status and structure (Bhatnagar et al., 2001).

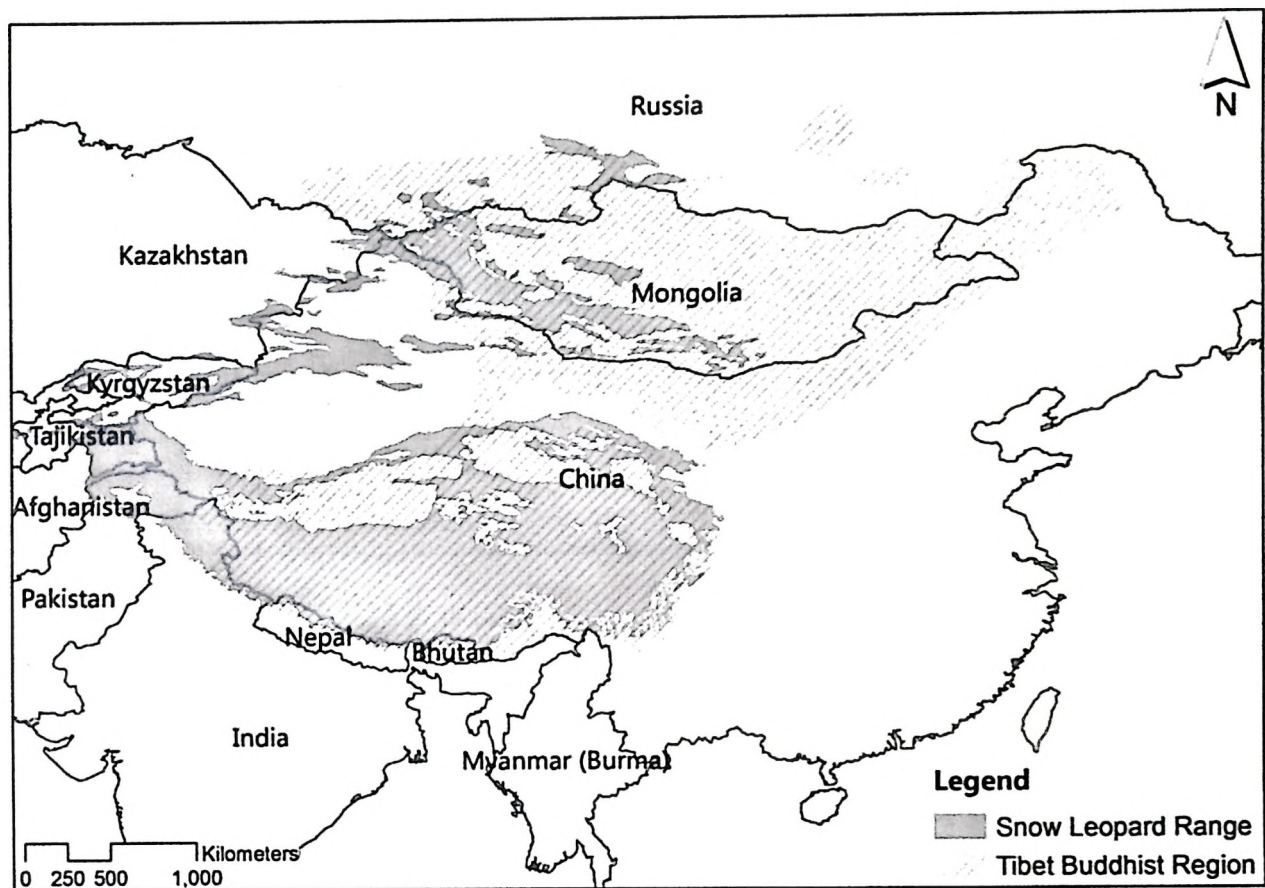
Snow leopards play an important role in their ecosystem as a keystone species (Lovaris et al., 2008). As a top carnivore, snow leopard's absence or abundance directly affects prey populations. Predation on wild ungulates such as blue sheep or markhor alters the vegetation cover, which has impacts on the niches for all animals

and plants in the habitat (Snow leopard Working Secretariat, 2013). Long-term absence in snow leopards could disrupt trophic cascades as ungulate populations would likely increase, leading to depletion of vegetation cover (Silliman & Angelini, 2012). Little is known about any interaction between snow leopards and sympatric carnivores. However, it is expected that snow leopard presence can have an effect on the abundance or absence of other carnivores such as lynx and wolves (Chetri, Odden & Wegge, 2017). Protecting snow leopard abundance will result in a cascade of benefits to the diversity of plant and animal life in the habitat (Chetri, Odden, & Wegge., 2017).

The snow leopard is an indicator species of Asia's high-altitude ecosystems, which support the snow leopard itself, its prey, and a diverse range of biodiversity that contributes to ecosystem services that promote human well-being on a local, regional, and global scale (Lovari, S et al., 2009). These ecosystems are significant not only as the home of this magnificent cat but also as the environmental and natural resources. The Himalayan glaciers are the source of ten major river systems in Asia. The Qinghai-Tibetan plateau serves as the source of three main rivers, while the Altai and Sayan mountains serve as the watershed between Central Asia and the Arctic Ocean. Water towers formed by high-mountain ecosystems to generate electricity downstream, maintain fisheries, support industry, and irrigate agriculture (Jackson et al., 2016). Hundreds of millions of people also depend on water for hydropower and agriculture, forage for livestock and food for themselves, medicinal supplies and products, mineral resources, cultural traditions and spiritual values, and inspiration that draws an increasing number of people (Snow leopard WS 2013).

1.2 Religious significance

Nature worship is prevalent in Central and South Asian traditional religions and cultures such as Buddhism, Daoism, Hinduism, Jainism, Shinto, Sikhism, and Zoroastrianism. These religions consider nature a crucial component of divinity treated with reverence (Nasr 1996). Thus, they play a crucial role in biodiversity conservation, which comes mainly in two forms. To begin, it is tradition to safeguard sacred species and sacred natural sites in the vicinity of religious structures. Second, religions indirectly maintain biodiversity by shaping their followers' view and behavior toward nature (Dudley et al., 2009). In both Buddhist and Taoist traditions, specific mountains and sites are embodied as shrines or pilgrimage destinations, and many mountain communities have strong spiritual beliefs that come from the mountain environment. There are many monasteries within snow leopard habitat (Li et al., 2014). It is believed that the presence and teachings within these monasteries encourages a passive approach to snow leopards, with 42% of local herders believing harming a snow leopard to be a sin in Buddhism (Li et al., 2014). The historical petroglyphs of snow leopards found in Tien Shan can be used in teachings to demonstrate the long existing coexistence of people and snow leopards (Salopek, 2017).



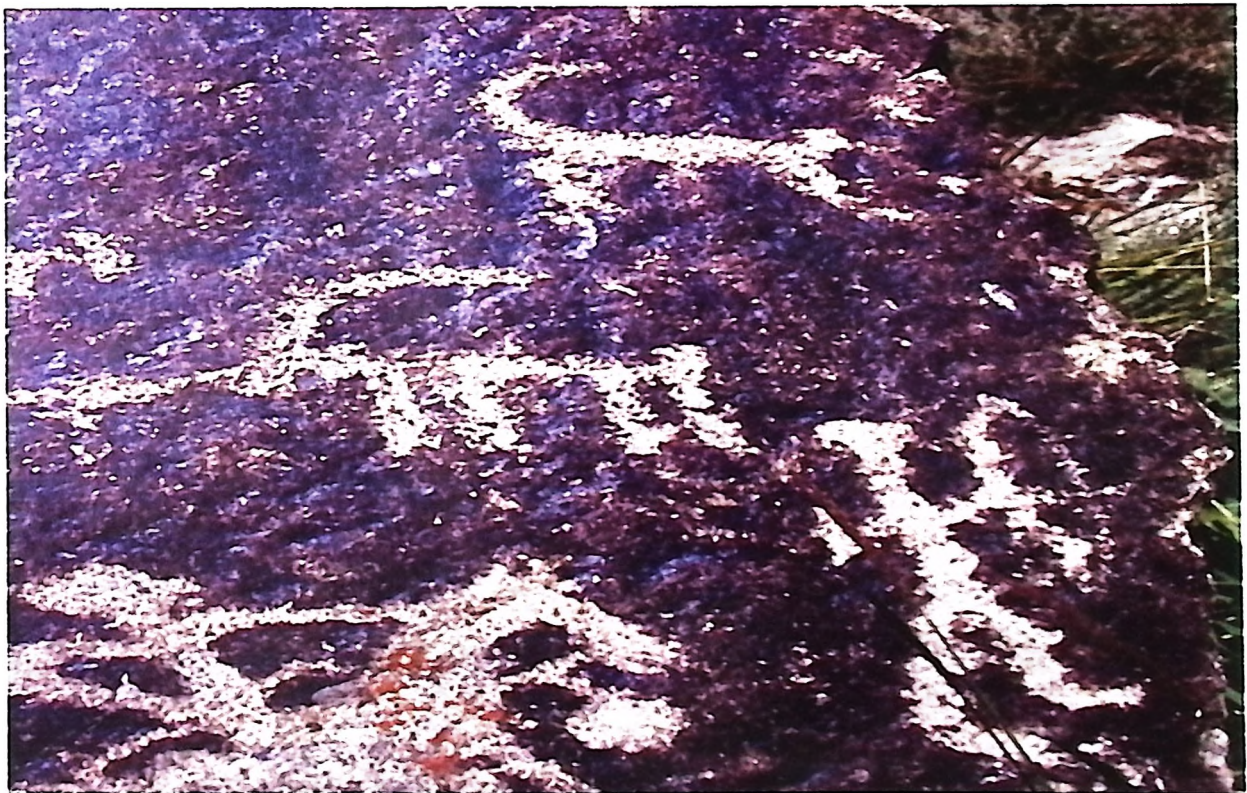
@Encyclopedia Britannica, 2003, Atlas of Faiths

Plate 1: Global range of snow leopards and Tibetan Buddhist regions. The regions under the influence of Tibetan Buddhism could cover about 80% of global snow leopard range.

1.3 Cultural significance

The symbolic relationships between biological and cultural diversity reflected in indigenous Wakhi people's stories and art (Mock 2011). In Wakhan District of northeast Afghanistan, numerous depictions of wild ungulates, and hunters with spears and bows on foot and on horseback observed as a canvas to tell stories grounded (Mock 2013). Snow leopards, in particular, offer a symbolic representation of the locations, appearing in coats of arms and other emblems of certain nations and places within the snow leopard range (Loginova 2016). The snow leopard is the state animal of Himachal Pradesh in India, as well as an emblem of Bishkek, Kyrgyzstan, Samarqand, Uzbekistan, Astana, and Almaty, Kazakhstan (Loginova 2016). The

image of a snow leopard also appears on state awards and currency of Kazakhstan and Russia. The mountains themselves that form the habitat of the snow leopard have a special role in cultures and beliefs of peoples in (McCarty and Mallon 2016). Through the years, the magnificence of Central and South Asia's great mountains has inspired painters, poets, and mountaineers. Human cultural variety is also abundant in the Central and South Asian highlands (McCarty and Mallon 2016). Snow leopards live in vast, rugged landscapes that also support some of the most remote human cultures of the world. It is an important cultural symbol in Central and South Asia, featuring widely in folklore and beliefs.



@ John Mock

Plate 2: Rock art depicting Snow leopard in Wakhna district of northeast Afghanistan.

1.4 Aesthetic significance

Snow leopard habitats have incalculable cultural and aesthetic significance. The way of life, religious and spiritual beliefs, traditional agriculture, cuisine, marital patterns, and administration of the cultures that occupy these locations are all distinct (Zakharenka et al., 2016). The Himalayan ranges, including several sacred mountains, have numerous mystical and holy connections to various religions and faiths. Scholars, artists, poets, spiritualists, and ordinary inhabitants are all inspired by the region. Art, photography, and literature have long caught the human imagination with visual depictions and written descriptions of the nature (Quammen et al., 2016).

1.5 Economic importance

The ecosystems that support snow leopards are precious economically. A quick estimate of the economic value of some prominent services generated by snow leopard habitats in India is nearly \$4 billion per year, the majority of which comes from hydropower and electricity generation (US\$3 billion), tourism (US\$0.4 billion) and livestock and agriculture (US\$0.5 billion) (SLWS 2013). Ecotourism in snow leopard ranges has become a popular and particularly successful in Nepal and India (Theile, 2003). Alongside creating an opportunity to educate people about the plight and importance of snow leopards, ecotourism creates an income for local people and instills the need to protect the species and their habitat (Vannelli et al., 2019). This can contribute to the promotion of sustainable living and development.

1.6 Ecological importance

The plant and animal species in snow leopard ecosystems are incredibly varied. Snow leopard habitat supports 350 species of animals and 1,200 species of birds in India alone, whereas the Altai Mountains sustain approximately 4,000 plant species, 143 species of animals, and 425 species of birds. Among them, numerous flagship species that occupy all or parts of the range are at least two species of bear, red panda, and wolf. Mountain ungulates such as wild yak, chiru or Tibetan antelope, Ladakh urial, Tibetan argali, Tibetan gazelle, four species of musk deer, the Hangul or Kashmir red deer, three species of Goral, Himalayan tahr, Serow and Takin also more than 20 species of birds of prey, including Griffons, Imperial eagles and Lammergeiers (Network, S.L. 2014). There are several endangered and therapeutic plants in these places, including over 675 food plants and almost 1,743 medicinal plant species in the Indian Himalayan region alone, many of which are employed by the pharmaceutical industry. Apart from its intrinsic value, biological diversity underpins agriculture. The area is home to around 335 kinds of wild relatives of cultivated crops. This region is also home to wild cousins of all major domesticated animals, including cattle, horses, sheep, and goats. Thus, the area's biodiversity resources not only provide life support to the people who live there and serve as a source of wild genetic material for plant and animal breeders (Zakharenka et al., 2016). Despite these immensely diverse values, snow leopards and their ecosystems are threatened throughout their range, their prey is declining in many areas, and the grasslands that support these prey, as well as the lives and livelihoods of pastoralists who share these mountain habitats, are deteriorating. The snow leopard is an

ecologically, culturally, and commercially significant icon of healthy high-mountain ecosystems and the communities who live there, yet it has a high risk of extinction across its range.

1.7 Importance of genetics in conserving the species

A critical challenge faced by the species in today's world is environmental changes. Species have to cope up with the ever-changing environment to survive. As such, the adaptation to environmental change becomes paramount to avoid extinction. Biodiversity conservation is one of humanity's most pressing issues, as wild species and domestic breeds and strains are vanishing at an exponential rate, and a growing number of them need human intervention to ensure their survival. About 99% of species that had ever existed are now extinct (Frankham et al., 2002). Several hundred species have disappeared over the past 400 years or so. Genetic diversity is the foundation for a higher level of Biodiversity, and it is the basis of environmental changes and, as such, become an essential pillar in conservation genetics.

Genetic diversity is required for the population to adapt to environmental changes. It is measured using an array of molecular tools and quantitative methods. Genetic diversity is usually high in large number of the naturally out bred population, but it is generally low in isolated small populations and species. Knowledge of genetics helps in conservation by assisting in the genetic management of small populations, taxonomic uncertainty resolution, and identifying management units, and applying molecular genetics research in interpreting species biology. It deals with the genetic factors that affect extinction risk and the genetic management regimes required to minimize these risks.

Globally, only a few studies have been conducted on snow leopard genetics so far, and many of these studies highlighted less genetic diversity and cryptic population genetic structure observed between the populations across its distribution range. The only extensive molecular study conducted by Janecka et al., (2017) using bi-parental nuclear microsatellite analysis categorized snow leopards into three sub-species belonging to:

- I Northern cluster, consisting of samples of western and southern magnolia.
- II Central group, including representatives from China, Bhutan, and Nepal.
- III The western cluster includes samples from Kyrgyzstan, Tajikistan, Pakistan, and India (including Ladakh).

To date, no molecular study understanding its genetic status and assessment of gene flow between the populations has been conducted on snow leopards in India, thereby creating lacunae in understanding the species biology. Therefore, evaluation of genetic diversity and population genetic structure is imperative for the conservation of snow leopard. This study aims to address this void on snow leopard genetics using molecular tools in a region of its range the Ladakh where the species is reasonably abundant.

My study has relevance and is timely in the following context: snow leopard habitat is contiguous across the Himalayan ranges and the animal has great dispersal abilities. Yet, the geographical orientation of High Mountain ranges can be a formidable barrier to even highly adapted species (Janecka et al., 2017). Besides, the region of Ladakh is earmarked for high investments in infrastructure development and

urbanization. These human induced changes in the landscape have the potential to become barriers to dispersal to many mountain species including the snow leopard. My study will provide a baseline in understanding the natural inherent genetic structuring (if any) in snow leopard populations across Ladakh and allow comparisons in the future if gene flow has been impaired by intensified human use and development. Such an understanding is essential for bringing in a balance between development and conservation and designing developmental projects that have the least impact on the conservation of our biological heritage.

2 Review of literature

2.1 Taxonomy and systematic

The snow leopard (*Panthera uncia Schreber 1776*) is a member of the Order Carnivora, Family Felidae and sub-family Pantherinae (Blomqvist 1978, Corbert and Hill 1986, Ellerman and Morrison Scott 1951). Some authors follow the monotypic nomenclature and use the name *Uncia uncia Schreber 1775* (Hemmer 1967, 1972, Rieger 1980). However, the former nomenclature appears to be used more widely than the latter.

Historically, several subspecies have been proposed including the nominated *Panthera uncia uncia* (Stroganov 1962), *Panthera uncia uncioides* in Nepal (Horsfield 1855), *Panthera uncia schneideri* in Sikkim (India, Zukowsky 1950), and *Panthera uncia baikalensis-romanii* in the Transbaikai (Russia, Medvedev 2000) based on coat color differences. Range-wide snow leopard phylogeography study address the taxonomic question regarding subspecies designation of the snow leopard using genetic data from non-invasive scat samples (Janecka et al., 2017). Based on the differentiation of nuclear loci snow leopard is classified into three subspecies. *P. u. uncia* (Restricted to Central Asia including Tian Shan, Alay, Pamir, Karakoram, and trans-Himalaya), *P. u. uncioides* with *schneideri* (core Himalaya and the Tibetan Plateau), and *P. u. irbis* with *baikalensis-romanii*(the Altai and Southern Gobi of Mongolia) (Janecka et al., 2017).

2.2 Etymology

The snow leopard is known in its native lands as Shan (Ladakhi, India); Bharal he, Barfani Chita (Hindi, Urdu, Pakistan); Chen (Bhutan), Sarken (Tibetan); Xue Bao (Chinese); Palang-i-berfy (Dari Afganisthan); snow leopard (English); Panthere des Neiges. Leopard des Neiges; Once (French); Schneelopard, Irbis (German) and Leopardo Nival, Pantera LasNieves (Spanish).

2.3 Species biology

2.3.1 Physical characteristics

The coat color is white to cream-yellow with grey to black spots and rosettes (Fox 1989). Spots on the head, neck and lower limbs are solid, whereas large rings or rosettes, some small enclosing spots, run along the side of the body and on the tail. A row of compact and elongated spots and two lateral rows of elongated rings extend on the back of the tail (Hemmer 1972). Individual snow leopards can be identified based on the distribution and arrangement of these spots along the body (Blomqvist and Nystrom 1980). The body length of adult leopards ranges from one to 1.3 m and the tail from 0.8 to 1 m. They stand about 0.6 m at the shoulder and weigh between 25 to 75 kg (Hemmer 1972).

2.3.2 Global distribution

The range of the snow leopard is restricted to the mountainous regions of Central Asia, including the Altai, Tien Shan, Kun Lun, Pamir, Hindu Kush, Karakoram, and Himalaya ranges. It is known to occur in twelve countries, namely Afghanistan,

Pakistan, India, Nepal, China, Bhutan, Mongolia, Russia, Tajikistan, Uzbekistan, Kazakhstan, and Kyrgyzstan, with an occupied habitat of about 1.6 million Km². snow leopards most frequently occur at elevations between 2,000 and 5,500 m, in areas of steep and broken rocky slopes that support shrub, grass, or steppe vegetation. On the northern limits of their range in Mongolia and Russia, they may use elevations as low as 600 m in hilly and occasionally occur in forested habitat. Range-wide, population estimates vary from 4,500 to 7,500.

2.3.3. Home range

Snow leopard home ranges range from 12 to 39 km² in productive habitat in Nepal (Jackson and Ahlborn 1989) to 500 km² or more in Mongolia (McCarthy et al., 2005). Some telemetry investigations show that male and female home ranges overlap, however use of a certain area is normally separated by time. Four radiocollared individuals in Nepal often used core area intersected with most suitable local topography, habitat and prey base. Snow leopard select Mountain ridges, cliff edges, and well-defined drainage lines as travel routes and deposit sites for scrapes, scats, and scent marks (Jackson and Ahlborn 1989). Snow leopards moved up to 7 km per day in Nepal's rugged terrain, but only around 1 km on average (Jackson and Ahlborn 1989), whereas in Mongolia, their daily movements were much greater (approximately 12 km), with one female covering 28 km in a single (McCarthy et al., 2005). However, in Ladakh region, density estimates (Fox et al., 1991) gave relatively low results (e.g., 1–2/100 km²) compared to later more intensive studies done by Chundawat (1992) and Jackson et al., (2006). Mallon (1988) in the same general area.

Table 1: Country wise population estimates of Snow leopard.

Country	Population estimate	Range	Density	Estimation year
Afghanistan	50 - 200	50,000Km ²	-	2013
Bhutan	100 - 200	15,000 Km ²	1/100 Km ²	1994
China	2000 - 2500	1,100,000 Km ²	0.3 – 0.4/ 100Km ²	1998
India	200 - 600	75,000 Km ²	0.5 – 0.9/ 100Km ²	2016
Kazakhstan	100 – 200	50,000 Km ²	-	2002
Kyrgyzstan	150 - 200	105,000 Km ²	2.35/100Km ²	2005
Mongolia	500 - 1000	101,000 Km ²	-	2000
Nepal	300 - 500	27,000 Km ²	0.1-10 / 100km	2009
Pakistan	200 - 420	80,000 Km ²	0.4 / 100Km ²	2003
Russia	70 - 90	12,000 Km ²	-	2012
Tajikistan	200 - 220	100,000 Km ²	-	2004
Uzbekistan	20 - 50	10,000 Km ²		2003

(McCarthy & Chapron, 2003; Muratov, 2004; Snow leopard Working Secretariat, 2013; Uneland, 2005; Valentová, 2017; WWF-Nepal, 200

2.3.4 Habitat

Snow leopards live in rugged mountainous terrain and are associated through most of their range with arid and semi-arid shrub land, grassland or steppe (Fox 1989; Jackson 1992). They are generally found in elevations between 3000 to 4500m, although they occasionally go above 5500m in the Himalayas and are found between 600 to 1500m at the northern limit of their range (Theile 2003). They are usually associated with steep rocky slopes with arid and semi-arid shrub land, grassland, or steppe vegetation. They have been observed in coniferous forests but generally, avoid dense forest. They are found at altitudes of 3,000 to 4,500 m., with a high of 5,500 m. and low elevations of 600 to 1,500 m. they are being observed. Generally, this species prefers areas of cliffs, gullies, rocky outcroppings, and ridges.

2.3.5 Vegetation

Snow leopards inhabit the high mountainous regions of central Asia. Vegetation in areas where snow leopards occur range from timberline alpine ecotones in the heavily forested southern slopes of the Himalaya, the mountains of western Sichuan (China). Parts of the Soviet Union ranges through open forests and woodland habitats in many mountain systems on the outer arc of snow leopard distribution, alpine zones in all-mountain systems, to scrubland and desert mountain habitats in the central portion of snow leopard range as one approaches the Gobi desert region (Novikov 1956, Schaller 1977, Jackson 1979a, Koshkarev 1984, Mallon 1984b). A large portion of their range is predominantly treeless due to either alpine or desert conditions. Snow leopards occur at elevations of about 600- 4000 m in the northern

part of their range to 1800-5800 m in the southern portions (Fox and Chundawat 2016, Stroganov 1962, Dang 1967).

2.3.6 Terrain

The terrain used by snow leopards is typically extremely rugged (Schaller 1977, Koshkarev 1984, Mallon 1984b, Fox et al., 1988, Jackson and Ahlborn 1988). In Ladakh, India, snow leopard travel routes occurred on terrain averaging 24° in slope angle and 35 m from steep cliffs or other sharp breaks in terrain; 50% of all travel route locations were within 5 m of such cliffs (Fox et al., 1988). Snow leopards in western Nepal showed a preference for cliffs, areas with snow leopards in excess of 40°, and areas within 25 m of edges such as cliffs. Preferred bedding sites situated on or near ridges, cliffs and other sites with good views. Snow leopards preferred to move, bed, and mark along with linear topographic features such as major ridgelines, bluff edges, gullies, and the base or crest of broken cliffs (Jackson and Ahlborn 1988).

2.3.7 Wild prey species

The snow leopard primary prey includes wild sheep and goats, whose typical habitat is the rugged terrain of mountainous regions. The most common of these species throughout the snow leopard range is the blue sheep (*Pseudois nayaur*), Asiatic ibex (*Capra sibirica*), markhor (*Capra falconeri*), and several forms of argali sheep (*Ovis ammon*). Wild sheep and goat species with more restricted distributions within the snow leopard range include the urial (*Ovis vignei*), Himalayan tahr (*Hemitragus jemlahicus*), serow (*Capricornis sumatraensis*), and goral (*Naemorhedus goral*).

Other ungulates which have been reported to constitute snow leopard prey include the Tibetan antelope (*Pantholops hodgsoni*), Alpine musk deer (*Moschus chrysogaster*), wild boar (*Sus scroa*), Tibetan gazelle (*Procapra picticaudata*), goitered gazelle (*Gazella subgutturosa*), wild ass (*Equus hemionus*), and wild yak (*Bos mutus*). Snow leopards are also reported to have hunted or killed langur (*Presbytis entellus*) (Green 1982) and bear (*Ursus sp.*) (Schaposchnikov 1956). Smaller mammals preyed on by snow leopard include marmots (*Marmota himalayana*), hares (*Lepus spp.*), pikas (*Ochotona spp.*), and voles and mice. Snow leopards also prey on birds, including the snowcock (*Tetrogallus spp.*) and chukar (*Alectoris chukar*). Relatively long periods between kills of large prey suggest that small mammals and birds may be important in meeting overall energy requirements (Jackson and Ahlborn 1988). Domestic animals such as sheep, goats, donkeys, horses, and cattle also constitute snow leopard prey and may be important components of their diet in some areas (Schaller 1977).

2.3.8 Diet

Snow leopards prey primarily on mountain ungulates and domestic livestock, supplemented by smaller mammals and game birds throughout their range (Bannikov 1954, Blanford, 1888–1891, Heptner and snow leopardudskii 1972, Roberts 1977, Schaller 1977). Blue sheep (*Pseudois nayaur*) were the main prey in Shey and Lapche of Nepal (Schaller 1977), Taxkorgan, Qinghai, and Gansu of China (Schaller et al., 1987, 1988 a, 1988 b), whereas principally ibex (*Capra ibex*) were primary in Tomar Feng, China (Schaller et al., 1987, 1988 b), and the Sanctuary of Alma Ata, USSR (Zhirjakov 1990). Markhor (*Capra faiconeri*) formed the bulk of their diet in

Chitral, Pakistan (Schaller 1977). Other prey species include argali (*Ovis ammon*), urial (*Ovis vignei*), tahr (*Hemitragus jemlahicus*), goral (*Naemorhedus goral*), serow (*Capricornis sumatraensis*), takin (*Budorcas taxicolour*), musk deer (*Moschus chrysogaster*), Cervus deer, roe deer (*Capreolus capreolus*), kulan (*Equus hemionus Kulan*), goitered gazelle (*Gazella subgutturosa*), wild boar (*Sus scrofa*), hare (*Lepus spp*), squirrel (*Sciurus vulgaris*), Red fox (*Vulpes vulpus*), wolf (*Canis lupus*), snowcock (*Tetrogallus spp.*), and chukar partridge (*Alectoris chukar*) (Bannikov 1954, Dang 1967, Dash et al., 1978, Jackson and Ahlborn 1988, Mallon 1984 a, Schaller 1977, Schaller et al., 1987, 1988 a, 1988 b, Zhirjakov 1990). Snow leopards are reported to scavenge on domestic yak Siberian ibex and roe deer carcasses (Schaller 1977, Zhirjakov 1990). Predation on domestic livestock occurs in most, if not all, of its range (Fox et al., 1988, 1991, Mallon 1984 a, Schaller 1977, Schaller et al., 1987, 1988 a, 1988 b, Sherpa and Oil 1988).

Genetic work of snow leopard

In spite of being a charismatic species, its cryptic nature and remote habitat makes it difficult to obtain information on snow leopard. Furthermore, studies on genetics of snow leopard are complicated due to its wide range of habitat and difficulty in transportation of biological material from sampling sites. Zhang et al., (2007) was the first to develop snow leopard species-specific primers to amplify a portion of the control region in the mitochondrial region. These primers used for wildlife forensics, species identification, and population genetic studies from China using skin and hair samples via non-invasive sampling technique. This study identified six haplotypes and six parsimony-informative sites within their 411 base pair (bp) sequence, and

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concluded that these primer sets are good for studying mitochondrial diversity and phylogeographic patterns of snow leopards in other portions of their range.

Wie et al., (2009) amplified whole mitochondrial genome of the snow leopard, and performed phylogenetic analysis using 10 other felid species and revealed snow leopards as sister taxa of lions. Davis et al., (2010) contradicted the previous study by using super-matrix of nuclear and mitochondrial genes with sex chromosome sequences and identified snow leopard and tigers as a sister taxa species. Subsequent study by Tseng et al., (2013) analyzed relationship between Snow leopard and tiger by using morphological characters and molecular data of Davis et al., (2010). In contrast to earlier studies, Cargiulo et al., (2014b), used whole mitochondrial genomes from all Pantherine lineages and identified snow leopard as sister taxa to leopard and lion.

Demographic history acts as vital aid towards conservation and management of snow leopard. However, mitochondrial DNA (mtDNA) provides information at the population and species level, therefore fine-scale genetic analysis through individual identification is important for examining snow leopard. The estimation of population or individuals in a given study area can be achieved by using combination of multiple nuclear microsatellite loci and mark-recapture statistics. The study done by Waist et al., 2007 used polymorphic microsatellite loci, originally developed for domestic cats for individual identification of snow leopard and reported 48 polymorphic microsatellite loci with 2-11 alleles per locus. Furthermore, they identified 10 loci with significant power to distinguish among individuals (Waist et al., 2007).

The elusive nature and difficulty of direct encounters of snow leopard in the wild, most of the studies emphasizes non-invasive techniques for better understanding of this charismatic felid. The development of polymorphic microsatellite loci was the first step in snow leopard genetics, leading to refinement of primers for snow leopard specific-primers (Janecka et al., 2008). The snow leopard-specific microsatellite primers had a higher success rate when used with scat samples because they amplified shorter DNA regions and primed to a more specific sequence, rather than to the closely related sequence of the domestic cats. The study done by Janecka et al., (2008) on wild snow leopard genetics was the pioneer and extensive study on snow leopard genetics. This study used noninvasive samples from different parts of its range (North West India, Central China, and southern Mongolia) and surveyed total transect length of 26.2 km and genetically identified 31 snow leopard samples out of 85 samples collected. Out of which 2-4 were found males and 2 females in southwestern India; 1 unknown sample in Central china, and 3-5 males and 2 females in southern Mongolia. Overall, they found low genetic diversity among the nine analysed individuals, with 2.5-3 mean allele per locus. Examination of four MtDNA region showed little genetic variability, with only two haplotype exhibited in the control region. All individual samples in the South Western India exhibited the two haplotypes, while central China and southern Mongolia samples exhibited the same single haplotype. This study provides the first baseline molecular information on the snow leopard. However, due to small sample size the study was purely descriptive (Janecka et al., 2008). Followed by Karmacharya et al., (2011) which focus on Shey Phoksundo national park and conservation reserve in Nepal, a total of 71 samples were collected out of which 19 of them genetically identified as snow leopard (three

males and six females) using these genotypes were generated using six microsatellite loci described in Janecka et al., (2008).

Janecka et al., (2017) studied population structure of snow leopard using microsatellite analysis and categorized into three sub-species; 1) Northern cluster, consisting of western and southern magnolia; 2) Central cluster, including china, Bhutan, and Nepal; and 3) western cluster, includes Kyrgyzstan, Tajikistan, Pakistan, and northern India (Ladakh). Snow leopard exhibited low genetic diversity at microsatellite loci ($AR=5.8$, $H_o=0.43$, $H_e=0.56$) and absence of mtDNA variation. Aruge et al., (2019) conducted a pilot study to access genetic diversity and population structure of snow leopard of Gilgit-Baltisthan, Pakistan using molecular markers, shows low heterozygosity and high gene flow between populations. The study by Shrestha and Kindlmann (2020), using habitat suitability map examined the importance of corridors for maintaining the genetic structure and gene flow between the population (Shrestha and Kindlmann, 2020). The recent study by Aruge et al., 2019 which focused on snow leopard population genetics in the northern area of its distribution and is one of the few fine-scale surveys of the species based on representative samples (Aruge et al., 2019, Shrestha and Kindlmann 2020). The results of the study alternatively suggest a subspecies division of the snow leopard (Zvychnaynaya et al., 2012, Janecka et al., 2017, 2018) with the delineation of the northern populations of Russia and Mongolia as a separate subspecies proposed by Janecka et al., (2017). In the Indian context, there are no genetic studies done in India apart from the range wide phylogeographic study on snow leopard global range countries (Janecka et al., 2017).

This study addresses the need for genetic diversity and population genetic structure to conserve natural heritage animal snow leopard by using non-invasive samples. Non-invasive sampling methods used instead of sampling populations directly by capturing individuals for sampling to avoid harming and disturbing the animal. In India among all the natural heritage sites, Nanda Devi and valley of flowers, Great Himalayan national park and kanchendzonga world heritage site holds potential snow leopard populations (WWF 2010). Snow leopard is also one of outstanding universal value, selected under the criteria number 10 (“to contain the most important and significant natural habitats for in-situ conservation of biological diversity, including those containing threatened species of outstanding universal value from the point of view of science or conservation”) of the world heritage sites inscription. Therefore, genetic diversity and population structure of snow leopard will helps in maintaining viable population as well as integrity of declared site.

3 Study hypothesis

Snow leopard, being a ubiquitous species with large home ranges (Jackson and Hunter 1996) and high mobile nature, can disperse long distances leading to high gene flow between the populations. However, due to natural barriers like permafrost at higher altitudes and large water bodies, which inhibits their dispersal patterns, the snow leopard populations can be genetically structured (Janecka et al., 2017). Moreover, in recent times, due to substantial increase in the anthropogenic activities (tourism), habitat alteration and decline in prey populations, can affect the genetic structure. Decline in snow leopard populations over long-term further might result in decreased genetic diversity impacting survival of snow leopard populations (Janecka et al., 2017, Shrestha and Kindlmann 2020).

Ho: Natural barriers along with human mediated activities that modify habitats might cause reduction in gene flow between the snow leopard populations leading to genetically structured populations.

Ha: Kargil and Hemis populations being in close proximity to each other with continuous habitat might have good gene flow between them in comparison to other (Nubra and Changthang) populations with poor intertwining habitats and farther away resulting in genetic structure.

Main aim: To estimate levels of genetic diversity and identify genetic structure of snow leopards using non-invasive samples within Ladakh region of the Trans-Himalaya, India.

3.3 Objectives

1. To estimate the levels of genetic diversity of the endangered snow leopard within Ladakh region of Trans-Himalaya.
2. To evaluate genetic structure of snow leopards, assess gene flow and understand barriers to movement within Ladakh region of Trans- Himalaya.

3.4 Research questions

1. Whether the nuclear microsatellite markers used in this study have sufficient power to discriminate individuals and siblings? These could then be used for estimating population of snow leopards. What is the sex ratio in snow leopard populations?
2. How many genetically identified populations exist in Ladakh and what is the level of gene flow between them?

4 Study area

The Trans-Himalaya is a cold desert, with an altitudinal range from 2,200 m to above 7,000 m, and winter temperatures may drop to below -40°C . The Trans-Himalaya receives annual precipitation of 100-1000 mm, which decreases to north and east (Fox et al., 1994). The Trans-Himalayan region in India is divided into three biogeographic provinces – the “Ladakh Mountains” or Zone 1A, the “Tibetan Plateau” or Zone 1B and the “Himalaya Sikkim” or Zone 1C (Panwar and Mathur 1992). Most of the Trans-Himalaya in the country falls in the Ladakh region of Ladakh union territory. The unique ecosystem of Ladakh, nestled in the lofty Himalayas and the mighty Karakoram lying between $32^{\circ} 15'$ to 36°N and $75^{\circ} 15'$ to $80^{\circ} 15' \text{E}$.

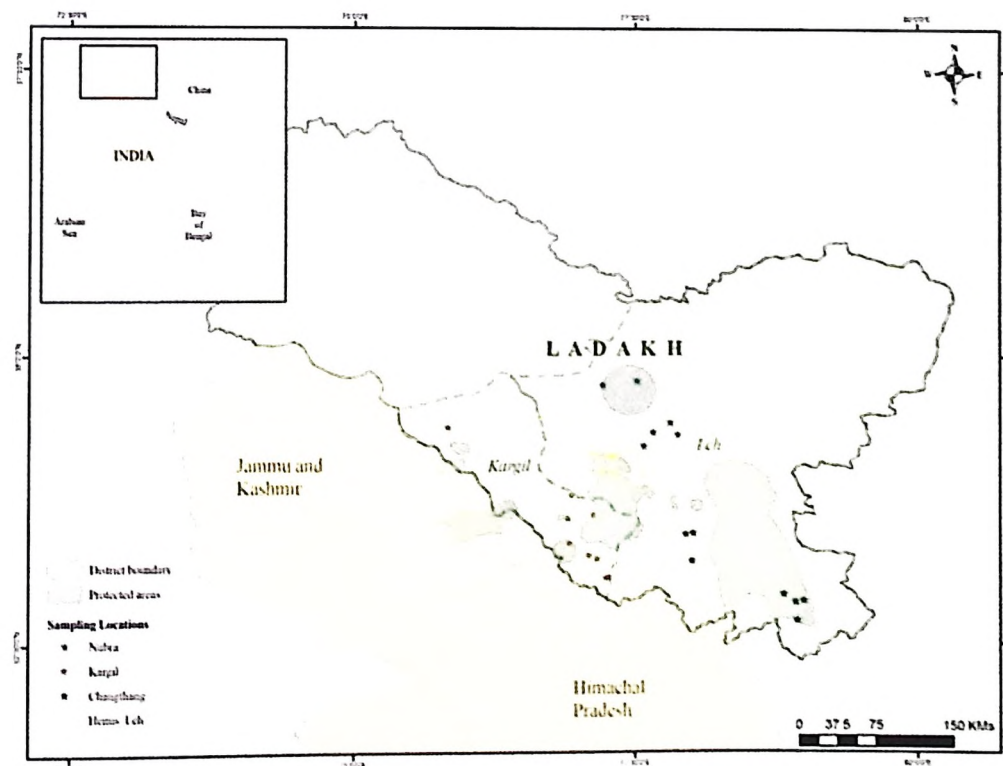


Fig 1: Study area map showing sampling locations (n=56) individuals used in this study.

The study area comprises of whole Ladakh union territory, which comprises of Hemis National park and Leh (Hemis-Leh), Kargil District (Kargil), Changthang wildlife sanctuary (Changthang) and Nubra Shayok wildlife sanctuary (Nubra). Ladakh region falls under Ladakh Mountains or zone 1A, and the Tibetan plateau or zone 1B biogeographic provinces. The Ladakh Mountains constitute approximately 60% of the Trans-Himalayan zone and spread in the Kargil, Nubra and Lahul spiti region of Ladakh union territory and Himachal Pradesh. These areas are mostly rugged mountains and valleys and have a large altitudinal range from 2200 m in the Kargil and Nubra region to over 7000 m in Karakorum Range. The second province is the Tibetan plateau that constitutes the remaining 40% of the Trans-Himalaya. This region includes Changthang in Ladakh parts of spiti and northern plateau of Sikkim and characterized by vast plains, rolling mountains and some large high altitude lakes. Most of this region lies above an elevation range of 4200 m.

5 Methodology

The study was conducted in collaboration and sponsored by the Forest Department of Ladakh. Samples were collected by the Forest department personnels. The study was permitted under the Wildlife Protection Act J&K, 1978 and approved by the dissertation evaluation committee of the Wildlife Institute of India.

5.1 Sample collection and DNA extraction

Putative snow leopard fecal samples (n=391) were collected from the Ladakh union territory which includes Hemis National Park and Leh (n=136), Changthang cold desert wildlife sanctuary (n=89), Nubra Shayok wildlife Sanctuary (n=72), and Kargil district (n=94). Samples were collected along ridgelines, cliff bases and outcrops, including livestock trails and identified in the field based on the size, shape, odour, and other associated field signs (i.e. proximity to leopard tracks, scrapes, and sprays). GPS location of each sample was noted and samples were preserved in zip locks filled with silica and later transported to Conservation genetic lab, Wildlife Institute of India for DNA extraction.

Genomic DNA was extracted from non-invasive samples using QIAamp DNA stool mini kit (Qiagen, Germany) following manufacturer's instructions. Similarly, DNA was extracted from tissue and blood snow leopard samples (n=5) using DNeasy Blood and Tissue Kit (QIAGEN, Germany) which were collected as part of telemetry study in Ladakh region. All the extractions were carried out in a dedicated room in batches and a negative control was included in every batch of extraction to monitor for any potential contamination.

5.2 Species identification

Following DNA extraction, species identification was carried out from non-invasive samples by amplifying a carnivore specific primer covering 148 bp region of mitochondrial cytochrome *b* gene (Ferral et al., 2000). Polymerase chain reaction (PCR) amplification was carried out in a volume of 10 µl reaction mixture containing 1.0 µl of 10 × PCR Buffer, 0.3 µl of 25 mM dNTPs, 1.5 µl of 20 µg/µl BSA, 0.2 µl of 5 U/µl Pfu Taq enzyme (dream Taq), 0.50 µl of each 20 µM primer, and 2.0 µl of the DNA extract. The thermocycling conditions include: initial denaturation 95°C for 15 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 60 seconds with a final extension at 72°C for 10 minutes. The amplified PCR products were visualized under ultraviolet light on a 2% agarose gel and sequenced bi-directionally using ABI 3500 XL sequencer (Applied Biosystems, USA). Finally, the generated sequences were manually inspected and validated using GenBank BLAST tool to confirm the species (<http://blast.ncbi.nlm.nih.gov/>).

5.3 Individual identification

A panel of sixteen polymorphic microsatellites (PUN82, PUN100, PUN327, PUN1283, PUN1293, PUN229, PUN834, PUN225, PUN132, PUN272, PUN124, PUN894, PUN1131, PUN1138, PUN1157 and PUN1262) were amplified (Janecka et al., 2008, Janecka et al., 2014). The polymorphic microsatellites were labelled at 5' region of forward primer with fluorescent dye 6-FAM, NED, PET and VIC (detailed in Appendix I Table S1). All the positive snow leopard samples were amplified using

multi-tube approach with a minimum of two to four replicates to limit genotyping errors (Taberlet et al., 2000). The consensus genotypes were generated with at least three similar genotypes to be considered as heterozygotes and two to be considered as homozygotes.

PCR amplification was carried out in a volume of 10 μ l reaction mixture containing 4 μ l of Qiagen PCR Buffer, 1 μ l of 20 μ g/ μ l BSA, 0.50 μ l of each 20 μ M labelled primer, and 2.0 μ l of the DNA extract. The thermocycling conditions include: initial denaturation 95°C for 15 minutes followed by 45 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 60 seconds with a final extension at 60°C for 45 minutes. The PCR products were visualized under ultraviolet light on a 2% agarose gel to verify amplification, and the amplicons were then fractionated and sized on ABI 3500 XL sequencer using (Applied Biosystems, USA) with Genescan 500 Liz (Applied Biosystems, USA) as internal size standard and all the alleles were scored manually using GeneMapper v 4.0 (<https://genemapper.software.informer.com/4.0>). A negative control (without DNA extract) and positive control (Snow leopard tissue or blood sample) was included in every PCR amplification to monitor for any potential contamination.

5.4 Sex identification

Sex identification was carried out for individually identified snow leopard samples using primers that amplified an intron region of the AMELY gene (Murphy et al., 2002). PCR amplification was achieved in male samples yielding a single band on gel electrophoresis with a fragment size of around 200 bp, while no amplification was observed in females. PCR amplification was carried out in a volume of 10 μ l reaction mixture containing 4 μ l of Qiagen PCR Buffer, 1 μ l of 20 μ g/ μ l BSA, 0.50 μ l of each 20 μ M primer pair, and 2.0 μ l of the DNA extract. The thermocycling conditions include: initial denaturation 94°C for 2 minutes followed by 50 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds with a final extension at 60°C for 45 minutes. The PCR products were later visualized under ultraviolet light on a 2% agarose gel. The PCR amplification for each individual was carried twice with known male and female positive samples to confirm the sex.

6 Data analysis

6.1 Genetic diversity estimates

Program CREATE was used to create input file for all the population genetic analysis (Coombs, J 2008). MICRO CHECKER v 2.2 was used to check for presence of null alleles and typographic errors due to stuttering patterns and small allele dominance (Oosterhout 2004). GIMLET v 1.3 was used for quantifying False alleles (FA) and allelic dropout rates (ADO) (Valiere 2002). Individuals were identified using identity analysis option implemented in program CERVUS v 3.0 (Kalinowski et al., 2007). Similarly, I also calculated $P_{(ID)}$ and $P_{(ID-SIBS)}$ using CERVUS v 3.0 to ensure the microsatellite markers used in this study have enough power to discriminate individuals and siblings. Standard genetic diversity estimates like expected (H_e) and observed (H_o) heterozygosity, mean number of alleles per locus (MNA), Allelic richness (AR), number of alleles (N), Polymorphic information content (PIC) was estimated for each sampling site and overall using software CERVUS v 3.0 (Kalinowski et al., 2007) and ARLEQUIN v 3.5 (Excoffier et al., 2005). Deviations from Hardy-Weinberg equilibrium (HWE) at each loci and Linkage disequilibrium (LD) among all the loci combinations were estimated using GENEPOP v 1.26 (Raymond & Rousset 1999). Wright's inbreeding coefficient (F_{IS}) was estimated using Weir and Cockerham method (Weir & Cockerham 1984) implemented in program FSTAT v 2.9 (Goudet 1995).

6.2 Population genetic structure

Population genetic structure was assessed for snow leopard sampling locations using Wright's F statistics using software GenAlEx v 6.5 (Peakall & Smouse 2006) with statistical significance tested using Weir and Cockerham estimator (Weir & Cockerham 1984). Alternative genetic differentiation estimators like G''_{ST} (Meirmans & Hedrick 2011) and D_{EST} (Jost 2008) were also calculated using GenAlEx v 6.5 which corrects for dependency of F_{ST} estimator on the amount of within population variation. Additionally, Analysis of molecular variance (AMOVA) was used to test the genetic variation between and or within the sampling sites using software ARLEQUIN v 3.5 (Excoffier et al., 2005).

The spatially non-explicit Bayesian clustering algorithm, STRUCTURE v 2.4 (Pritchard et al. 2000) was used to identify the number of genetic clusters (k). Analyses was carried out using population admixture model along with correlated allele frequencies within populations. Run parameters includes 500000 MCMC iterations after a burn-in period of 50000 replicates without (LOCPRIOR=0) and with location information (LOCPRIOR=1). Values of each K was allowed to vary between 1 to 20 independent runs. STRUCTURE HARVESTER v 0.6.94 (Earl and vonHoldt 2012) was used to infer the optimal K value by calculating the second order rate of change in the likelihood values of K values (ΔK) (Evanno et al., 2005).

Multivariate analysis are suggested as an alternative method to Bayesian clustering analysis and holds significance especially when the populations are deviating from HWE and are under LD due to presence of null alleles as they do not require any such population genetics assumptions. Discriminant analysis of principal components

(DAPC) was conducted using package Adegenet v 2.1.1 (Jombart 2008) in R ver. 3.0.1 (R Core Team, 2013) (Edwards 1971). Presence of Isolation by Distance (IBD) across study areas was also tested using Mantel test in GenAlEx v 6.5 (Peakall & Smouse 2006). Mantel test was conducted with 9,999 permutations to test for significance between the matrix of pairwise Edwards's genetic distance (Edwards 1971) and Euclidian geographic distance.

Finally, program BayesAss+ v 1.3 (Wilson & Rannala 2003) was used to trace an individual's immigrant ancestry over the last 5-6 generations. Analysis was run with a total run length of 9×10^7 MCMC iterations of which 3×10^7 MCMC iterations were discarded as burn-in phase with the MCMC phase being sampled at every 2,000 steps. Individual assignments and immigrant ancestries were calculated at a migration rate prior of 0.05 with all other settings kept at default values.

6.3 Bottleneck events

Wilcoxon's sign rank test and mode shift test implemented in program BOTTLENECK v 1.2 (Piry et al., 1999) was used to detect evidence of past genetic bottleneck events (if any) in the sampled populations and overall. Data was analysed with 100,000 simulations using mutation models: single step wise model (SMM) and two-phase model (TPM) with 0.95 and 0.12 generic values for frequency of single step mutations and variance respectively as recommended (Piry et al., 1999). Garza-Williamson index (G-W) or M ratio, was also estimated using Arlequin v 3.5 (Excoffier 2010).

7 Results

7.1 Species identification

Out of total 396 non-invasive and tissue samples from dead snow leopards, I could successfully isolate DNA from only 225 samples (56.8% success rate; Fig 2). From these, only 95 samples were identified as snow leopards based on mtDNA Cyt *b* region. The NCBI BLAST results confirmed the snow leopards with a similarity index of 99-100% matching with other snow leopard sequences available on Genbank (Fig 3 a, b, and c). The other species identified based on NCBI BLAST includes red fox, Himalayan wolf, dog, lynx, and pallas cat.

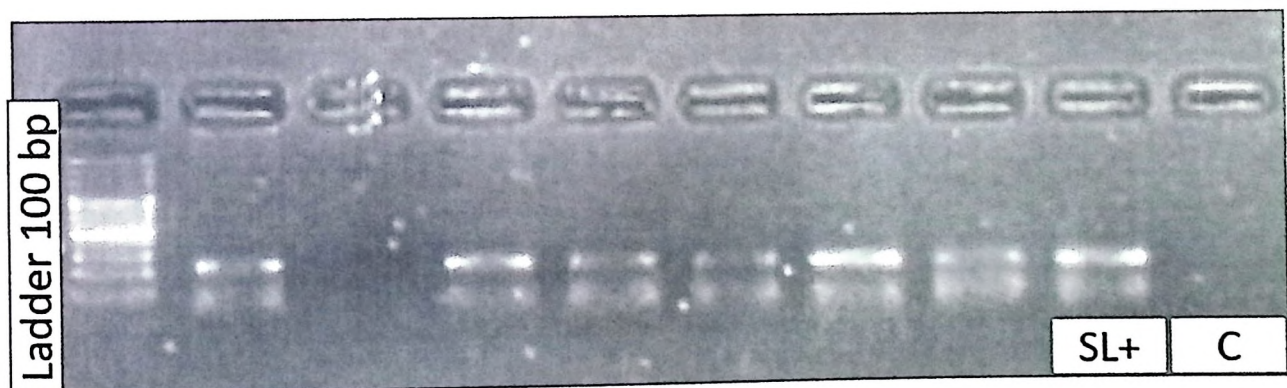


Fig 2: Agarose gel (2%) image showing the result of positive amplification of mtDNA Cyt *b* in carnivore samples with snow leopard samples used as positive control.

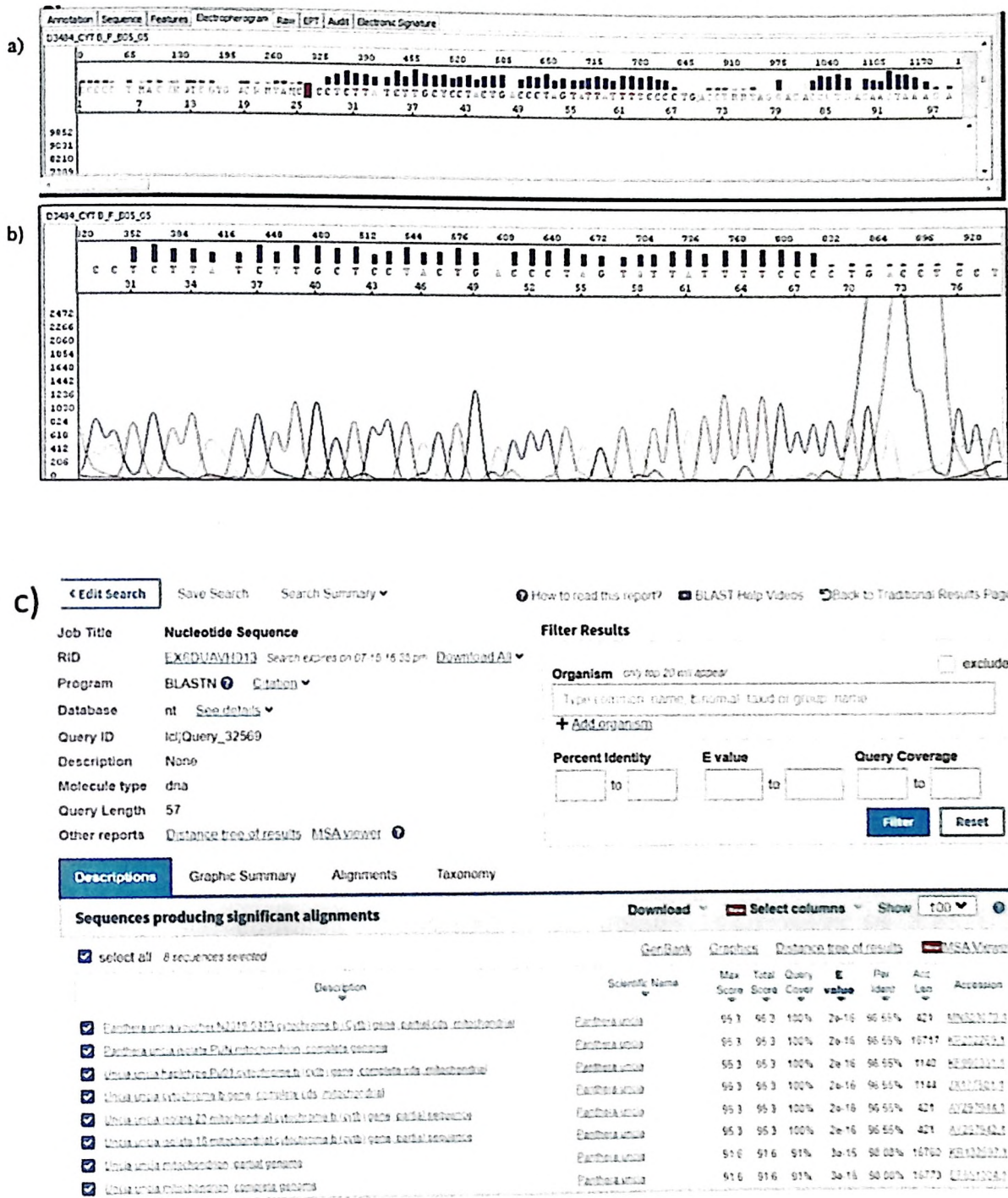


Fig 3: Sanger sequencing resultsa) mtDNA Cyt *b* nucleotide sequence of snow leopard sample, b)electropherogram of snow leopard sample and c)NCBI BLAST result of putative snow leopard sample showing 100 % similarity with other snow leopard sequence available on GenBank.

7.2 Individual identification and genetic diversity estimates

I attempted to amplify 16 microsatellites from the 95 positive snow leopard samples. Out of 95 confirmed snow leopard samples, 76 samples yielded microsatellite data at 10 loci with high amplification success with mean proportion of alleles typed at 97.5 %. Since the possibility of sampling the same individual arises while working with non-invasive samples (feces), the 76 samples were considered for Identity analysis and finally identified 56 individuals (feces=51, tissue=5) with 27 recaptures.

All the ten microsatellite loci were identified to be polymorphic with alleles range between three (Pun 82) to seven (Pun 229) with the 4.9 mean number of alleles observed overall (Fig 4, Table 2, Appendix I Fig 1). The PIC value of the microsatellite loci also varied from 0.460 (Pun 1293) to 0.837 (Pun 229) with a mean value of 0.622 (Table 2). The panel of ten microsatellite markers used for individual identification had cumulative probability of identity (P_{ID}) value of 3.8×10^{-9} and probability of identifying between full siblings ($P_{ID(sib)}$) value of 3.5×10^{-4} . The PIC, P_{ID} (Probability that two individuals will share the same genotypes), and $P_{ID(sib)}$ (probability that siblings will share the same genotype) values had sufficient power to discriminate between two siblings in a population of over hundred thousand snow leopards. Given the guesstimate population size of snow leopards in Ladakh (~ 200 individuals), the loci used in this study would more than suffice to determinate between individuals and siblings. (Table 2).

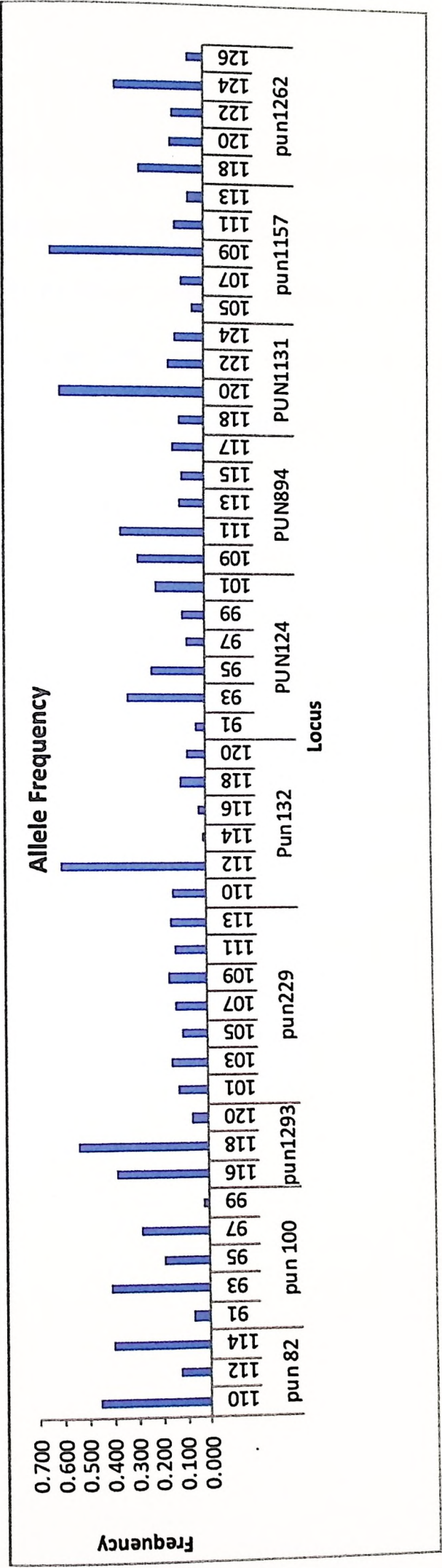


Fig 4: Plot showing the frequency of each allele at each microsatellite loci (n=10)

Genotyping errors rates varied among loci with Allelic dropout (ADO) (0-40%) and false allele rates (FA) (0-48%) but overall results indicated the presence of ADO and FA rates to be lower than 20% (Table 2). Presence of null alleles were detected at single loci (Pun 229) with no evidence of stuttering and small allele dominance (Table 2). Mean genetic diversity estimates for snow leopards including the Mean number of alleles (MNA), Allelic richness (AR), Observed heterozygosity (H_o), and Expected heterozygosity (H_e) values were 4.90 (1.28), 7.80 (2.53), 0.58 (0.07), and 0.67 (0.10) respectively (Table 3). Population wise genetic diversity estimates indicates all the sampling locations have similar values (Table 3). After sequential Bonferroni corrections, none of the loci deviated from the Hardy Weinberg Equilibrium (HWE), but detected Linkage disequilibrium (LD) across four pairs of loci combinations (Pun 82-Pun 229, Pun 100-Pun 229, Pun 100-Pun 894, Pun 1131-Pun 1262). The overall mean inbreeding coefficient value was 0.111 (Table 3).

Table 2: Loci wise (n=10) genetic diversity estimates of snow leopards from Ladakh, India.

Locus	K	N	Ho	He	PIC	P _(D Sib)	HWE	F (Null)	F _{IS}	ADO	FA
Pun 82	3	53	0.679	0.61	0.521	0.508	NS	-0.0571	-0.123	0.327	0.000
Pun 100	5	54	0.648	0.706	0.648	0.435	NS	0.0316	0.074	0.151	0.000
Pun 1293	3	55	0.564	0.556	0.46	0.547	NS	0.0208	-0.022	0.000	0.481
Pun 229	7	54	0.519	0.863	0.837	0.332	NS	0.2451	0.394	0.000	0.000
Pun 132	6	55	0.491	0.593	0.556	0.507	NS	0.0684	0.165	0.389	0.000
Pun 124	6	56	0.679	0.78	0.738	0.385	NS	0.0639	0.122	0.000	0.418
Pun 894	5	53	0.623	0.755	0.708	0.402	NS	0.0893	0.167	0.000	0.385
Pun 1131	4	55	0.545	0.582	0.537	0.516	NS	0.0124	0.055	0.407	0.000
Pun 1157	5	55	0.473	0.554	0.519	0.534	NS	0.0647	0.139	0.056	0.000
Pun 1262	5	55	0.636	0.748	0.701	0.406	NS	0.0718	0.142	0.259	0.000
Mean	4.9	56	0.585	0.674	0.622	3.53x10 ⁻⁴			0.111	0.159	0.128

K= Number of alleles, N=Sample size, Ho= Observed Heterozygosity, He=Expected Heterozygosity, PIC= polymorphic information content, P_(D Sib) = Probability of siblings, HWE= Hardy Weinberg Equilibrium (NS= non-significant), F (Null) = frequency of null allele F_{IS}= Inbreeding coefficient, ADO= Allelic Dropout, and FA= False alleles.

Table 3: Mean genetic diversity estimates across four sampling locations based on 10 microsatellites data.

Snow leopard Populations	Microsatellites					
	N	MNA	Ho	He	ARA	F_{IS}
Hemis-Leh	34	4.900	0.582	0.663	7.800	0.078
Kargil	09	4.300	0.673	0.687	7.200	-0.044
Changthang	07	3.900	0.488	0.703	6.600	0.242
Nubra	06	3.800	0.580	0.650	6.800	0.010
Overall mean (SD)	56	4.900 (1.287)	0.585 (0.077)	0.674 (0.109)	7.800 (2.573)	0.111 (0.021)

N=Number of samples, MNA=Mean no. of alleles, Ho=Observed heterozygosity, He= Expected heterozygosity, ARA=Allelic range, F_{IS} =Inbreeding coefficient.

7.3 Population genetic structure

The pairwise genetic differentiation values including Wright's F'_{ST} , Hedrick's G'_{ST} and Jost's D_{EST} were observed to be low and statistically non-significant between populations (Table 4). All the populations were found to exhibit low levels of genetic differentiation between them across all the three estimators (<0.03). The highest level of genetic differentiation was observed between Nubra-Kargil (0.030) and Nubra- Hemis-Leh (0.027). Further AMOVA test revealed the most observed variation was within individuals (85.97%) with significant F_{ST} ($p=0.01$), followed by Among individuals (13.02%) and among populations (1.02%) indicating high levels of gene flow between the sampling locations (Table 5).

Table 4: Pair wise genetic differentiation values between the four sampling locations.

	Hemis-Leh	Kargil	Changthang	Nubra
Hemis-Leh	-	0.017*/0.024*	0/0*	0.051*/0.075*
Kargil	0.024*	-	0.002*/0.003*	0.050*/0.072*
Changthang	0.013*	0*	-	0*/0*
Nubra	0.027*	0.030*	0.006*	-

F_{ST} values were shown below diagonal, whereas Hedrick's G'_{ST} and Jost's D_{EST} values were shown above diagonal. Non-significant values were denoted with * symbol.

Table 5: Results of analysis of molecular variance (AMOVA) comparing genetic variation using ten microsatellites across 4 sampling locations.

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variation
Among populations	3	10.435	0.026	1.02
Among individuals within populations	52	151.226	0.338	13.02
Within individuals	56	125.0	2.23	85.97

The Bayesian clustering analysis in STRUCTURE v 2.3 suggested the most probable number of genetic clusters based on delta K (ΔK) values to be two ($k=2$) for both models, one with no prior location (LOCPRIOR=0) and another with prior location information (LOCPRIOR =1) following criterion of Evanno method (Fig 5a, Fig 6a). No genetically distinct population was observed in both the no prior and prior location information models suggesting high levels of genetic admixture between the populations (Fig 5b, Fig 6b). DAPC plot also supported the results of Bayesian clustering STRUCTURE analysis where the genetic ellipses of samples from all the four populations were substantially overlapped without showing any clear evidence of distinct population (Fig 7a, 7b). Further, mantel test did not show any pattern of isolation by distance (IBD) with a non-significant and weak correlation value ($r=0.17$, $p=0.08$) between genetic distance and geographic distances (Fig 8). BayesAss analysis also suggested high amount of migration rates between the

sampling populations (Table 6). Mximum dispersal was observed from Hemis-Leh to Kargil (0.20, Standard deviation=0.08, Confidence interval=0-0.31) and from Changthang to Hemis-Leh (0.18, Standard deviation=0.07, Confidence interval=0.02-0.30).

Table 6: Contemporary migration rates in snow leopards identified by Bayes Ass+ between 4 sampling sites with standard deviation and 95% confidence intervals in brackets.

Recipient population	Host population			
	Kargil	Hemis-Leh	Changthang	Nubra
Kargil	0.72 (0.06, 0.66-0.90)	0.20 (0.08, 0.00-0.31)	0.04 (0.04, 0-0.18)	0.02 (0.02, 0-0.09)
Hemis-Leh	0.06 (0.05, 0-0.20)	0.73 (0.05, 0.66-0.87)	0.18 (0.07, 0.02-0.30)	0.01 (0.01, 0-0.07)
Changthang	0.04 (0.04, 0-0.14)	0.07 (0.07, 0-0.24)	0.85 (0.08, 0.67-0.98)	0.03 (0.03, 0-0.12)
Nubra	0.03 (0.04, 0-0.16)	0.12 (0.07, 0-0.28)	0.10 (0.07, 0-0.26)	0.72 (0.05, 0.66-0.86)

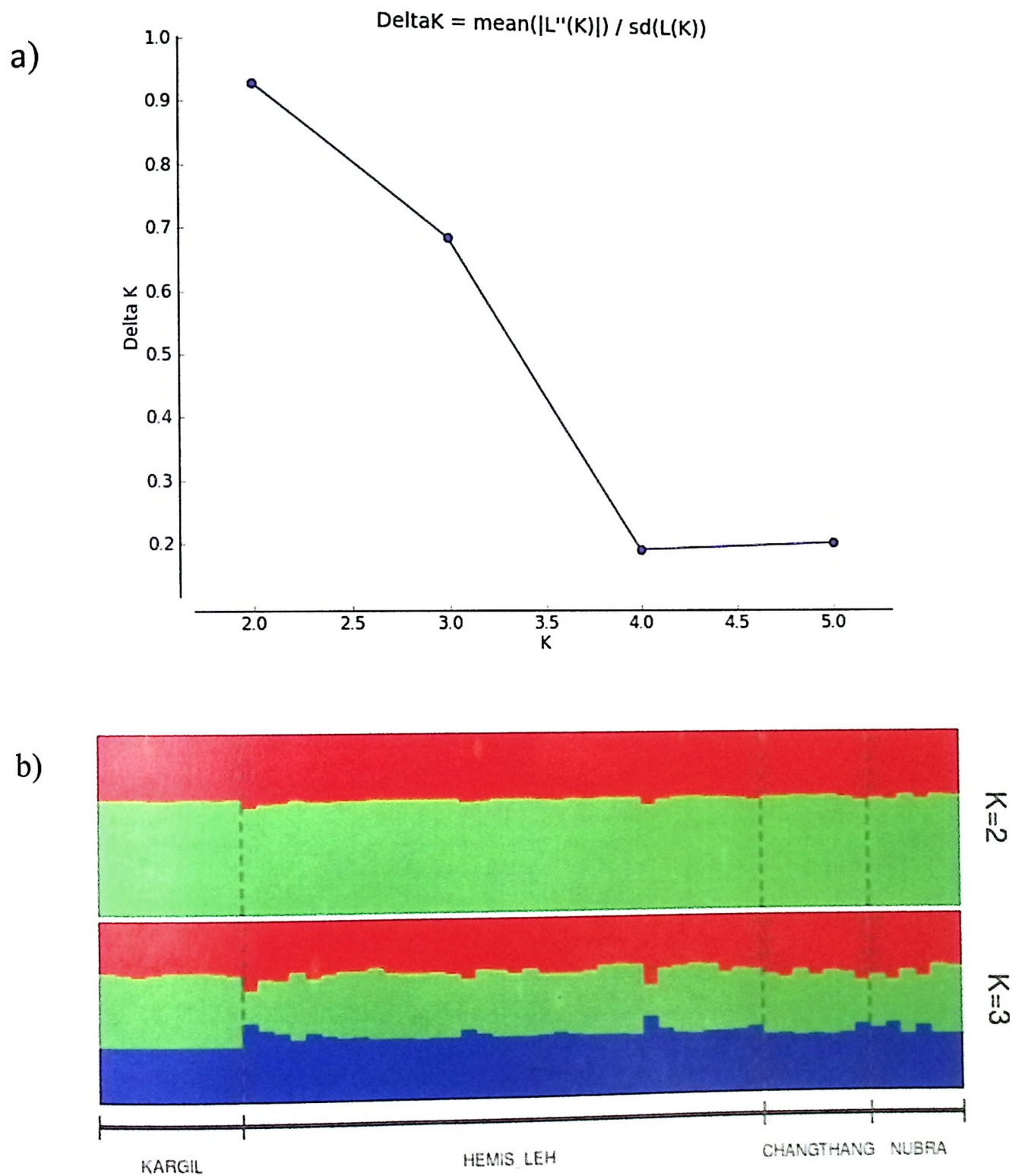


Fig 5: Results of Bayesian clustering analysis based on admixture model using no prior location model (LOCPRIOR=0). a) Delta k plot showing $k=2$ as best supported value, b) Summary bar plot of STRUCTURE runs at $k=2$ and 3 showing population assignments for each individual using no prior sampling location information.

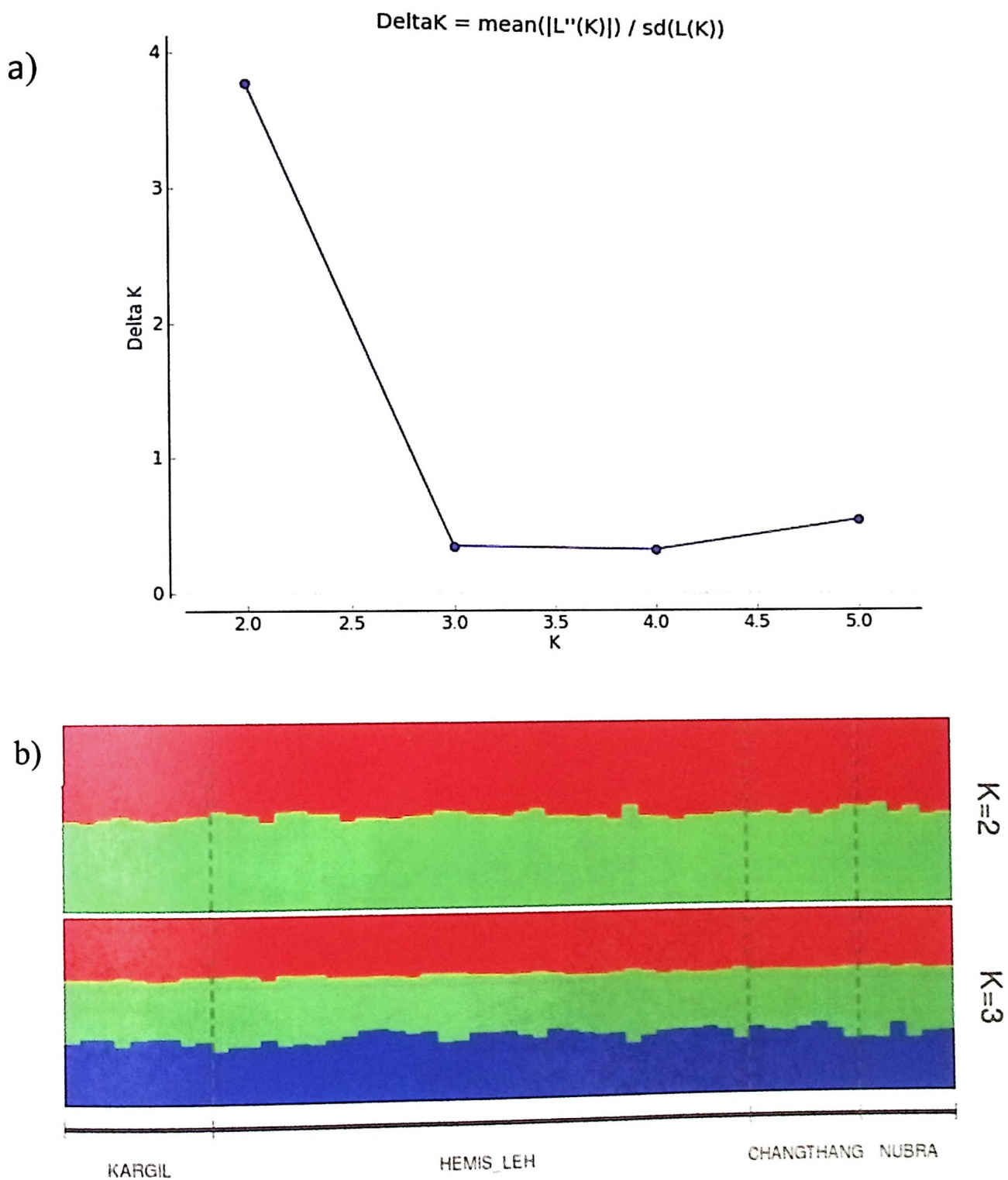


Fig 6: Results of Bayesian clustering analysis based on admixture model using prior location model (LOCPRIOR=1). a) Delta k plot showing $k=2$ as best supported value, b) Summary bar plot of STRUCTURE runs at $k=2$ and 3 showing population assignments for each individual using prior sampling location information.

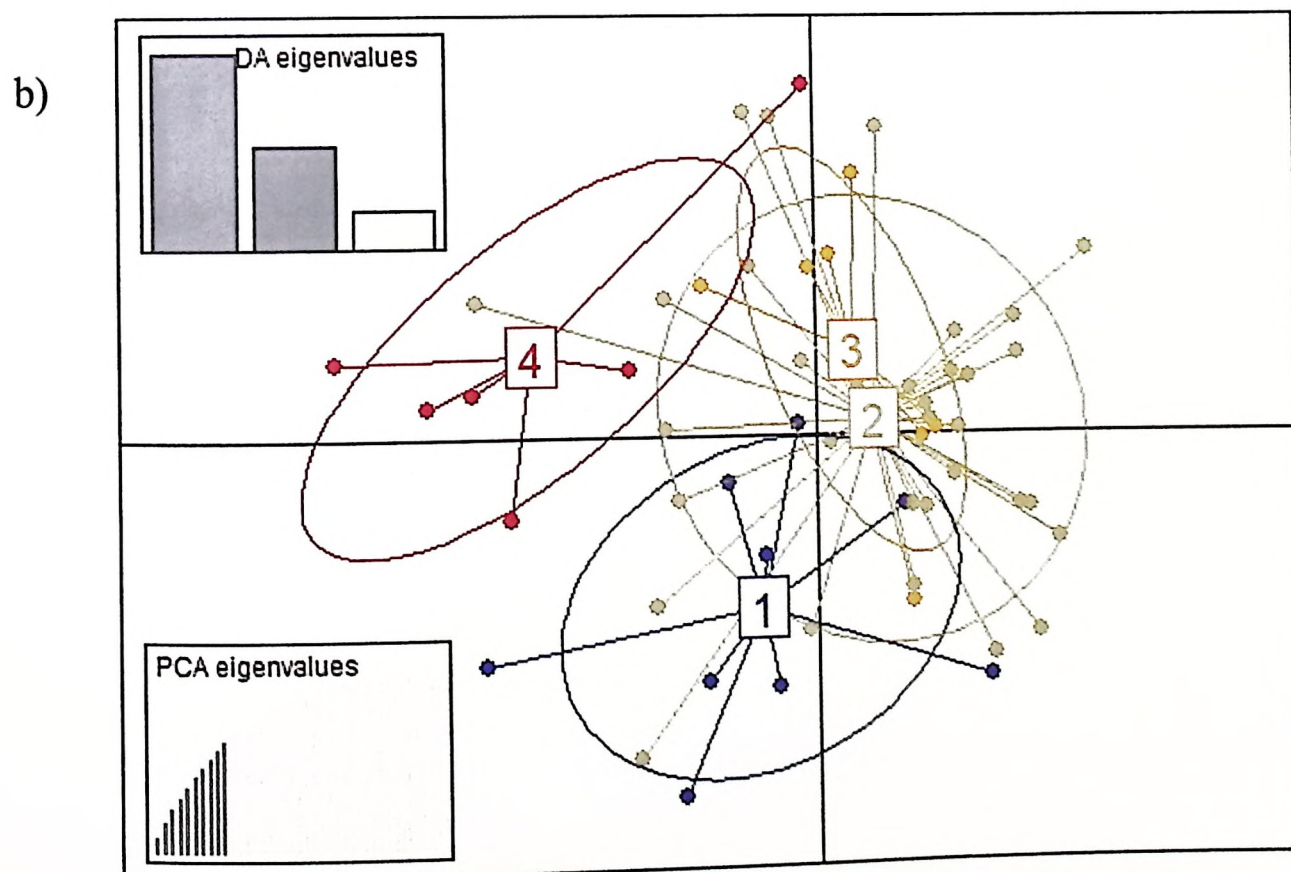
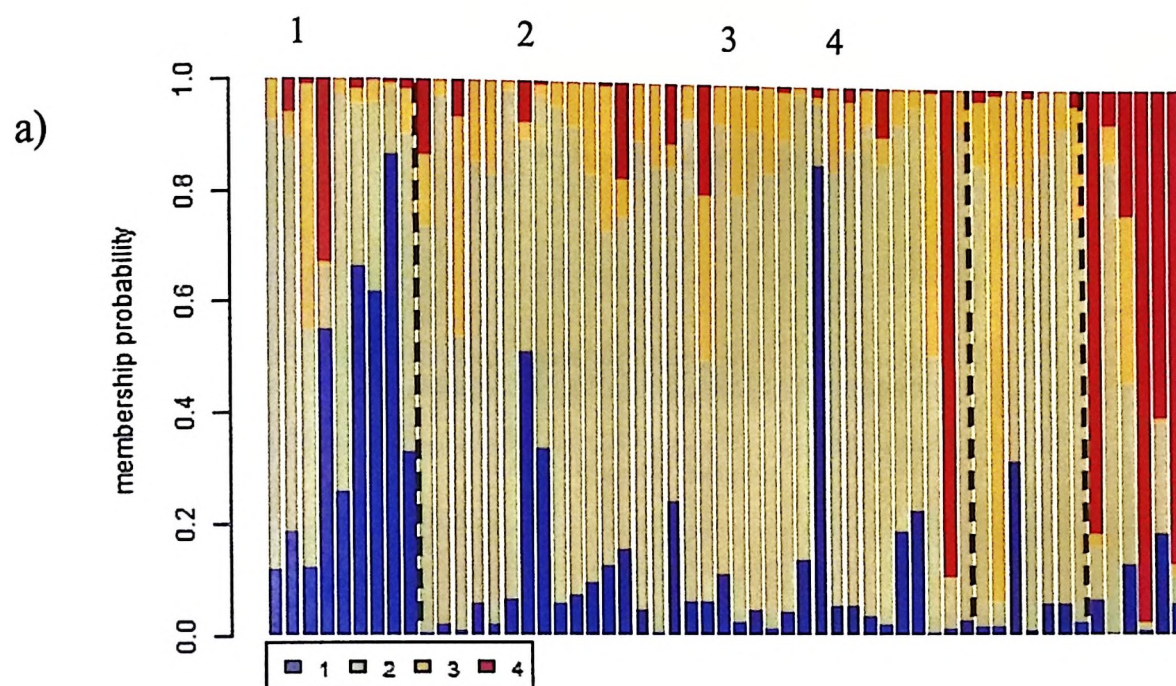


Fig 7: Compo plot (a) and scatterplot (b) of DAPC with each population indicated by different colour codes and inertia ellipses shown on first two axes(1=Kargil; 2=Hemis-Leh; 3=Changthang and 4=Nubra)).

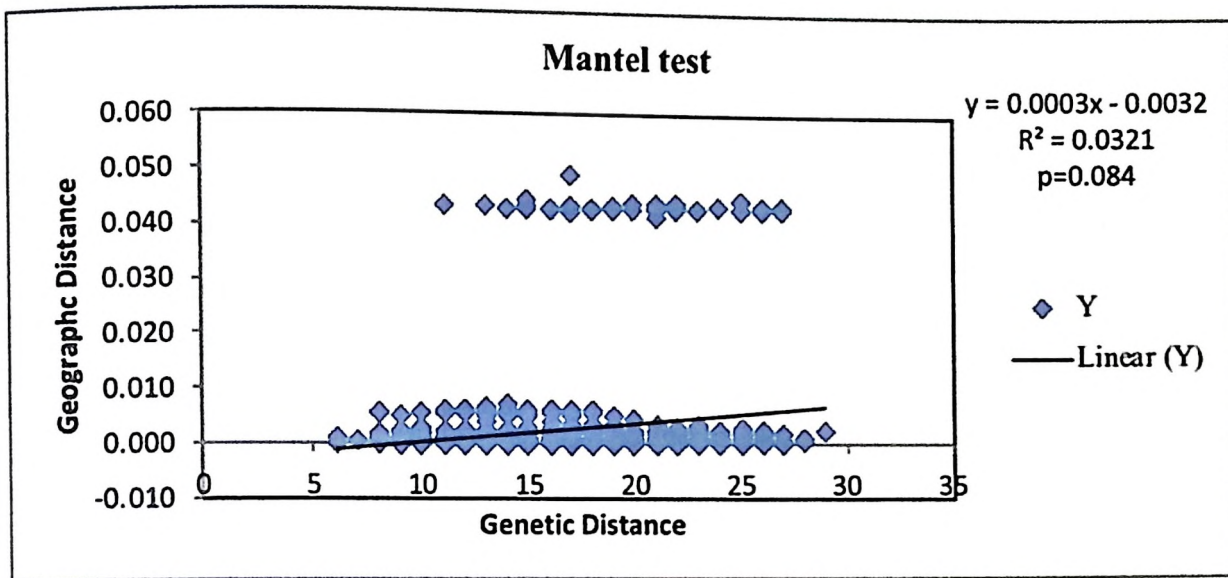


Fig 8: Image showing results of Mantel test plotted between genetic distance and geographic distance matrices for testing Isolation by Distance.

7.4 Bottleneck results

Bottleneck analysis using Wilcoxon sign rank test identified no significant bottleneck events observed using both the SMM and TPM microsatellite mutation models (Table 7). Allele frequency distribution tests on the other hand showed shifted mode curve indicative of genetic bottleneck events in the recent past. Similarly, the M-ratio or G-W index values for overall and population wise showed values less than 0.68 indicative of genetic bottleneck (Table 7).

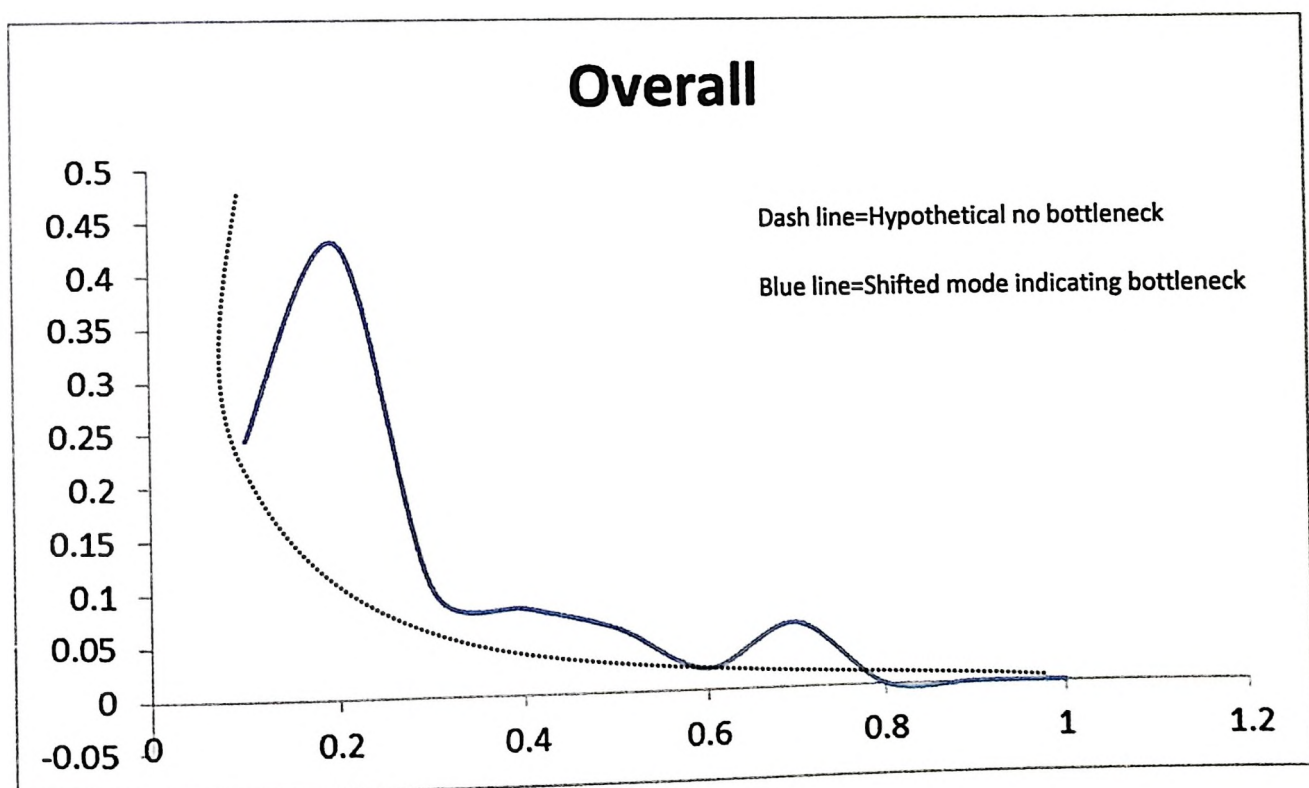


Fig 9: Bottleneck results using microsatellites showing shifted mode curve pattern.

Table 7: Summary of bottleneck analyses results.

Population	Mutation model	Sign test	Wilcoxon test H deficiency/ h excess/ h excess or deficiency	Mode shift	G-W index (M ratio)
Hemis-Leh	TPM	P=0.37	P=0.81/0.21/0.43	Shifted mode	0.56 (0.02)
	SMM	P=0.37	P=0.78/0.24/0.49		
Kargil	TPM	P=0.15	P=0.86/0.16/0.32	Shifted mode	0.53 (0.09)
	SMM	P=0.37	P=0.83/0.18/0.37		
Changthang	TPM	P=0.05	P=0.98/0.04/0.08	Shifted mode	0.53 (0.08)
	SMM	P=0.04	P=0.98/0.04/0.08		
Nubra	TPM	P=0.10	P=0.05/0.95/0.10	Shifted mode	0.56 (0.02)
	SMM	P=0.12	P=0.05/0.95/0.10		
Overall	TPM	P=0.15	P=0.91/0.96/0.19	Shifted mode	0.56
	SMM	P=0.36	P=0.81/0.21/0.43		

TPM = two phase mutation model, SMM = stepwise mutation model, and G-W index=Garza Williamson index (standard deviation)

7.5 Sex identification

Sex identification using PCR amplification revealed that out of 25 snow leopard samples, thirteen were identified as males (54.2%) and eleven as females (45.8%).

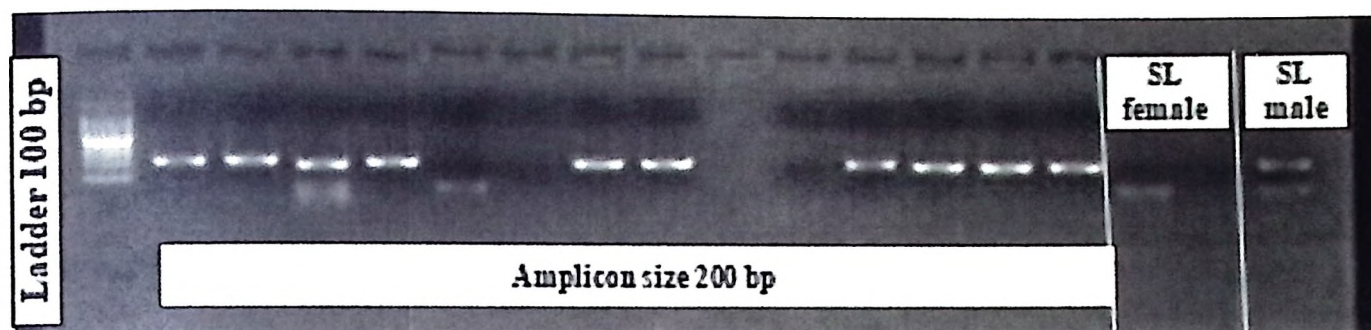


Fig 10: Agarosegel picture (2%) highlighting the males with PCR amplification (200 bp) in the AMELY gene and females with no amplification.

8 DISCUSSION

The present study illustrates the general strengths and challenges in using non-invasive genetic samples to assess the population status of snow leopards in Ladakh. Out of the 391 scat samples collected for my study, 95 were identified snow leopard scat. A large proportion of the samples collected were misidentified in the field as snow leopard scat or were too degraded for genetic analysis. 45 scat samples were identified which belonged to other species likely to be canids, red fox, Pallas cat, and lynx. The misidentification of carnivore scats in the field is a problem frequently encountered and has been addressed in many studies (Janecka et al., 2008, Murphy et al., 1999, Farrel et al., 2000). The improper storage of scat samples could be the reason for the less proportion of snow leopard than the previous studies. Which there was no amplification in the PCR using carnivore specific primer designed for identification of carnivores for 166 samples. These scats were probably of species apart from snow leopard or the DNA yield was not appropriate for genetic analysis. The presence of DNA was verified by electrophoresis on 0.8% agarose gel.

In most mammalian species the sex ration is biased towards females (Caughley 1977). Male biased sex ratio is rare but observed in snow leopards (Aruge et al., 2019, Shrestha and Kindalmanm 2020). The possible reasons could be the ease of their hunting due to their attachment with cubs, which makes it difficult for them to escape when hunted. Post- parturition vulnerability of females to different mortality factors increase manifold due to increased ecological and energetic cost of parturition and lactation. In this study, the sex ratio though biased towards males was

statistically not different from parity with 13 males and 11 females (from 24 snow leopard individuals).

Snow leopards in general have low genetic diversity compared to other species in the same genus. This is suggestive of a major historical bottleneck for the species. The microsatellite markers are usually effective in detecting genetic bottlenecks in the recent history of the species while Mt-DNA is a better marker for detecting ancient demographic events. Further analysis using full genome or Mt-DNA markers will shed light on the ancient events that shaped the current low diversity observed in the species. In the current study using microsatellites has shown low genetic variation ($H_o = 0.585$) as compared to expected heterozygosity ($H_e = 0.674$). Low heterozygosity has been reported in snow leopards previously in Nepal by Karmacharya et al., 2011 and Shrestha and Kindermann et al., 2020, in Pakistan by Aruge et al., 2019 and within global range Janecka et al., 2008, 2011, 2017. The snow leopard population of Ladakh has similar genetic diversity as recorded from a global analysis for the species (Janecka et al., 2017).

The ability of the 10 marker panel to distinguish between individual siblings ($P_{ID\ sib}$) value of this study is was 3.53×10^{-4} which was more than that reported by Janecka et al., 2017 ($P_{ID\ sib} 9.7 \times 10^{-3}$) that used a panel of 6-microsatellite marker. My data show high gene flow between all regional populations in my study area. However, the pairwise differentiation of regional populations showed that there was comparatively low gene flow between Nubra and Kargil population, which were geographically the most distant.

9 CONCLUSION

Snow leopards inhabit the high mountains of Central and South Asia and are found in 12 countries including India. They have an extremely patchy and fragmented distribution throughout their range with low density of less than one adult per 100 km² (Mallon d 1998; McCarthy & Chapron, 2003; Muratov, 2004). The snow leopard population in Ladakh ranges from 100 to 200 individuals (Mallon 1984), which is relatively high for a habitat where climate and altitudinal factors contribute to the barrenness and low productivity of the region. Due to resource limitation snow leopards ranges are large and density is low this poses a conservation problem as vast areas are required to hold a viable population. However, Ladakh is home to a large population of snow leopard compared to other parts of India and as per my study, forms a contagious single population. The populations of Ladakh are highly admixed with no genetic differentiation between all four regional populations. This suggests that existing natural barriers of high altitude peaks and large waterbodies have not hampered gene flow, but with increased anthropogenic activities and habitat fragmentation, we cannot be certain that genetic linkages will remain between these regional populations. A large diverse genepool allows species to avert extinctions by allowing them to adept to changes in climate and the environment (Frankham 2002). While small, fragmented populations have higher probability of extinctions due to demographic and environmental stochasticity (Soule 1985) and are less capable of adapting to a changing environment. Therefore, the understanding genetic status of snow leopards will help to better manage and conserve the species. Conserving the snow leopard in will in turn protect the whole ecosystem as well as the cultural

values associated with the landscape. My thesis shows the relevance and importance of considering within species genetic diversity and gene flow to conserve iconic species of outstanding universal value of the natural heritage site. It advises heritage site managers to use molecular tools for evaluating and monitoring this value.

Also, Some of the UNESCO world heritage sites such as Golden Mountains of Altai (Russian federation) (Bekut and Knapp 2012), Kanchendzonga National Park (India), Sagarmata National Park (Baral et al., 2017), Nandadevi and Valley of flowers National Park (Pananjay et al., 2011) and many other sites hold good number of snow leopards which are listed as outstanding universal value. Evaluating the status of the elusive snow leopard requires innovative approaches; my study shows the potential of using genetic mark-recapture to estimate populations at landscape scales. This study forms the baseline for future monitoring in the advent of accelerated anthropogenic development of the region. Motorways, army installations, intensification of agriculture are on the increase in this remote region. These developmental activities may pose insurmountable barriers to gene flow and alter structure in the future. To prevent fragmentation of snow leopard populations as well as those of other species mitigation measures should be implemented to promote green infrastructure. My study highlights the intactness of the Trans Himalayan ecosystems that needs to be maintained while planning the development of the region to ensure the legacy of our natural heritage for future generations.

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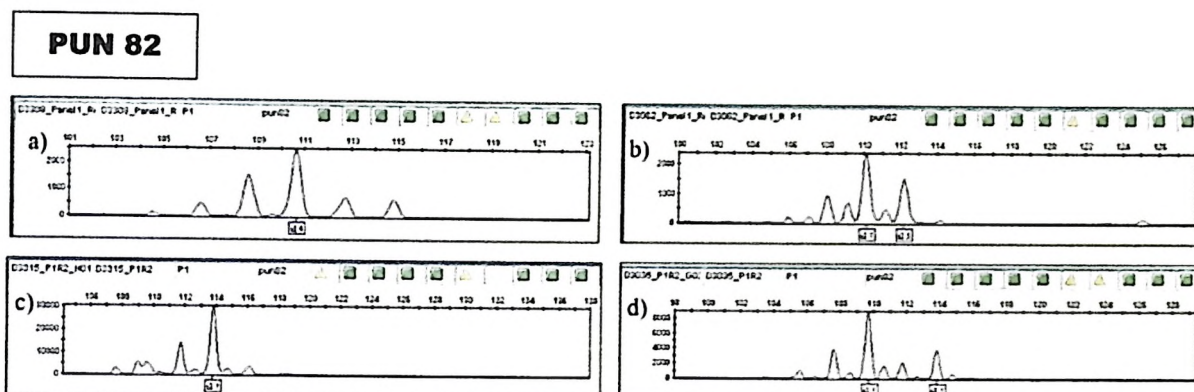
Appendix I

Table S1: Details of microsatellite markers used in the study

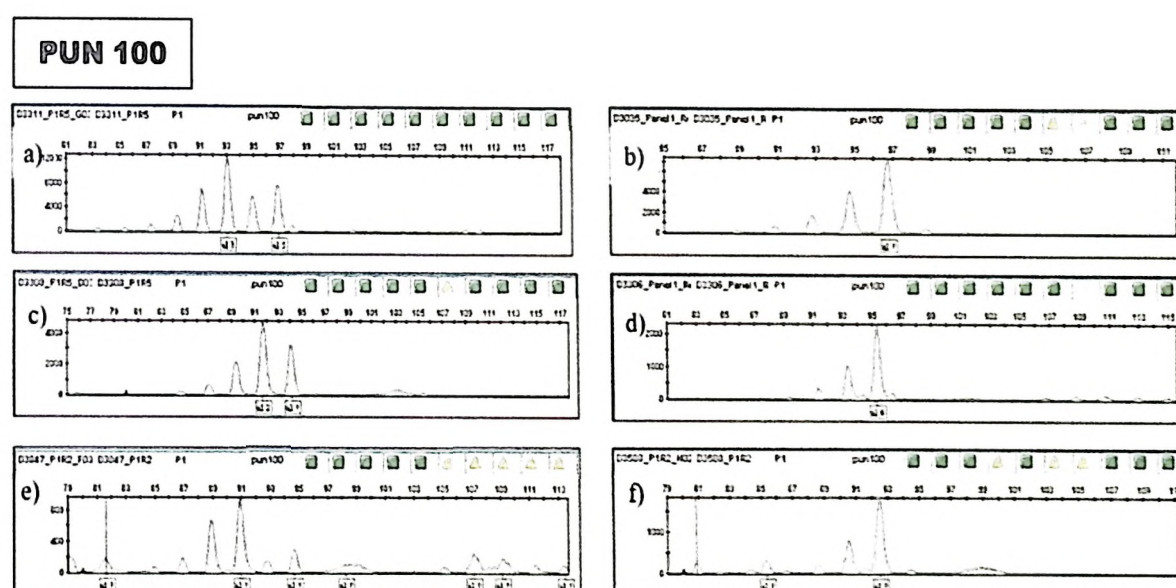
Loci	Primer sequence	Dye	Repeat	Allele size	Reference
PUN82	F: CCGCTTAAGAAAGAGGCTAAAAA R: TCAAAGAGTGTTGTTAGATAATATGGA	FAM	Di (2)	110–115	Janecka et al., (2008)
PUN100	F: GATTGTGAACCCCAAGAAAAAGA R: ATCCCTCTAGACATCTTAGTCACC	VIC	Di (2)	88-96	Janecka et al., (2008)
PUN124	F: CCATTCCCTCCCTGTCTGTA R: TGTCTCTCAAACCATAGACAGTTTC	PET	Di (2)	90-100	Janecka et al., (2008)
PUN225	F: CTGAGGCCAGCAATCATTC R: CCCACAGAACTTCCTGCTT	NED	Di (2)	177-183	Janecka et al., (2008)
PUN132	F: CGAAATGCAGTAATGTTAGTTTACA R: CACGGGTCGTCTCTTTTG	FAM	Di (2)	113-123	Janecka et al., (2008)
PUN229	F: AGACAAACTGACAAGCTTAGAGG R: TCATGTCTTTACATTCATTCTTTT	VIC	Di (2)	103-113	Janecka et al., (2008)

PUN327	F: TTAATTTGGGGAATGTTAGGG R: TGCAACATCTATCAGTGAAATAACA	PET	Di (2)	79-91	Janecka et al., 2014b
PUN1283	F: TCATCACCAACCTTGCAATT R:ACCAAGGATGTCTGCGTCTT	NED	Di (2)	125-129	Janecka et al., 2014b
PUN1293	F: GCACCCCTTCTTCTTGTAGTG R:AGAACTCACAGGGGCACAAT	FAM	Di (2)	116-118	Janecka et al., 2014b
PUN272	F: CACCTTTGCATCCAATAAATTC R:AACTACCTTTACCTCCTTCCAAA	VIC	Di (2)	123-129	Janecka et al., 2014b
PUN834	F: AAACACGTGCGCATGTAGAC R:TGGAAAGTGTGTTTGACAGC	PET	Di (2)	109-113	Janecka et al., 2014b
PUN894	F: CATGCCAGACTGCATTTGTT R:CCCACACATGACAATCCTGTT	NED	Di (2)	110-118	Janecka et al., 2014b
PUN1131	F: TGGACAAATAGACCCAAGACTG R:TAACCCCATTTGATGCTGTT	FAM	Di (2)	118-124	Janecka et al., 2014b
PUN1138	F: TTGAAAAGCAAGACACTAATACATTTC R:TGAGATATTTCGTGATAGCTTGTTT	VIC	Di (2)	114-124	Janecka et al., 2014b

Fig S1: Graph showing the allelic profiles of each microsatellite markers.

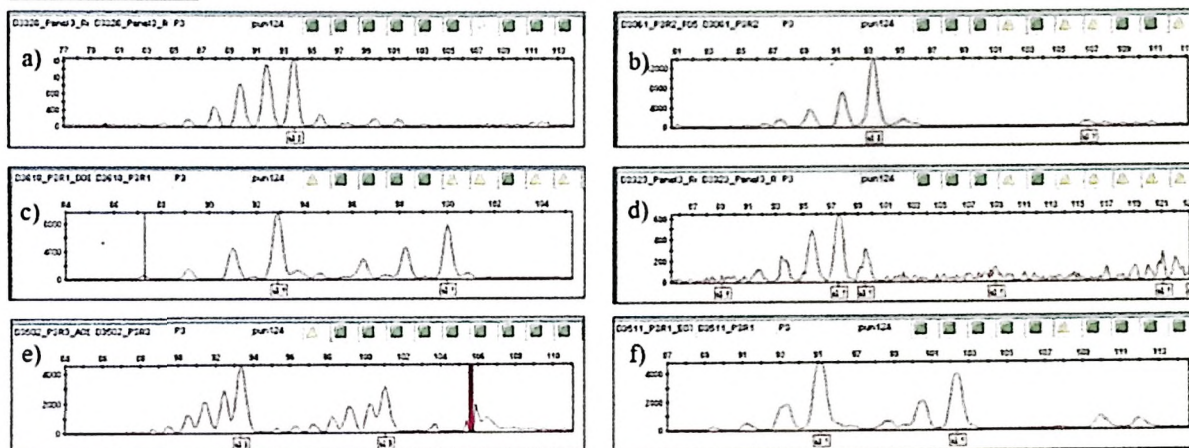


Allele scores: a)110; b)110-112; c)114 and d)110-114.



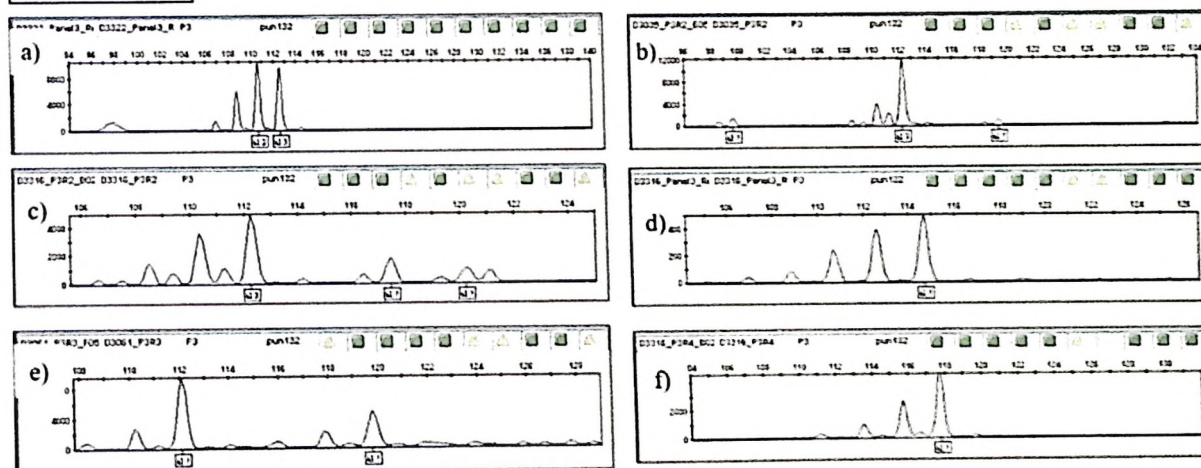
Allele scores: a)93-97; b)97; c)91-93; d)95; e)91 and f)93.

PUN 124



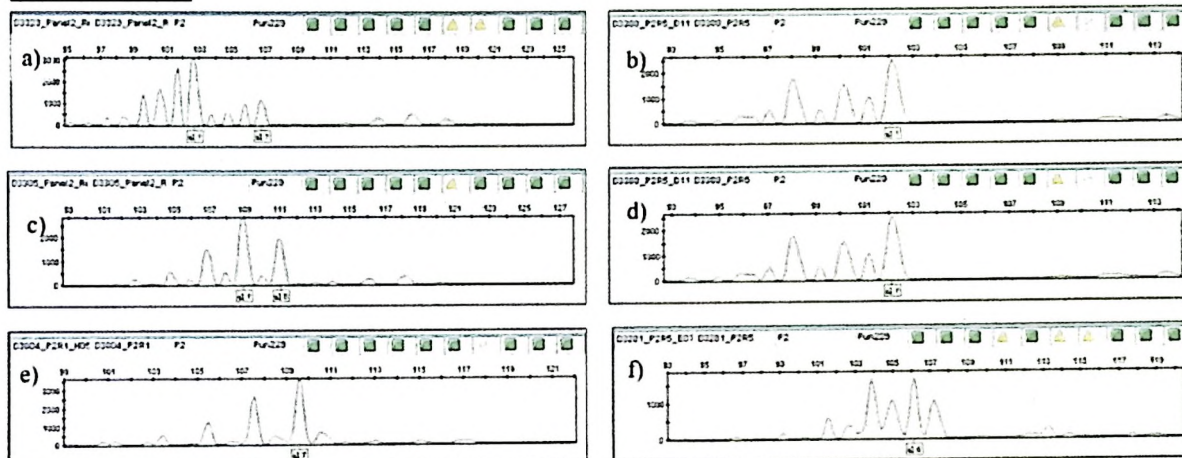
Allele scores: a)93; b)93; c)93-99; d)97 e)93-101 and f)95-101.

PUN 132



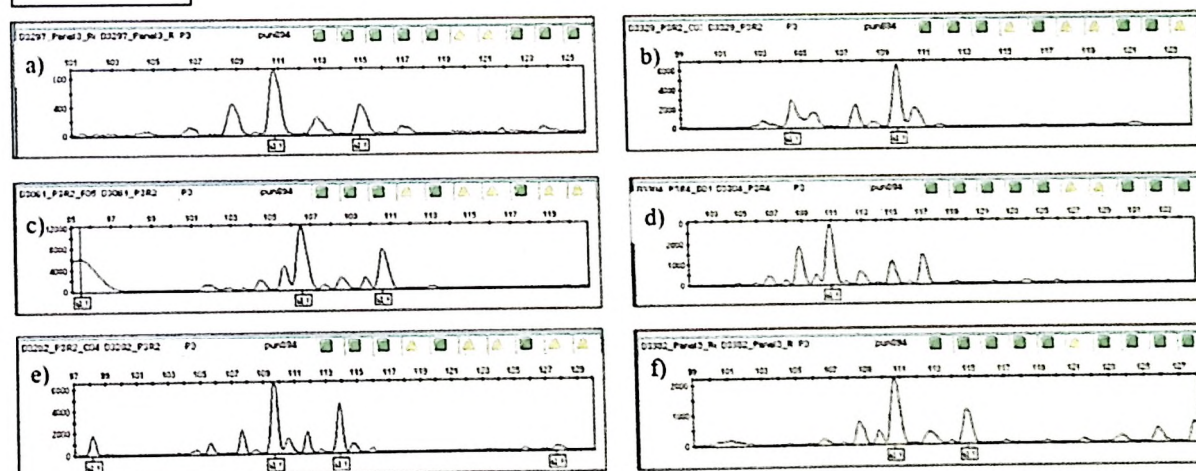
Allele scores: a)110-112; b)112; c)112-120; d)114; e)112-120 and f)118.

PUN 229



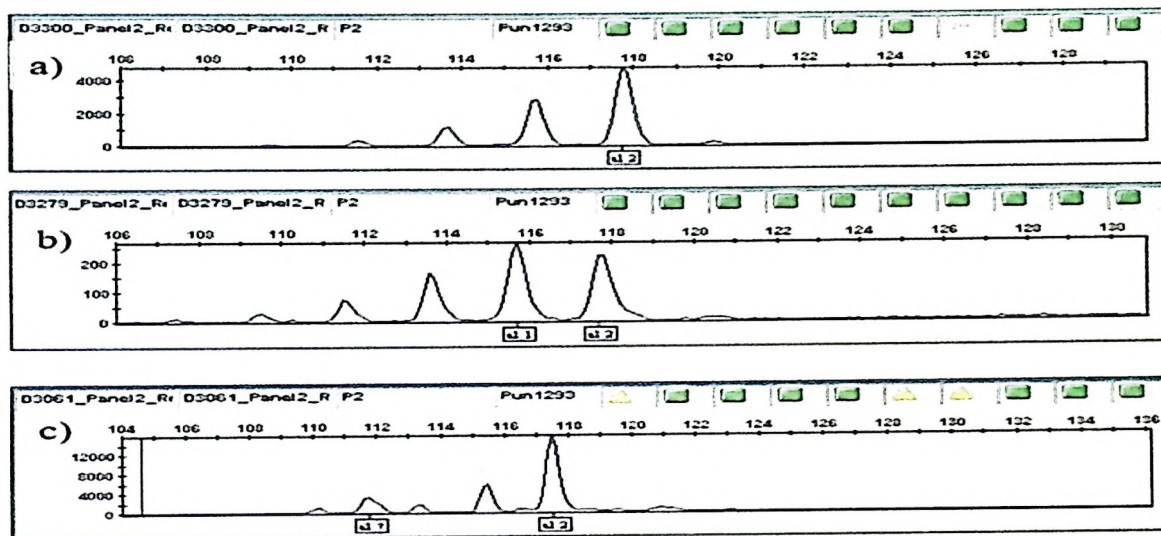
Allele scores: a)103-107; b)103; c)109-111; d)103; e)109 and f)103-105.

PUN 894



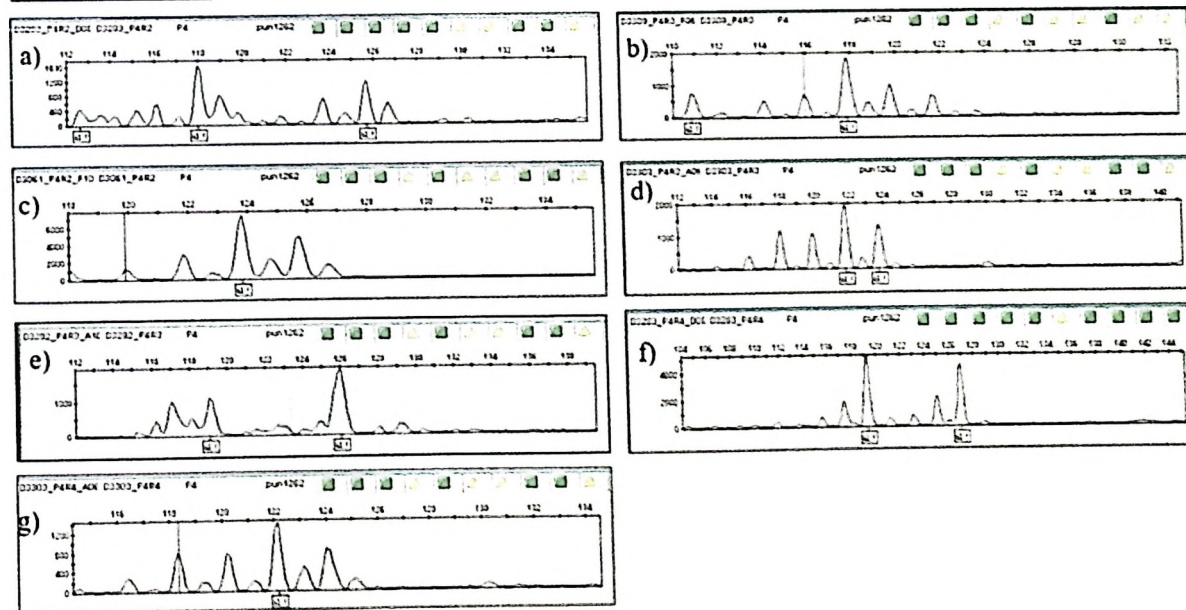
Allele scores: a)110-114; b)110; c)107-111; d)111-117; e)109-113 and f)111-115.

PUN 1293



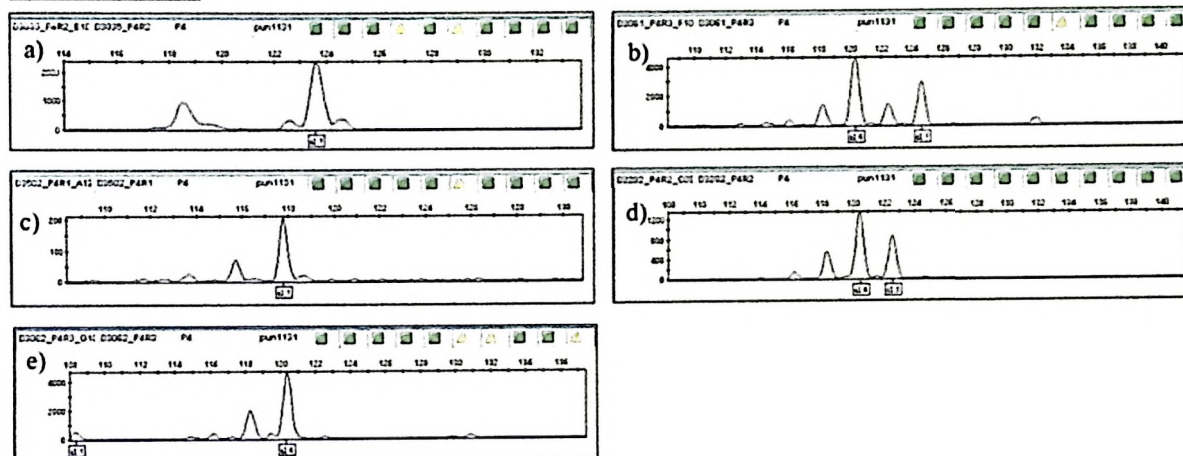
Allele scores: a)118; b)116-118 and c)118.

PUN 1262



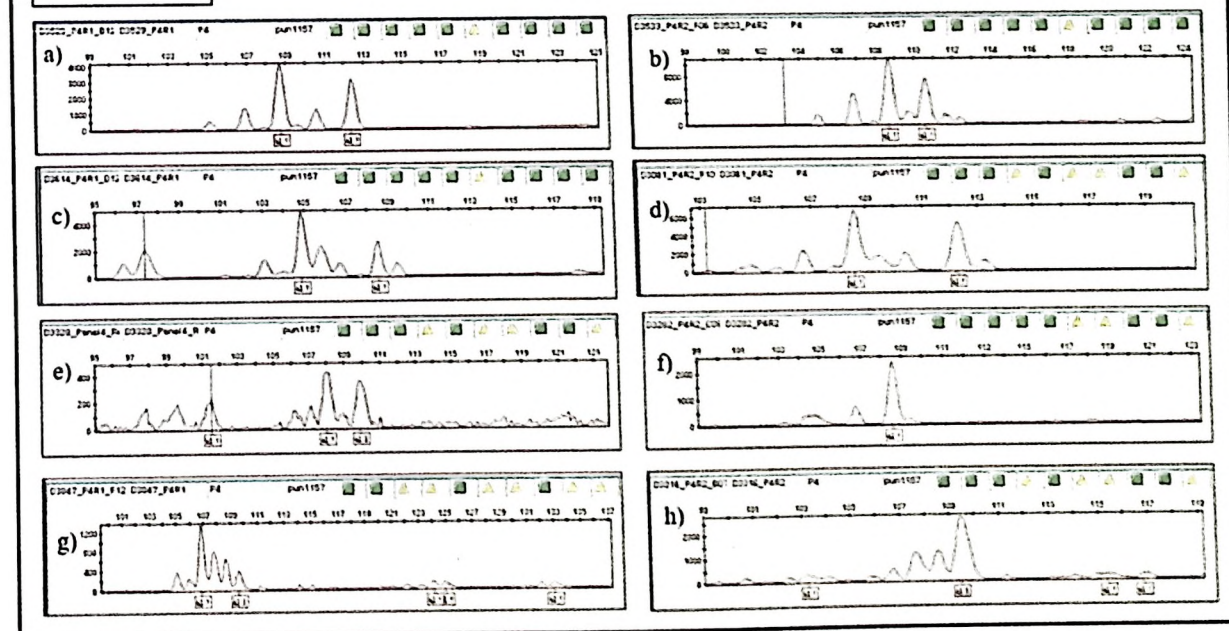
Allele scores: a)118-126; b)118-120; c)124; d)122-124; e)126; f)120-126 and g)122.

PUN 1131



Allele scores: a)124; b)120-124; c)118; d)120-122 and e)120.

PUN 1157



Allele scores: a)109-113; b)109-111; c)105-109; d)109-111; e)107-109; f)109; g)107 and h)109.