



**Phylogeography and population genetics of  
leopards (*Panthera pardus fusca*) in India**

**Thesis submitted for the award of the degree of**

**Doctor of Philosophy  
in  
WILDLIFE SCIENCE**

**by**

**SUPRIYA BHATT**

**to**

**Saurashtra University  
Rajkot-360005 (Gujarat)**

**Under the Supervision of**

**Dr. Samrat Mondol, Scientist-F**



**भारतीय वन्यजीव संस्थान  
Wildlife Institute of India**

**July 2024**



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**Citation:**

Bhatt, S. (2024). Phylogeography and population genetics of leopards (*Panthera pardus fusca*) in India. Ph.D. Thesis. Wildlife Institute of India, Dehradun, India, and Saurashtra University, Rajkot, India. Pp- 1-117.



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## DECLARATION

I hereby declare that the work conducted under the thesis entitled “**Phylogeography and population genetics of leopards (*Panthera pardus fusca*) in India**”, is a record of original and independent research work done by me and subsequently submitted for the award of the degree of **Doctor of Philosophy in Wildlife Science** to the **Saurashtra University, Rajkot (Gujarat)**. This research work has been carried out under the guidance and supervision of Dr. Samrat Mondol, Scientist-F, and co-supervision of Dr. Bivash Pandav, Scientist-G of Wildlife Institute of India, Dehradun. The work has not formed the basis for the award of any other degree, diploma, or any other qualification. I also declare that the thesis embodies my own work, analysis, observation, understanding, and the particulars given in it are true to the best of my knowledge.

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## CERTIFICATE

This is to certify that the thesis by **Ms. Supriya Bhatt** entitled “**Phylogeography and population genetics of leopards (*Panthera pardus fusca*) in India**” is an original and independent research work submitted to the **Saurashtra University, Rajkot (Gujarat)**, for the award of the degree of **Doctor of Philosophy in Wildlife Science**.

**Ms. Supriya Bhatt** has put more than six semesters of research work embodied in this thesis under my guidance and supervision. The work presented in this thesis has not been submitted to any other University or Institute for the award of any degree, diploma or distinction.

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I certify that the research work was appreciated by all who were present, and the comments made by the faculty and researchers have been appropriately included in the thesis.

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## **Acknowledgment**

*“All our dreams can come true if we dare to pursue them and have supportive persons around you”*. Not one there are many to whom I am deeply grateful to all individuals who have supported me throughout my PhD journey and take this chance to thank them all.

First and foremost, I would like to thank the Director and Dean of WII for providing me the opportunity to carry out my doctoral work at WII. I extend my sincere thanks to the Research Coordinator and the Nodal Officer, Wildlife Forensics & Conservation Genetics Cell (WFCGC) for their invaluable guidance and assistance in various aspects of my PhD work. I would like to thank Dr. V.P. Uniyal, Dr. Samrat Mondol, and Mr. M.M. Uniyal for their help in making the administrative work with Saurashtra University more conducive. I extend my thanks to Mr. Madhanraj for his timely help with the sequencing and genotyping facility. I am also deeply grateful to the Uttarakhand Forest Department, Uttar Pradesh Forest Department, and Bihar Forest Department for providing me with the necessary research permits for sampling. I am grateful to my field assistants Imam, Bhura, Ammi, Ranjhu, and Annu for their assistance in data collection.

I extend my deepest gratitude to both of my supervisors Dr. Samrat Mondol (Scientist-F) and Dr. Bivash Pandav (Scientist-G), for their expert guidance and invaluable support throughout my research journey. I especially thank Dr. Samrat Mondol for his patience in teaching me the fundamentals of conservation genetics, help in devising the PhD proposal, and for his insightful contributions at every stage of my PhD work. I am deeply grateful to him for arranging the lab logistics, research funding, and student support throughout the PhD duration, enabling the smooth execution of this

work. I am equally grateful to Dr. Bivash Pandav for facilitating research permissions and guiding the field surveys and field logistics. My sincere appreciation goes to Dr. Vishnupriya Kolipakam (Scientist D) for her help with the analyses. I extend my thanks to my external reviewers Dr. Jahanavi Joshi and Dr. Dipankar Ghose for their invaluable input in my thesis.

I would like to acknowledge all the *Meercat* lab members for their valuable comments especially Shrewshree, Ankit, and Sohini as well as my seniors Dr. Anant and Dr. Sitendu. I am extremely thankful to Dr. Suvankar Biswas for his unwavering support and partnership in both lab and fieldwork throughout my PhD work. I extend my thanks to Mr. Harshvardhan, Dr. Shiv, Dr. Debanjan, Ms. Anubhuti, and dissertation students Chayan and Zenab for their help in various aspects of field and lab work.

I am indebted to my friends cum labmates, Dr. Shrushti, Dr. Shiv, Dr. Shrutarshi, Dr. Tista, Dr. Suvankar, and Dr. Rahul for being strong pillars of my life, and their regular scientific inputs have made this work more meaningful. I am thankful to Ms Ankita, Dr. Shrushti, Dr. Shiv for being the constant person to hold me strong. I am thankful to my friends Dr. Meghna, Dr. Dibyadeep, Dr. Bheem, Dr. Mirza, Dr. Surya, Ms. Sarbjeet, Dr. Swapnali, Dr. Urjit for their constant support throughout the journey.

I acknowledge all the teachers who have guided me during my academic journey. I sincerely thank my teacher Dr. K.L.Bisht (Professor), HOD Biotechnology (former), PG College Kotdwar for providing me the opportunity to experience academia as guest faculty. I also like to thank the funding resources i.e. DST and Panthera. I also thank Dr. Vandana Singh, Scientist-F (DST WOS-A scheme) for her timely support for the smooth continuation of this grant.

Finally, I am eternally grateful to my family for their unconditional love, patience, and support. I would like to thank my mother, Sushma Bhatt, my father, Harish Chandra Bhatt, and my siblings Ankita, Jyoti, and Suraj. I especially thank Ankita who always supported me through constant support and motivation throughout my PhD. I also like to thank my mother-in-law, Savitri Jadli, and Father-in-law, Capt. Rajendra Prasad Jadli and family for their constant support and motivation. Their belief in my abilities and constant encouragement have been my driving force. I would like to thank my better half and my childhood friend, Surendra Jadli for his understanding, patience, and enduring support throughout this journey. I would like to thank my child, Adhyansh, for being a constant source of joy and stress relief with his smiles, making this journey truly wonderful.

Lastly, I acknowledge the Almighty for blessing me with a wonderful life, surrounding me with incredible people, and giving me the strength and determination to carry out and complete this work.

## List of publications:

Biswas S, **Bhatt S**, Paul S, Modi S, Ghosh T, Habib B, Nigam P, Talukdar G, Pandav B, Mondol S. 2019. A practice faeces collection protocol for multidisciplinary research in wildlife science. *Current Science* 116:1878-1885.

**Bhatt S**, Biswas S, Karanth K, Pandav B, Mondol S. 2020. Genetic analyses reveal population structure and recent decline in leopards (*Panthera pardus fusca*) across the Indian subcontinent. *PeerJ*, 8, p.e8482.

## List of conferences:

### *International/National conferences*

**Bhatt S**, Biswas S, Pandav B, Mondol S. 2019. Genetic analyses reveal population structure and recent decline in Indian leopards (*Panthera pardus fusca*) at ATBC-AP 2019 Sri Lanka, 11<sup>th</sup> to 13<sup>th</sup> September 2019.

**Bhatt S**, Biswas S, Pandav B, Mondol S. 2020. Phylogeography and population structure of Indian leopard, *Panthera pardus fusca* at 14<sup>th</sup> Uttarakhand State Science and Technology Congress 2019-20 Dehradun, 27<sup>th</sup> to 29 February 2020.

**Bhatt S**, Biswas S, Kolipakam V, Pandav B, Mondol S. 2023. Mitochondrial phylogeography and demographic history of leopard (*Panthera pardus fusca*) India. Oral presentation at 1<sup>st</sup> Indian Conservation Conference (ICCON-2023), Mysuru, Karnataka, 9- 11 April, 2023.

### *Institutional conferences*

**Bhatt S**, Biswas S, Pandav B, Mondol S. 2018. Genetic analyses reveal population structure and demographic decline in leopards (*Panthera pardus fusca*) across Terai-Arc Landscape. Oral presentation at XXXII<sup>nd</sup> Annual Research Seminar, September 10-September 20, Wildlife Institute of India, Dehradun, India.

**Bhatt S**, Biswas S, Pandav B, Mondol S. 2019. Genetic analyses reveal population structure and recent decline in leopards (*Panthera pardus fusca*) of India. Oral presentation at XXXIII<sup>rd</sup> Internal Annual Research Seminar, August 19-August 27, Wildlife Institute of India, Dehradun, India.

**Bhatt S**, Biswas S, Kolipakam V, Pandav B, Mondol S. 2023. Phylogeography and demography history of leopards (*Panthera pardus fusca*) in India based on mitochondrial DNA. Oral presentation at XVI<sup>th</sup> Internal Annual Research Seminar, September 12- September 14, Wildlife Institute of India, Dehradun, India.

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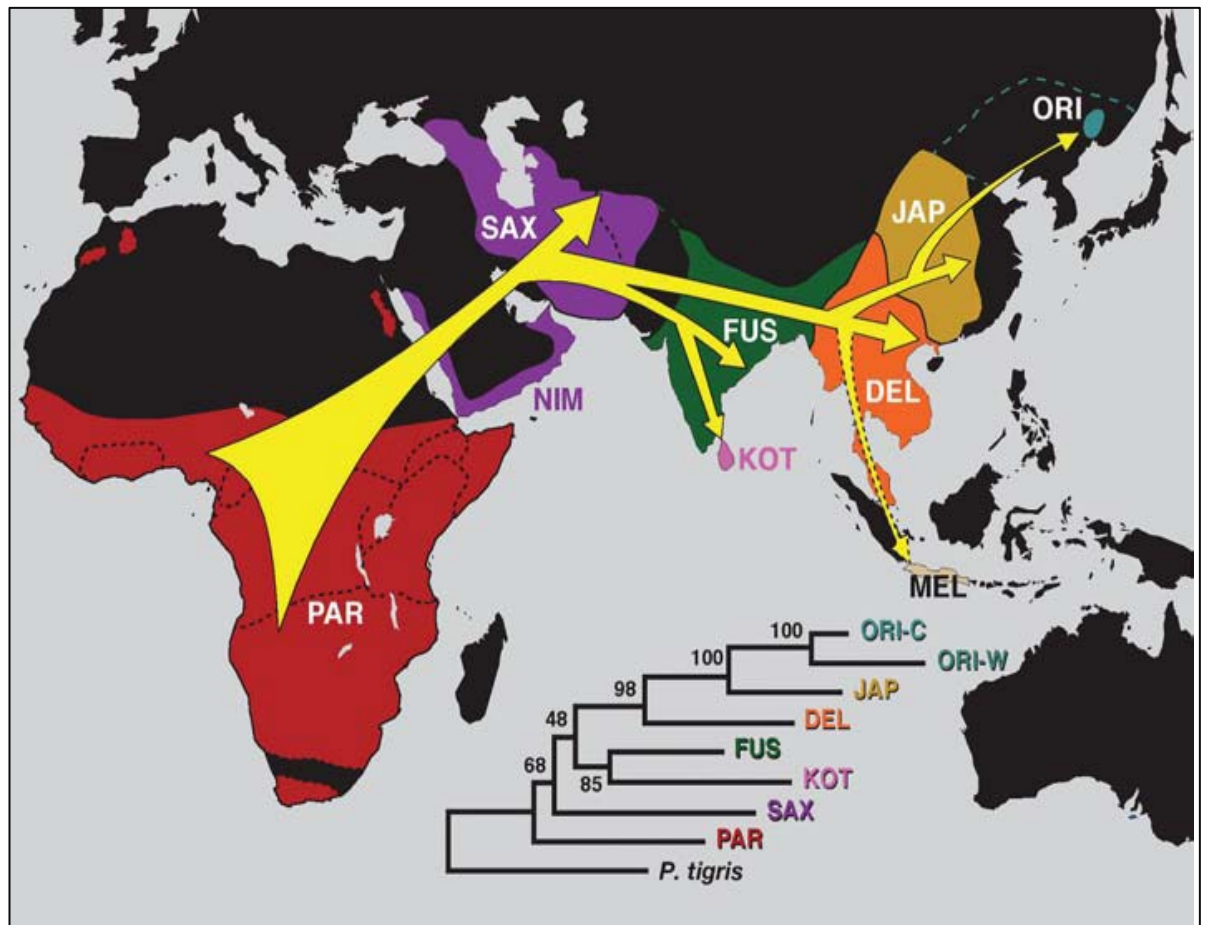
## Introduction

## Introduction

Large carnivores are critical to ecosystem structure and functioning (Sergio et al., 2008) and their absence can lead to significant changes in trophic cascades (Terborgh et al., 2001, Steneck et al., 2005, Estes et al., 2011, Ripple et al., 2014). Growing natural and anthropogenic pressures in the form of climate change, habitat loss and prey depletion, wildlife trade, and human-wildlife conflicts are pushing large carnivores into ever-shrinking habitat islands and severely exacerbating their endangered status, and in some cases extinction (Sillero-Subiri & Laurenson, 2001, Ceballos et al., 2005, Schipper et al., 2008, Karanth & Chellam, 2009, Karanth et al., 2009). Recent assessments of the conservation status indicate alarming rates of population decline for many carnivores at a global scale (Ceballos et al., 2005, Schipper et al., 2008, Karanth & Chellam, 2009, Jacobson et al., 2016, Wolf & Ripple, 2017). Specifically, the families *Felidae*, *Canidae*, and *Ursidae* are under severe threat across the globe (Ceballos et al., 2005, Schipper et al., 2008, Karanth et al., 2010, Wolf & Ripple, 2017).

Leopard (*Panthera pardus*, Linnaeus, 1758) represents the most widely distributed and adaptable member of the family *Felidae* and is part of the genus *Panthera*. Genomic studies suggest that leopards are closer to lions (*Panthera leo*) as compared to phenotypically similar jaguars (*Panthera onca*) (Figueiro et al., 2017). The genomic analyses estimated that lions and leopards first diverged from their common ancestors around 2.57 million years ago (Ma) (Figueiro et al., 2017). The modern-day leopard originated in Africa and further migrated out of Africa to Europe and Asia around 710-483 thousand years ago (Ka) (Uphyrkina et al., 2001, O'Brien & Johnson 2007,

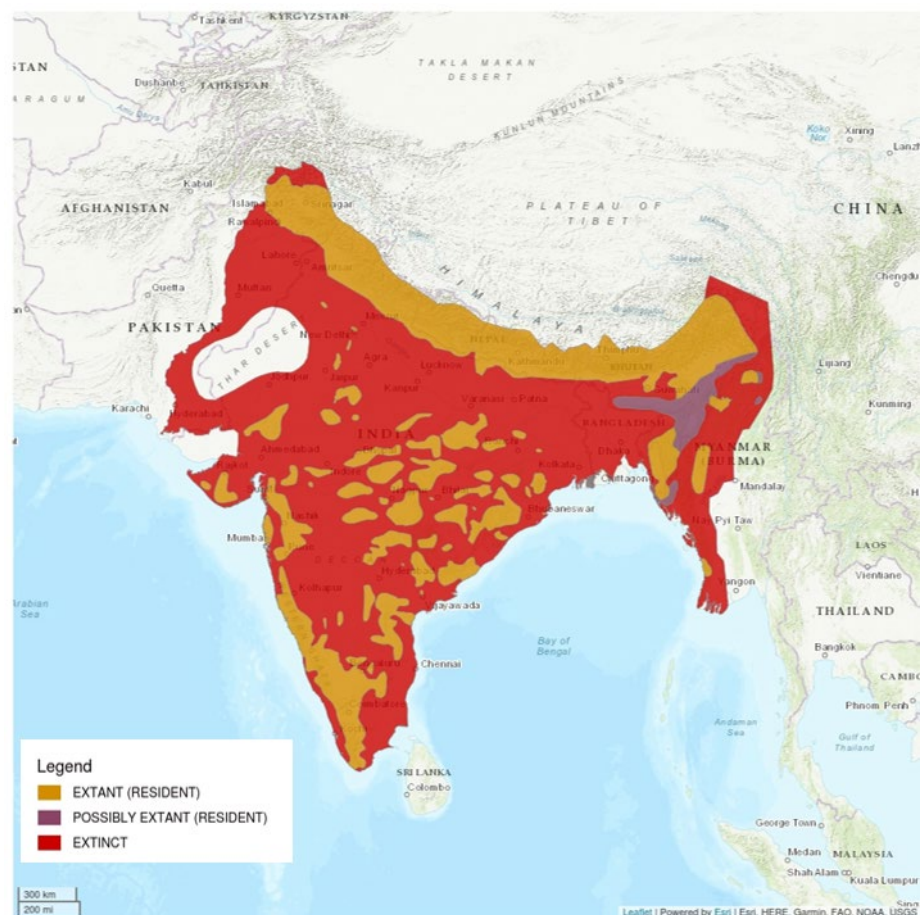
Pajimans et al., 2018) (Figure 1). The European subspecies went extinct during the Last Glacial Maximum (LGM) (Pajimans et al., 2018). The historical range of leopards spanned across nearly 35,000,000 km<sup>2</sup> area covering all of sub-Saharan and North Africa, the Middle East and Asia Minor, South and Southeast Asia, and the Russian Far East (Uphyrkina et al., 2001, Jacobson et al., 2016). However, the current distribution and numbers have significantly decreased across their range due to habitat loss, prey depletion, conflict, and poaching over the last century (Shepherd, 2001, Banks & Newman 2004, Jacobson et al., 2016). Recent meta-analyses of leopard status and distribution suggest 48-67% range loss for the species in Africa and 83-87% in Asia (Jacobson et al., 2016), making them among the top ten large carnivore species most affected by range contraction (Wolf & Ripple, 2017). Initially, 27 leopard subspecies were recognized based on coat colour patterns and morphological characteristics that were further re-categorised into nine subspecies based on molecular genetic methods (Pocock 1930, Uphyrkina et al., 2001). Out of the nine recognized leopard subspecies, two (*P. p. orientalis* (ORI) and *P. p. nimr* (NIM)) are classified as Critically Endangered, while two (*P. p. saxicolor* (SAX) and *P. p. melas* (MEL)) are considered Endangered, one Vulnerable (*P. p. kotiya* (KOT)) and remaining four (*P. p. japonensis* (JAP), *P. p. delacouri* (DEL), *P. p. fusca* (FUS) and *P. p. pardus* (PAR)) are categorized as Near Threatened by IUCN (Stein et al., 2022, Shivakumar et al., 2023) (see Figure 1). Despite continuously decreasing numbers and range, their ubiquitous presence across human habitations leads to misconceptions regarding their current abundance.



**Figure 1.** The figure represents the evolutionary route and distribution range of all modern leopard subspecies based on mitochondrial & microsatellite DNA (O'Brien & Johnson, 2005). The yellow arrow indicates the route & arrow thickness indicates relative genetic diversity.

The Indian subspecies (*P.p.fusca*) is distributed among India, Nepal, Bhutan, Bangladesh, and part of Pakistan in all forested habitats, and the Himalayas except Sundarbans and desert habitats (Daniel 1996, Shivakumar et al., 2023) (Figure 2). India harbours more than 75% of the extant range of *P.p. fusca* and this species is considered endangered in Appendix I by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and Schedule I category by Wildlife Protection Act 1972 (WPA 1972). Still, this species faces significant threats

such as habitat loss, human-wildlife conflict, poaching, and illegal trade for its body parts (Athreya et al., 2010, Mondol et al., 2015, Raza et al., 2010). A total of 12,616 – 15,132 individual leopards are estimated across four major tiger conservation landscapes i.e., Shivalik Hills & Gangetic Plains, Central India & Eastern Ghats, Western Ghats, North Eastern Hills, & Brahmaputra Floodplains (Qureshi et al., 2024). However, the available population estimates are from the tiger-bearing areas only and as leopards do survive in highly human-populated and modified areas (Athreya et al., 2013, 2014) this estimate is likely to be minimal and incomplete.



**Figure 2.** The figure represents the past and present distribution range of *Panthera pardus fusca* (source-IUCN).

Unfortunately, there is still a paucity of information on their population status and demography at regional and global scales. Much of our knowledge of leopard ecology and demography in the Indian subcontinent comes from location-specific studies. Density estimates are available from northern India (Harihar et al., 2009, Wang and Macdonald 2009, Thapa et al., 2014, Habib et al., 2014, Noor et al., 2020, Yadav et al., 2024), central India (Edgaonkar 2008, Mondal et al., 2011, Mondal et al., 2012a,b, Athreya et al., 2013, Jhala et al., 2015, Zehra et al., 2016, Chauhan et al., 2005, Pawar et al., 2019, Rather et al., 2021), western Ghats (Kalle et al., 2011, Ramesh et al., 2012, Gubbi et al., 2021) and northeast (Borah et al., 2013, Goswami and Ganesh 2014, Selvan et al., 2014). Further, recent studies in the Indian subcontinent provide contradictory patterns of local population trends. E.g., historical records and occupancy estimation models based on ecological data and field observations Karanth et al., (2010) estimated high local extinction probabilities of leopards across the subcontinent, and Athreya et al., (2010) reported higher rates of recent conflict incidences and related mortality at local scales. Other ecological (Harihar et al., 2011) as well as population genetic studies of demographic history (Dutta et al., 2013 a,b) suggest stable or increased leopard populations at local scales. Therefore, the information on population and demography changes for Indian leopards need to be investigated to plan appropriate conservation measures. As leopards are elusive in behavior and found ubiquitously across human habitations, that makes it difficult to generate systematic data for population estimation and demographic patterns at landscape levels. As, traditional approaches such as photographic capture-recapture methods require years of intensive fieldwork and associated technical and logistical concerns and are difficult to implement around human-dominated regions. Other

invasive approaches such as radio collaring are feasible for a small number of individuals, limiting their utility to study such endangered species at large scales.

To overcome that, non-invasive genetic sampling has proven advantageous over traditional methods because of its ease of collection, ability to access a large sample size, and broader spatial coverage to study such elusive and wide-ranging species. The non-invasive genetic samples along with molecular tools have been utilised for population genetics (Biswas et al., 2022, Modi et al., 2021), evolutionary history (Ghosh et al., 2022), demography (Mondol et al., 2009, Mondol et al., 2013), and wildlife forensics (Mondol et al., 2015a, Ghosh et al., 2021). Further, these approaches altogether allowed us to understand the genetic diversity, its spatial distribution, and the impact of Anthropocene on the species biology.

Till now the available genetic studies have focused on subspecies delimitation, diversity, and ascertaining the origin of the species (Miththapala, 1956, Uphyrkina et al., 2001, Asad et al., 2010) (Table 1). In the Indian context, studies also have focused on the estimation of the genetic variation, population connectivity, and demographic history the central India population of leopards (Dutta et al., 2013a, b, Thatte et al., 2020,) (see Table 1). Mondol et al., 2015 utilised genetic tools to identify and trace the poaching hotspots of leopards in India. A recent study by Singh et al., 2020 has looked into the genetic diversity and demographic history of leopards of Western Himalaya based on both the mitochondrial and microsatellite markers.

Table 1. *Available genetic studies on Indian leopards.*

Subject	Objectives	Study area	Key findings	Citations
<b>Phylogenetics</b>	Ascertain subspecies recognition	Leopard distribution range	Subspecies delimited	Mithapala, 1996
	Phylogenetic position, diversity and origin of leopard	Leopard distribution range	Subspecies delimited & origin. Genetic variation based on 9 haplotypes for Indian leopard	Uphyrkina et al., 2001
<b>Population genetics</b>	Genetic variation and population structure in Central Indian landscape	Central India	High genetic diversity & low genetic differentiation among leopards of central India	Dutta et al., 2013a
	Gene flow and demographic history	Central India	Reduced gene flow and no evidence of genetic bottleneck	Dutta et al., 2013b
	Estimated population genetic attributes and demographic history (Microsatellite and mitochondrial DNA)	Western Himalayas	Moderate genetic diversity based on the markers, substructuring and demographic history	Singh et al., 2020
	Genetic variation and landscape connectivity	Central India	High genetic diversity and reduced gene flow due to isolation by distance	Thatte et al., 2020
<b>Wildlife Forensics</b>	DNA-based identification of geographic origin of traded leopard part	Central India	Central India identified as poaching hotspot	Mondol et al., 2015

Given the above background, there is still a dearth of knowledge on leopard genetic makeup at the subspecies level, population level, individual level, and demographic history of the species. Therefore, in this thesis, I have utilised non-invasive genetic approaches to understand the various aspects of species biology focusing on phylogeography, demography, and local population dynamics.

## **1.1 Objectives:**

Considering all the above-mentioned gaps, the major objectives of this proposed thesis are:

- a) To assess the phylogeography of the Indian leopards using mtDNA and microsatellite markers
- b) To evaluate the population structure and demographic history of leopards in the Indian subcontinent using both mtDNA and microsatellite markers
- c) To investigate leopard social dynamics using genetic data at the local level and examine how it varies across different densities.

## **1.2 Thesis organisation:**

Considering that there are limited genetic studies available for leopards and the available ones have their geographical limitations (Dutta et al., 2013, Singh et al., 2022). This doctoral thesis is the first to provide a pan-India study on leopard population genetics and demography. This thesis also has attempted to understand the population dynamics of leopards at a local scale with genetic relatedness as a proxy approach.

Chapter 1 deals with the assessment of genetic variation, phylogeography & demographic history among Indian leopards. In this chapter, I have used mitochondrial markers to address objectives a and b.

Chapter 2 deals with the evaluation of genetic variation, population subdivision, and population changes among Indian leopards. In this chapter, I have utilised microsatellite markers to address parts of Objective a and b.

Chapter 3 deals with the local population dynamics of leopards in the Rajaji tiger reserve where utilisation of microsatellite markers was done to assess relatedness to achieve objective c.

Synthesis deals with a summary of the key findings of this thesis preceded by references.

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**Chapter 1. Mitochondrial DNA phylogeography of leopard (*Panthera pardus fusca*) across the Indian subcontinent**

## Introduction

Leopards typify one of the widely distributed and adaptable members among large felids (Jacobson et al., 2016). As a top predator, leopards play a crucial role in shaping ecological interactions within biological communities, thereby maintaining the structure and diversity of these ecosystems (Terborgh et al., 2001, Steneck, 2005, Ripple et al., 2014). Burgeoning anthropogenic pressure and key threats like habitat loss, poaching, and human-wildlife conflict have resulted in a drastic decline in their historic distribution range (~70%) (Jacobson et al., 2016, Ripple et al., 2017). They are listed under the ‘Vulnerable’ category by the IUCN Red List (Stein et al., 2022) and listed as Schedule I under the Wildlife (Protection) Act, 1972 of India.

Leopards followed a complex evolutionary history and they (leopard and lion) diverged from a common ancestor around 2.57 million years ago (mya) (Figueiro et al., 2017). The leopard mtDNA clade with the highest genetic diversity occurs in Africa indicating the origin of the species and around 710 and 483 thousand years ago they migrated towards Europe and Asia (Uphyrkina et al., 2001, O'Brien & Johnson 2007, Figueiro et al., 2017). They migrated to India through the western corridor. In India, ~75% extant range of *Panthera pardus fusca* is represented by a current estimate of ~14000 individuals (Qureshi et al., 2024). They are found everywhere in India except desert habitats and Sunderbans (Daniel JC, 2009, Qureshi et al., 2024). However, they faced a huge loss due to trophy hunting (Rangarajan, 2005), and in the current era due to human-wildlife conflict (Athreya et al., 2011, Navya et al., 2014, Sidhu et al., 2017, Naha et al., 2018) poaching (Wildlife Protection Society of India

(WPSI) reports, Raza et al., 2012). However, there is a paucity of information on the genetic makeup and demographic history of the Indian leopard.

Available genetic studies on Indian leopards are done based on microsatellites data and mitochondrial DNA data are limited in their geographic scope (see Table 1). Till now the available studies based on mitochondrial DNA study on Indian leopards utilised NADH5-611bp and CR-116bp (six haplotypes, South: Nagarhole National Park, North: Shakkarbaug Zoo, Uphyrkina et al., 2001), and the control region (CR-236bp) (three haplotypes, Uttarakashi district of Uttarakhand, Singh et al., 2020) and have limitations in terms of extent of sampling. Therefore, to fill this gap, we have sampled all across the different biogeographic zones and examined genetic variation, population structure, and demographic history in the mitochondrial DNA of leopards. In more detail, we investigated the following: (1) the amount of genetic variation in leopards that persists across the Indian subcontinent; and (2) the population structure of leopards at the national level. (3) the leopard population's demographic history through analysing past population changes

## **Methods**

### *Research permission and ethical considerations*

All required permissions for our field surveys and biological sampling were provided by the Forest Departments of Uttarakhand (Permit no: 90/5-6), Uttar Pradesh (Permit no: 1127/23-2-12(G) and 1891/23-2-12) and Bihar (Permit no: Wildlife-589). Due to the non-invasive nature of sampling, no ethical clearance was required for this study.

### *Biological sampling*

To understand the phylogeographic pattern and demographic history, it is important to obtain samples across the species distribution in the study area. We used non-invasive sample data from the Indian subcontinent to generate leopard genetic data for this investigation. Our intensive field surveys include the Terai-Arc landscape (TAL) in India, covering the northern Indian states of Uttarakhand, Uttar Pradesh, and Bihar, for a duration of 2016-2018.

India retains >75% of *P. p. fusca* distribution and they are present ubiquitously across diverse habitat types (Jacobson et al., 2016). These samples covered major landscapes including the Himalayas, Shivalik hills and Gangetic plains, Central India landscape and Eastern Ghats, Western Ghats landscape, North Eastern hills and Brahmaputra flood plains, where leopards are distributed (Jhala et al., 2018, Jhala et al., 2020, Qureshi et al., 2024). During the surveys, we opportunistically collected a total of 778 fresh large carnivore faecal samples. These samples were collected from both inside (n=469) and outside (n=309) protected areas from different parts of this landscape. In the field, the samples were judged as large carnivores based on several physical characteristics such as scrape marks, tracks, faecal diameter, etc. All faecal samples were collected in wax paper and stored individually in sterile zip-lock bags and stored inside dry, dark boxes in the field for a maximum of two weeks period (Biswas et al., 2019). All samples were collected with GPS locations and were transferred to the laboratory and stored in -20°C freezers until further processing.

In addition to the north Indian samples collected in this study, we have also used leopard genetic samples (n=139) by Mondol et al., (2014), representing the Northeast, Western

Ghats, and central Indian landscape to generate mitochondrial DNA data. These samples were utilised for microsatellite data generation for subsequent utilisation in the forensics analysis of seized leopard samples (Mondol et al., 2014).

#### *mtDNA markers and PCR amplification*

Due to the poor quality of the source DNA (faecal samples), it was necessary to amplify short fragments of selected mtDNA genes and we designed all necessary markers in this study. To achieve this, we retrieved all leopard mtDNA sequences (of different lengths) available in NCBI and selected NADH5, NADH4, and Cytochrome b (Cytb henceforth) as target genes for primer design (based on sequence variation by comparing other studies such as Miththapala et al., 1996 and Uphyrkina et al., 2001). All relevant sequences were aligned and screened visually to identify variable sites. Primers were designed manually considering the following criteria: (i) amplicon size of  $\leq 250$  bp to assure higher amplification success and (ii) design multiple, overlapping primers for each gene for testing with faecal DNA samples before final selection. A total of 16 primer pairs were designed: 8 for NADH5, 4 for NADH4, and 4 for Cytb (Table 1).

**Table1.** *List of primers designed in this study.*

S.No.	Primer	Sequence
1	ParCytb F1	CCCATATCTGCCGCGATG
2	ParCytb R1	AAGCCCCCTCAGATTCAT
3	ParCytb F2	CAATCCCATACATTGGGA
4	ParCytb R2	GACGAGTAGTATGAGTGC
5	ParND5 F1	GGACCAAAAAATTGGTGC
6	ParND5 R1	GCCAATTGTTTCTTGTCC
7	ParND5 F2	ATAAGCATAATCCCGGCT
8	ParND5 R2	CCCTACTTGAAAAATCGG
9	ParND5 F3	CGAACCTCAAATTGTCA

10	ParND5 R3	GGTTGTTGGCGGTTACTA
11	ParND5 F4	CCCCGTATTTCTAATCAC
12	ParND5 R4	AGCGAGGAATCATGCTAT
13	ParND4 F1	CCTTAACAACATGACTCC
14	ParND4 R1	ATGCGGATTAATGTGGCT
15	ParND4 F2	CAGCCCTTATAATTGCTC
16	ParND4 R2	GTTTGTGCCTATAAGGAC

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For marker standardization, PCR reactions were performed in 10 µl reaction volumes containing 4 µl of Qiagen master mix (Qiagen Inc., Hilden, Germany), 4 µM of BSA, 0.3 µM primer mix, and 2 µl of template leopard DNA (1:10 dilution). The PCR conditions included denaturation at 95 °C for 15 minutes, followed by 50 cycles of denaturation (95 °C for 30 seconds), annealing (54 °C for 30 seconds), and extension (72 °C for 30 seconds), with a final extension of 72 °C for 15 minutes (Table 1). PCR reactions included extraction and PCR negatives to monitor contamination. The PCR products were visualized with a 2% agarose gel. The amplified PCR products were cleaned using an Exonuclease-Shrimp Alkaline Phosphatase mixture (New England Biolabs, Ipswich, Massachusetts) and sequenced bidirectionally using BigDye v3.1 Terminator kit in ABI 3500XL Genetic Analyzer (Applied Biosystems, California, United States).

## **Data Analysis**

### *Genetic diversity and population structure*

All leopard mtDNA sequences from the target genes were checked manually and cleaned for any nucleotide ambiguities using MEGA 7 (Kumar et al., 2016). Further, each gene sequence and the concatenated version were aligned in CLUSTAL W. We calculated various genetic diversity indices (number of haplotypes, haplotype diversity

(h), nucleotide diversity ( $p$ ), number of polymorphic sites ( $s$ ), etc.) using program DnaSP v.6.12.01 (Rozas et al., 2017). We constructed a median-joining haplotype network using the program PopART version 1.7 (Leigh & Bryant, 2015) to assess the distribution of Indian leopard genetic variations. The median-joining haplotype network calculations ( $\epsilon = 0$ ) were carried out for both analyses by assigning equal weights to all the variable sites. Further, to test the presence of population genetic structure AMOVA (Analysis of Molecular Variance) was performed in Arlequin (Excoffier et al., 2005). This F-statistics-based analysis assumes that genetic diversity within two populations is not significantly different from grouping the populations as a whole.

We estimated any potential genetic structure within the leopard mtDNA data using a Bayesian approach implemented in Bayesian Analysis of Population Structure (BAPS) version 6.0 (Corander et al., 2011). In this approach, prior information regarding the number of estimates is considered to be an unknown parameter, and ecologically meaningful upper and lower bounds are specified. Populations are assigned to  $n$  panmictic clusters using a stochastic partitioning model. For mitochondrial DNA data, estimating admixture ancestry requires a different approach, as the DNA sequence cannot be treated as an unlinked locus. A special approach, ‘clustering of linked loci’ (Corander J & Tang J, 2007), where the joint probability of the genetic partitioning is considered across the linked loci, instead of the individual locus. For the Markov Monte Carlo analysis, analytical factorization is implemented to sample alleles across the gene. Priors for population clustering were set with  $K$  values between 1 and 10. Each value of  $K$  was then analysed using 500 iterations and 100 burn-ins for each

referenced individual per population. The population clusters were determined by using the logarithm ( $\log(\text{ml})$ ) of the Bayes factor (Corander J & Tang J, 2007).

### *Demographic history*

We used both qualitative and quantitative approaches to detect historical trends in the Indian leopard population. The qualitative approach used frequency statistics to signal population size changes using the test of neutrality and nucleotide mismatch distribution while the quantitative approaches deal with likelihood-based (implemented in program BEAST, Boukaert et al., 2014) or approximate-based (implemented in program DIYABC, Cornuet et al., 2008) Bayesian algorithms to estimate time and change in population size.

We used Tajima's D and Fu's Fs as appropriate statistics of neutrality implemented in DnaSP 5.0 (Rozas et al., 2010) where the significant negative value of Tajima's D and Fu's Fs tests are an indication of demographic expansion. The significance is obtained based on 1000 coalescent simulations. Tajima's D value of zero implies that the population is experiencing an equilibrium state due to selective neutral variations, whereas a negative value of D suggests demographic expansion or mutational selection, and a positive D value suggests demographic contraction. Similarly, in the case of Fu's Fs statistics (Fu, 1997), negative Fs values are seen during demographic expansion or departure from a null hypothesis of neutral selection and population equilibrium. Further, we computed mismatch distributions for Indian leopards using Arlequin (Excoffier et al., 2005) to understand population demography. Here expanding populations show right-skewed unimodal peaks whereas populations in demographic equilibrium display ragged and multimodal peaks (Rogers &

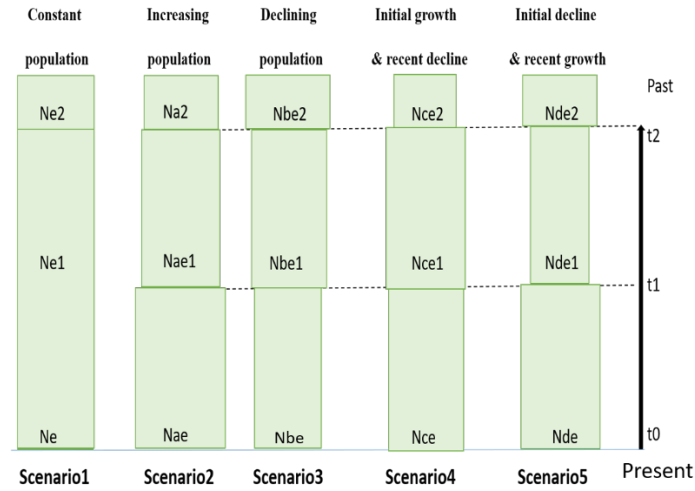
Harpending, 1992). The distribution patterns were assessed by estimating the sum of squared deviation (SSD) and Harpending's raggedness index (Rg), tested for significance with 1000 bootstraps in Arlequin (Excoffier et al., 2005).

For the quantitative approach, we investigated historical population size change signatures of *P. p. fusca* using a coalescent-based Bayesian Skyline plot (BSP) (Drummond et al., 2005) implemented in BEAST v.2.3.6 (Bouckaert et al., 2014). The piecewise-linear skyline model was implemented with the number of groups ( $m$ ) set to 7. Site model parameters were determined by jModelTest v2.1.10 (Darriba et al., 2012, Darriba et al., 2014) (based on Akaike information criterion (AIC) and Bayesian information criterion (BIC)) for all three selected genes (Cytb, NADH4, and NADH5). Substitution rates per site per million years of 1.38% (Cyt) and 1.22% (NADH5) were used in analyses (Mukherjee et al., 2010), whereas for NADH4 it was estimated as 1.38% by specifying a broad uniform prior. Further, analyses were conducted with three independent runs, using uniform distribution under a strict molecular clock. MCMC runs included  $10^8$  steps with a 10% burn-in. Data convergence of effective sample sizes (ESS > 500) was checked with Tracer version 1.7.1 (Rambaut et al., 2018).

Further, we used an approximate Bayesian approach implemented in the program DIYABC (Cornuet et al., 2008) to understand and determine the most plausible demographic history of the Indian leopards. The probabilities of the different plausible scenarios of demographic history were determined by defining a range of priors ( $R$ ) for the different population histories using population size, admixture ratios, timing of events, where the value of each prior was drawn from a vector hyperprior ( $R_I$ ), such

that the  $R_I = p(R)$ . Genetic data ( $G$ ) was then simulated, drawing values from within the boundaries set in  $R$ , from  $R_I$ .  $G$  was then summarised using a set of summary statistics ( $S_I$ ) and compared to the summary statistics of the actual dataset ( $S$ ) (Joyce & Marjoram, 2008). A rejection method (Pritchard et al., 1999) was used to estimate the conditional density of the posterior distribution  $p(R|S = S_I)$ , by determining the values of  $R_I$  which were within distance  $D$  from  $R$ , and  $|S-S_I|$  was small. The posterior distribution was then obtained from the retained data, of each scenario of population history, and the most plausible scenario was then determined using a regression framework with a set of criteria of  $D$ . We considered multiple parameters to model leopard demography. In the first case (named as Recent demographic model), the parameters obtained from BSP analysis were used as prior (see Annexure I), whereas the second case (Old demographic model) had a different set of parameters considering much older events in leopard demography (Table S2). Overall, we modelled five different scenarios (presented in Figure 2) as following:

- Scenario 1 (constant): constant population size
- Scenario 2 (growth): growing population
- Scenario 3 (decline): declining population
- Scenario 4 (growth\_decline): initial growing population till time  $t$  and recent decline
- Scenario 5 (decline\_growth): initial declining population and recent growth of population



**Figure 1.** Schematic representation of five possible demographic scenarios for Indian leopards. Here,  $N$  indicates genetic effective population size, and time is denoted by  $t$ .

During modelling, initial control checks were performed by simulating 100,000 runs for each scenario in Recent (Model 1) and old demographic model (Model 2) to assess parameter ranges. After parameter modifications final simulations were conducted with 1 million runs for all scenario-parameter combinations. The best scenario was selected based on the comparison of relative posterior probabilities of individual scenarios calculated based on 0.1% and 1% of simulated datasets most closely matching the data.

## Results

### *Leopard Genetic variation, phylogeography and population structure*

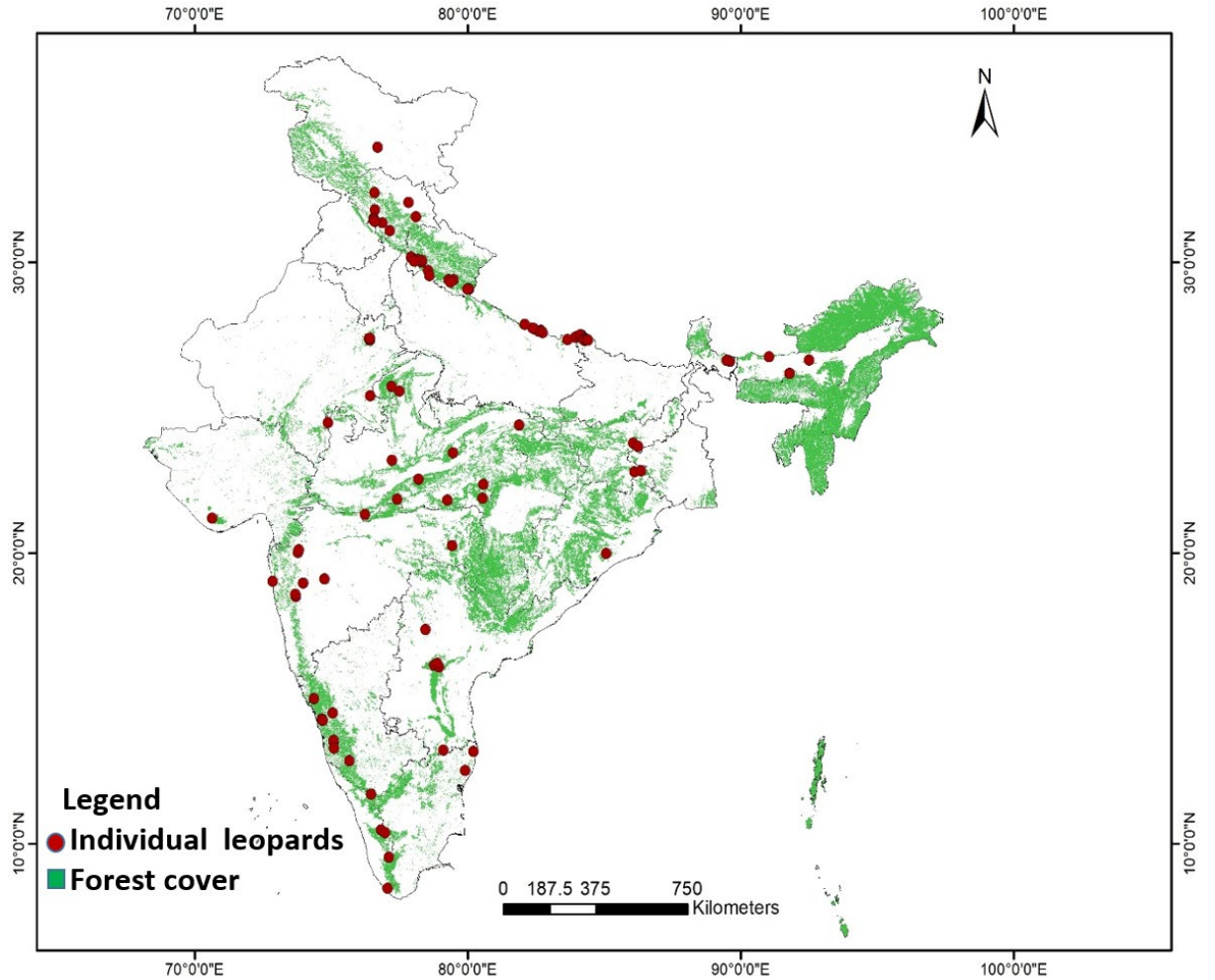
Of the 778 large carnivore faecal samples collected from TAL, we identified 195 faeces to be of leopard origin (25%) using species-specific PCR assays (Mondol et al., 2014, Maroju et al., 2016). In addition, 457 samples were ascertained to be of tiger

(59%) and the remaining 126 faecal samples did not produce any result (16%) for either of these large felids, possibly due to poor-quality DNA. Out of the total confirmed leopard samples, we used a total of 225 individual leopard samples (86 samples from Bhatt et al., 2020 and 139 samples from Mondol et al., 2014, respectively). Only 112 samples (50% success rate) showed successful amplifications for all three targeted mtDNA segments (NADH4, NADH5, and Cytb).

The remaining samples were removed as required data was not produced. We generated a concatenated 1265 bp sequence data (NADH4- 494 bp, NADH5- 548 bp, and Cytb- 223 bp, respectively) from the sampled individuals. The Genbank accession numbers for the submitted sequences are OQ420769-OQ420880 (Cytb), OQ420881-OQ420992 (NADH4), OQ420993-OQ421104 (NADH5) (Annexure II). Overall, we found 46 haplotypes with high haplotypic diversity ( $0.913 \pm 0.019$ ) and low nucleotide diversity ( $0.00343 \pm 0.01$ ) in Indian leopards and with a high number of segregating sites ( $n=57$ ) (Table 2 for individual gene summary).

**Table 2.** Genetic summary statistics for 112 leopard's mitochondrial DNA sequences.

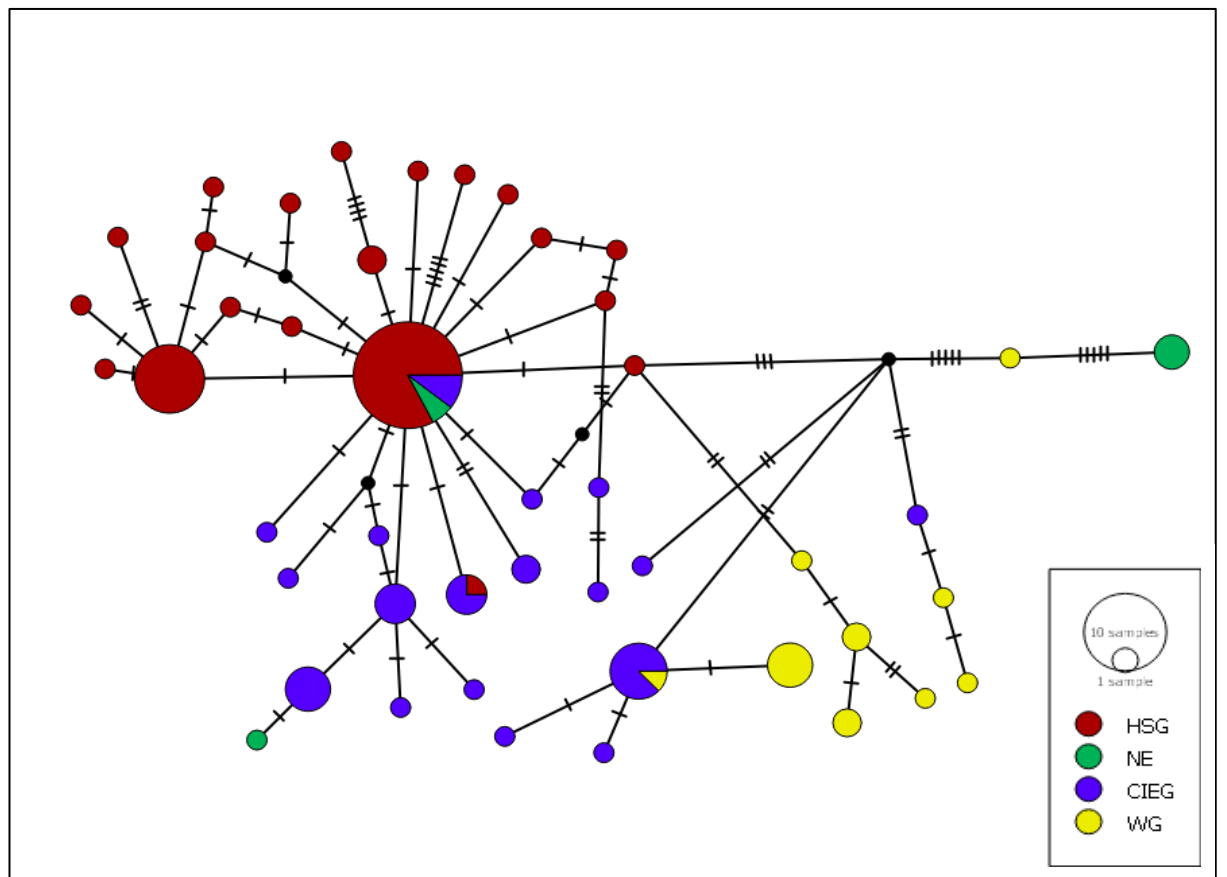
Genetics diversity indices	CytB (494bp)	NADH4	NADH5
Number of haplotypes, h	27	4	18
Haplotype diversity, $H_d$	0.802	0.337	0.631
Haplotype diversity variance	0.00112	0.00239	0.0025
Nucleotide diversity, $P_i$	0.00354	0.00155	0.00396
Variable (polymorphic) sites	31	3	20
Singleton variable sites	11	1	8
Parsimony informative sites	20	2	12



**Figure 2.** Location of individual leopard samples ( $n=112$ ) used in this study.

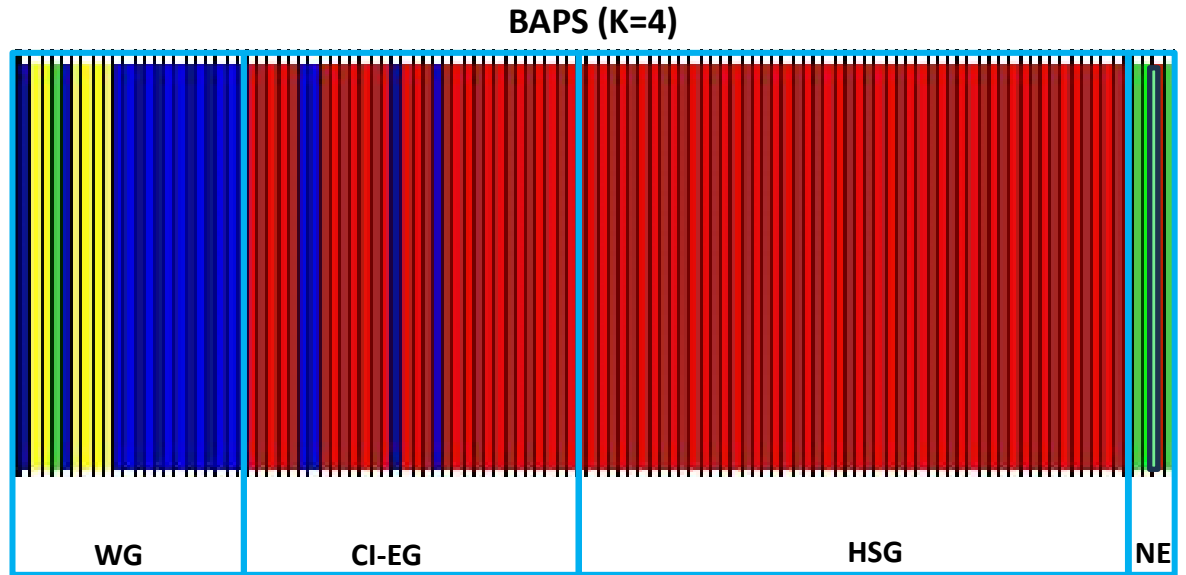
In total, we identified the highest number of unique haplotypes from the Himalayas, Shivalik hills, and Gangetic plains (HSG), followed by the Central India landscape and Eastern Ghats (CI-EG), Western Ghats (WG), and North Eastern hills and Brahmaputra flood plains (NE), respectively. Only three haplotypes were shared among the sampled regions: one (haplotype 9) between CIEG and WG, one (haplotype 22) among HSG, CIEG, and NE, and one (haplotype 30) among HSG and CIEG. The

network analyses indicate no strong phylogeographic structure among different leopard haplotypes across India (See Figure 3). This result was also supported by AMOVA where the population as a whole showed a low  $F_{st}$  value of 0.13. We identified the source of variation was only 13% among the population and found 86% within the population. This suggested high gene flow for the leopards and indicated no genetic structure.



**Figure 3.** Median Joining network based on 1265bp sequence data (Cytb, NADH4, NADH5) of 112 Indian leopard samples. Here, the size of each circle is proportional to the frequencies of each haplotype. The small black dots indicate the missing haplotypes, and the small line between the haplotype represents the mutational steps.

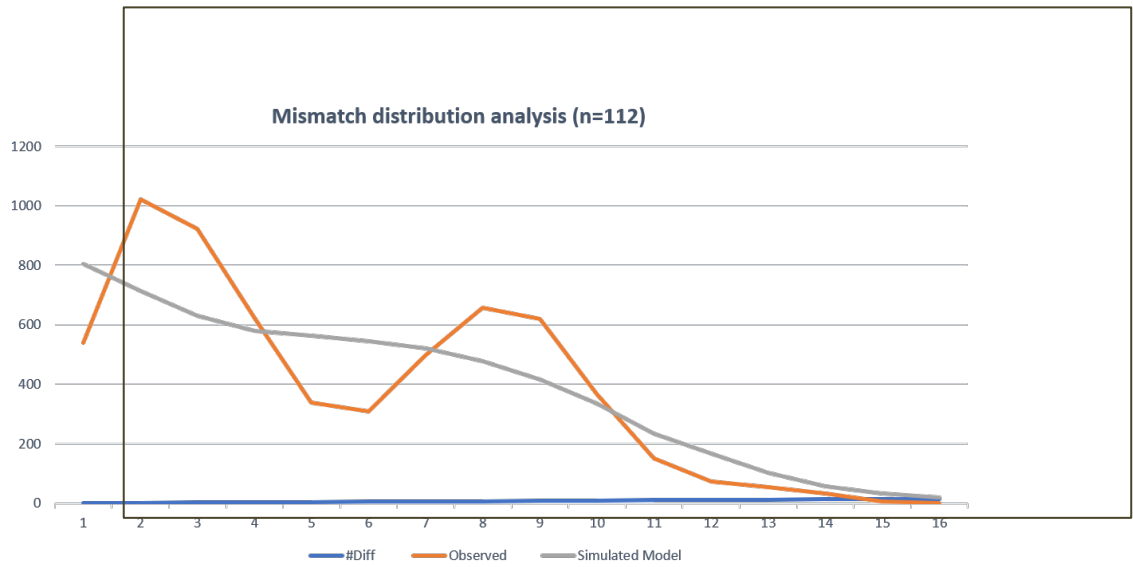
Further, BAPS analyses showed four genetically intermixed clusters ( $K=4$ ) with a log- $(ml)$  value of -864.2843. However, this signature is intermixed geographically with the dominance of the northern signature (Figure 4).



**Figure 4.** The BAPS indicates overall ( $K=4$ ). Here, different colours indicate different lineages.

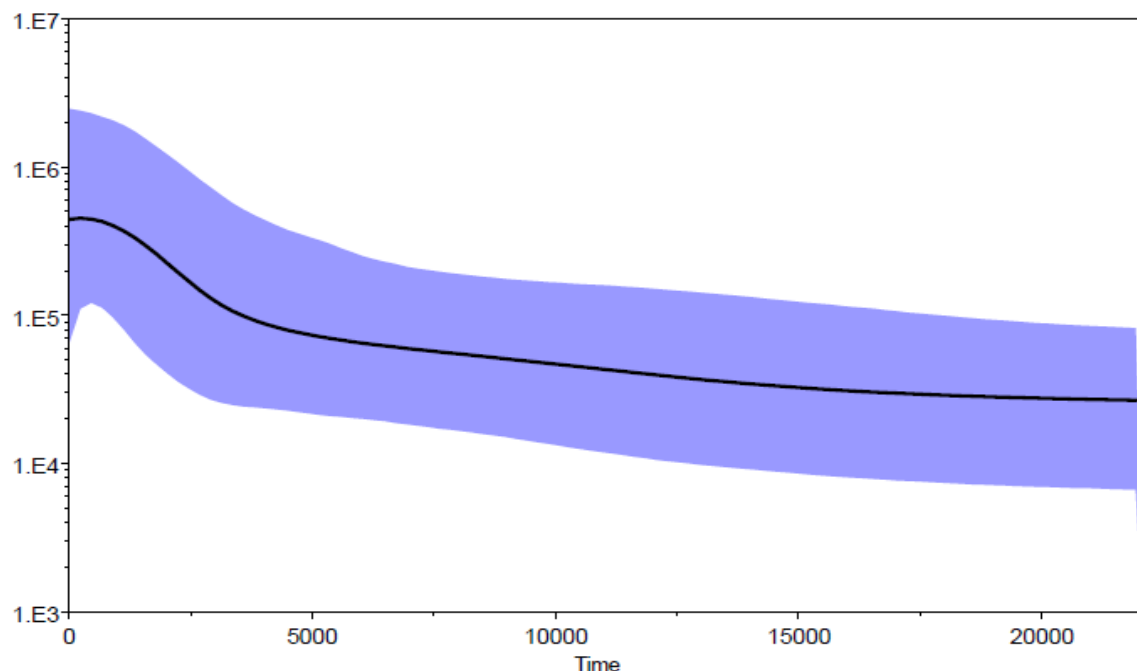
#### *Patterns of historical demography*

The qualitative and quantitative approaches showed contrasting patterns of population demographic scenarios. The leopard samples showed negative values for Tajima's  $D$  ( $-0.30895$ ,  $p < 0.05$ ) and Fu's  $F$  ( $-0.2335$ ,  $p < 0.05$ ), indicating population expansion or selection. On the other hand, mismatch analyses showed multimodal peaks under both constant population size and population growth-decline models, indicating past demographic equilibrium (Figure 5). The observed mismatch values showed a non-significant value of Harpending's raggedness index,  $r = 0.015$  ( $p = 0.85$ ) ( $SSD = 0.011$ ,  $p = 0.65$ ), indicating population equilibrium.



**Figure 5.** The given figure indicated multimodal peaks for the observed data set (denoted by orange line) and simulated data by grey line.

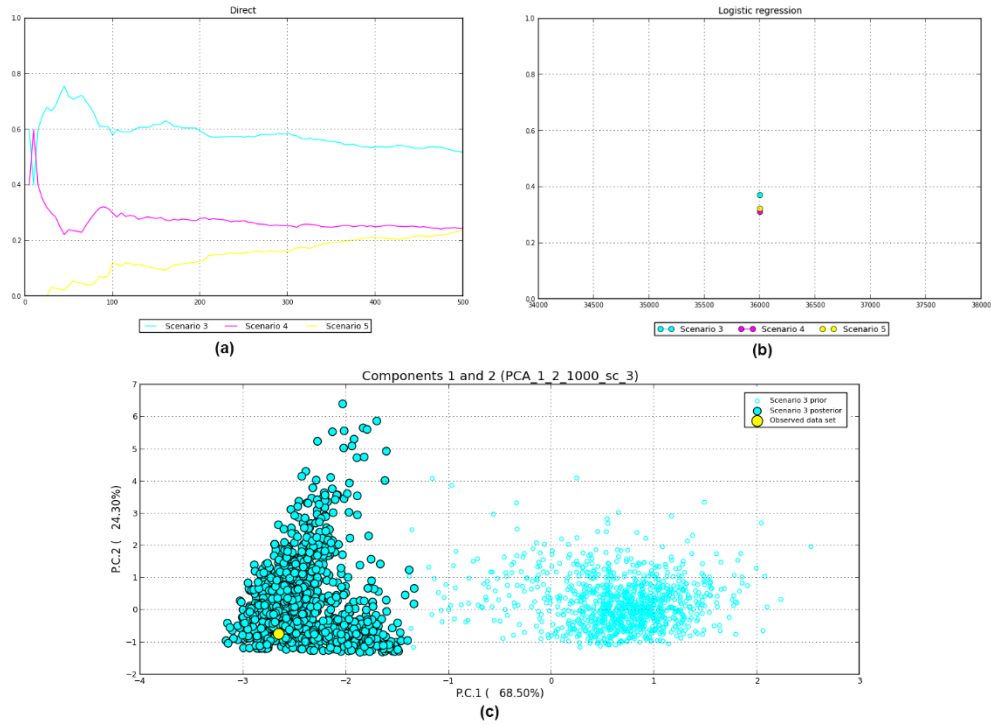
The quantitative BSP approach showed a similar pattern where an increase in female effective population size ~3000-4000 years ago (during the mid-Holocene period) was observed, followed by equilibrium in the population ~200 years ago (223-448 years) with a wide confidence interval (Figure 6).



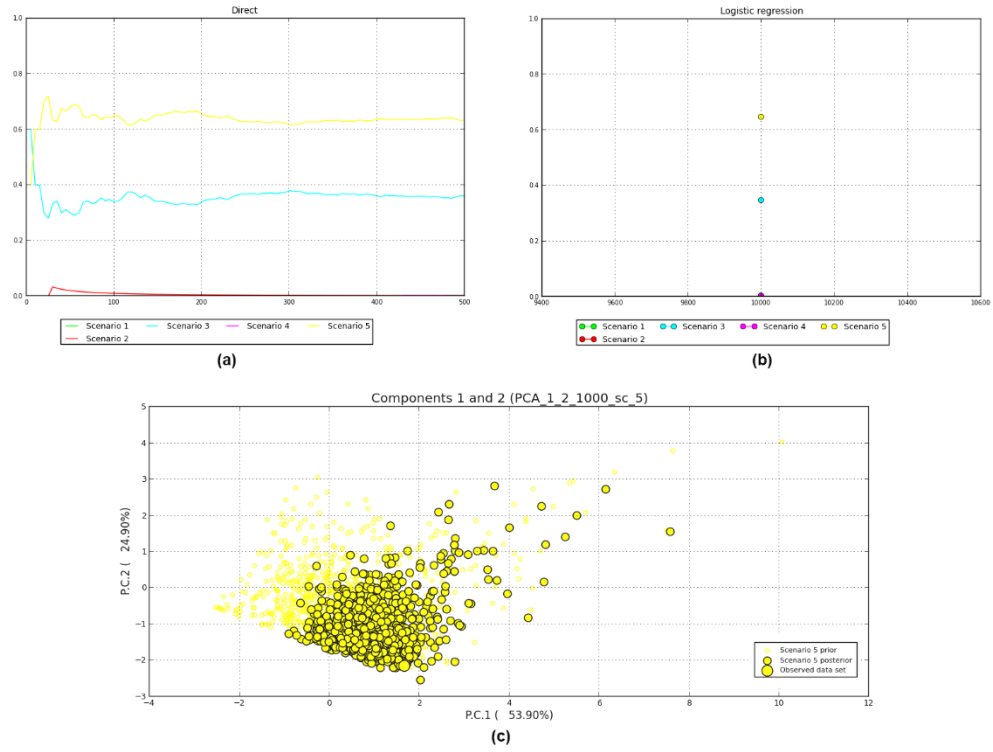
**Figure 6.** *Bayesian skyline plot (BSP) indicating a change in the female effective population size of the leopard (black line). Here, the shaded area shows the 95% HPD interval.*

Recent and Old demographic models tested under the Approximate Bayesian framework supported a scenario of leopard population decline. Evaluation of prior and scenario combinations under the recent demographic model revealed that only Scenario 3 (declining population) (0.65 [0.44-0.86]), 4 (initial growing population till time  $t$  and recent decline) (0.31 [0.00-0.65]), and 5 (initial declining population and recent growth of population) (0.03 [0.00-0.82]) produced data close to the observed dataset, indicating best possible demographic history scenarios. Interpretation through logistic regression determined that all three Scenarios (Scenario 3 (0.36 [0.36-0.38]), 4 (0.30 [0.30-0.31]), and 5 (0.32 [0.31-0.33])) were equally plausible and supported a population decline ~500 years ago from present with large confidence interval.

Analyses of the past demographic history model showed that scenario 3 (0.40 [0.33-0.46]), and 5 (0.60[0.53 – 0.66]) were most plausible (Figure 7). The logistic regression analyses demonstrated the higher likelihood of scenario 5 (0.64[0.63 – 0.66]) over scenario 3 (0.36 [0.34 – 0.37]).



**Figure 7.** (a) Model comparison among scenarios 3 & 4 using a direct approach, (b) Model comparison among scenarios 3 & 4 using a logistic approach. For both approaches, scenario 3 was better than scenario 4. (Here, each scenario is colour-labelled.) (c) A model-checking procedure was applied using a PCA on test statistic vectors to visualize the fit between the simulated and observed datasets. Note the large cloud of data from the posterior and observed datasets centered on a small cluster but far from the prior predictive distribution, suggesting that the best-supported scenario does not explain the observed dataset with high confidence interval.



**Figure 7.** (a) Model comparison among scenarios 3 & 5 using a direct approach, (b) Model comparison among scenarios 3 & 5 using a logistic approach. For both approaches, scenario 3 was better than scenario 5. (Here, each scenario is colour-labelled.) (c) A model-checking procedure was applied using a PCA on test statistic vectors to visualize the fit between the simulated and observed datasets. Note the large cloud of data from the prior and observed datasets centered on a small cluster from the posterior predictive distribution, suggesting that the best-supported scenario explained the observed data well.

The fifth demographic scenario supports a decline in the female effective population size (Nd2 ~60000) declining about 50000 years ago to an effective population size (Nd1~10000) around 10000 years ago. Further, it was followed by a population recovery to an effective population size of ~90000 in the current time (Table 3).

**Table 3.** *Parameter estimation of variables for different models (a & b) with mtDNA for the demographic history of Indian leopards using Approximate Bayesian Computation based coalescent modelling. a). Model 1 (Recent demographic history) based on the most likely scenario (3) b). Model 2 (Old demographic history) is based on the most likely scenario (5).*

	Parameters	Mode	2.5% quantile	95% quantile
<b>Model 1</b>	Nbe2	25000	9130	64200
<b>(Recent demographic history)</b>	Nbe1	2310000	1060000	78000000
	Nbe	96100000	10800000	97400000
	t2	809	165	16300
	t1	257	6.43	409
<b>Model 2</b>	Nde2	57600	10700	95400
<b>(Old demographic history)</b>	Nde1	6130	537	45300
	Nde	99600	74200	99400
	t2	49000	30300	65300
	t1	11600	4130	22500

## Discussion

### *Genetic diversity, Haplotype relationship, and population structure*

To the best of our knowledge, this is probably the first study focused on the mitochondrial DNA phylogeography and matrilineal demographic history of the leopards in India. The results from this study using partial fragments of NADH5, NADH4, and CytB genes suggested a high haplotype diversity and low nucleotide diversity, which is comparable to the African subspecies of leopard (Ropiquet et al., 2015; Morris et al., 2022). The reported haplotype and nuclear diversity are higher than as reported by earlier studies, most probably due to their limitations in terms of sample coverage (Singh et al., 2022; Uphyrkina et al., 2001). The unique haplotypes were found for all the landscapes. There were some missing haplotypes as well, showing the gaps in the sampling. Some haplotypes were not shared exclusively with their geographic location, which may be attributed to the natural long dispersal ability of the species (Sunquist, 1983; Bailey, 1993).

Our genetic analyses suggested no phylogeographic structure for Indian leopards, and this was also supported by AMOVA, which indicated high gene flow in the past. The northern haplotype signature seems to be the central node. The most probable reason behind this can be attributed to the migration history of leopards as revealed by molecular genetics (O'Brien and Johnson, 2005). The study leopards migrated to India using the western corridor and further followed two different routes, i.e., one from the Northwest part of India to the northern part of India and the other route from the southwest of India to the southern part (O'Brien and Johnson, 2005). However, one of the limitations of our study is the limited sampling from the north-eastern part of the

country. Therefore, future efforts should focus on covering this region to assess complete phylogeographic patterns for the species. These patterns may also arise due to the limitations associated with the use of linked loci, i.e., mitochondrial DNA markers. Mitochondrial markers help us to understand only the matrilineal history, which is mostly under genetic drift due to a low effective population size ( $N_e$ , one-fourth of nuclear DNA) as compared to the nuclear DNA (Frankham, 1996). So, it is better to utilise the biparental markers to explore the phylogeographic patterns.

#### *Demographic history of leopards*

The demographic history is relevant in understanding the population status of a species in the broader ecological and evolutionary context, especially for the wild ones for which comprehensive estimates are unavailable. For leopards, we got very contrasting patterns based on the qualitative and quantitative approaches. The high haplotype diversity with low nucleotide diversity also indicates the sudden expansion in the population. In this study haplotype network is star-shaped, where haplotypes radiate outside, also indicating probable expansion in the past. Also, the qualitative approaches indicate either signals of population or equilibrium in the past, probably due to demographic expansion from a population of low effective population size (Frankham, 1996).

The quantitative analyses revealed a drastic decline in female effective size ( $N_e$ ) around the Last Glacial Maximum (LGM), which is attributed to the extreme environmental changes that occurred during that period (Figueiro et al., 2017). This was the period when the European subspecies of leopards went extinct (Pajimans et al., 2018). Nonetheless, the timing of the population decline is similar for other

endangered species like lions (Figueiro et al., 2017), pangolins (Hu et al., 2020), and other large megafauna (Mahmood et al., 2021). The quantitative study suggested population recovery around the mid-Holocene for leopards due to a favourable climate. This also suggests that intensive climatic fluctuations during the LGM have resulted in the decline of the Indian leopard populations as observed for other large mammals (Mahmood et al., 2021). The population also faced a drastic reduction due to bounty hunting in the Mughal era, as suggested by our recent model demographic history (Mani, 1974), however, the range of the confidence interval was high, so this effect was further explored with the utilisation of microsatellite markers in Chapter 2. The demographic changes observed by Singh et al. (2021) suggested demographic decline around 1000 years ago due to habitat destruction and increased pastoralism as a result of domestication by humans. As older demographic history was interpreted as probably due to low mutation rates of mitochondrial DNA as compared to microsatellite DNA.

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## **Chapter 2. Assessment of population structure and demographic history of Indian leopard using nuclear DNA (*Panthera pardus fusca*)**

## Introduction

The Indian leopard (*P.p.fusca*) has the largest population size and range outside of Africa (Jacobson et al., 2016). However, poaching and conflict pose serious risks to leopard populations in the Indian subcontinent (Edgaonkar & Chellam, 1998; Athreya et al., 2010; Karanth et al., 2010, 2012, 2013a, b; Raza et al., 2012; Mondol et al., 2014). Leopards are also commonly found outside of protected areas, making them more susceptible to human conflict (Karanth et al., 2009; Athreya et al., 2010; Karanth et al., 2010, 2012a, b, 2013; Naha et al., 2018). Unfortunately, there is still a lack of information on their population and demography at regional and global scales (see Chapter 1).

In this chapter, we used non-invasively collected samples to generate genetic data to investigate leopard genetic variation, population structure, and demographic history among leopards in the Indian subcontinent using microsatellite markers. More specifically, we assessed (1) the extent of genetic variation leopard persists across the Indian subcontinent; (2) the population structure of leopards at the country scale; (3) the demographic history of leopards by assessing recent changes in population size, and finally (4) compared the finding of genetic decline analyses with countrywide ecological extinction probabilities. We interpreted our results in the context of local extinction probabilities as estimated by Karanth et al. (2010). We addressed these questions using genetic data generated using 13 polymorphic microsatellite loci from leopard faecal samples collected across different landscapes of India.

## **Methods**

### ***Research permissions and ethical considerations***

All required permissions for our field surveys and biological sampling were provided by the Forest Departments of Uttarakhand (Permit no: 90/5-6), Uttar Pradesh (Permit no: 1127/23-2-12(G) and 1891/23-2-12), and Bihar (Permit no: Wildlife-589). Due to the non-invasive nature of sampling, no ethical clearance was required for this study.

### ***Sampling***

To detect population structure and past population demography, it is important to obtain genetic samples from different leopard habitats all across the study area. In this study, we used leopard genetic data generated from non-invasive samples collected across the Indian subcontinent. We carried out extensive field surveys between 2016-2018 covering the North-Indian states of Uttarakhand, Uttar Pradesh, and Bihar, Terai-Arc landscape (TAL). During the surveys, we opportunistically collected a total of 778 fresh large carnivore faecal samples. These samples were collected from both inside (n=469) and outside (n=309) protected areas from different parts of this landscape. In the field, the samples were judged as large carnivores based on several physical characteristics such as scrape marks, tracks, faecal diameter, etc. All faecal samples were collected on wax paper and stored individually in sterile zip-lock bags and stored inside dry, dark boxes in the field for a maximum of a two-week period (Biswas et al., 2019). All samples were collected with GPS locations and were transferred to the laboratory and stored in -20°C freezers until further processing.

Further, we added north Indian samples collected in this study with leopard genetic data previously described in Mondol et al. (2014), representing mostly the Western Ghats and central Indian landscape. The data was earlier used in forensic analyses to

assign seized leopard samples to their potential geographic origins in India (Mondol et al., 2014). Out of the 173 individual leopards described in the earlier study, we removed data from related individuals and samples with insufficient data (n=30) and used the remaining 143 samples for analyses in this study. These samples were collected from the states of Kerala (n=5), Tamil Nadu (n=4), Karnataka (n=53), Andhra Pradesh (n=3), Madhya Pradesh (n=12), Maharashtra (n=46), Gujarat (n=2), Rajasthan (n=5), Himachal Pradesh (n=8), Jharkhand (n=1), West Bengal (n=2) and Assam (n=2), respectively. The sample locations across the Indian subcontinent used in the final analyses are provided in Figure 1.

#### ***DNA extraction, species, and individual identification***

For all field-collected faecal samples, DNA extraction was performed using protocols described in Biswas et al., (2019). For that, every frozen faeces was brought to room temperature followed by swabbing the top layer twice using sterile cotton swabs soaked in phosphate buffer saline (PBS) (HiMedia) was twice. The swabs were lysed at 56°C using 30 µl of Proteinase K (20 mg/ml) and 300 µl of ATL buffer (Qiagen Inc., Germany) followed by Qiagen DNeasy tissue DNA kit extraction protocol. Elution of DNA were performed twice in 100 µl of preheated 1X TE buffer. Extraction negatives were included for each set of samples to monitor contaminations.

Species identification was performed using leopard-specific multiplex PCR assay described in Mondol et al., (2014) and Maroju et al., (2016). PCR reactions were done in 10 µl volumes containing 3.5 µl multiplex buffer mix (Qiagen Inc., Germany), 4 µM BSA, 0.2 µM primer mix and 3 µl of scat DNA with conditions including initial denaturation (95°C for 15 min); 40 cycles of denaturation (94°C for 30 s), annealing ( $T_a$  for 30 s) and extension (72°C for 35 s); followed by a final extension (72°C for 10

min). Negative controls were included to monitor possible contamination. Leopard faeces were identified by viewing species-specific bands of 130 and 190 bp (Mondol et al., 2014) and 277 bp (Maroju et al., 2016) in 2% agarose gel.

For individual identification, we used the same panel of 13 microsatellite loci previously used in Mondol et al., (2014) (Table 1). To generate comparable data with the samples used from earlier study by Mondol et al., (2014) we employed stringent laboratory protocols. All PCR amplifications were performed in 10 µl volumes containing 5 µl Qiagen multiplex PCR buffer mix (QIAGEN Inc., Germany), 0.2 µM labelled forward primer (Applied Biosystems, USA), 0.2 µM unlabelled reverse primer, 4 µM BSA and 3 µl of the faecal DNA extract. The reactions were performed in an ABI thermocycler with conditions including initial denaturation (94°C for 15 min); 45 cycles of denaturation (94°C for 30 sec), annealing ( $T_a$  for 30 sec) and extension (72°C for 30 sec); followed by final extension (72°C for 30 min). Multiple primers were multiplexed to reduce cost and save DNA (Table 1). PCR negatives were incorporated in all reaction setups to monitor possible contamination. The PCR products were analysed using an automated ABI 3500XL Bioanalyzer with LIZ 500 size standard (Applied Biosystems, USA) and then alleles were scored with GENEMAPPER version 4.0 (Softgenetics Inc., USA). During data generation from field-collected samples, we used one reference sample (genotyped for all loci) from the earlier study for genotyping. As the entire new data was generated along with the reference sample and the alleles were scored along with the reference genotypes, the new data (allele scores) was comparable with the earlier data for analyses.

To ensure good quality multi-locus genotypes from faecal samples, we followed a modified multiple-tube approach in combination with quality index analyses (Miquel

et al., 2006) as described previously for leopards by Mondol et al., (2009, 2014). All faecal samples were amplified and genotyped four independent times for all the loci. For further analysis, samples that yielded identical genotypes for three independent amplifications (a quality index of 0.75 or higher for each locus) were deemed reliable; the remaining samples were excluded for further analysis.

### *Analysis*

For each locus, we calculated average amplification success as the percent positive PCR (Broquet & Petit 2004) after four repeats across all samples. We quantified allelic dropout and false allele rates manually as the number of dropouts or false alleles over the total number of amplifications, respectively (Broquet & Petit, 2004), as well as using MICROCHECKER v 2.2.3. (Oosterhout et al., 2004). The Allele dropout rate (ADO) is the ratio of the observed number of amplifications with loss of one allele to the number of positive amplifications of the heterozygous individuals. False allele frequency is calculated for both homozygous and heterozygous genotypes as the ratio of the number of amplifications having one or more false alleles at a specific locus and the total number of amplifications.

Post data quality assessment and finalization of consensus genotypes for all samples, we selected good quality data samples having at least nine or more loci (out of 13) for further analyses. We used the identity analysis module to identify identical genotypes (or recaptures) by comparing data from all samples implemented in program CERVUS (Kalinowski, Taper & Marshall, 2007). Further, all genetic recaptures were removed from the data set. We calculated the  $PID_{(sibs)}$  for all the unique individuals using GIMLET (Valiere, 2002). Further, alleles having less than 10% frequency across all amplified samples were rechecked. We determined Hardy Weinberg equilibrium and

linkage disequilibrium for all the loci using ARLEQUIN (Excoffier, Laval & Schneider, 2005). Finally, we select out related individuals in our samples to avoid the effects of related individuals in all analyses in program GENECLASS 2.0 (Piry et al., 2004).

To determine the leopard genetic structure across the Indian subcontinent, we used a Bayesian clustering approach implemented in program STRUCTURE (Pritchard et al., 2000, Falush et al., 2003). We performed 10 independent analyses for each K values ( $n=1-10$ ), with 450,000 iterations and a burn-in of 50,000 assuming correlated allele frequencies. We used STRUCTURE HARVESTER web version (Earl & vonHoldt, 2012) to determine optimal value of K. Subsequent summary statistics were calculated in ARLEQUIN 3.1 (Excoffier et al., 2005) and indices of overall genetic differentiation (pairwise  $F_{st}$ ) were estimated using GenAlEx version 6.5 (Peakall and Smouse 2012), dividing the leopard populations according to the STRUCTURE results across the Indian subcontinent. The divisions were based on Q-values (estimated proportions of ancestry) estimated in STRUCTURE, where we used  $Q > 0.75$  as threshold for individual assignment to a particular population (Mora et al., 2010). Additionally, a comparison of population differentiation (or  $G_{st}$ ) (Nei, 1987) between four leopard sub-populations was calculated in GenAlEx version 6.5 (Peakall and Smouse 2012).

### ***Demography analyses***

Demographic analyses were performed with different genetic subpopulations of leopards based on the results from STRUCTURE analyses. We used a combination of different approaches to detect leopard population demography. The first two qualitative approaches use summary statistics to detect population size changes,

whereas the quantitative approach is a likelihood-based Bayesian algorithm. We used summary statistic-based methods including the Ewens, Watterson, Cornuet and Luikart method implemented in the program BOTTLENECK (Cornuet & Luikart 1996), and the Garza-Williamson index or M ratio (Garza & Williamson 2001) implemented in program ARLEQUIN 3.1. The quantitative Bayesian approach was implemented in program MSVAR 1.3 (Storz & Beaumont 2002).

*a) The Ewens, Watterson, Cornuet and Luikart (EWCL) approach:*

This approach uses two summary statistics of the allele frequency spectrum, number of alleles ( $N_A$ ), and expected heterozygosity ( $H_e$ ) across different mutational models to detect the population size changes. Simulations are performed to obtain the expected distribution of  $H_e$  for a demographically stable population under three mutation models: infinite allele model (IAM), single stepwise model (SMM) and two-phase model (TPM) and these values are then compared to the real data values. This method detects a deviation from mutation-drift equilibrium and neutrality, suggesting selection, population growth, or decline. More importantly, consistent results from independent loci could be attributed to demographic events over selection. For simulations with TPM model, we used two different (5% and 30%) multi-step mutation events for leopards.

*b) The Garza-Williamson index/M ratio approach:*

This approach allows the detection of population decline using two summary statistics of the allele frequency spectrum, number of alleles ( $N_A$ ) and the allelic size range. The basic principle states that in a declining population, the expectation of the reduction of number of alleles is much higher than the reduction of allelic size range. Thus, the ratio

between the number of alleles and the allelic size range is expected to be smaller in populations undergoing recent decline than in equilibrium populations.

c) *The Storz and Beaumont approach:*

This approach is an extended version of Beaumont's approach (Beaumont 1999), which takes the assumption that a stable population of size  $N_1$  began to fluctuate (either increase or decrease)  $T$  generations ago, reaching its current size of  $N_0$ . Under the stepwise mutation model (SMM), this change in population size is expected to occur at an exponential scale at a rate of  $y=2N_0m$ , where  $m$  is the mutation rate per locus per generation. Further, this approach uses information in the full allelic distribution in a coalescent framework to estimate the posterior probability distribution, allowing quantification of effective population sizes  $N_0$  and  $N_1$  along with  $T$ , time, rather than their ratio (as in Beaumont 1999). In this approach, log normal prior distributions for  $N_0$ ,  $N_1$ ,  $T$  and  $\mu$  (mutation rate) are assumed. The mean and the standard deviations of these prior log normal distributions are drawn from prior (or hyperpriors) distributions. A Markov Chain Monte Carlo (MCMC) algorithm is used to generate samples from the posterior distribution of these parameters. We used wide uninformative priors to perform multiple runs for this approach. For minimal effect towards the posterior distribution variances for the prior distributions were kept large. A total number of 2 million iterations were performed for each run. We utilized a five-year generation period since the leopard is known to have a generation time of roughly 4-5 years (Dutta et al., 2012b).

***Estimation of leopard extinction probability***

To understand extinction probability across various biogeographic zones of India we analysed patterns and determinants of leopard occurrence as described in Karanth et al., (2009, 2010). We applied a grid-based approach to determine current distribution patterns for leopards, where the selection of grids was based on prior information of leopard presence. This involved collating presence-absence information from more than 100 Indian wildlife experts along with historical information of leopard presence involving hunting locations and other taxidermy and museum records. Each grid cell was an average of 2818 km<sup>2</sup> in size and we used data from 1229 grid cells covering 3,46,3322 km<sup>2</sup> area of the Indian subcontinent. To examine the influence of ecological and social covariates on leopard occupancy, we applied occupancy modelling in this study. We used a maximum likelihood approach for leopard occupancy in PRESENCE. V.2.0 program (Hines 2006). Covariates likely to influence leopard distribution modelled included presence and extent of protected area, land cover-land use characteristics, human cultural tolerance and population density. Data for protected areas was retrieved from the World Database on protected areas ([www.unep-wcmc.org](http://www.unep-wcmc.org)) and topographic maps. Land cover- land use data were derived from Global Land Cover Facility (2000) and further refined based on Roy et al., (2006) and Joshi et al., (2006). A human tolerance index that characterized different Indian states from most to least tolerant was developed based on knowledge about society-culture, law enforcement, hunting patterns and prior field experiences (for details see Karanth et al., 2009, 2010). We derived human population density data from LandScan Global Population Data 2000 ([www.ornl.gov/gist](http://www.ornl.gov/gist)). Based on existing information on species' ecology we predicted higher occupancy in protected areas, deciduous-grass-scrub land cover types, and lower occupancy in less tolerant states and highly populated areas

because of direct competition for food and space (Rangarajan 2001). We performed pair-wise correlation tests to screen variables for multicollinearity. The occupancy approach accounts for non-detection of species during surveys and the inability to survey some sites (see Karanth et al., 2009, 2010 for additional details). The probability of extinction was calculated as (1- probability of occurrence). We derived leopard extinction probabilities for three separate major landscapes (Western Ghats, Central India, and North India) as these regions strongly represented our genetic sampling. These extinction probabilities were compared to the genetically derived estimates.

## **Results**

### ***Individual identification of leopards from the north Indian landscape***

Of the 778 large carnivore faecal samples collected from TAL, we identified 195 faeces to be of leopard origin (25%) using species-specific PCR assays (Mondol et al., 2014; Maroju et al., 2016). In addition, 457 samples were ascertained to be of tiger (59%) and remaining 126 faecal samples did not produce any result (16%). We amplified 13 microsatellite loci panel on these 195 genetically confirmed leopard faecal samples, and after data validation through multiple repeats generated seven or more loci data from 65 faecal DNA. Subsequently, we identified 56 unique leopard individuals from the 65 samples, whereas nine individuals were ascertained as 'genetic recaptures'. The mean allelic dropout (ADO) rate was found to be 0.05 for these loci, whereas the mean false allele (FA) rate was 0.04. for all the 13 loci, indicating low genotyping error rates for this 13 loci panel. The success of amplification ranged between 41% to 100% from leopard faecal DNA. No loci were found to deviated from the Hardy-Weinberg equilibrium with no evidence of strong linkage disequilibrium

between any pair of loci. The cumulative  $PID_{sibs}$  and  $PID_{unbiased}$  values for leopard individuals were found to be  $3.91 \times 10^{-6}$  and  $2.73 \times 10^{-16}$ , respectively, indicating a strong statistical support for unambiguous individual identification. Summary statistics for these samples collected across the Terai-Arc landscape are provided in Table 1. We identified 26, 21, and nine unique leopard individuals from the states of Uttarakhand, Uttar Pradesh, and Bihar, respectively. As the data generated from north India is comparable to the earlier data, we added these 56 unique leopard data to 143 individual genotypes described in Mondol et al., (2014), and overall, 199 unique unrelated leopards were used in subsequent population structure, genetic variation, and demography analyses.

**Table 1:** Genetic diversity and genotyping error details for the leopard samples collected across Terai Arc landscape ( $n=56$ ) in this study. A total of 13 microsatellite loci were used. Data from these samples have been added to earlier leopard forensic data described in Mondol et al., (2014).

Locus	Repeat length	$N_A$	Allele size range	$H_E$	$H_o$	Null Allele	Allelic dropout	False allele	HWE	Reference
FCA230	2	16	44	0.87	0.69	0.18	0.001	0.005	Yes	Menotti-Raymond et al., (1999)
FCA309	2	17	42	0.85	0.70	0.22	0.004	0.004	Yes	Menotti-Raymond et al., (1999)
FCA232	2	15	36	0.83	0.68	0.19	0.007	0.013	Yes	Menotti-Raymond et al., (1999)
FCA090	2	16	34	0.87	0.66	0.30	0.007	0.002	Yes	Menotti-Raymond et al., (1999)
FCA052	2	14	32	0.85	0.77	0.19	0.004	0.006	Yes	Menotti-Raymond et al., (1999)
FCA672	2	20	40	0.87	0.74	0.05	0.0	0.001	Yes	Menotti-Raymond et al., (1999)
FCA279	2	16	30	0.88	0.76	0.08	0.001	0.003	Yes	Menotti-Raymond et al., (1999)
FCA126	2	16	32	0.89	0.70	0.36	0.004	0.001	Yes	Menotti-Raymond et al., (1999)
msFCA391	4	10	36	0.86	0.64	0.19	0.009	0.007	Yes	Mondol et al., (2011)

<b>msHDZ 170</b>	2	13	42	0.83	0.53	0.30	0.002	0.002	Yes	Mondol et al., (2011)
<b>msFCA 441</b>	4	12	52	0.82	0.52	0.23	0.006	0.003	Yes	Mondol et al., (2011)
<b>msFCA 506</b>	2	19	56	0.89	0.69	0.25	0.008	0	Yes	Mondol et al., (2011)
<b>msFCA 453</b>	4	7	32	0.68	0.61	0.25	0.006	0.007	Yes	Mondol et al., (2011)
<b>Mean (SD)</b>		<b>14.69 (3.41)</b>	<b>39.0 8 (7.71)</b>	<b>0.84 (0.05)</b>	<b>0.67 (0.07)</b>	<b>0.21</b>	<b>0.005</b>	<b>0.004</b>		

$N_A$  - No. of alleles,  $H_E$  – Expected heterozygosity,  $H_O$  – Observed heterozygosity, HWE – Hardy-Weinberg Equilibrium

### *Leopard population structure and genetic variation across India*

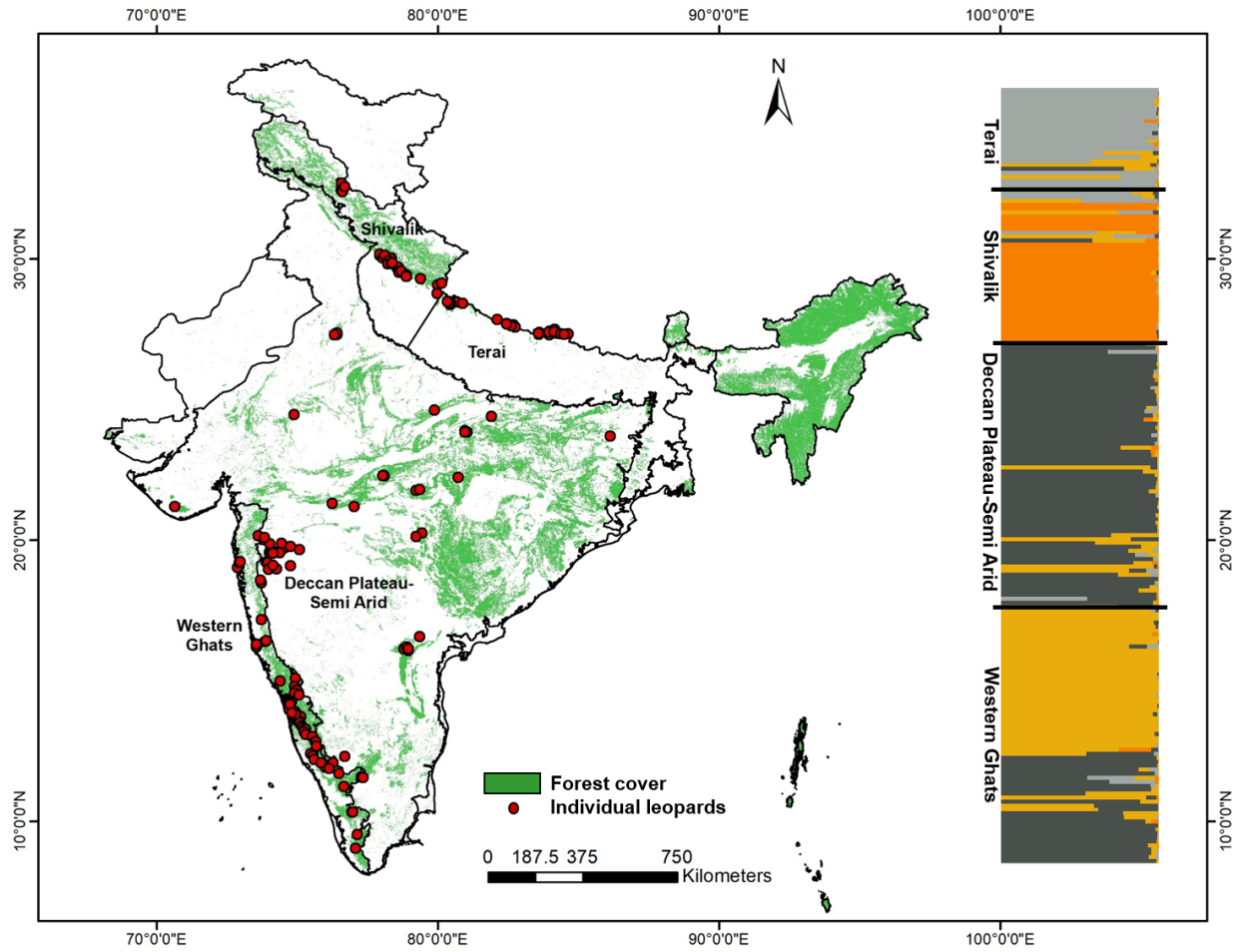
Our sampling strategy targeted country-wise leopard populations to assess population structure and genetic variation. From 199 final unique leopard genotypes we removed four samples representing the eastern and northeast India (n=2 from the states of West Bengal and Assam each, respectively) from further analyses as they represented inadequate sampling from these regions. Genetic clustering analysis using 13 microsatellite data from the remaining 195 wild leopard individuals showed four distinct genetic subpopulations (K=4), as presented in Figure 1. Majority of the samples showed respective group-specific ancestry, with Western Ghats samples representing the first group (henceforth WG, n=65), the Deccan Plateau-Semi Arid region forming the second cluster (henceforth DP-SA, n=66), the samples from Shivalik region covering parts of Himalaya and western parts of upper Gangetic plains making the third group (henceforth SR, n=38), and finally samples from the Terai region covering eastern part of upper and western part of the lower Gangetic Plains samples forming the fourth cluster (henceforth TR, n=26), respectively (Figure 1). However, small number of samples (n=18) distributed among the four subpopulations

showed mixed ancestry. Subsequent analyses revealed that these leopard subpopulations are genetically differentiated ( $F_{st}$  and  $G_{st}$ ) at low, but significant levels (Table 2) for all four populations. The  $F_{st}$  value among these populations ranged between 0.028-0.115, whereas the  $G_{st}$  value range between 0.023-0.104 (Table 2).

**Table 2:** Genetic differentiation (pairwise  $F_{st}$  and  $G_{st}$ ) for four leopard subpopulations in the Indian subcontinent. The upper diagonal presents the pairwise  $G_{st}$  values whereas the lower diagonal presents the pairwise  $F_{st}$  values.

	Western Ghats	Deccan Plateau-Semi Arid	Shivalik	Terai
<b>Western Ghats</b>	--	0.023*	0.039*	0.091*
<b>Deccan Plateau - Semi Arid</b>	0.028*	--	0.045*	0.104*
<b>Shivalik</b>	0.048*	0.05*	--	0.073*
<b>Terai</b>	0.103*	0.115*	0.089*	--

\* p value =0.001



**Figure 1.** Genetic sampling and leopard population structure across the Indian subcontinent with forest cover map and leopard sampling locations were used in this study. The map also shows the inferred bio-geographic leopard habitats based on genetic structure, as found in this study, and corroborative leopard genetic clusters indicated by program STRUCTURE (based on 13 microsatellite loci).

Analyses with 13 microsatellite loci among the four genetic subpopulations showed a higher mean number of alleles ( $NA_{WG}=11.77$  (S.D. 3.85),  $NA_{DP-SA}=10.46$  (S.D. 2.71)) and observed heterozygosity ( $H_{oWG}=0.81$  (S.D. 0.08),  $H_{oDP-SA}=0.8$  (S.D. 0.08)) in Western Ghats and Deccan Plateau-Semi Arid subpopulations, when compared with samples from Shivalik and Terai region subpopulations ( $NA_{SR}=08.46$  (S.D. 2.41),  $NA_{TR}=05.00$  (S.D. 1.84) and  $H_{oSR}=0.40$  (S.D. 0.14),  $H_{oTR}=0.36$  (S.D. 0.28),

respectively) (see Table 3 for details). However, the allelic size range values were similar in all populations (Table 3).

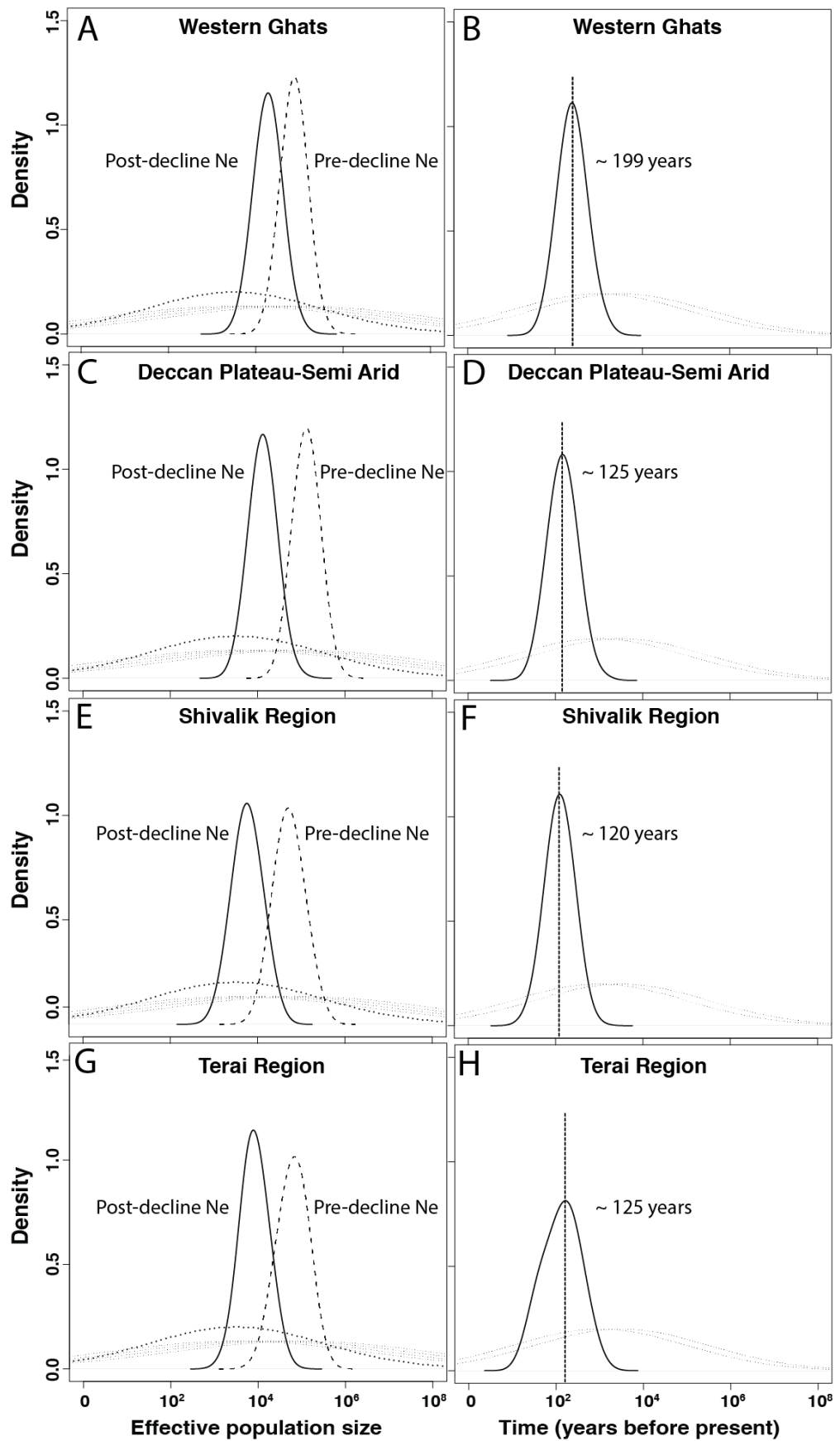
**Table 3:** Subpopulation-wise summary statistics (based on 13 microsatellite loci) for Indian leopards

	Western Ghats (n=65)				Deccan Plateau-Semi Arid (n=66)				Shivalik (n=38)				Terai (n=26)			
Locus	N <sub>A</sub>	AS R	H <sub>E</sub>	H <sub>O</sub>	N <sub>A</sub>	ASR	H <sub>E</sub>	H <sub>O</sub>	N <sub>A</sub>	ASR	H <sub>E</sub>	H <sub>O</sub>	N <sub>A</sub>	AS R	H <sub>E</sub>	H <sub>O</sub>
<b>FCA230</b>	13	36	0.8 8	0.8 6	10	22	0.7 8	0.8 0	08	24	0.8 3	0.2 3	05	26	0.52	0.6 5
<b>FCA309</b>	17	42	0.9 0	0.8 7	11	30	0.7 8	0.8 1	08	18	0.8 1	0.4 6	06	10	0.82	0.3 2
<b>FCA232</b>	13	36	0.8 5	0.8 4	09	18	0.6 8	0.7 2	09	26	0.7 8	0.4 2	07	26	0.78	0.4 6
<b>FCA090</b>	14	30	0.8 5	0.8 4	08	18	0.7 8	0.8 7	09	30	0.8 6	0.3 6	02	10	0.47	0.0 0
<b>FCA052</b>	12	32	0.8 4	0.8 9	11	22	0.8 2	0.8 4	08	20	0.8 3	0.4 8	06	22	0.84	0.4 3
<b>FCA672</b>	19	40	0.9 0	0.8 9	10	26	0.8 2	0.7 5	06	16	0.6 5	0.5 0	07	20	0.64	0.7 7
<b>FCA279</b>	11	26	0.8 1	0.7 5	14	26	0.8 7	0.8 1	15	28	0.9 0	0.6 7	06	18	0.78	0.9 2
<b>FCA126</b>	14	26	0.8 7	0.8 3	13	30	0.8 8	0.8 9	09	22	0.7 6	0.1 8	04	12	0.11	0.7 4
<b>msFCA391</b>	07	28	0.8 3	0.6 7	08	32	0.8 1	0.8 1	07	24	0.7 8	0.5 7	07	32	0.75	0.1 3
<b>msHDZ170</b>	09	20	0.8 4	0.7 9	10	22	0.7 5	0.8 8	10	36	0.7 6	0.1 6	02	02	0.29	0.0
<b>msFCA441</b>	07	36	0.7 5	0.7 9	08	28	0.6 5	0.5 5	08	40	0.8 2	0.3 8	05	28	0.66	0.4 0
<b>msFCA506</b>	12	32	0.8 6	0.9 0	17	56	0.8 3	0.8 2	09	24	0.8 3	0.3 3	06	22	0.79	0.4 0
<b>msFCA453</b>	05	20	0.6 3	0.6 7	07	32	0.6 9	0.8 0	04	20	0.6 5	0.3 6	02	16	0.43	0.1 4
<b>Mean (SD)</b>	<b>11.77 (3.85)</b>	<b>31.08 (6.69)</b>	<b>0.83 (0.07)</b>	<b>0.81 (0.08)</b>	<b>10.46 (2.71)</b>	<b>27.85 (9.36)</b>	<b>0.78 (0.07)</b>	<b>0.80 (0.08)</b>	<b>08.46 (2.41)</b>	<b>25.23 (6.64)</b>	<b>0.79 (0.07)</b>	<b>0.40 (0.14)</b>	<b>05.00 (1.84)</b>	<b>18.77 (8.21)</b>	<b>0.65 (0.17)</b>	<b>0.66 (0.28)</b>

$N_A$  - No. of alleles, ASR- Allelic size range,  $H_E$  – Expected heterozygosity,  $H_O$  – Observed heterozygosity.

### ***Detection of demographic change***

We used the microsatellite data to investigate signals of demographic changes in each of the four leopard genetic subpopulations across the subcontinent. Both of the qualitative approaches, the EWCL and the M-ratio methods indicate signatures of population bottleneck. The EWCL approach implemented in the program BOTTLENECK shows 8-10 loci with heterozygote excess based on the different mutation models used, implying a loss of rare alleles through population decline for all four subpopulations. Similarly, the M-ratio approach also shows a low ratio between number of alleles ( $N_A$ ) and the allelic size range in all four populations (M-ratio<sub>WG</sub>- 0.37 (S.D. 0.09); M-ratio<sub>DP-SA</sub>- 0.38 (S.D. 0.09); M-ratio<sub>SR</sub>- 0.33 (S.D. 0.09); M-ratio<sub>TR</sub>- 0.29 (S.D. 0.15)), indicating signatures of population bottleneck. However, both of these approaches cannot quantify the extent and timing of the bottleneck events. We used the Storz and Beaumont approach for the quantification and dating of any such events through coalescent simulations. Models with exponential decline scenarios show consistently that the posterior distributions for  $\log(N_0)$  is always lower than  $\log(N_1)$  for all four subpopulations, indicating population decline for leopards across the subcontinent (Table 4 and Figure 2). Further quantification revealed that the current effective size is varying low (12-25%) than the historical effective size, with Western Ghats, Deccan Plateau-Semi Arid, Shivalik, and Terai regions losing approximately 75%, 90%, 90% and 88% of their leopard population, respectively (Table 4 and Figure 2).



**Figure 2.** Demographic history of Indian leopards (*Panthera pardus fusca*). A, C, E, and G show the posterior distributions for leopard population size changes for different subpopulations, based on 13 microsatellite loci using the Storz and Beaumont approach. The dashed and solid lines represent posterior distributions of ancestral and present effective population sizes. The priors are represented by the dotted line. B, D, F, and H represent the posterior distribution for the time since the leopard population decline started for corresponding subpopulations. The priors are shown by the dotted lines.

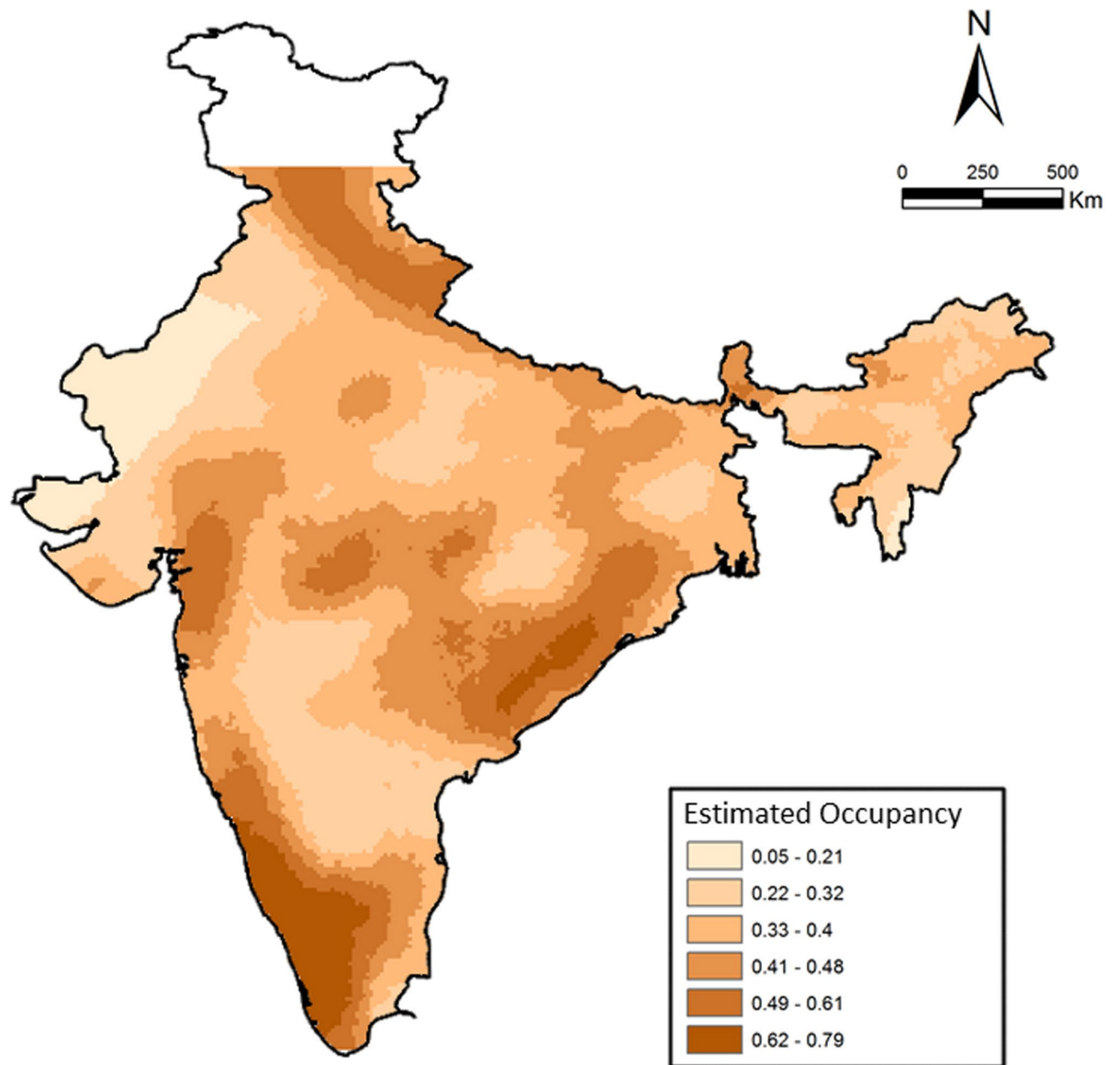
Our method also enabled us to date the population collapse by providing a posterior distribution for the timing of decline. Our analyses revealed distributions that suggested recent time of declines in all four populations of leopards (Table 4, Figure 2). The north Indian subpopulations (Shivalik and Terai) and the Deccan Plateau-Semi Arid population showed the most recent decline occurred about 120-125 years before present, respectively. However, the Western Ghats population indicated potential decline around 200 years ago (Table 4 and Figure 2).

**Table 4:** Comparison of different demographic decline analyses results for different subpopulations of leopards across India.

Method	Analysis type	Demographic signal				
		Model	Western Ghats	Deccan Plateau-Semi Arid	Shivalik	Terai
Bottleneck	Qualitative	IAM	Heterozygosity excess for 13 loci	Heterozygosity excess for 10 loci	Heterozygosity excess for 12 loci	Heterozygosity excess for 11 loci
		SMM	Heterozygosity excess for 01 loci	Heterozygosity excess for 02 loci	Heterozygosity excess for 06 loci	Heterozygosity excess for 08 loci
		TPM	Heterozygosity excess for 07 loci	Heterozygosity excess for 07 loci	Heterozygosity excess for 09 loci	Heterozygosity excess for 10 loci
M ratio			0.37 (SD 0.09)	0.38 (SD 0.09)	0.33 (SD 0.09)	0.29 (SD 0.09)
Storz-Beaumont method	Quantitative		Decline- 75% Time- ~200 years ago	Decline- 90% Time- ~125 years ago	Decline- 90% Time- ~125 years ago	Decline- 88% Time- ~125 years ago

### ***Leopard occurrence and distribution***

We examined the factors influencing leopard distribution at a countrywide scale, where the top ranked model incorporating 28 covariates suggested a wide distribution of habitat types (described in Karanth et al., 2009, 2010). The model also indicated a positive influence of protected areas and negative influence of higher human population densities and higher cultural tolerance of people (details in Karanth et al., 2009). Areas with cultivated land, barren areas, deciduous forests, and rural-urban were strongly associated with higher leopard occurrence. Naive estimated occupancy was 0.52, whereas model estimated probability of occupancy was significantly higher at 0.68, suggesting that leopards are still widely distributed (Figure 3) in India compared to most other large mammals (as suggested in Karanth et al., (2010)). When compared among the overall three major sub-regions (North India (NI), Deccan Plateau-Semi Arid and Western Ghats), we find that average estimated occurrence was lowest in the North India ( $\Psi_{NI}=0.63$ , Range: 0.05-1.00, Standard error=0.01, Number of cells=384) compared to Western Ghats ( $\Psi_{WG}=0.83$ , Range: 0.23-1.00, Standard error=0.02, Number of cells=90) and Deccan Plateau-Semi Arid ( $\Psi_{DP-SA}=0.79$ , Range:0.25-1.00, Standard error=0.005, Number of cells=818). Overall, average estimated  $\Psi$  was 0.74 (Standard error=0.006, Number of cells=1292).



**Figure 3.** *Patterns of leopard occurrence in India based on the analysis of questionnaire surveys. The map shows a gradient of estimated cell-wise occupancy probabilities created through spatial kriging.*

## Discussion

To our knowledge, this is likely the first and most thorough sampling-based study conducted at the national level on the demographic trends and genetics of the leopard population in the Indian subcontinent. Except the eastern and northeast Indian landscape, where our sampling intensity was less all other regions are well covered in

this study. Our genetic analyses with microsatellite data collected across the subcontinent reveal four genetic subpopulations of leopards in India: The Western Ghats, Deccan Plateau-Semi Arid landscape, hill region of north India (Shivalik) and Terai or flat region of north India. While there was some amount of mixed genetic signal across different genetic subpopulations, they were separated as different clusters (Figure 1). These genetic clusters mostly correspond to respective biogeographic zones of India, with the Western Ghats and a combination of Deccan Plateau-Semi Arid forming two clusters, whereas the north Indian clusters of Shivalik and Terai are parts of the Himalayan and Gangetic Plains zones, respectively. This is possibly due to the species distribution across various habitat types in different biogeographic zones across the Indian subcontinent. For example, an earlier study has reported differences in leopard occupancy in ‘Bhabar’ habitats in the foothills of Shivalik (high abundance) and flat Terai region (low abundance) due to the presence of socially dominant tigers and the absence of rugged terrain to escape of leopards (Johnsingh et al., 2004). Such habitat heterogeneities might have resulted in developing genetic differences across these landscapes.

Overall, these four subpopulations were genetically differentiated by low, but significant  $F_{st}$  and  $G_{st}$  values across all comparisons ( $F_{st}$  and  $G_{st}$  values ranging from 0.028-0.115 and 0.023-0.104, respectively, see Table 2). Previous studies on tigers (Mondol et al., 2009, 2013, Kolipakam et al., 2019, subcontinent scale), as well as leopards (Dutta et al., 2012b, central Indian landscape), suggested long-distance movement as a potential cause for low genetic differentiation between populations. Leopards are more widely distributed than other sympatric large carnivores (tiger, lion) in the Indian subcontinent due to their general adaptability and wider diet spectrum

(Seidensticker et al., 1990, Edgaonkar & Chellam 1998, Athreya et al., 2010), and disperse long distances (Sunquist 1983, Bailey 1993) often through human habitats. In addition, human-leopard conflict-driven translocation is common in many parts of India (Athreya et al., 2010). Together, natural dispersal abilities and ‘human mediated gene flow’ because of translocation might be responsible for the low genetic differentiation among leopard subpopulations across the subcontinent. Earlier work in central Indian landscape (Dutta et al., 2012b) suggested a reduction in gene flow at recent times due to habitat destruction, but our study did not focus to answer such questions. Future studies should focus on using historical samples (e.g. museum skins, bones etc.) to assess any possible change in gene flow among leopard populations (For e.g. see Martinez-Cruz et al., 2007, Valdiosera et al., 2008, Lorenzen et al., 2011, Mondol et al., 2013) at subpopulation levels across the country.

Our demography analyses with genetic data across all four genetic subpopulations indicate a strong decline in leopard population size. Results with both qualitative (bottleneck and M-ratio approach) as well as quantitative (Storz and Beaumont approach) analyses revealed strong, but varying signals of population decline in all four subpopulations (Table 4). The Deccan Plateau-Semi Arid, Shivalik, and Terai subpopulations show a 90%, 90%, and 88% decline in population size, respectively, whereas the Western Ghats population shows relatively less (75%) decline in population size (Table 4). This pattern is possible as the Western Ghats still retains possibly the largest continuous forested landscape with multiple interconnected protected areas, whereas the other regions have a lot of anthropogenic activities, thus affecting leopard populations living in them. Further, the ecological data-based occupancy analysis showed extinction probabilities of 0.37, 0.21, and 0.17 for North

India, Deccan Plateau-Semi Arid, and Western Ghats landscape, respectively (Table 4). This is not surprising as throughout their distribution leopards are closely associated with the human population, making them vulnerable to conflict and poaching (Gavashelishvili & Lukarevskiy 2008, Athreya et al., 2010, Balme et al., 2010). While there is a discrepancy between the magnitudes of decline based on genetic and ecological models, such pattern may be because the ecological methods are more spatial, and inference is based on how much area leopards occupied in the past and how this has changed. However, if densities of leopards were high in the past loss of even small habitat areas could result in the loss of many individuals. Since no quantitative comparisons for leopard density between the Western Ghats, Deccan Plateau-Semi Arid and North India are currently available, we cannot conclusively infer the former, but further research should investigate leopard densities and their temporal changes across the country. Finally, this decline pattern also roughly corroborates with 83-87% leopard range loss in Asia, indicating that habitat loss is possibly leading to population decline.

The magnitude of decline for leopards found in this study contrasts to some of the earlier leopard studies in the subcontinent (for e.g. ecological work by Harihar et al., 2011, and genetic work by Dutta et al., 2012a) and Africa (Spong et al., 2000b), which suggest stable or increasing local leopard population trends. This is certainly possible as many of these studies were conducted inside protected areas, where leopard population dynamics depend on presence/absence of other large carnivores (tiger, dhole etc.) and other ecological factors. However, only 11% of Indian leopard distribution is within the protected area network (Jacobson et al., 2016), and our sampling at subcontinent scale is thus probably indicating the decline patterns at a

much larger scale. Nevertheless, this pattern of population decline is consistent with many other endangered species in the Asian region (for e.g. tiger-98% decline, Mondol et al., 2009; giant panda-90% decline, Zhu et al., 2010; orangutan-95% decline, Goossens et al., 2006; red panda-98% decline, Hu et al., 2011; Prezwalski's gazelle-99% decline, Yang and Jiang 2011 etc.) as well as top carnivore species across the globe (for e.g. Finnish wolf-92% decline, Aspi et al., 2006; Otter-75% decline, Hajkova et al., 2007; Golden eagle-94% decline, Bourke et al., 2010; African wild dog-70% decline, Marsden et al., 2012; Fisher-90% decline, Tucker et al., 2012 etc.). Our results pointed out another important finding is the recent decline for all the leopard subpopulations. Our results suggest median leopard decline timing between 120-200 years across four genetic subpopulations (Table 4). Except Western Ghats (decline timing of ~200 years), all other subpopulations indicate much recent population decline (Central India-Deccan Plateau ~125 years, Shivalik ~120 years and Terai ~125 years). When compared with other sympatric, endangered species in the subcontinent (for e.g. tiger decline ~200 years ago; Mondol et al., 2009) or in the region (for e.g. Orangutan- ~210 years, Goossens et al., 2006; Giant panda- ~250 years, Zhu et al., 2010) this still seems to be much recent event. Other wide-ranging carnivores across the globe (for e.g. European wolves Aspi et al., 2006; African wild dog- Marsden et al., 2012; Eurasian badgers- Franz et al., 2014 etc.) too faced a much longer decline period than leopards. One plausible explanation could be the recent increase in leopard-human conflict (Athreya et al., 2010) and poaching intensity due to large demand for leopard body parts in the illegal wildlife markets (Raza et al., 2012, WPSI 2014). Historically, major leopard-hunting events were recorded across the Indian subcontinent during Mughal times (about 500-600 years ago), followed by

colonial British bounty-hunting rule between 1850-1920 (Rangarajan 2006). However, large-scale landscape modification and fragmentation by human during the last century (central India- Rangarajan 1999, north India- Rangarajan 2006), coupled with poaching and conflict has possibly resulted in much recent loss of leopard populations across the country. Apart from sporadic information, we lack comprehensive data, both at historical as well as modern scales to investigate the true causes behind such patterns of differential population decline timing. For e.g., Dutta et al., (2012b) showed that during the last three centuries, severe changes in landscape characteristics (Settlement, villages, wild lands, human density) have occurred in the central Indian leopard habitats. However, we lack information on hunting and conflict levels in these regions. Future efforts should generate this important information to get an idea of the scenarios leading to such strong decline in a wide-ranging species like the leopard. Finally, it is important to point out that in this study we have only explored relatively simple decline scenarios, and future studies should evaluate more detailed, computationally intensive demographic analyses with genome-wide molecular markers (For e.g. see Frantz et al., 2014, Nater et al., 2015) for better understanding of complex decline scenarios. Additionally, more SNPs would provide clearer patterns on the genetic variation and evolutionary history of the species and underlying processes behind such patterns (see Armstrong et al., 2021).

The study's results also highlight the fact that, despite a severe decline (Table 4) and small, but significant population structure (Figure 1B, Table 2) leopards still retain high genetic variation in the Indian subcontinent. We found that leopard genetic variation across four genetic subpopulations is similar and comparable to Africa (Spong et al., 2000b, Uphyrkina et al., 2001), and higher than Arabian (Ilani 1981,

Perez et al., 2006) and Amur leopards (Uphyrkina et al., 2001, 2002, Sugimoto et al., 2014). The higher levels of variation among Indian leopards could be attributed to still relatively large population size, high pre-bottleneck genetic variation, and potential historical gene flow across large landscapes.

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**Chapter 3: - Evaluating patterns of leopard genetic relatedness under different tiger densities in Rajaji Tiger Reserve, Uttarakhand**

## **4.1 Introduction**

Top predators are critical for the shaping the trophic cascades by prey predator dynamics (Terborgh et al., 2001, Ripple et al., 2014). However, their range overlap leads to direct or indirect interactions among them leading to intraguild competition (Karanth and Sunquist 1995, Creel et al., 2008, Droge et al., 2017, Newsome et al., 2017, Ramesh et al., 2017). Increasing conservation efforts in India have led to a substantial rise in populations of big cats (tigers and leopards) in the last decade (Jhala et al., 2014, Jhala et al., 2018, Qureshi et al., 2022). This increase along with shrinking habitats has led to the areas having variable population densities for these big cats (tigers and leopards). Both sympatric species have significant overlap among them that further leads to intraguild competition (Karanth and Sunquist 1995). In the given scenario, leopards being sympatric to tigers survive through spatio-temporal avoidance, changes in prey preferences (Harihar et al., 2011, Rathore 2015, Krishna 2021), or displacement (Harihar et al., 2011, Ramesh et al., 2012). This often leads to physical, behavioural, and physiological consequences on the subordinate (McDougal 1988, Odden et al., 2010, Malviya et al., 2018, Patel et al., 2023). Further, they change their social and spatial patterns in order to maximize their fitness with resources and minimise interspecific competition (Mitchell & Powell 2012). The sociobiology of these solitary carnivores is determined by their territorial behaviour, availability of prey, and kinship (Elbroch et al., 2016). The kinship hypothesis suggest that tolerance

and cooperation with related ones and this inclusive fitness increases continuation of genetic lineage (Hamilton 1964).

Leopards are one of the solitary carnivores and have complex social structure (Sunquist & Sunquist, 2002). Their social organisation comprises varying overlaps of resident males, resident females, and floaters (males/females) (Bailey 1993, Sandell 1989). Their females are philopatric in nature while males are known to disperse (Bailey 1993, Sandell 1989). The social dynamics and mating system of the species lead to the spatial pattern of genetic relatedness that ultimately plays a significant contribution to evolutionary processes (Fattebert et al., 2016). To study the impact of dominant-subordinate interactions on the local population dynamics of subordinate species like leopards we used genetic relatedness as proxy approach. In this study, we addressed this question within Rajaji Tiger Reserve (RTR) with subsequent impact of tiger competition. Rajaji Tiger Reserve has one of the highest densities of leopard  $16.90 \pm 1.44 / 100 \text{ sq.km}$  (Jhala et al., 2021). Both areas are connected by a narrow corridor i.e. Chilla-Motichur which is adversely affected by high human pressure (Johnsingh et al., 2004, Harihar et al., 2009, Biswas et al., 2022). As of now, the eastern part with estimates of  $8.0 \pm 1.40 / 100 \text{ sq. km}$  has higher tiger density compared to the western part with estimates of  $0.28 \pm 0.23 / 100 \text{ sq. km}$  (Jhala et al., 2021). The tiger population in the western part of RTR has almost no connectivity with the eastern part (Jhala et al., 2014, Jhala et al., 2018). Both WRTR and ERTR have a prey density of  $41.22 \pm 6.65 / 100 \text{ sq.km}$  and  $39.23 \pm 4.76 / 100 \text{ sq.km}$  respectively (Harihar et al., 2020). This situation makes the RTR a unique natural experimental setup to assess the impacts of competition from a dominant predator (tiger) on the subordinate co-predator (leopard), where the western part of RTR (WRTR, with almost no tigers) provide one

extreme situation and the eastern part of RTR (ERTR, with high tiger density) provide the other extreme end. In this chapter, we used genetic relatedness (within each population) as a proxy to assess potential differences in leopard social dynamics against a tiger competition gradient between these two areas. We hypothesise that WRTR, having almost no tiger presence will have higher frequencies of related individuals (parent-offspring, full-siblings, half-siblings etc.) compared to the ERTR.

In this chapter, we assessed within population dynamics of leopards in the Rajaji National Park. More specifically we addressed 1) pattern of relatedness among leopards and 2) pattern of genetic relatedness vary with varying density of tiger? We addressed these questions using genetic data generated for 13 microsatellites from faecal samples.

## **4.2 Methods**

### **4.2.1 *Research permissions & ethical considerations***

All required permissions for our field surveys and biological sampling were provided by the Uttarakhand Forest Department (Permit no: 90/5-6). No ethical clearance was required as non-invasive samples were used for the study.

### ***Study area***

This study was conducted in Rajaji Tiger Reserve (RTR) which is a part of Rajaji–Corbett Tiger Conservation Unit (RCTCU) and one of the recognised globally important tiger TCU (Dinerstein et al., 1997). It is spread over an area of 820.42 sq.km and along the Shivalik foothills lying between the lesser Himalayas and the upper Gangetic plains. The forest is broadly classified as the Northern Tropical Moist deciduous Forest type (Champion and Seth, 2005). The river Ganga flows through the

RTR for about 20 km. dividing the park into two parts i.e. the Western part of RTR (570 Km<sup>2</sup>) and the Eastern part of RTR (250 Km<sup>2</sup>). Both areas are connected by a narrow corridor i.e. Chilla-Motichur which is adversely affected by high human pressure (Johnsingh et al., 2004, Harihar et al., 2009, Biswas et al., 2022). The tiger population has almost no connectivity between the western part of RTR with the eastern part (Jhala et al., 2014, Jhala et al., 2020). Both WRTR and ERTR have a prey density of  $41.22 \pm 6.65 / 100 \text{ sq.km}$  and  $39.23 \pm 4.76 / 100 \text{ sq.km}$  respectively (Harihar et al., 2020). This situation makes the RTR a unique natural experimental setup to assess the impacts of competition from a dominant predator (tiger) on the subordinate co-predator (leopard), where the western part of RTR (WRTR, with almost no tigers) provide one extreme situation and the eastern part of RTR (ERTR, with high tiger density) provide the other extreme end.

#### **4.2.2 Sampling, DNA extraction and Individual Identification**

To understand the local population dynamics, it is important to have good sample coverage across the study area. We conducted an extensive field survey across Rajaji National Park from 2015 to 2020. We foot-surveyed all existing trails covering the entire area to collect faecal samples. The number of trails walked in a particular area was decided based on existing knowledge of leopard presence by the frontline staff members of the sampling team. In ERTR, buffer areas such Haridwar Forest Division (HFD) and Lansdowne Forest Division (LFD) are also incorporated.

In the field, the samples were judged as large carnivores based on several physical characteristics such as scrape marks, tracks, faecal diameter etc. All faecal samples were collected in wax paper and stored individually in sterile zip-lock bags and stored

inside dry, dark boxes in the field for a maximum of two weeks period (Biswas et al., 2019). All samples were collected with GPS locations and were transferred to the laboratory and stored in  $-20^{\circ}\text{C}$  freezers until further processing. All the field-collected samples were subjected to DNA extraction followed by species identification and individual identification as mentioned in the second Chapter. These samples were compiled with already identified utilised samples in first ( $n=8$ ) and second chapter ( $n=21$ ) with an overlap of two samples. These compiled samples were further utilised for downstream analyses.

#### **4.2.3 Individual Identification analysis**

We selected only those samples with good-quality data for at least nine or more loci (out of 13) for further analyses. We used the identity analysis module implemented in the program CERVUS (Kalinowski, Taper & Marshall, 2007) to identify identical genotypes (or recaptures) by comparing data from all samples. All genetic recaptures were removed from the data set. GIMLET (Valiere, 2002) was used to calculate the PID (sibs) for all the unique individuals. Following this, any allele having less than 10% frequency across all amplified samples was rechecked for allele confirmation.

#### **4.2.4 Relatedness Analysis**

To understand the relationships among the individual leopards, we utilised a sibship inference analysis using COLONY (Jones et al., 2010), a maximum-likelihood approach to test for the occurrence of siblings in individual leopards. Now, the relationships were further categorised into full sibs and half sibs based on the sibship values. The related individuals and their relationships were compiled for leopards of

both WRTR and ERTR complex separately to understand their local population dynamics across the tiger density gradient.

#### **4.2.5 Social Network Analysis**

We visualised the relatedness within the individuals in the form of a network using the software GEPHI 0.9.2 (Bastian et al., 2009). Here, we represented the individuals as nodes of the networks, and the magnitude of the relatedness was used as the strength of the edges. We computed the network density, modularity, and mean as well as node (individual) level estimates of degree, weighted degree, and Eigen centrality to understand the relative importance of the nodes/individual leopards to the network. We have also visualised static network structure based on relatedness to understand the probable impact of a dominant predator.

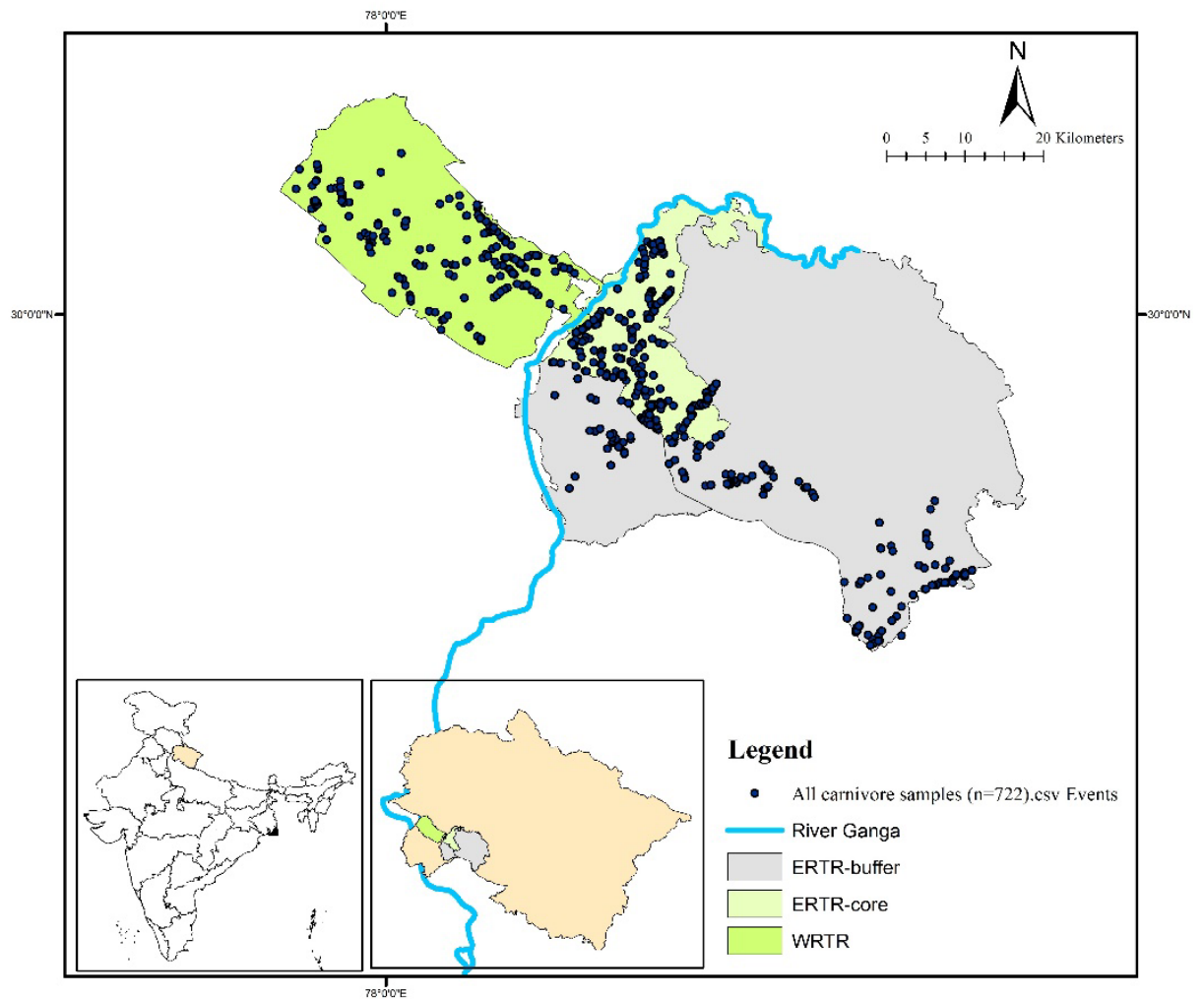
Further, to understand the spatial pattern of the genetic relatedness and estimate the extent of difference among Western and Eastern parts of Rajaji, GENALEX 6.5 (Peakall & Smouse 2006) was utilised. Here, distance classes are based on the pairwise geographic distance among the leopard samples and performed with 10,000 permutations and 1,000 bootstrap iterations to estimate the 95% confidence intervals for each distance class.

### **4.3 Result**

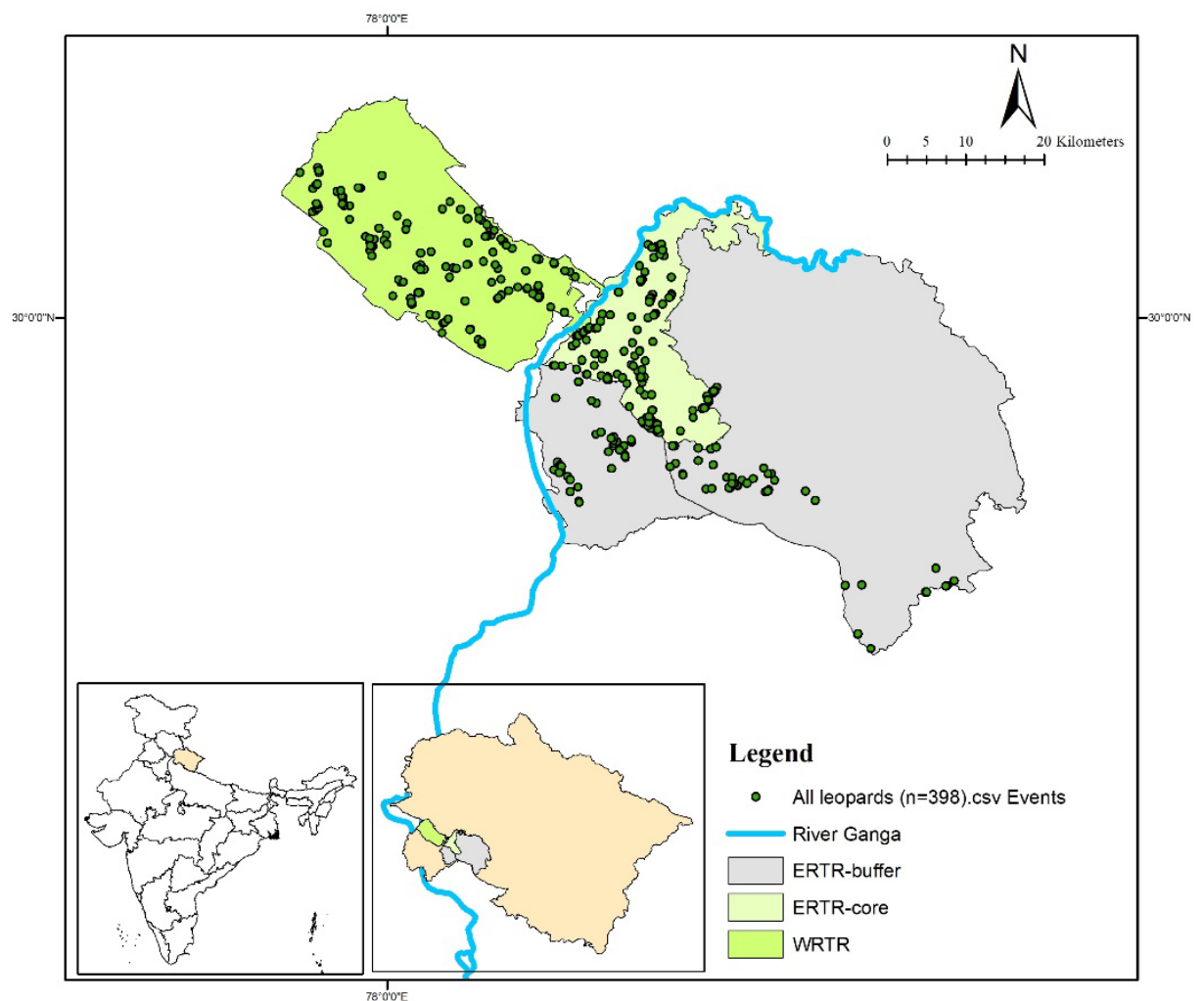
#### **4.3.1 Sampling Efforts and Individual Identification**

With a total sampling effort of ~800 Km<sup>2</sup>, we collected a total of 722 large carnivore faecal samples (Figure 1) from the study area. Following species identification, we confirmed 398 leopard (Figure 2) and 242 tiger samples (with an overall success rate of 88.64%). For individual identification (n=332) samples were utilised after removing

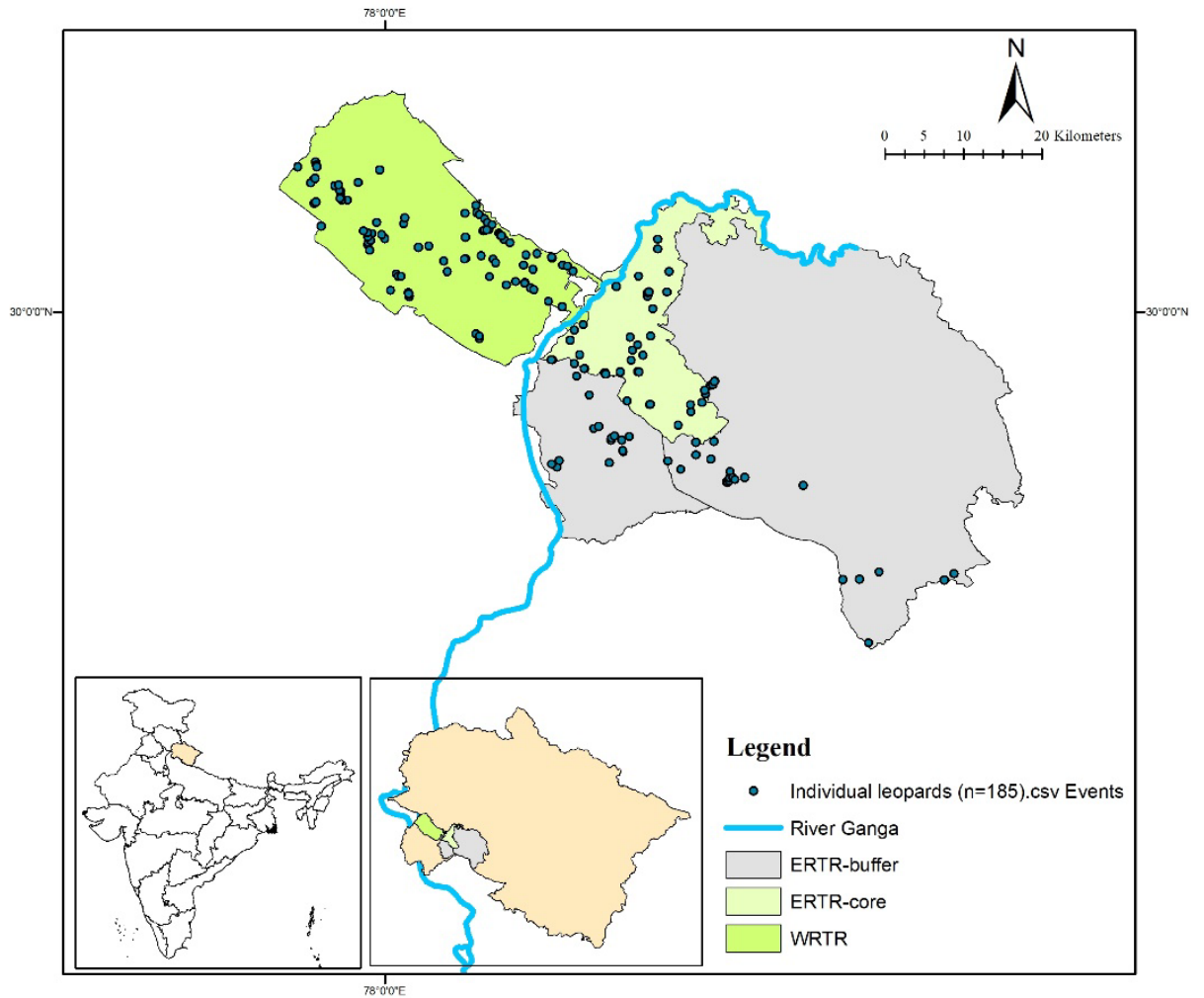
old samples. We generated >9 loci data for 206 samples which were subsequently utilised for the downstream analyses. A total of 158 unique leopard individuals (Figure 3) were identified after removing recaptures (n=13) and data deficit samples (n=8). These samples were further compiled with individual leopard genotypes (n=27) from earlier chapters. The cumulative probability of identity values for 185 (158+27) individual leopards was  $1.09 \times 10^{-13}$  (PID<sub>unbiased</sub>) and  $1.5 \times 10^{-5}$  (PID<sub>sibs</sub>), providing high probability of unambiguous. A total of 97 individual leopards were confirmed for the Western part of Rajaji and 88 individual leopards were confirmed for the Eastern part of Rajaji.



**Figure 4.1.** *The figure shows the distribution of carnivore faecal samples collected across RTR (n=722).*



**Figure 4.2.** The figure shows the distribution of confirmed leopard samples across RTR ( $n=398$ ).

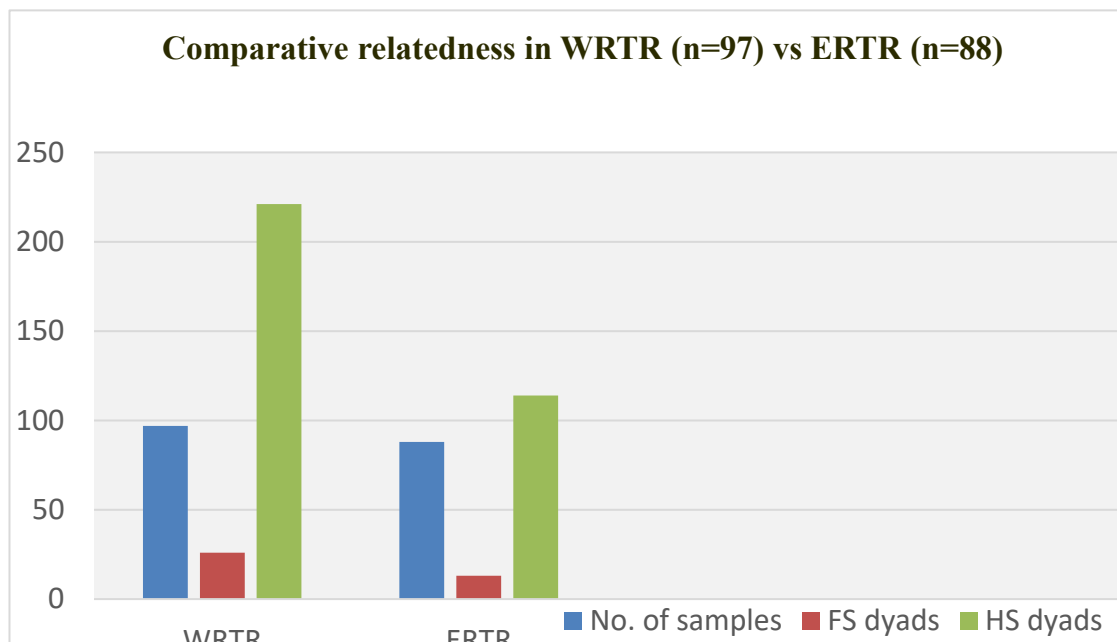


**Figure 3.** The figure shows the distribution of individual leopards across RTR (n=185).

#### 4.3.2 Comparative Relatedness Analysis

The sibship analyses from both the parts of Rajaji showed more related dyads in the Western part compared to the Eastern Rajaji. In Western Rajaji, we got 26 dyads for full sibs (FS) and 221 dyads for half sibs (HS). In Eastern Rajaji, we got 13 dyads for full sibs (FS) and 114 dyads for half sibs (HS) (Figure 4). To further visualise the social network of these FS and HS, overall same weights were utilised for all the

interactions and WR provided us more connected dyads, triads, and tetrads as well compared to ER. In ER dyadic and triadic interaction were observed as well.

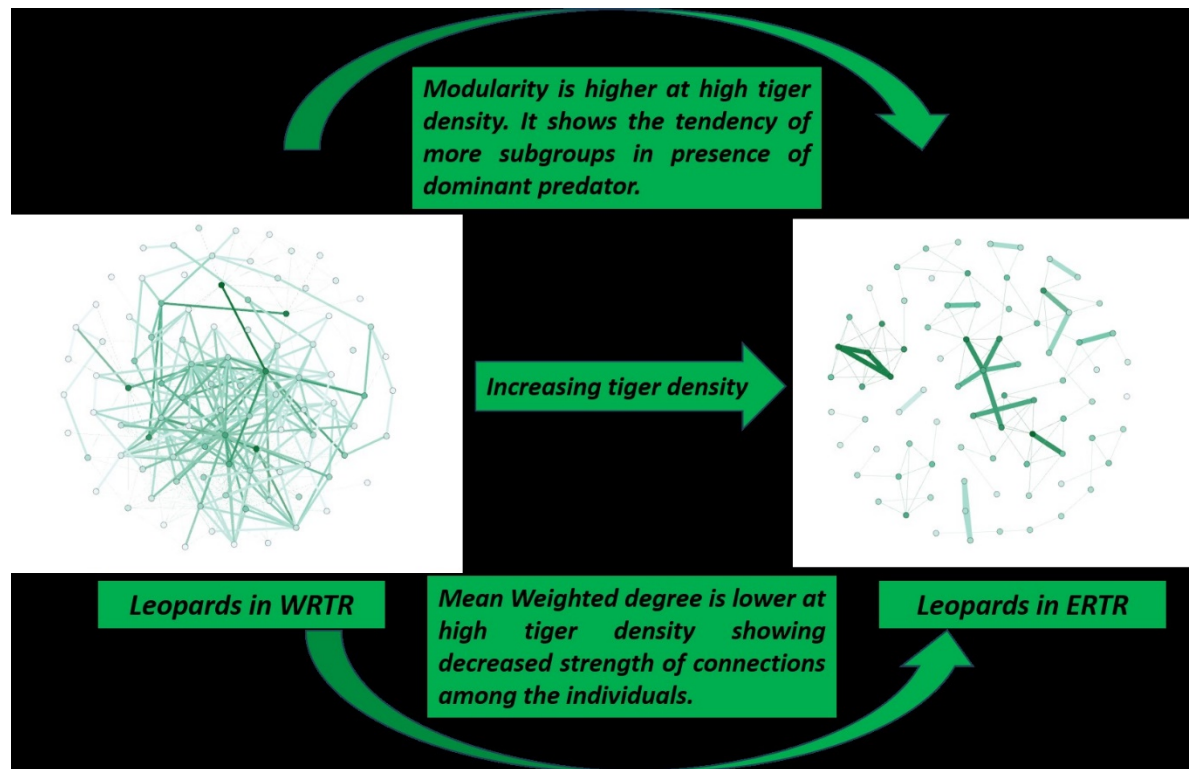


**Figure 4.4.** The graph is a comparative representation of the number of individuals and their related dyads both full sib dyads (FS) and half sib dyads (HS) in WRTR and ERTR.

#### 4.3.3 Social network analysis (SNA)

The estimates of network density, modularity and mean as well as node (individual) level assessments of degree, weighted degree, and eigen-centrality are provided in the table (Table 1). The average degree, average weighted degree, and average clustering coefficient were higher in low tiger density area showing more relatedness in Western part of Rajaji while in Eastern Rajaji the related values were quite low (Table 1). Eigenvector centrality (estimate of nodes with high connectivity) is much higher in Western Rajaji compared to Eastern Rajaji. The factors such as modularity, number of communities, and connected components were higher in high tiger density areas,

showing the tendency of leopards to form more subgroups in Eastern Rajaji compared to Western Rajaji (Figure 5).



**Figure 4.5.** The figure represents the snapshot of social network where each individual (node) is represented by green dots and the relationships (edges) by lines. Here the darker the lines more the extent of relationships.

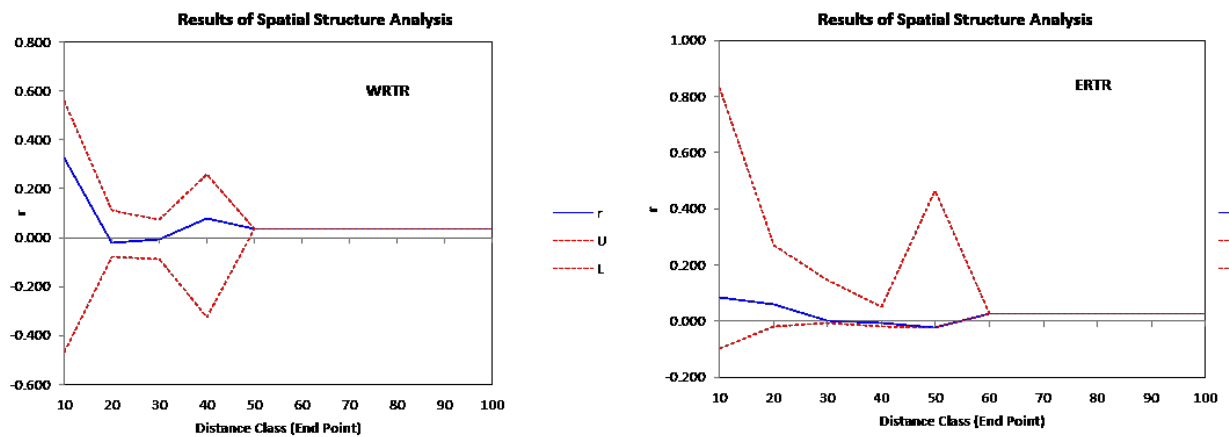
**Table 4. 1.** The table indicates various estimates of Social Network Analysis (SNA) for both WRTR & ERTR.

Statistics	WRTR	ERTR
Average Degree	9.979	3.125
Avg. Weighted Degree	3.495	0.906
Avg. Clustering Coefficient	0.413	0.558
Eigenvector centrality	0.0036	0.00012
No. of communities	7	14
Community detection/modularity	0.356	0.793
Connected components	1	7

Higher value indicates strength of connections among individuals

Higher value indicates tendency of subgrouping among individuals

The spatial autocorrelation analysis for leopards from both WRTR and ERTR was found non-significant (Figure 6).



**Figure 4.6.** The figure represented here is a correlogram showing a spatial autocorrelation coefficient ( $r$ ) across distance classes (each 10 km) for leopards in WRTR and ERTR.

## 4.4 Discussion

In this chapter, we integrate non-invasive genetic methods into social network analysis (SNA) to understand the impact of the dominant-subordinate dynamics. We attempted to understand these using relatedness as proxy approach in an elusive, endangered, and solitary species (leopard). Here, these type of patterns are otherwise impossible to decipher with traditional approaches such as camera trap and radiotelemetry (Carter et al., 2023). The spatial distribution of kin groups decides patterns of genetic relatedness in an area. These patterns further decide the local population structure for the species, ultimately leading to the evolutionary patterns for the species. From our results, we inferred that the null hypothesis is accepted that states that there is an impact of varying density of tigers on the pattern of leopard genetic relatedness. Our results have shown that a low tiger density area (WRTR) leopard has high genetic relatedness as compared to a high tiger density area (ERTR) showing the probable impact of the dominant predator.

Our results reveal that the leopard shows a complex social structure in WRTR where they form interconnected social units (dyads and triads) which are possibly due to the philopatric females and relaxed territorial behaviour of males towards kins (Macdonald, 1983, Fattebert et al., 2015, 2016, Verschueren et al., 2023). It is known that carnivore societies are majorly regulated by the vegetation, prey density, the density of the conspecific, and presence/absence of the dominant species (Macdonald, 1983). In this study, all conditions being similar to both the WRTR and ERTR we found out that the high density of the dominant predator probably played a critical role in deciding the extent of relatedness for the subordinate one. The presence of large predator controls the population of the subordinate and their absence may lead to a

significant increase in the population of later. Our results indicate that they seem to be forming more sub-groups in ERTR as compared to WRTR. Considering WRTR, where there is almost no pressure from the dominant predators more individuals are found to be more closely linked as compared to the ERTR. Therefore, in the Western part, the leopard population seems to have more stepping stones in terms of genetic connectivity as compared to the Eastern part. Further, the individuals with more connected nodes can be identified as key/central individuals and therefore their removal can hamper/disturb the whole dynamics of the species in that area (Fattebert et al., 2016). The removal of central individuals has often resulted in more intraspecific competition among the subadults to establish a territory in the vacant area (Fattebert et al., 2016). Therefore, this study in combination with other approaches such as camera trap and telemetry can help us identify such hotspots to propose site-specific management.

Also, in terms of spatial autocorrelation, we did not find any significant pattern for the changes in relatedness. This pattern has also been evident in many studies where the pattern of relatedness is not spatially oriented supporting the random distribution of kins (Bartolommei et al., 2016). Also, one of the limitations of the study is that identification of the males and females could not be achieved in this study, possibly females could have shown positive spatial autocorrelation due to their philopatric nature while males as they disperse could have shown negative spatial autocorrelation (Gour et al., 2013, Ratnayeke et al., 2002, Fattebert et al., 2016). However, some of the studies, show no correlation between patterns of genetic relatedness and their spatial orientation (Schmidt et al., 2016).

Earlier studies on intraguild competition majorly suggested the importance of landscape connectivity and prey availability in direct relation to the strength of the competition (Karanth et al., 2017, Harvey et al., 2017, Kumar et al., 2019). In recent times, with successful tiger recovery in many areas, the tiger density and demography has depressed leopard demography (Kumar et al., 2019, Yadav et al., 2024). Therefore, despite the species-centric conservation focus, the efforts should focus on the sustenance of the entire guild as a whole.

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## **Key finding and conservation implications**

## 5.1 Conclusion

The leopard, *Panthera pardus*, is a member of family *Felidae* and is most widely distributed and highly adaptable persisting in most of its historic range (Daniel, 1993, Jacobson et al., 2016). The species has lost ~70% of its historical distribution due to past climatic fluctuations, increasing anthropogenic pressure, and threats like habitat loss, poaching, and human-wildlife conflict (Jacobson et al., 2016, Pajimans et al., 2018, Ripple et al., 2017). These factors have majorly affected the current distribution, genetic variation, and demography patterns of the species (Hewitt 2000). Therefore, understanding phylogeographic patterns and demographic history is essential for planning effective management plans for the long-term survival of the species (Pajimans et al., 2018). However, such information is still lacking for the elusive and endangered species like leopards. Therefore, the main goal of the thesis was to investigate the phylogeography and demographic history of Indian leopards, which was explored in Chapter 1 & Chapter 2 with the help of mitochondrial and microsatellite markers, respectively. Within the Indian landscape, leopards share habitats with the tigers that are behaviourally dominant to them (Qureshi et al., 2022, Srivathsa et al., 2023). To understand the impacts of dominant-subordinate interactions, we looked into the leopard population dynamics in the presence of variable tiger densities at a local scale. Here, we combined population genetics and social network analysis approaches and used genetic relatedness as a proxy approach to understanding these dynamics.

In the thesis, Chapter 1 and 2 describes the information on the genetic variation of extant Indian leopards using both mitochondrial and microsatellite DNA data. Our

results revealed moderate diversity in microsatellite DNA which is comparable to the African leopards (Ropiquet et al., 2015, Morris et al., 2022) and high diversity for mtDNA (Anco et al., 2017, Morris et al., 2022, 2024) (see Table 1). This high genetic variation may be attributed to high genetic variation before undergoing decline, large population size, and historical gene flow across their range. Earlier available genetic studies on Indian leopards have their limitation in geographic scope and have less focus on maternal genetic variation (for details see Table 1)

A genomic DNA-based study by Pajimans et al., (2018) revealed Asian leopards being monophyletic across the nuclear genomes due to high population differentiation, stronger isolation by distance, and low heterozygosity compared to African leopards. In the current scenario, where all other Asian leopards (except the Indian subspecies) are facing a severe reduction in their population across their range (Stein et al., 2022), our results indicate that Indian leopards are doing much better in terms of genetic variability among the Asian subspecies of leopards and therefore deserves strong conservation attention.

The first two chapters also describe the phylogeography of Indian leopards based on mitochondrial (Chapter 1) and microsatellite (Chapter 2). The results based on the mitochondrial DNA data in Chapter 1 revealed a weak phylogeographic structure. Contrasting to that Chapter 2 revealed the presence of four genetic subpopulations of Indian leopards but with a low genetic differentiation value ( $F_{st}$ ) with mixed genetic signals among the subpopulations. This may be attributed to the natural dispersal ability of leopards to disperse longer distances (Sunquist 1983, Bailey 1993). The subpopulations correspond majorly to their landscapes including Western Ghats,

Central India Landscape, Shivalik, and Terai. However, the impact of changes in terrain, structural features of the landscape, and anthropogenic disturbance lead to a reduction in the gene flow in the Terai arc landscape (TAL). Also, the discordance between Chapter 1 and Chapter 2 results may be due to differences in the resolution of chosen markers in each chapter. This pattern could be due to low effective population size (one-fourth), no lineage sorting, low mutation of mitochondrial DNA as compared to the microsatellite DNA, and being more subject to genetic drift.

**Table 5.1.** Comparison of available population genetics estimates of Indian and African leopard subspecies with current study.

Subspecies	Study area	H <sub>o</sub> /H <sub>E</sub>	Haplotype diversity	Nucleotide diversity	Citation
<i>P.p.pardus</i>	Africa	0.66/0.69	N.A. (28 haplotypes, NADH5, CytB)	N.A.	Ropiquet et al., 2015
<i>P.p.pardus</i>	African continent (Historical samples as well)	N.A.	0.841 (30 haplotypes, NADH5)	0.004	Anco et al., 2017
<i>P.p.pardus</i>	Mpumalanga province, Africa	0.75/0.73	0.94 (33 haplotypes NADH5, CytB)	0.127	Morris et al., 2023
<i>P.p.pardus</i>	African continent	N.A.	0.085-0.89 (47 haplotypes, NADH5)	0.016	Morris et al., 2024
<i>P.p.fusca</i>	Maharashtra, India	0.57/0.66	N.A.	N.A.	Mondol et al., 2009
<i>P.p.fusca</i>	Central India, India	0.74/0.84	N.A.	N.A.	Dutta et al., 2013
<i>P.p.fusca</i>	Western Ghats, India	0.70/0.85	N.A.	N.A.	Mondol et al., 2014a
<i>P.p.fusca</i>	India (reference sample)	0.60/0.85	N.A.	N.A.	Mondol et al., 2014b
<i>P.p.fusca</i>	Central Indian landscape, India	0.61/0.78	N.A.	N.A.	Thatte et al., 2019
<i>P.p.fusca</i>	Western Himalayas, India	0.60/0.79	0.56 (3 haplotypes, Control region)	0.001	Singh et al., 2022
<i>P.p.fusca</i>	Himalayas, Shivalik hills, Central India, Western Ghats, part North East	0.67/0.84	0.913 (46 haplotypes, NADH4, NADH5, CytB)	0.00343	This study

Chapters 1 & 2 also investigated *demographic history* and revealed a decline in the leopard populations around the Last Glacial Maxima (LGM) and these results can be corroborated by the collapse of other large mammals due to extreme climatic fluctuation around that time (Hewitt 2000, Mahmood et al., 2021). The results are similar to the African subspecies where there was a population decline around the same time i.e. 20 kya (thousand years ago) (Morris et al., 2024). In the same timeframe, the extreme climatic conditions led to the extinction of European leopards (Pajimans et al., 2018). Further, our study also suggested the population recovery of the leopards in the mid-Holocene due to favourable climate conditions (Mahmood et al., 2021). Our results based on microsatellite data revealed signals of around 75-90% population decline in all four subpopulations. The decline was relatively less for the Western Ghats as compared to the other populations. The extent of the decline for these subpopulations is corroborated by extinction probabilities deduced by the ecological data-based occupancy analysis. These patterns were congruent with leopard range loss (around 83-87%) in Asia, indicating that habitat loss, prey depletion, conflict, and poaching lead to population decline (Raza et al., 2012, Selvan et al., 2014, Jacobson et al., 2016). The extent and timing of population decline among leopards are similar to other endangered Asian species for example tiger (Mondol et al., 2009), giant panda (Zhu et al., 2010), orangutan (Goossens et al., 2006), and red panda (Hu et al., 2011). Based on our result, the timing of the decline was between 120-200 years across all four genetic subpopulations. This may be a result of major leopard hunting around the Mughal era (500-600 years ago) followed by bounty hunting during the British colonial period (100-170 years ago) (Rangarajan 2006). The demographic history revealed by the microsatellite markers detected comparatively recent population

changes compared to the mitochondrial DNA. This is probably due to the low effective population size ( $N_e$ ) and slower mutation rate of mitochondrial DNA markers as compared to the microsatellite markers that led to mtDNA coalescing back to much older times than the microsatellite ones. For further studies, historical museum samples can be studied to understand the extent of change in the genetic variability among past and extant populations.

Chapter 3 deals with the integration of genetics and social network theory to understand the local population dynamics of leopards. RTR has one of the highest leopard densities with similar habitat, prey density, and varying density of tigers across the western and eastern parts as separated by the Ganges. Thus, RTR is a unique natural experimental setup to assess the impacts of competition from a dominant predator (tiger) on the subordinate co-predator (leopard), where the western part of RTR (WRTR, with almost no tigers) provides one extreme situation and the eastern part of RTR (ERTR, with high tiger density) provide the other extreme end. Leopards are known to show spatiotemporal segregation, changes in prey preferences, and move to less-quality habitats to avoid tigers (Karanth and Sunquist 1995, Harihar *et al.*, 2011, Rathore, 2015, Krishna, 2021, Patel *et al.*, 2023). This results in physical, behavioural, and physiological impacts on subordinates. We found high genetic relatedness at a low tiger density area (WRTR) as compared to a high tiger density area (ERTR) showing the probable impact of the dominant predator. This study suggests tiger presence affects the leopard's social dynamics.

## **5.2 Conservation Implications**

1. This thesis provides baseline information on Indian leopard genetic variation based on mitochondrial DNA and nuclear DNA. The results indicate that the

subspecies retain a moderate genetic diversity within India, which is comparable to the other flourishing species of leopard i.e. the African subspecies. The thesis provides major management units i.e. Western Ghats, Central Indian Landscape, Shivalik, and Terai that need major conservation attention.

2. This thesis suggests the need of more conservation attention towards the linear landscapes as they are more sensitive towards habitat fragmentation leading to discontinuity of the gene flow of the species that has already been established for tigers as well in Terai Arc Landscape (TAL).
3. Our results indicate the impacts of climatic fluctuations as well as human-mediated population loss of the species. The current trends toward population decline are driven by poaching and human-wildlife conflict (as reported by *the Wildlife Protection Society of India (WPSI)*). This suggests that population decline in a widely distributed adaptable species like the leopard.
4. This thesis underlies the probable impact of dominant species on the local dynamics of the subordinate. This thesis suggests that despite planning single-species-centric conservation plans, we should focus on a multispecies conservation protocol to conserve the entire guild.

Overall, this thesis provides comprehensive information on genetic variation, population structure, demographic changes, and extinction probability of leopards. Due to their widespread presence, species like as leopards are comparatively easier to study than other sympatric carnivores like tigers; yet, research on the size, trend, and dynamics of leopard populations is scarce, especially outside of protected regions. Actually, because of their wide geographic range, leopard populations are thought to

be stable, as evidenced by their current IUCN Red List status of ‘Near threatened’ (Shivakumar et al., 2023). However, genetic data revealed decline in the population as a result of climatic fluctuations in the past around the LGM followed by population recovery around the mid-Holocene and further impact of the Anthropocene has resulted in a drastic population decline across different habitats in the Indian subcontinent.

In addition, our study showed a decline in the population of a species that is ubiquitously distributed and often perceived as abundant locally: the leopard. This suggests that leopards in India should receive the same level of conservation attention as sympatric tigers. Our results strengthen the relevance of non-invasive genetic approaches for elusive species like the leopard. We also suggest that thorough ecological research on leopard populations at the landscape level is essential for planning future conservation efforts. This study also highlights the significance of conducting comparable research on other widely distributed animals, since populations of other species, such as leopards, may be declining, particularly in light of the Anthropocene.

Further, we suggest mitigation of human-leopard conflict with leopard awareness programs and incentives among the locals, especially the ones living in close vicinity of forested areas. For future studies, high-resolution molecular markers can be utilised to explore different aspects of species biology, such as population connectivity, population dynamics, and adaptation. In future studies, population dynamics along with genetic studies can be compiled with the conventional monitoring approaches such as telemetry and camera traps to better understand the local factors deciding its social organisation.

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## **ANNEXURE- I**

### **Permits and Ethical Clearance:**

All required permissions for our field surveys and biological sampling were provided by the Forest Departments of Uttarakhand (Permit no: 90/5-6), Uttar Pradesh (Permit no: 1127/23-2-12(G) and 1891/23-2-12) and Bihar (Permit no: Wildlife-589). Due to the non-invasive nature of sampling, no ethical clearance was required for this study.

## ANNEXURE- II

### Conference certificates:





## Certificate of Participation



This is to certify that

SUPRIYA BHATT

has actively participated in

14<sup>th</sup> Uttarakhand State Science and Technology Congress 2019-2020

27<sup>th</sup> - 29<sup>th</sup> February, 2020

presented a research paper (Oral/Poster) entitled

PHYLOGEOGRAPHY AND DEMOGRAPHIC HISTORY OF

INDIAN LEOPARD, PANTHERA PARDUS FUSCA

under the discipline

ZOOLOGY

at Uttarakhand Council for Science and Technology, Vigyan Dham, Dehradun.

**Dr Ashutosh Mishra**  
Organizing Secretary  
14<sup>th</sup> USSTC  
Council for Science and Technology  
Uttarakhand

**Dr Rajendra Dobhal FNASc**  
Chairman - 14<sup>th</sup> USSTC  
Director General  
Council for Science and Technology  
Uttarakhand



# CERTIFICATE Of Participation

Presented to

Supriya Bhatt

for an Oral Presentation  
on the topic

Mitochondrial phylogeography and demographic history of  
leopard (*Panthera pardus fusca*) India

at the 50 Years of Project Tiger  
& First Indian Conservation Conference,  
Mysuru, Karnataka, India on 10th April 2023



**Bilal Habib**

Coordinator ICCON-2023  
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**Virendra Tiwari**

Director,  
Wildlife Institute of India

**S.P. Yadav**

ADG - Project Tiger & Elephant Ministry of  
Environment, Forest & Climate Change



भारतीय वन्यजीव संस्थान  
Wildlife Institute of India







# Genetic analyses reveal population structure and recent decline in leopards (*Panthera pardus fusca*) across the Indian subcontinent

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## ABSTRACT

**Background.** Large carnivores maintain the stability and functioning of ecosystems. Currently, many carnivore species face declining population sizes due to natural and anthropogenic pressures. The leopard, *Panthera pardus*, is probably the most widely distributed and highly adaptable large felid globally, still persisting in most of its historic range. However, we lack subspecies-level data on country or regional scale on population trends, as ecological monitoring approaches are difficult to apply on such wide-ranging species. We used genetic data from leopards sampled across the Indian subcontinent to investigate population structure and patterns of demographic decline.

**Methods.** We collected faecal samples from the Terai-Arc landscape of northern India and identified 56 unique individuals using a panel of 13 microsatellite markers. We merged this data with already available 143 leopard individuals and assessed genetic structure at country scale. Subsequently, we investigated the demographic history of each identified subpopulations and compared genetic decline analyses with countrywide local extinction probabilities.

**Results.** Our genetic analyses revealed four distinct subpopulations corresponding to Western Ghats, Deccan Plateau-Semi Arid, Shivalik and Terai region of the north Indian landscape, each with high genetic variation. Coalescent simulations with microsatellite loci revealed a possibly human-induced 75–90% population decline between ~120–200 years ago across India. Population-specific estimates of genetic decline are in concordance with ecological estimates of local extinction probabilities in these subpopulations obtained from occupancy modeling of the historic and current distribution of leopards in India.

**Conclusions.** Our results confirm the population decline of a widely distributed, adaptable large carnivore. We re-iterate the relevance of indirect genetic methods for such species in conjunction with occupancy assessment and recommend that detailed, landscape-level ecological studies on leopard populations are critical to future conservation efforts. Our approaches and inference are relevant to other widely distributed, seemingly unaffected carnivores such as the leopard.

Submitted 9 September 2019

Accepted 29 December 2019

Published 4 February 2020

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Academic editor

Jan Schipper

Additional Information and  
Declarations can be found on  
page 18

DOI 10.7717/peerj.8482

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## OPEN ACCESS

**Subjects** Biogeography, Conservation Biology, Ecology, Evolutionary Studies, Genetics

**Keywords** Endangered species, Population subdivision, Genetic variation, Leopard phylogeography, Demographic history, Carnivore conservation

## INTRODUCTION

Large carnivores are critical to ecosystem structure and functioning and their absence can lead to significant changes in them and may affect trophic dynamics, resulting in cascading effects ([Estes et al., 2011](#); [Ripple et al., 2014](#)). Growing natural and anthropogenic pressures in the form of climate change, habitat loss and prey depletion, wildlife trade and human-wildlife conflicts are pushing large carnivores into ever-shrinking habitat islands and severely exacerbating their endangered status, and in some cases extinction ([Schipper et al., 2008](#); [Karanth et al., 2010](#)). Recent assessments of the conservation status indicate alarming rates of population decline for many carnivores at a global scale. Specifically, the families *Felidae*, *Canidae* and *Ursidae* are under severe threat across the globe ([Wolf & Ripple, 2017](#)).

The leopard (*Panthera pardus*) represents the most widely distributed and adaptable member of the family *Felidae*. The historical range of leopards spanned across nearly 35,000,000 km<sup>2</sup> area covering all of sub-Saharan and north Africa, the Middle East and Asia Minor, South and Southeast Asia, and the Russian Far East ([Uphyrkina et al., 2001](#); [Jacobson et al., 2016](#)). However, their current distribution and numbers have significantly decreased across the range due to habitat loss, prey depletion, conflict and poaching over the last century ([Jacobson et al., 2016](#)). Recent meta-analyses of leopard status and distribution suggest 48–67% range loss for the species in Africa and 83–87% in Asia ([Jacobson et al., 2016](#)), making them among the top ten large carnivore species most-affected by range contraction ([Wolf & Ripple, 2017](#)). This has resulted in changing the species status from ‘Near Threatened’ to ‘Vulnerable’ by IUCN ([Stein et al., 2016](#)). Despite continuously decreasing numbers and range, their ubiquitous presence across human habitations leads to misconceptions regarding their current abundance.

Among all the subspecies, the Indian leopard (*P. p. fusca*) retains the largest population size and range outside Africa ([Jacobson et al., 2016](#)). In the Indian subcontinent poaching and conflict are major threats to leopard populations ([Athreya et al., 2010](#); [Raza et al., 2012](#)). Leopards also frequently occur outside protected areas, increasing their vulnerability to conflict with humans ([Athreya et al., 2010](#); [Naha, Sathyakumar & Rawat, 2018](#)). Unfortunately, there is still a paucity of information on their population and demography at regional and global scales. Few earlier studies have assessed the subspecies status ([Asad et al., 2019](#); [Farhadinia et al., 2015](#); [Paijmans et al., 2018](#)) and genetic diversity ([Uphyrkina et al., 2001](#); [Dutta et al., 2013](#); [Mondol et al., 2014](#)) of leopards in the Asian region including India, but comprehensive data is lacking. Much of our knowledge on leopard ecology and demography in the Indian subcontinent come from location-specific studies ([Karanth & Sunquist, 2000](#); [Chauhan et al., 2005](#); [Harihar, Pandav & Goyal, 2009](#); [Wang & Macdonald, 2009](#); [Kalle et al., 2011](#); [Grant, 2012](#); [Mondal et al., 2012](#); [Dutta et al., 2012](#); [Dutta et al., 2013](#); [Thapa et al., 2014](#); [Borah et al., 2014](#); [Selvan et al., 2014](#);

*Pawar et al., 2019*). In India, the latest estimate of leopards in the forested habitats of 14 tiger-inhabiting states is 7910 (SE 6566-9181) (*Jhala, Qureshi & Gopal, 2015*). As leopards do survive in highly human populated and modified areas (*Athreya et al., 2010*) this estimate is likely to be minimal and incomplete. Further, recent studies in the Indian subcontinent provide contradictory patterns of local population trends. For example, historical records and occupancy estimation models based on ecological data and field observations by *Karanth et al. (2010)* estimated high local extinction probabilities of leopards across the subcontinent, and *Athreya et al. (2010)* reported higher rates of recent conflict incidences and related mortality at local scales. Other ecological (*Harihar, Pandav & Goyal, 2011*) as well as population genetic studies of demographic history (*Dutta et al., 2013*) suggest stable or increased leopard populations at a landscape scale. However, lack of detailed, systematic field data makes it difficult to generate accurate population estimates as well as demographic patterns at landscape scales.

In this paper, we used faecal samples to assess leopard genetic variation, population structure and demographic history in the Indian subcontinent. More specifically, we investigated (1) extent of genetic variation in leopard that persists across the Indian subcontinent; (2) population structure of leopards at country scale; (3) the demographic history of leopards by assessing recent changes in population size and finally (4) compared the finding of genetic decline analyses with countrywide local extinction probabilities. We interpreted our results in the context of local extinction probabilities as estimated in *Karanth et al. (2010)*. We addressed these questions using genetic data generated using 13 polymorphic microsatellite loci from leopard faecal samples collected across different landscapes of India.

## METHODS

### Research permissions and ethical considerations

All required permissions for our field surveys and biological sampling were provided by the Forest Departments of Uttarakhand (Permit no: 90/5-6), Uttar Pradesh (Permit no: 1127/23-2-12(G) and 1891/23-2-12) and Bihar (Permit no: Wildlife-589). Due to non-invasive nature of sampling, no ethical clearance was required for this study.

### Sampling

To detect population structure and past population demography it is important to obtain genetic samples from different leopard habitats all across the study area. In this study, we used leopard genetic data generated from non-invasive samples collected across the Indian subcontinent. We conducted extensive field surveys across the Indian part of Terai-Arc landscape (TAL) covering the north-Indian states of Uttarakhand, Uttar Pradesh and Bihar between 2016–2018. This region has already been studied for large carnivore occupancy using traditional camera trapping as well as field surveys (*Johnsingh et al., 2004; Harihar, Pandav & Goyal, 2009; Jhala, Qureshi & Gopal, 2015; Chanchani et al., 2016*). We foot surveyed all existing trails covering the entire region to collect faecal samples. Number of trails walked in a particular area was decided based on existing knowledge of leopard presence by the local people and frontline staff members of the sampling team. We

collected a total of 778 fresh large carnivore faecal samples. These samples were collected from both inside ( $n = 469$ ) and outside ( $n = 309$ ) protected areas from different parts of this landscape. In the field, the samples were judged as large carnivores based on several physical characteristics such as scrape marks, tracks, faecal diameter etc. All faecal samples were collected in wax paper and stored individually in sterile zip-lock bags and stored inside dry, dark boxes in the field for a maximum of two weeks period (Biswas *et al.*, 2019). All samples were collected with GPS locations and were transferred to the laboratory and stored in  $-20^{\circ}\text{C}$  freezers until further processing.

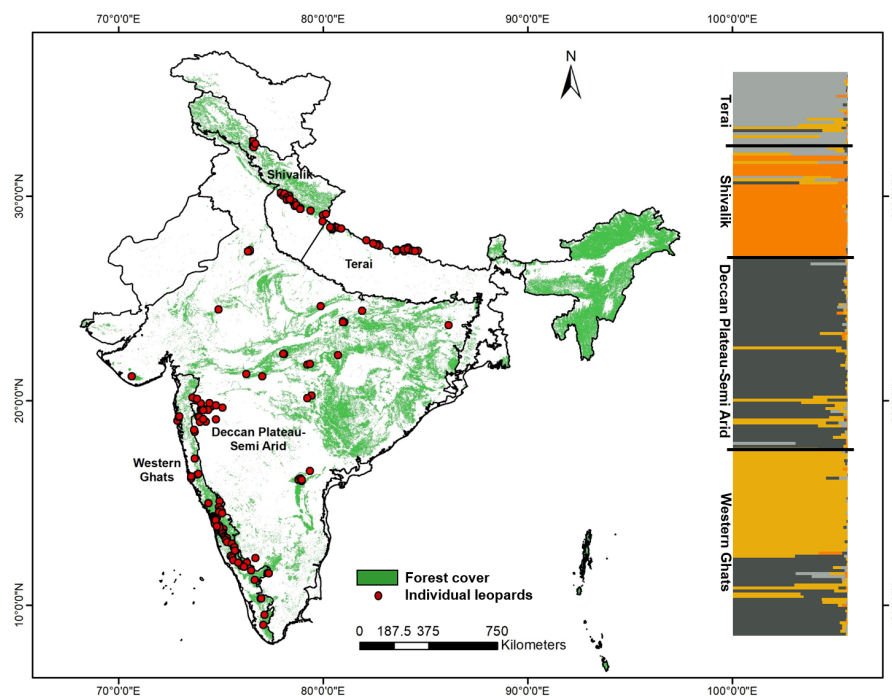
In addition to the north Indian samples collected in this study, we used genetic data previously described in Mondol *et al.* (2015), representing mostly the Western Ghats and central Indian landscape. The data was earlier used in forensic analyses to assign seized leopard samples to their potential geographic origins in India (Mondol *et al.*, 2015). Out of the 173 individual leopards described in the earlier study, we removed data from related individuals and samples with insufficient data ( $n = 30$ ) and used the remaining 143 samples for analyses in this study. These samples were collected from the states of Kerala ( $n = 5$ ), Tamil Nadu ( $n = 4$ ), Karnataka ( $n = 53$ ), Andhra Pradesh ( $n = 3$ ), Madhya Pradesh ( $n = 12$ ), Maharashtra ( $n = 46$ ), Gujarat ( $n = 2$ ), Rajasthan ( $n = 5$ ), Himachal Pradesh ( $n = 8$ ), Jharkhand ( $n = 1$ ), West Bengal ( $n = 2$ ) and Assam ( $n = 2$ ), respectively. The sample locations are presented in Fig. 1.

### DNA extraction, species and individual identification

For all field-collected faecal samples, DNA extraction was performed using protocols described in Biswas *et al.* (2019). In brief, each frozen faeces was thawed to room temperature and the upper layer was swabbed twice with Phosphate buffer saline (PBS) saturated sterile cotton applicators (HiMedia). The swabs were lysed with  $30\ \mu\text{l}$  of Proteinase K (20 mg/ml) and  $300\ \mu\text{l}$  of ATL buffer (Qiagen Inc., Hilden, Germany) overnight at  $56^{\circ}\text{C}$ , followed by Qiagen DNeasy tissue DNA kit extraction protocol. DNA was eluted twice in  $100\ \mu\text{l}$  preheated 1X TE buffer. For every set of samples, extraction negatives were included to monitor possible contaminations.

Species identification was performed using leopard-specific multiplex PCR assay with NADH4 and NADH2 region primers described in Mondol *et al.* (2014) and cytochrome b primers used in Maroju *et al.* (2016). PCR reactions were done in  $10\ \mu\text{l}$  volumes containing  $3.5\ \mu\text{l}$  multiplex buffer mix (Qiagen Inc., Hilden, Germany),  $4\ \mu\text{M}$  BSA,  $0.2\ \mu\text{M}$  primer mix and  $3\ \mu\text{l}$  of scat DNA with conditions including initial denaturation ( $95^{\circ}\text{C}$  for 15 min); 40 cycles of denaturation ( $94^{\circ}\text{C}$  for 30 s), annealing ( $T_a$  for 30 s) and extension ( $72^{\circ}\text{C}$  for 35 s); followed by a final extension ( $72^{\circ}\text{C}$  for 10 min). Negative controls were included to monitor possible contamination. Leopard faeces were identified by viewing species-specific bands of 130 bp (NADH4) and 190 bp (NADH2) (Mondol *et al.*, 2014) and 277 bp (cytochrome b) (Maroju *et al.*, 2016) in 2% agarose gel.

For individual identification, we used the same panel of 13 microsatellite loci previously used in Mondol *et al.* (2014) (Table 1). To generate comparable data with the samples used from earlier study by Mondol *et al.* (2014) we employed stringent laboratory protocols. All PCR amplifications were performed in  $10\ \mu\text{l}$  volumes containing  $5\ \mu\text{l}$  Qiagen multiplex PCR



**Figure 1** Genetic sampling and leopard population structure across the Indian subcontinent with forest cover map and leopard sampling locations used in this study. The map also shows the inferred biogeographic leopard habitats based on genetic structure, as found in this study and corroborative leopard genetic clusters indicated by program STRUCTURE (based on 13 microsatellite loci).

Full-size [DOI: 10.7717/peerj.8482/fig-1](https://doi.org/10.7717/peerj.8482/fig-1)

buffer mix (QIAGEN Inc., Hilden, Germany), 0.2  $\mu$ M labelled forward primer (Applied Biosystems, Foster City, CA, USA), 0.2  $\mu$ M unlabelled reverse primer, 4  $\mu$ M BSA and 3  $\mu$ l of the faecal DNA extract. The reactions were performed in an ABI thermocycler with conditions including initial denaturation (94 °C for 15 min); 45 cycles of denaturation (94 °C for 30 s), annealing ( $T_a$  for 30 s) and extension (72 °C for 30 s); followed by final extension (72 °C for 30 min). Multiple primers were multiplexed to reduce cost and save DNA (Table 1). PCR negatives were incorporated in all reaction setups to monitor possible contamination. The PCR products were analyzed using an automated ABI 3500XL Bioanalyzer with LIZ 500 size standard (Applied Biosystems, Foster City, CA, USA) and alleles were scored with GENEMAPPER version 4.0 (Softgenetics Inc., State Collage, PA, USA). During data generation from field-collected samples we used one reference sample (genotyped for all loci) from the earlier study for genotyping. As the entire new data is generated along with the reference sample and the alleles were scored along with the reference genotypes, the new data (allele scores) were comparable with earlier data for analyses.

To ensure high quality multi-locus genotypes from faecal samples, we followed a modified multiple-tube approach in combination with quality index analyses (Miquel *et al.*, 2006) as described previously for leopards by Mondol *et al.* (2009a) and Mondol *et al.* (2014). All faecal samples were amplified and genotyped four independent times for all the

**Table 1** Genetic diversity and genotyping error details for the leopard samples collected across Terai-Arc landscape ( $n = 56$ ) in this study. A total of 13 microsatellite loci were used. Data from these samples have been added to earlier leopard forensic data described in [Mondol et al. \(2014\)](#).

Locus	Repeat length	N <sub>A</sub>	Allelic size range	H <sub>E</sub>	H <sub>O</sub>	Null allele	Allelic dropout	False allele	HWE	Reference
FCA230	2	16	44	0.87	0.69	0.18	0.001	0.005	Yes	<a href="#">Menotti-Raymond et al. (1999)</a>
FCA309	2	17	42	0.85	0.70	0.22	0.004	0.004	Yes	<a href="#">Menotti-Raymond et al. (1999)</a>
FCA232	2	15	36	0.83	0.68	0.19	0.007	0.013	Yes	<a href="#">Menotti-Raymond et al. (1999)</a>
FCA090	2	16	34	0.87	0.66	0.30	0.007	0.002	Yes	<a href="#">Menotti-Raymond et al. (1999)</a>
FCA052	2	14	32	0.85	0.77	0.19	0.004	0.006	Yes	<a href="#">Menotti-Raymond et al. (1999)</a>
FCA672	2	20	40	0.87	0.74	0.05	0.0	0.001	Yes	<a href="#">Menotti-Raymond et al. (1999)</a>
FCA279	2	16	30	0.88	0.76	0.08	0.001	0.003	Yes	<a href="#">Menotti-Raymond et al. (1999)</a>
FCA126	2	16	32	0.89	0.70	0.36	0.004	0.001	Yes	<a href="#">Menotti-Raymond et al. (1999)</a>
msFCA391	4	10	36	0.86	0.64	0.19	0.009	0.007	Yes	<a href="#">Mondol et al. (2012)</a>
msHDZ170	2	13	42	0.83	0.53	0.30	0.002	0.002	Yes	<a href="#">Mondol et al. (2012)</a>
msFCA441	4	12	52	0.82	0.52	0.23	0.006	0.003	Yes	<a href="#">Mondol et al. (2012)</a>
msFCA506	2	19	56	0.89	0.69	0.25	0.008	0	Yes	<a href="#">Mondol et al. (2012)</a>
msFCA453	4	7	32	0.68	0.61	0.25	0.006	0.007	Yes	<a href="#">Mondol et al. (2012)</a>
Mean (SD)		14.69 (3.41)	39.08 (7.71)	0.84 (0.05)	0.67 (0.07)	0.21	0.005	0.004		

**Notes.**

N<sub>A</sub>, No. of alleles; H<sub>E</sub>, Expected heterozygosity; H<sub>O</sub>, Observed heterozygosity; HWE, Hardy-Weinberg Equilibrium.

loci. Samples producing identical genotypes for at least three independent amplifications (or a quality index of 0.75 or more) for each loci were considered reliable and used for all further analysis, while the rest were discarded.

## Analysis

For each locus, we calculated average amplification success as the percent positive PCR ([Broquet & Petit, 2004](#)) after four repeats across all samples. We quantified allelic dropout and false allele rates manually as the number of dropouts or false alleles over the total number of amplifications, respectively ([Broquet & Petit, 2004](#)), as well as using MICROCHECKER v 2.2.3 ([Van Oosterhout et al., 2004](#)). The false allele frequency is calculated for both homozygous and heterozygous genotypes as the ratio of the number of amplifications having one or more false alleles at a particular locus and the total number of amplifications while allele dropout rate (ADO) is calculated as the ratio between the observed number of amplifications having loss of one allele and the number of positive amplifications of heterozygous individuals.

Post data quality assessment we selected only those samples with good quality data for at least nine or more loci (out of 13) for further analyses. We used the identity analysis module implemented in program CERVUS ([Kalinowski, Taper & Marshall, 2007](#)) to identify identical genotypes (or recaptures) by comparing data from all samples. All genetic recaptures were removed from the data set. GIMLET ([Valiere, 2002](#)) was used to calculate the PID<sub>(sibs)</sub> for all the unique individuals. Following this, any allele having less than 10% frequency across all amplified samples were rechecked for allele confirmation. ARLEQUIN ([Excoffier, Laval & Schneider, 2005](#)) was used to determine Hardy Weinberg

equilibrium and linkage disequilibrium for all the loci. Finally, to avoid the effects of related individuals in all analyses, we used the program GENECLASS 2.0 (Piry et al., 2004) to select out related individuals in our samples.

To determine the genetic structure of leopards across the Indian subcontinent we used a Bayesian clustering approach implemented in program STRUCTURE (Pritchard, Stephens & Donnelly, 2000; Falush, Stephens & Pritchard, 2003). We performed 10 independent analyses for each K values between one and ten, using 450,000 iterations and a burn-in of 50,000 assuming correlated allele frequencies. The optimal value of K was determined using STRUCTURE HARVESTER web version (Earl & VonHoldt, 2012). Further, we used multivariate analyses approach implemented in program Discriminant Analysis of Principal Component (DAPC) (Jombart, Devillard & Balloux, 2010) to identify genetic clusters in our data. This approach transforms the genetic data into principal components, followed by clustering to define group of individuals with a consideration of minimum within group variation and maximum between group variations among the clusters. The analyses were conducted using adegenet package 2.1.1 in R studio 1.1.453 (R Development Core Team, 2014) where optimal number of clusters were determined through the Bayesian Information Criterion (Jombart, Devillard & Balloux, 2010). Subsequent summary statistics were calculated in ARLEQUIN 3.1 (Excoffier, Laval & Schneider, 2005) and indices of overall genetic differentiation (pairwise  $F_{st}$ ) were estimated using GenAEx version 6.5 (Peakall & Smouse, 2012), dividing the leopard populations according to the STRUCTURE results across the Indian subcontinent. The divisions were based on Q-values (estimated proportions of ancestry) calculated in STRUCTURE, where we used  $Q > 0.75$  as threshold for assigning individuals to a particular population (Mora et al., 2010). Additionally, compression of expected heterozygosity (or  $G_{st}$ ) (Nei, 1973) between four leopard subpopulations was calculated in GenAEx version 6.5 (Peakall & Smouse, 2012). Finally, HP-RARE 1.0 (Kalinowski, 2005) was used to estimate private alleles within each subpopulation.

## Demography analyses

Demographic analyses were performed with different genetic subpopulations of leopards based on the results from STRUCTURE analyses. We used a number of different approaches to detect past population demography for leopards. The first two qualitative approaches use summary statistics to detect population size changes, whereas the quantitative approach is a likelihood-based Bayesian algorithm. The summary statistic-based methods used were the Ewens, Watterson, Cornuet and Luikart method implemented in program BOTTLENECK (Cornuet & Luikart, 1996), and the Garza-Williamson index or M ratio (Garza & Williamson, 2001) implemented in program ARLEQUIN 3.1. The quantitative Bayesian approach used was implemented in the program MSVAR 1.3 (Storz & Beaumont, 2002).

### (a) The Ewens, Watterson, Cornuet and Luikart (EWCL) approach:

This approach allows the detection of population size changes using two summary statistics of the allele frequency spectrum, number of alleles ( $N_A$ ) and expected heterozygosity ( $H_e$ ) across different mutational models. Simulations are performed to obtain the expected

distribution of  $H_e$  for a demographically stable population under three mutation models: infinite allele model (IAM), single stepwise model (SMM) and two-phase model (TPM) and the values are then compared to the real data values. This method can detect departures from mutation-drift equilibrium and neutrality, which can be explained by any departure from the null model, including selection, population growth or decline. More importantly, consistent results from independent loci could be attributed to demographic events over selection. For simulations with TPM model, we used two different (5% and 30%) multi-step mutation events for leopards.

**(b) The Garza-Williamson index/M ratio approach:**

This approach allows the detection of population decline using two summary statistics of the allele frequency spectrum, number of alleles ( $N_A$ ) and the allelic size range. The basic principle behind this approach is in a reducing population, the expectation of the reduction of number of alleles is much higher than the reduction of allelic size range. Thus, the ratio between the number of alleles and the allelic size range is expected to be smaller in recently reduced populations than in equilibrium populations.

**(c) The Storz and Beaumont approach:**

This approach is an extension of Beaumont's approach (Beaumont, 1999) that assumes a stable population of size  $N_1$  started to change (either decrease or increase)  $T_a$  generations ago to the current population size  $N_0$ . This change in the population size is assumed to be at an exponential scale under stepwise mutation model (SMM), at a rate  $\gamma = 2N_0m$ , where  $m$  is the mutation rate per locus per generation. This Bayesian approach uses the information from the full allelic distribution in a coalescent framework to estimate the posterior probability distribution, allowing quantification of effective population sizes  $N_0$  and  $N_1$ , rather than their ratio (as in Beaumont, 1999) along with  $T$ , time since the population change. In this approach, prior distributions for  $N_0$ ,  $N_1$ ,  $T$  and  $\mu$  (mutation rate) are assumed to be log normal. The mean and the standard deviations of these prior log normal distributions are drawn from prior (or hyperpriors) distributions. A Markov Chain Monte Carlo (MCMC) algorithm is used to generate samples from the posterior distribution of these parameters. We used wide uninformative priors to perform multiple runs for this approach (Table S1). For minimal effect towards the posterior distributions variances for the prior distributions were kept large. A total number of 2 million iterations were performed for each run.

The generation time for leopards are known to be about 4–5 years (Dutta et al., 2013) and we used a five-year generation time for all analyses.

**Estimation of leopard extinction probability**

To understand extinction probability across various biogeographic zones of India we analysed patterns and determinants of leopard occurrence as described in Karanth et al. (2009) and Karanth et al. (2010). In this study, we have just divided the earlier information available for leopards for different genetic subpopulations. We applied a grid-based approach to determine current distribution patterns for leopards, where the selection of grids was based on prior information of leopard presence. This involved

collating presence-absence information from more than 100 Indian wildlife experts along with historical information of leopard presence involving hunting locations and other taxidermy and museum records. Each grid cell was an average of 2,818 km<sup>2</sup> in size and we used data from 1,229 grid cells covering 3,463,322 km<sup>2</sup> area of the Indian subcontinent. This study applied occupancy modelling to examine the influence of ecological and social covariates on patterns of leopard occupancy. We used a maximum likelihood approach for leopard occupancy in PRESENCE. V.2.0 program (Hines, 2006). Covariates likely to influence leopard distribution modelled included presence and extent of protected areas, land cover-land use characteristics, human cultural tolerance and population density. Data for protected areas was retrieved from the World Database on protected areas (<http://www.unep-wcmc.org>) and topographic maps. Land cover- land use data were derived from Global Land Cover Facility (2000) and further refined based on Roy *et al.* (2006) and Joshi *et al.* (2006). A human tolerance index that characterized different Indian states from most to least tolerant was developed based on knowledge about society-culture, law enforcement, hunting patterns and prior field experiences (for details see Karanth *et al.*, 2009; Karanth *et al.*, 2010). Human population density data were derived from LandScan Global Population Data 2000 (<http://www.ornl.gov/gist>). Based on existing information on species' ecology we predicted higher occupancy in protected areas, deciduous-grass-scrub land cover types and lower occupancy in less tolerant states and highly populated areas because of direct competition for food and space (Brashares, Arcese & Sam, 2001; Rangarajan, 2001; Parks & Harcourt, 2002; Karanth *et al.*, 2010). We performed pair-wise correlation tests to screen variables for multicollinearity. The occupancy approach accounts for non-detection of species during surveys and inability to survey some sites (see Karanth *et al.*, 2009; Karanth *et al.*, 2010 for additional details). The probability of extinction was calculated as (1- probability of occurrence) (Karanth *et al.*, 2010). We derived leopard extinction probabilities for three separate major landscapes (Western Ghats, central India and north India) as these regions strongly represented our genetic sampling. These extinction probabilities were compared to the genetically derived estimates.

## RESULTS

### Individual identification of leopards from north Indian landscape

Of the 778 large carnivore faecal samples collected from TAL, we identified 195 faeces to be of leopard origin (25%) using species-specific PCR assays (Mondol *et al.*, 2014; Maroju *et al.*, 2016). In addition, 457 samples were ascertained to be of tiger (59%) and remaining 126 faecal samples did not produce any result (16%) for either of these large felids, possibly due to poor quality DNA. We amplified 13 microsatellite loci panel on these 195 genetically confirmed leopard faecal samples, and after data validation through multiple repeats generated nine or more loci data from 65 faecal DNA. Subsequently, we identified 56 unique leopard individuals from the 65 samples, whereas nine individuals were ascertained as 'genetic recaptures'. The mean allelic dropout rate for these loci was found to be 0.05, whereas mean false allele rate for all the 13 loci was 0.04, indicating this 13 loci panel has low genotyping error rates. Amplification success ranged between 41–100% from

leopard faecal DNA. None of the loci were found to deviate from the Hardy-Weinberg equilibrium and there were no evidence for strong linkage disequilibrium between any pair of loci. Cumulative  $PID_{sibs}$  and  $PID_{unbiased}$  values were found to be  $3.91 \times 10^{-6}$  and  $2.73 \times 10^{-16}$ , respectively, indicating a strong statistical support for unambiguous individual identification. Summary statistics for these samples collected across Terai-Arc landscape is provided in Table 1. We identified 26, 21 and nine unique leopard individuals from the states of Uttarakhand, Uttar Pradesh and Bihar, respectively. As the data generated from north India is comparable to the earlier data, we added this 56 unique leopard data to 143 individual genotypes described in Mondol et al. (2014), and overall 199 unique unrelated leopards were used in subsequent population structure, genetic variation and demography analyses.

### Leopard population structure and genetic variation across India

Our sampling strategy targeted countrywide leopard populations to assess population structure and genetic variation. From 199 final unique leopard genotypes we removed four samples representing the eastern and northeast India ( $n = 2$  from the states of West Bengal and Assam each, respectively) from further analyses as they represented inadequate sampling from these regions. Bayesian clustering analysis using 13 microsatellite data from the remaining 195 wild leopard individuals showed four distinct genetic subpopulations ( $K = 4$ , see Fig. S1), as presented in Fig. 1. The DAPC analyses identified five different clusters ( $K = 5$ ) using the Bayesian Information Criterion (Fig. S2). However, out of these genetic clusters two of them were overlapping with each other. Overall, both analyses showed the same pattern of four genetic subpopulations. Majority of the samples showed respective group-specific ancestry, with Western Ghats samples representing the first group (henceforth WG,  $n = 65$ ), the Deccan Plateau-Semi Arid region forming the second (henceforth DP-SA,  $n = 66$ ), the samples from Shivalik region covering parts of Himalaya and western parts of upper Gangetic plains making the third group (henceforth SR,  $n = 38$ ), and finally samples from the Terai region covering eastern part of upper and western part of the lower Gangetic Plains samples forming the fourth one (henceforth TR,  $n = 26$ ), respectively (Fig. 1). However, small number of samples ( $n = 18$ ) distributed among the four subpopulations showed mixed ancestry. Subsequent analyses revealed that these leopard subpopulations are genetically differentiated ( $F_{st}$  and  $G_{st}$ ) at low, but significant levels (Table 2) for all four populations. The  $F_{st}$  value among these populations ranged between 0.028–0.115, whereas the  $G_{st}$  value between 0.023–0.104 (Table 2).

Analyses and with 13 microsatellite loci among the four genetic subpopulations showed a higher mean number of alleles ( $NA_{WG} = 11.77$  (S.D. 3.85),  $NA_{DP-SA} = 10.46$  (S.D. 2.71)) and observed heterozygosity ( $H_{oWG} = 0.81$  (S.D. 0.08),  $H_{oDP-SA} = 0.8$  (S.D. 0.08)) in Western Ghats and Deccan Plateau-Semi Arid subpopulations, when compared with samples from Shivalik and Terai region subpopulations ( $NA_{SR} = 08.46$  (S.D. 2.41),  $NA_{TR} = 05.00$  (S.D. 1.84) and  $H_{oSR} = 0.40$  (S.D. 0.14),  $H_{oTR} = 0.36$  (S.D. 0.28), respectively) (see Table 3 for details). However, the allelic size range values were similar in all populations (Table 3). Western Ghats and Deccan Plateau- Semi Arid subpopulations

**Table 2** Genetic differentiation (pairwise  $F_{st}$  and  $G_{st}$ ) for four leopard subpopulations in the Indian subcontinent. The upper diagonal presents the pairwise  $G_{st}$  values whereas the lower diagonal presents the pairwise  $F_{st}$  values.

	Western Ghats ( $n = 65$ )	Deccan Plateau-Semi Arid ( $n = 66$ )	Shivalik ( $n = 38$ )	Terai ( $n = 26$ )
Western Ghats ( $n = 65$ )	–	0.023*	0.039*	0.091*
Deccan Plateau-Semi Arid ( $n = 66$ )	0.028*	–	0.045*	0.104*
Shivalik ( $n = 38$ )	0.048*	0.05*	–	0.073*
Terai ( $n = 26$ )	0.103*	0.115*	0.089*	–

Notes.

\* $p$  value = 0.001.

showed higher number of private alleles (2.38 and 0.85, respectively) when compared to Shivalik and Terai subpopulations (0.46 and 0.15, respectively) (Table 3).

## Detection of demographic change

We used microsatellite data to investigate signals of demographic changes in each of the four leopard genetic subpopulations across the subcontinent. Both qualitative approaches, the EWCL and the M-ratio methods indicate signatures of population bottleneck. The EWCL approach implemented in the program BOTTLENECK shows 8-10 loci with heterozygote excess depending on the mutation models used, suggesting a loss of rare alleles through population decline for all four subpopulations. Similarly, the M-ratio approach also shows a low ratio between number of alleles ( $N_A$ ) and the allelic size range in all four subpopulations (M-ratio<sub>WG</sub>-0.37 (S.D. 0.09); M-ratio<sub>DP-SA</sub>-0.38 (S.D. 0.09); M-ratio<sub>SR</sub>-0.33 (S.D. 0.09); M-ratio<sub>TR</sub>-0.29 (S.D. 0.15)), indicating signatures of population bottleneck.

In the quantitative MSVAR approach, models with exponential decline scenarios show consistently that the posterior distributions for log ( $N_0$ ) is always lower than log ( $N_1$ ) for all four subpopulations, indicating population decline for leopards across the subcontinent (Table 4 and Fig. 2). Further quantification revealed that the current effective size is varying low (12–25%) than the historical effective size, with Western Ghats, Deccan Plateau-Semi Arid, Shivalik and Terai regions losing approximately 75%, 90%, 90% and 88% of their leopard population, respectively (Table 4 and Fig. 2).

Our subsequent analyses also revealed distributions that suggested recent time of declines in all four populations of leopards (Table 4, Fig. 2). The north Indian subpopulations (Shivalik and Terai) and the Deccan Plateau-Semi Arid population showed the most recent decline occurred about 120–125 years before present, respectively. However, the Western Ghats population indicated potential decline around 200 years ago (Table 4 and Fig. 2).

## Leopard occurrence and distribution

We examined the factors influencing leopard distribution at a countrywide scale, where the top ranked model incorporating 28 covariates suggested a wide distribution of habitat types (described in Karanth *et al.*, 2009; Karanth *et al.*, 2010). The model also indicated a positive influence of protected areas, higher cultural tolerance of people and negative influence of higher human population densities and (details in Karanth *et al.*, 2009).

**Table 3** Subpopulation-wise summary statistics (based on 13 microsatellite loci) for Indian leopards.

Locus	Western Ghats ( <i>n</i> = 65)					Deccan Plateau-Semi Arid ( <i>n</i> = 66)					Shivalik ( <i>n</i> = 38)					Terai ( <i>n</i> = 26)				
	N <sub>A</sub>	ASR	H <sub>E</sub>	H <sub>O</sub>	PA	N <sub>A</sub>	ASR	H <sub>E</sub>	H <sub>O</sub>	PA	N <sub>A</sub>	ASR	H <sub>E</sub>	H <sub>O</sub>	PA	N <sub>A</sub>	ASR	H <sub>E</sub>	H <sub>O</sub>	PA
FCA230	13	36	0.88	0.86	03	10	22	0.78	0.80	01	08	24	0.83	0.23	00	05	26	0.52	0.65	00
FCA309	17	42	0.90	0.87	05	11	30	0.78	0.81	00	08	18	0.81	0.46	00	06	10	0.82	0.32	00
FCA232	13	36	0.85	0.84	03	09	18	0.68	0.72	00	09	26	0.78	0.42	01	07	26	0.78	0.46	00
FCA090	14	30	0.85	0.84	02	08	18	0.78	0.87	00	09	30	0.86	0.36	02	02	10	0.47	0.00	00
FCA052	12	32	0.84	0.89	02	11	22	0.82	0.84	01	08	20	0.83	0.48	00	06	22	0.84	0.43	00
FCA672	19	40	0.90	0.89	09	10	26	0.82	0.75	01	06	16	0.65	0.50	00	07	20	0.64	0.77	00
FCA279	11	26	0.81	0.75	01	14	26	0.87	0.81	00	15	28	0.90	0.67	01	06	18	0.78	0.92	00
FCA126	14	26	0.87	0.83	03	13	30	0.88	0.89	00	09	22	0.76	0.18	00	04	12	0.11	0.74	00
msFCA391	07	28	0.83	0.67	00	08	32	0.81	0.81	01	07	24	0.78	0.57	00	07	32	0.75	0.13	01
msHDZ170	09	20	0.84	0.79	00	10	22	0.75	0.88	01	10	36	0.76	0.16	02	02	02	0.29	0.0	00
msFCA441	07	36	0.75	0.79	02	08	28	0.65	0.55	00	08	40	0.82	0.38	00	05	28	0.66	0.40	01
msFCA506	12	32	0.86	0.90	01	17	56	0.83	0.82	04	09	24	0.83	0.33	00	06	22	0.79	0.40	00
msFCA453	05	20	0.63	0.67	00	07	32	0.69	0.80	02	04	20	0.65	0.36	00	02	16	0.43	0.14	00
Mean (SD)	11.77 (3.85)	31.08 (6.69)	0.83 (0.07)	0.81 (0.08)	2.38	10.46 (2.71)	27.85 (9.36)	0.78 (0.07)	0.80 (0.08)	0.85	08.46 (2.41)	25.23 (6.64)	0.79 (0.07)	0.40 (0.14)	0.46	05.00 (1.84)	18.77 (8.21)	0.65 (0.17)	0.36 (0.28)	0.15

**Notes.**

N<sub>A</sub>, No. of alleles; ASR, Allelic size range; H<sub>E</sub>, Expected heterozygosity; H<sub>O</sub>, Observed heterozygosity; PA, Private alleles.

**Table 4** Comparison of different demographic decline analyses results for different subpopulations of leopards across India.

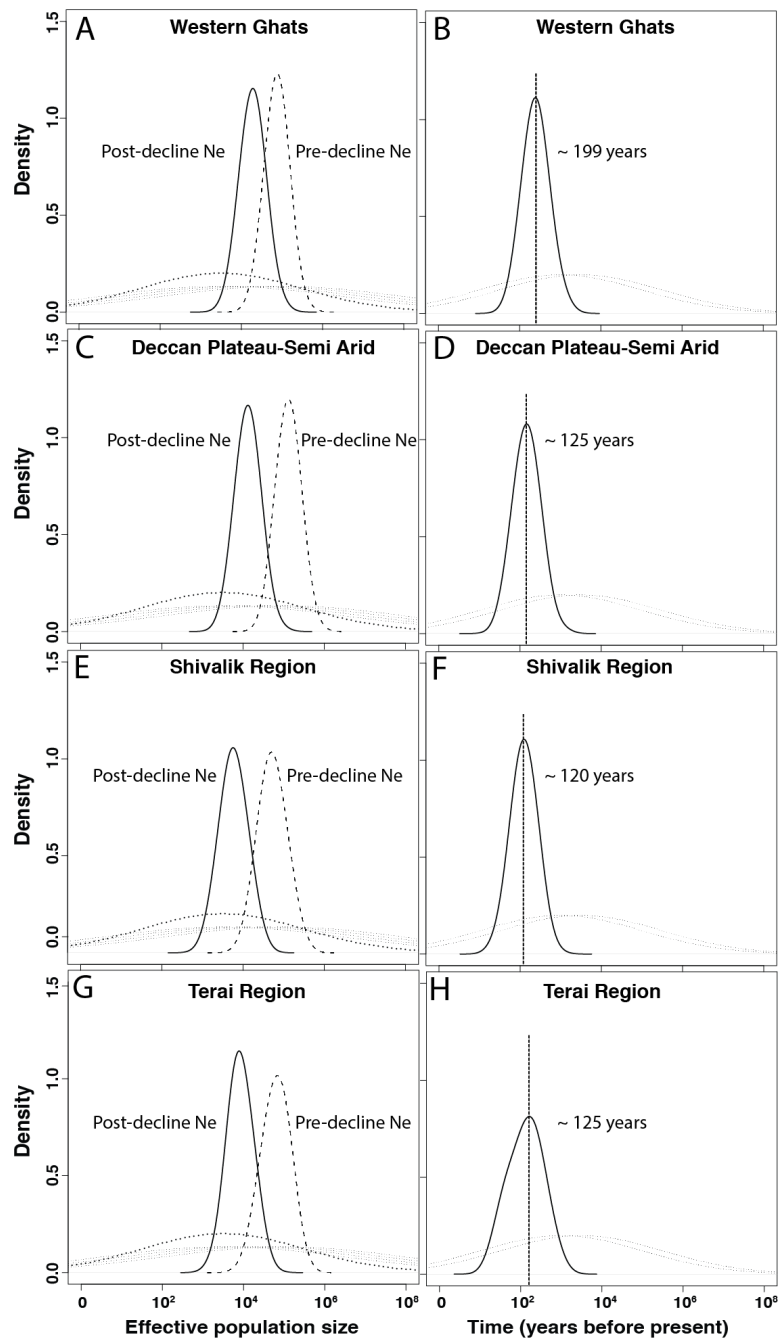
Method	Analysis type	Demographic signal				
		Model	Western ghats	Deccan plateau-Semi arid	Shivalik	Terai
Bottleneck	Qualitative	IAM	Heterozygosity excess for 13 loci	Heterozygosity excess for 10 loci	Heterozygosity excess for 12 loci	Heterozygosity excess for 11 loci
		SMM	Heterozygosity excess for 01 loci	Heterozygosity excess for 02 loci	Heterozygosity excess for 06 loci	Heterozygosity excess for 08 loci
		TPM	Heterozygosity excess for 07 loci	Heterozygosity excess for 07 loci	Heterozygosity excess for 09 loci	Heterozygosity excess for 10 loci
M ratio	Quantitative		0.37 (SD 0.09)	0.38 (SD 0.09)	0.33 (SD 0.09)	0.29 (SD 0.15)
Storz-Beaumont method			Decline—75% Time—~200 years ago	Decline- 90% Time—~125 years ago	Decline- 90% Time—~125 years ago	Decline—88% Time—~120 years ago
Extinction probability	Quantitative	Occupancy	0.17	0.21		0.37

Areas with cultivated land, barren areas, deciduous forests and rural–urban were strongly associated with higher leopard occurrence. Naive estimated occupancy was 0.52, whereas model estimated probability of occupancy was significantly higher at 0.68, suggesting that leopards are still widely distributed (Fig. 3) in India compared to most other large mammals (as suggested in [Karanth et al., 2010](#)). When compared among the overall three major sub-regions (north India (NI), Deccan Plateau-Semi Arid and Western Ghats), we find that average estimated occurrence was lowest in the north India ( $\text{Psi}_{\text{NI}} = 0.63 \pm 0.01$ , Range: 0.05–1.00, 384 cells) compared to Western Ghats ( $\text{Psi}_{\text{WG}} = 0.83 \pm 0.02$ , Range: 0.23–1.00, 90 cells) and Deccan Plateau-Semi Arid ( $\text{Psi}_{\text{DP-SA}} = 0.79 \pm 0.005$ , Range: 0.25–1.00, 818 cells). Overall, average estimated Psi was  $0.74 \pm 0.006$  (1,292 cells).

## DISCUSSION

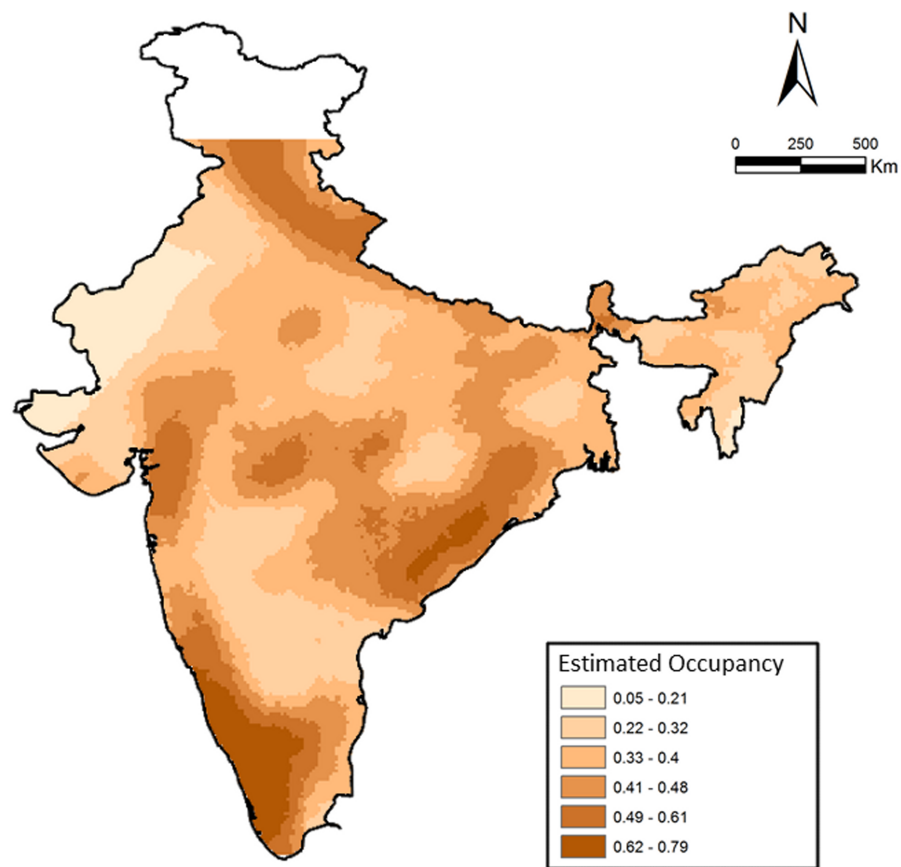
To the best of our knowledge, this is probably the first and most exhaustive study on leopard population genetics and demographic patterns in the Indian subcontinent. Except the eastern and northeast Indian landscape, where our sampling intensity was less all other regions are well covered in this study. Our genetic analyses with microsatellite data collected across the subcontinent reveal four genetic subpopulations of leopards in India: the Western Ghats, Deccan Plateau-Semi Arid landscape, hill region of north India (Shivalik) and Terai or flat region of north India. While there was some amount of mixed genetic signal across different genetic subpopulations, they were clearly separated as different groups (Fig. 1). These genetic groups mostly correspond to respective biogeographic zones of India, with Western Ghats and combination of Deccan Plateau-Semi Arid forms two subpopulations, whereas the north Indian subpopulation of Shivalik and Terai are parts of the Himalayan and Gangetic Plains zones, respectively.

Based on these patterns, we presume that these genetic clusters were formed due to restricted gene flow along major habitat type differences between these biogeographic



**Figure 2** Demographic history of Indian leopards (*Panthera pardus fusca*). A, C, E and G show the posterior distributions for leopard population size changes for different subpopulations, based on 13 microsatellite loci using Storz and Beaumont approach. The dashed and solid lines represent posterior distributions of ancestral and present effective population sizes. The priors are represented by the dotted line. B, D, F, and H represent the posterior distribution for the time since the leopard population decline started for corresponding subpopulations. The priors are shown by the dotted lines.

Full-size [DOI: 10.7717/peerj.8482/fig-2](https://doi.org/10.7717/peerj.8482/fig-2)



**Figure 3** Patterns of leopard occurrence in India based on the analysis of questionnaire surveys. The map shows a gradient of estimated cell-wise occupancy probabilities created through spatial kriging.

Full-size [DOI: 10.7717/peerj.8482/fig-3](https://doi.org/10.7717/peerj.8482/fig-3)

zones. For example, the difference between habitat types of large contiguously forested Western Ghats (largely moist deciduous habitat) and the Deccan Plateau-Semi Arid (mostly dry deciduous habitat) probably lead to genetic differences between leopards from these regions. Similarly, difference in habitat types in ‘Bhabar’ habitats of Shivalik (hilly rugged terrains, large trees, high leopard abundance) and Terai region (flat terrain, grassland, low leopard abundance) (*Johnsingh et al., 2004*), has possibly led to reduced gene flow between these two areas. Such habitat-driven reasons meaningfully explain the genetic differentiation between leopards from these landscapes. These four subpopulations were genetically differentiated by low, but significant levels (*Table 2*). Previous studies on tigers (*Mondol, Karanth & Ramakrishnan, 2009; Mondol, Bruford & Ramakrishnan, 2013* subcontinent scale) as well as leopards (*Dutta et al., 2013*, central Indian landscape) suggested long-distance movement as a potential cause for low genetic differentiation between populations. Leopards are known to disperse long distances (*Ropiquet et al., 2015; Farhadinia et al., 2018*) and human-leopard conflict driven translocation is common in many parts in India (*Athreya et al., 2010; Odden et al., 2014*). Together, natural dispersal abilities and ‘human mediated gene flow’ through translocations might be responsible for

the low genetic differentiation among leopard subpopulations across the subcontinent. Earlier work in central Indian landscape ([Dutta et al., 2013](#)) suggested a reduction in gene flow at recent times due to habitat destruction, but our study did not focus to answer such questions. Future studies should focus on using historical samples (museum skins, bones etc.) to assess any possible change in gene flow among leopard populations (For example see [Martinez-Cruz, Godoy & Negro, 2007](#); [Valdiosera et al., 2008](#); [Lorenzen et al., 2011](#); [Mondol, Bruford & Ramakrishnan, 2013](#)) at subpopulation levels across the country.

However, our demography analyses with genetic data indicate strong decline in leopard population size across all four genetic subpopulations. Results with both qualitative (bottleneck and M-ratio approach) as well as quantitative (Storz and Beaumont approach) analyses revealed strong, but varying signals of demographic decline in all four subpopulations ([Table 4](#)). The Deccan Plateau-Semi Arid, Shivalik and Terai subpopulations show 90%, 90% and 88% decline in population size, respectively, whereas the Western Ghats subpopulation show relatively less (75%) decline in population size ([Table 4](#)). Leopards are vulnerable to conflict and poaching due to their close associations with human habitations ([Gavashelishvili & Lukarevskiy, 2008](#); [Athreya et al., 2010](#); [Balme, Slotow & Hunter, 2009](#)). The Western Ghats retains possibly the largest contiguous forested landscape with multiple interconnected protected areas, whereas the other regions have lot of human activities, possibly affecting leopard populations living in them. Further, the ecological data based occupancy analysis showed extinction probabilities of 0.37, 0.21 and 0.17 for north India, Deccan Plateau-Semi Arid and Western Ghats landscape, respectively ([Table 4](#)). It is possible that this discrepancy in the magnitudes of decline based on genetic and ecological models is because the ecological methods are more spatial. The inferences from this model are dependent on temporal differences in leopard occupancy. However, if densities of leopards were high in the past, loss of even small habitats could result in the loss of many individuals. Since no quantitative comparisons for leopard density between the Western Ghats, Deccan Plateau-Semi Arid and north India is currently available, we cannot conclusively infer the former, but further research should investigate leopard densities and their temporal changes across the country. Finally, this decline pattern also roughly corroborates with 83–87% leopard range loss in Asia ([Jacobson et al., 2016](#)), indicating that habitat loss is a contributing factor towards the population decline.

The magnitude of decline for leopards found in this study is contrasting to some of the earlier leopard studies in the subcontinent (for example ecological work by [Harihar, Pandav & Goyal, 2011](#), and genetic work by [Dutta et al., 2013](#)) and eastern Africa ([Spong, Johansson & Bjorklund, 2000](#) in Tanzania), which suggest stable or increasing local leopard population trends. This is certainly possible as many of these studies were conducted inside protected areas, where leopard population dynamics depends on presence/absence of other large carnivores (tiger, dhole etc.). Given that only 11% of Indian leopard distribution is within protected area network ([Jacobson et al., 2016](#)), it is challenging to truly understand the population trends at country level. Our sampling at subcontinent scale is thus indicating the actual patterns of population demography that is difficult to assess based on ecological/genetic studies at local level.

Another important finding is the relatively recent timing of decline for all the leopard subpopulations in the subcontinent. Our results suggest median leopard decline timing between 120–200 years across four genetic subpopulations (Table 4). Except Western Ghats (decline timing of ~200 years), all other subpopulations indicate much recent population decline (Deccan Plateau-Semi Arid ~125 years, Shivalik ~120 years and Terai ~125 years). When compared with other sympatric, endangered species in the subcontinent (for example tiger decline ~200 years ago; Mondol, Karanth & Ramakrishnan, 2009) or Asian region (for example Orangutan- ~210 years, Goossens et al., 2006; Giant panda- ~250 years, Zhu et al., 2010) this still seems to be much recent event. Other wide-ranging carnivores across the globe (for example European wolves Aspi et al., 2006; African wild dog- Marsden et al., 2012; Eurasian badgers-Frantz et al., 2014 etc.) too faced much longer decline period than leopards. One plausible explanation could be recent increases in leopard-human conflict (Athreya et al., 2010; Karanth & Kudalkar, 2017) and poaching intensity due to large demand of leopard body parts in the illegal wildlife markets (Raza et al., 2012; WPSI, 2017). Historically, major leopard hunting events had been recorded across the Indian subcontinent during Mughal times (about 500–600 years ago), followed by colonial British bounty-hunting rule between 1850–1920 (Rangarajan, 2005). However, large-scale landscape modification and fragmentation by humans during the last century (central India-Rangarajan, 1999, north India-Rangarajan, 2005), coupled with poaching and conflict may have resulted in much recent loss of leopard populations across the country. We lack comprehensive data, both at historical as well as modern scales to investigate the true causes behind such patterns of differential population decline timing. For example, Dutta et al. (2013) showed that during last three centuries severe changes in landscape characteristics (Settlement, villages, wild lands, human density) have occurred in the central Indian leopard habitats. However, we lack information on hunting and conflict levels during this time from these regions. Future efforts should generate this important information to get an idea of the scenarios leading to such strong decline in a wide-ranging species like leopard. Finally, it is important to point out that in this study we have only explored relatively simple decline scenarios during demographic modelling. Future studies should evaluate more detailed, computationally intensive demographic analyses with genome wide molecular markers (For example, see Frantz et al., 2014; Nater et al., 2015) for better understanding of complex decline scenarios.

Finally, another important aspect of the results from this study is that despite severe decline (Table 4) and small, but significant population structure (Fig. 1B, Table 2) leopards still retain high genetic variation in the Indian subcontinent. We found that leopard genetic variation across four genetic subpopulations is similar and comparable to eastern and southern Africa (Spong, Johansson & Bjorklund, 2000; Uphyrkina et al., 2001; McManus et al., 2015), and higher than Arabian (Ilani, 1981; Perez, Geffen & Mokady, 2006) and Amur leopards (Uphyrkina et al., 2001; Sugimoto et al., 2014). The higher levels of variation could possibly be attributed to still relatively large population size, high pre-bottleneck genetic variation and potential historical gene flow across large landscapes.

## CONCLUSION

While leopards are relatively easier to study than other sympatric carnivores like tigers due to their ubiquitous presence, studies on their population size, trend and dynamics are limited, particularly in outside protected areas. In fact, due to their broad geographic distribution, leopard populations are perceived to be stable, with current IUCN Red List status of 'vulnerable'. However, both historical records and recent conflict with humans suggest potentially declining population trends. Using genetic data, we reveal a strong signal of population decline (between 75–90%) across different habitats in the Indian subcontinent over the last 120–200 years. We demonstrate population decline in a wide-ranging and, commonly perceived as locally abundant species like the leopard, suggesting that leopards demand similar conservation attention like tigers in India. While we are unable to corroborate these population decline patterns with leopard census data, our results suggest that it will be important to generate such ecological abundance estimates for leopard populations in the near future. This work also emphasizes the importance of similar work on wide-ranging species, as it is possible that other species like the leopard may show population decline, especially in the context of the Anthropocene.

## ACKNOWLEDGEMENTS

We acknowledge the Director, Dean and Nodal Officer of Wildlife Forensics and Conservation Genetics Cell of Wildlife Institute of India for their support in this work. We thank Dr. Uma Ramakrishnan of National Centre for Biological Sciences for providing reference leopard samples. Mr A. Madhanraj has provided critical support in genotyping facility and Mr. Debanjan Sarkar helped with GIS in the laboratory. We thank all the lab members of Wildlife Forensic and Conservation Genetics cell and especially Meercat lab for productive discussions and valuable comments. We also thank Dr. S.K. Gupta and Dr. S.P. Goyal for logistic support; our field assistants Annu, Bura, Abbhi, Ranjhu and Imam for their effort in the field. Finally, we thank two reviewers and the editor for their critical suggestions to improve our earlier version of the manuscript.

## ADDITIONAL INFORMATION AND DECLARATIONS

### Funding

This research was funded by Wildlife Conservation Trust-Panthera Global Cat Alliance Grants and Department of Science and Technology, Government of India grant no EMR/2014/000982. Samrat Mondol was supported by the Department of Science and Technology INSPIRE Faculty Award (No.IFA12-LSBM-47) and Krithi Karanth was supported by Centre for Wildlife Studies, Wildlife Conservation Society-New York and Oracle. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Grant Disclosures

The following grant information was disclosed by the authors:

Wildlife Conservation Trust-Panthera Global Cat Alliance Grants and Department of Science and Technology, Government of India: EMR/2014/000982.  
Department of Science and Technology INSPIRE Faculty Award: IFA12-LSBM-47.  
Centre for Wildlife Studies, Wildlife Conservation Society-New York and Oracle.

## Competing Interests

The authors declare there are no competing interests.

## Author Contributions

- Supriya Bhatt and Suvankar Biswas performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Krithi Karanth analyzed the data, prepared figures and/or tables, and approved the final draft.
- Bivash Pandav conceived and designed the experiments, authored or reviewed drafts of the paper, fieldwork supervision, and approved the final draft.
- Samrat Mondol conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, made earlier leopard data available, and approved the final draft.

## Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

The work conducted here is using non-invasive samples (faeces) collected from the wild with appropriate permissions. Due to the non-invasive nature of the work, no ethical approval is required.

## Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Forest Departments from the states of Uttarakhand, Uttar Pradesh and Bihar provided required permission for field sampling (90/5-6, Uttarakhand Forest Department) (1127/23-2-12(G) and 1891/23-2-12(G), Uttar Pradesh Forest Department) (Wildlife-589, Bihar Forest Department).

## Data Availability

The following information was supplied regarding data availability:

The raw data used in this study are available in the [Supplemental Files](#) and at Dryad: Bhatt, Supriya et al. (2020), Genetic analyses reveal population structure and recent decline in leopards (*Panthera pardus fusca*) across Indian subcontinent, v2, Dryad, Dataset, [10.5061/dryad.v6wwpzgrg](https://doi.org/10.5061/dryad.v6wwpzgrg).

## Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.8482#supplemental-information>.

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