RED PANDA STATUS IN BHUTAN

National Red Panda Survey Report 2023

Department of Forests and Park Services
Ministry of Energy and Natural Resources
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Report prepared by
Tashi Dhendup, Nature Conservation Division, Department of Forests and Park Services, Ministry of Energy and Natural Resources, Bhutan
Mukesh Thakur, PhD, Zoological Survey of India, Ministry of Environment, Forests and Climate Change, Government of India
Letro, Nature Conservation Division, Department of Forests and Park Services, Ministry of Energy and Natural Resources (now with Charles Sturt University)
Sonam Wangdi, Nature Conservation Division, Department of Forests and Park Services, Ministry of Energy and Natural Resources, Bhutan

Field coordination:
Tandin, Nature Conservation Division, former coordinator (now with WWF Bhutan); Letro, Nature Conservation Division (now with Charles Sturt University); Pema Dendup, Jigme Dorji National Park (now with Sarpang Forest Division); Ugyen, Jigme Khesar Strict Nature Reserve; Sonam Tobgay, Trashigang Forest Division; Rinzin Dorji, Gedu Forest Division; Karma Wangdi, Wangchuck Centennial National Park; Pema Rinzin, Paro Forest Division; Tashi Phuntsho, Wangdue Forest Division; Phurpa, Samdrup Jongkhar Division; Tenzin Rangay, Phrumsengla National Park; Sonam Yangden, Dagana Forest Division (now with Sarpang Forest Division); Phub Dorji, Zhemgang Forest Division (now with Nature Conservation Division); Yonten Jamtsho, Mongar Forest Division; Karma Chorten, Dendup, Jigme Singye Wangchuck National Park (now with FRPMD); Karma Gyeltshen, Royal Manas National Park; Kezang Dorjee, Sakteng Wildlife Sanctuary; Sangay Dukpa, Bumdeling Wildlife Sanctuary; Lekey Chaida, Jumotshagkha Wildlife Sanctuary.

Reviewed by:
Technical Advisory Committee, Department of Forests and Park Services, Ministry of Energy and Natural Resources, Royal Government of Bhutan

Cover Photography:
© Yejay/ Forest Ranger

Design and layout:
Dechen Wangmo, WWF-Bhutan

Citation: Nature Conservation Division. 2024. Red Panda Status in Bhutan: National Red Panda Survey Report 2023. Department of Forests and Park Services, Ministry of Energy and Natural Resources, Royal Government of Bhutan

ISBN: 978-99980-782-8-4

Foreword

The red panda, a creature of captivating charm and ecological significance, graces the Bhutanese Himalayas. Its rusty red fur and bushy tail are a familiar sight in our forests, a symbol of the rich biodiversity our country harbors. Yet, the red panda's future remains a cause for concern. Habitat loss, fragmentation, and climate change pose significant threats to this beloved species.

Bhutan, guided by its philosophy of Gross National Happiness, recognizes the intrinsic value of all living beings. The red panda, with its playful nature and vital role in the ecosystem, holds a special place in our hearts. For this reason, we have embarked on this comprehensive national red panda survey. This survey represents a significant step forward in our efforts to safeguard the red panda. The Department of Forests and Park Services in collaboration with the Zoological Survey of India have employed cutting-edge scientific methods to gain a clearer understanding of the red panda population size and distribution within Bhutan, identify areas of crucial habitat and potential threats to their survival and inform effective conservation strategies to ensure the long-term viability of red panda populations in Bhutan.

This national survey is not simply a scientific endeavor; it is a call to action. We invite all stakeholders – local communities, conservationists, and citizens alike – to join us on this journey. By working together, we can unlock the secrets of the red panda and ensure its continued presence in the tapestry of Bhutan's natural heritage. Let us rise to the challenge and ensure that the call of the red panda continues to echo through the majestic mountains of Bhutan for generations to come.

Lastly, I congratulate the Department of Forests and Park Services for executing this mammoth task and the partners at the Zoological Survey of India, WWF-Bhutan and GEF.

Tashi Delek!

Karma Tshering
Secretary

Preface

Bhutan, a land known for its breathtaking landscapes and rich cultural heritage, is also home to a captivating creature – the red panda. These charismatic mammals, with their fiery fur and playful demeanor, are not just a source of national pride but also an indicator of a healthy Himalayan ecosystem. However, red pandas face threats like habitat loss and climate change, necessitating immediate action.

Recognizing their importance, this national red panda survey represents a momentous undertaking for the Department of Forests and Park Services. We are deeply grateful for the invaluable collaboration with our partners the Zoological Survey of India, WWF-Bhutan and GEF for financially supporting the study. Their support has been instrumental in shaping this survey.

This survey has been a true testament to the tireless efforts of our dedicated rangers. Their unwavering commitment, braving challenging terrain and harsh weather, has been the backbone of this project. We owe them our deepest gratitude.

The findings of this survey are not just crucial for red panda conservation in Bhutan but also hold significance for the entire region. Understanding their population size, distribution patterns, and threats will guide us in developing targeted and effective conservation strategies. This, in turn, will benefit other species that share this ecosystem, contributing to a more holistic approach to biodiversity conservation. This document also serves as a comprehensive record of our efforts, outlining the methodologies employed, the challenges encountered, and the critical findings.

We believe this survey paves the way for a future where red pandas continue to thrive in the Bhutanese highlands. Their presence serves as a reminder of the delicate balance of nature, and it is our responsibility to ensure this balance is preserved. The knowledge gained through this survey will be our guiding light in achieving this goal.

Lobzang Dorji
Director

Acknowledgement

This national red panda survey represents a culmination of dedication and collaboration from numerous individuals and organizations. Our deepest gratitude goes to the very foundation of this endeavor – our rangers. Their unwavering commitment forms the bedrock of this study. They traversed diverse, often challenging terrains, braving harsh weather conditions, to collect scat samples across the entire country. Their tireless efforts are a testament to their passion for conservation, and we commend them for their invaluable contribution.

We are grateful to the Ministry of Energy and Natural Resources for recognizing the importance of red panda conservation and granting their approval for this crucial study. Their support has paved the way for this critical undertaking.

The Zoological Survey of India has been an invaluable partner in this endeavor. Their expertise in laboratory analysis of the collected samples and their willingness to share their knowledge in data analysis and co-writing this report have significantly enriched the study. We are deeply indebted to them for their critical contribution.

Our sincere appreciation goes to WWF-Bhutan, WWF-Germany and the Global Environment Facility (GEF) for providing the financial resources that made this nationwide survey a reality. Their support has enabled us to conduct a comprehensive study and gather vital data for red panda conservation.

By working together, we have taken a significant step forward in understanding the red panda population in Bhutan and safeguarding their future within our borders. We are confident that the findings presented in this report will serve as a valuable resource for red panda conservation efforts not only in Bhutan but across the entire Himalayan region.

Sonam Wangdi
Chief Forestry Officer

List of Acronyms

ADO : Allelic Dropout

BFL : Bhutan for Life

BLAST : Basic Local Alignment Search Tool

DAPC : Discriminant Analysis of Principal Component

DFO : Divisional Forest Office

DNA : Dioxyribonucleic acid

FA : False allele

FMID : Forest Monitoring and Information Division

HE : Expected Heterozygosity

HO : Observed Heterozygosity

HWE : Hardy-Weinberg Equilibrium

IBD : Isolation by Distance

IUCN : International Union for Conservation of Nature

MaxEnt : Maximum Entrophy

MoENR : Ministry of Energy and Natural Resources

NBC : National Biodiversity Centre

NCBI : National Centre for Biotechnology Information

NCD : Nature Conservation Division

NSB : National Statistical Bureau

PA : Protected Areas

PCR : Polymerase Chain Reaction

PID : Probability of identity of unrelated individuals

ZSI : Zoological Survey of India

WWF : Worldwide Fund for Nature

GEF : Global Environment Facility

List of Figures

Figure 1. Global distribution of red panda (Reproduced from IUCN red list, 2015).

Figure 2. A red panda camera trapped in their natural habitat. Red Pandas prefer areas with bamboo which are their preferred food source.

Figure 3. Protected area network in Bhutan with district

Figure 4. Red Panda habitat suitability map as obtained from MaxEnt modelling

Figure 5. 666 grids each measuring 2km x 2km were selected for red panda fecal sampling after a rigorous expert reviews and consultations.

Figure 6. A ranger examines a tree branch for red panda samples.

Figure 7. Red panda scats in the wild (left) and samples collected and stored in vials filled with silica desiccants to keep them dry(right)

Figure 8. A) Gel image of DNA extracted from red panda samples, B) D-Loop PCR amplified on 2% agarose gel

Figure 9. Number of red panda scat samples that successfully went through each stage, from DNA extraction to genotyping.

Figure 10. Haplotype network created using d-loop data. (a) various colours depicted the different geographic locations, and a corresponding geographical map is provided for reference. (b) distinct colours depicted the distinguish haplotype, and a corresponding geographical map to illustrate the distribution of the 14 haplotypes in 15 districts of Bhutan.

Figure 11. (a) Haplotypes of the control region in red pandas are represented by different colours. Yellow dots correspond to Chinese red pandas from China, red dots represent those from India, orange dots denote Himalayan red pandas from Bhutan (current study), blue dots represent Himalayan red pandas from Tibet, and green dots signify Himalayan red panda from India. (b) Map showing the geographic distribution of shared haplotypes of the Himalayan red panda in India and Bhutan.

Figure 12. Mismatch distribution modal using the D-Loop in DNA analysis through software DNASP

Figure 13. Bayesian skyline plot (BSP) of the control region sequence constructed in the BEAST

Figure 14. Microsatellite amplification success rate of nine microsatellites with red panda samples

Figure 15. Map showing the locations from where red pandas were recorded through scat sampling during the survey

Figure 16. Bhutan map showing the Area of Occupancy and Extent of Occurrence of red panda in Bhutan prepared using GeoCAT

Figure 17. Red Pandas can often be seen walking in the snow

Figure 18. (a) Bayesian STRUCTURE clustering results based on nine microsatellite genotypes among the red panda populations of Bhutan, (b) the ad hoc quantity (delta k) values k3, indicating the most likely number of three genetic clusters, (c) Discriminant Analysis of Principal Components (DAPC) of red panda population in Bhutan. Each circle represents a cluster and each dot represents an individual.

Figure 19. (a) Spatial PCA showing clusters in spatially distributed populations, (b) Scatterplot showing the result of mantel test for presence of IBD (isolation by distance) between significance of geographical distance on the genetic distance (r = 0.0163), (c) Detection of genetic barrier and landscape interpolation using Allele In Space (AIS).

Figure 20. Updated Red Panda distribution map with recent records from the nationwide tiger camera trap surveys in the last 10 years and the current scat survey of 2023.

List of Tables

Table 1. Variables influencing red panda habitat use as per MaxEnt modelling

Table 2. The size range of spindle-shaped pellets of adult and sub-adult/cub red panda (Yonzon, 1989).

Table 3. Microsatellites and their characteristics selected for red panda analysis

Table 4. Details of three multiplex panels designed for the multilocus genotyping of red panda

Table 5. Observed haplotypes in red panda across 15 districts of Bhutan

Table 6. Summary of genetic diversity indices and neutrality tests of demographic expansions of Himalayan red panda. N- Number of samples; P- Polymorphic sites; H- Number of Haplotypes; K- Average number of nucleotide differences; Hd-Haplotypes diversity; π- Nucleotide diversity; * not significant

Table 7. Number of grids, total number of samples collected and number of individuals (*includes recaptures from other sites)

Table 8. Genetic diversity estimates of sampled red panda population in Bhutan (Na- number of alleles; Ne- Effective number of alleles; Ho- Observed heterozygosity; He-Expected heterozygosity; Fis- Inbreeding coefficient)

Executive Summary

The red panda (*Ailurus fulgens*) is classified as Endangered by the International Union for Conservation of Nature (IUCN) Red List, reflecting the significant threats it faces globally. These threats include habitat loss and fragmentation due to deforestation for agriculture, logging, and development. Additionally, red pandas are subject to poaching and illegal trade, driven by demand for their distinctive fur and the pet trade. Climate change further exacerbates these threats by altering their habitat and food resources.

In Bhutan, the conservation status of the red panda is relatively more stable compared to other regions. The Bhutanese government has implemented substantial measures to protect red panda habitats through the establishment of protected areas and biological corridors. Bhutan also engages in community-based conservation efforts, involving local populations in sustainable practices that benefit both wildlife and communities. Moreover, the country enforces strict anti-poaching laws to protect red pandas from illegal hunting and trade. Despite these positive measures, red pandas in Bhutan still face challenges from habitat fragmentation and climate change.

Given the decline in wildlife populations and increasing habitat degradation, understanding the abundance and genetic health of the local red panda population is crucial. This report summarizes a recent survey conducted to investigate current red panda numbers in Bhutan, as well as gene flow and population connectivity within the country. The survey provides valuable insights into genetic diversity, population stability, and potential challenges facing the red panda population in the region. The study utilized non-invasive genetic tools, to assess the red panda population in Bhutan. The following are some of the key findings from the study:

- *Genetic Diversity and Haplotype Sharing:* The study generated 432 sequences of the control region of the red panda population in Bhutan, revealing haplotype sharing within Bhutan and between Bhutan and India, indicating ancestral gene flow shared across these regions. However, the existing red panda population in Bhutan exhibited relatively lower genetic diversity compared to previous studies, likely due to historical bottlenecks events.
- *Demographic Equilibrium, Bottleneck, and Recent Expansion:* Neutrality tests and a multimodal mismatch curve indicated that the red panda population in Bhutan is currently under demographic equilibrium. The study also found evidence of a bottleneck event approximately 5,000 years ago, followed by recent population expansion.
- *Haplotype Diversity and Regional Differences:* The study identified 21 haplotypes in Bhutan, with Haplotype H3 being common and distributed across thirteen districts. The Haa district exhibited the highest number of unique haplotypes (five), while other districts showed haplotype sharing. Haplotype sharing was also observed between the red panda populations of India and Bhutan. The sharing of haplotypes between regions is a reflection of the past gene flow facilitated by the red panda movement in the landscape.
- *Unique Individuals and Population Structure:* Microsatellite genotyping identified 302 unique red panda individuals using a panel of seven microsatellite loci. Both Bayesian and non-Bayesian structure analyses revealed three distinct clusters of red pandas in Bhutan following the metapopulation framework. Red Pandas from central and eastern Bhutan showed more genetic sharing among themselves compared to the central and western populations, indicating higher genetic facilitation between central and eastern populations and higher resistance between central and western populations.

These findings provide a comprehensive understanding of the genetic make up and population structure of red pandas in Bhutan, highlighting the importance of continued conservation efforts to ensure their long-term survival.

Table of Contents

Foreword i

Preface ii

Acknowledgement iii

List of Acronyms iv

List of Figures v

List of Tables vi

Executive Summary vii

CHAPTER 1 Introduction 1

1.1. Red Panda: Global Status and Distribution 1

1.2. Status of Red Panda in Bhutan 3

1.3. Nationwide Red Panda Survey 2023 4

CHAPTER 2 Materials and Methods 6

2.1. Study Area 6

2.2. Red Panda Faecal Sample Collection 10

2.3. Laboratory Analyses 12

2.4. Analysis of Genetic Data 16

CHAPTER 3 Results 19

3.1. Mitochondrial Markers 19

3.2. Microsatellite Analysis 23

CHAPTER 4 Discussion and Management Recommendations 30

4.1. Red Panda Numbers and Distribution 30

4.2. Genetic Diversity and Connectivity 31

4.3. Insights into Population History 32

4.4. Scat Sampling: A Powerful Tool for Red Panda Monitoring 33

4.5. Conservation Implications for Red Pandas 33

References 35

CHAPTER 1
Introduction

1.1. Red Panda: Global Status and Distribution

The red panda (*Ailurus fulgens*), with its captivating rusty-red fur and bushy tail, is an arboreal mammal endemic to the Eastern Himalayas. This seemingly adorable creature holds a unique distinction – it is the sole living member of the family Ailuridae (Pradhan et al., 2001). Despite its endearing appearance, the red panda faces significant threats in the wild, and is classified as "Endangered" on the IUCN Red List (Wang et al. 2008). Their distribution is characterized by patchiness and low population densities throughout their range (Wei et al. 1999; Thapa et al. 2018).

Figure 1. Global distribution of red panda (Reproduced from IUCN red list, 2015).

Native to temperate conifer and cool broadleaf forests with dense bamboo undergrowth, red pandas occupy a specific ecological niche at elevations ranging from 1,500 to 4,800 meters (Choudhury, 2001). Red pandas are habitat specialists, thriving in environments that cater to their specific needs. Dense bamboo undergrowth is crucial for both food and cover (Yonzon & Hunter, 1991). Fallen logs and stumps provide essential platforms for climbing, resting, and accessing food sources (Bista et al. 2017; Wei et al. 1999). Proximity to water sources is another key factor for their survival (Bista et al. 2017; Yonzon & Hunter, 1991). Interestingly, despite being classified as Carnivora, their diet primarily consists of bamboo leaves and shoots, supplemented by fruits, mushrooms, roots, and acorns (Yonzon & Hunter, 1991). Occasionally, they may consume insects and bird eggs.

The distribution of red panda historically spanned Nepal, southwest China, Bhutan, Myanmar, and the northeastern Indian states of Sikkim, West Bengal, Arunachal Pradesh, and Meghalaya (Choudhury, 2001) (Fig. 1). However, recent confirmations of their presence in Meghalaya are lacking.

Recent advancements in genetic analysis suggest the existence of two distinct species: the Himalayan red panda (*Ailurus fulgens*) and the Chinese red panda (*Ailurus styani*) (Hu et al. 2020). Previously, these were considered subspecies separated by the Salween River (Choudhury, 2001; Wang et al., 2008).

Red panda faces a multitude of threats in the wild. Habitat loss and fragmentation driven by human activities such as deforestation and infrastructure development pose the most significant challenge (Yonzon and Hunter 1991b; Glatston, 1994; Wei et al. 1999). Poaching remains a concern despite legal protections in all range countries (Yonzon & Hunter, 1991b; Glatston, 1994). The presence of free-roaming domestic dogs kept by herders for livestock protection can also pose a predation risk (Bista et al. 2017; Dendup et al., 2016). Climate change further complicates their situation by impacting vegetation composition and increasing the frequency of extreme weather events, potentially disrupting their food sources and habitat suitability (Glatston et al. 2015). The dependence of local human populations on natural resources can also lead to disturbances that negatively affect red panda habitats.

Global population estimates suggest there are less than 10,000 mature red panda individuals remaining (Glatston et al. 2015). The "Endangered" status on the IUCN Red List underscores the urgency of conservation efforts (Wang et al. 2008). Fortunately, the red panda is listed on Appendix I of CITES, prohibiting international commercial trade. Additionally, all range countries offer legal protection for this charismatic mammal.

1.2. Status of Red Panda in Bhutan

Red panda is widely distributed across Bhutan, inhabiting 19 out of 20 districts, with the exception of Pemagatshel. Their presence has been confirmed within numerous protected areas, such as Wangchuck Centennial National Park, Jigme Dorji National Park, Jigme Singye Wangchuck National Park, Phrumsengla National Park, Bumdeling Wildlife Sanctuary, Sakteng Wildlife Sanctuary, Jigme Khesar Strict Nature Reserve, and the Royal Botanical Park. Additionally, red pandas utilize biological corridors that connect several of these protected areas, which is crucial for maintaining genetic diversity and population health. Approximately 10,971.2km^2 is predicted to be potential red panda habitat in Bhutan which is 28.58% of the total country area (Letro et al. 2022).

In Bhutan, red pandas are found at elevations ranging from 1,515 to 4,389 masl (Dorji et al. 2011, Letro et al. 2022). This altitudinal variation is closely linked to the availability of bamboo, their primary food source (Fig. 2). Bamboo species such as *Yushania maling*, *Y. microphylla*, *Borinda grossa*, and *Arundinaria racemosa* are particularly favored. While previous studies reported a preference for mixed conifer and fir forests, recent surveys have documented red pandas in a broader range of habitats, including alpine scrub, meadows, and even areas near human settlements (Dendup et al. 2023).

Figure 2. A red panda camera trapped in their natural habitat. Red Pandas prefer areas with bamboo which are their preferred food source.

Despite their widespread distribution and legal protection under Schedule II of the Forest and Nature Conservation Act of Bhutan (2023), red pandas in Bhutan face several significant threats. One major threat comes from free-roaming dogs kept by herders for livestock protection (Letro et al. 2022). These dogs can predate on red pandas and potentially transmit diseases like rabies and canine distemper, to which red pandas are highly susceptible. Habitat degradation due to climate change is another critical concern, impacting vegetation composition and increasing the frequency of extreme weather events. Additionally, a lack of awareness about the red panda's ecological importance among local communities, including farmers, students, and monks, hinders effective conservation efforts.

Limited financial resources and technical expertise among frontline conservation officials present ongoing challenges. Securing funding specifically for red panda conservation is often more difficult compared to charismatic megafauna like tigers and snow leopards. A Red Panda Conservation Action Plan 2018-2023 has been developed to guide the conservation of this threatened species in Bhutan but not many actions have been implemented due to limited funding (NCD 2023).

Despite these challenges, there have been promising initiatives in recent years. For instance, projects like the sustainable rangeland management initiative in Sakteng Wildlife Sanctuary aim to protect red panda habitats while supporting local herder communities. The Bhutan for Life project also prioritizes the conservation of lesser-known species, including red pandas, through sustainable financing of protected areas.

1.3. Nationwide Red Panda Survey 2023

Objective 2 of Bhutan's Red Panda Conservation Action Plan (2018-2023) focuses on enhancing knowledge about red panda conservation and their habitat requirements. A key priority action identified is conducting nationwide red panda surveys to gain a comprehensive understanding of their distribution and population estimates. Therefore, the current survey was carried out to generate information on red panda abundance, distribution, genetic diversity and population structure in Bhutan which in turn is expected to inform red panda conservation programs in the country.

Due to the arboreal nature of red pandas, camera traps often face challenges in capturing clear images for population surveys. To address this limitation, the Department of Forests and Park Services carried out non-invasive fecal sampling across all potential red panda habitats in the country. This survey aimed to achieve the following objectives:

1. Estimate the minimum number of red pandas in Bhutan: By analyzing the presence and distribution of fecal samples, researchers can obtain valuable insights into red panda population size within the surveyed areas.
2. Understand the distribution pattern of red pandas across the country: Fecal samples can be geographically referenced, allowing researchers to map the presence of red pandas and identify areas with higher concentrations.

3. Gain insights into potential diversity and inbreeding: Genetic analysis of fecal samples can provide clues about the genetic makeup of the red panda population in Bhutan. This information can help identify potential issues like inbreeding that could be detrimental to population health.
4. Investigate population genetic structure: Analysis of fecal DNA can reveal information about gene flow and connectivity between different red panda subpopulations within Bhutan. This knowledge is crucial for designing effective conservation strategies.

A collaborative effort led by the Department of Forests and Park Services (Ministry of Energy and Natural Resources, Royal Government of Bhutan) conducted the red panda survey. The Zoological Survey of India (Government of India) provided expertise by performing the laboratory analyses of samples. Data analysis and the final report were produced jointly by both institutions.

CHAPTER 2
Materials and Methods

2.1. Study Area

2.1.1. Bhutan: Geographic Location

Bhutan, a small, landlocked nation nestled in the Eastern Himalayas, boasts a unique identity shaped by its geography, culture, and environmental stewardship. Wedged between the Tibetan Autonomous Region of China to the north and Indian states, Bhutan's 38,394 square kilometres are a treasure trove of biodiversity.

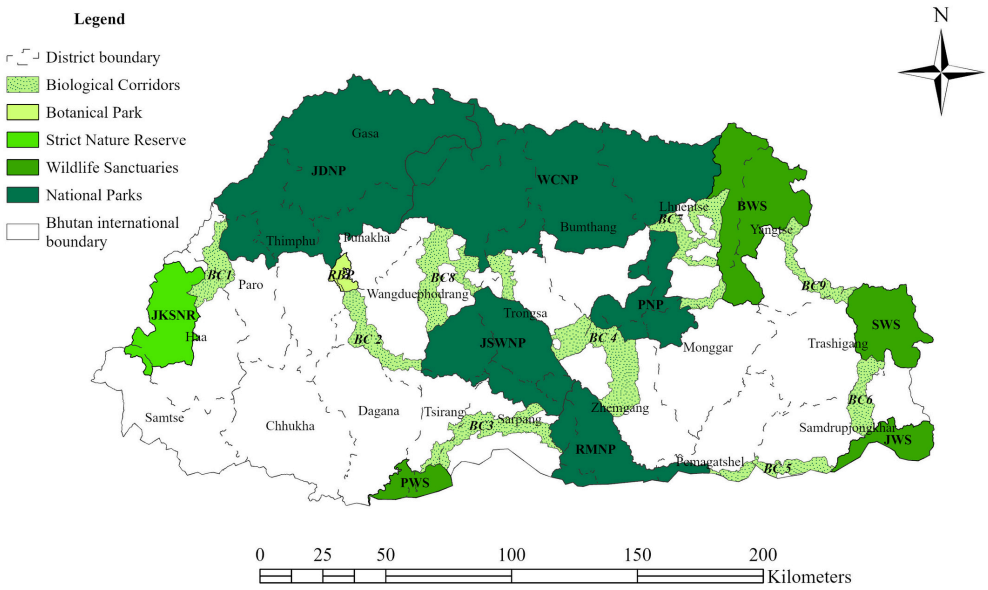

Figure 3. Protected area network in Bhutan with districts

Approximately 770,000 people call Bhutan home, residing primarily in the fertile valleys across the country. The country retains a strong sense of community, with a population density of 20.38 people per square kilometre (NSB, 2023). Despite being classified as developing, Bhutan prioritizes a unique philosophy - Gross National Happiness (GNH) - which emphasizes environmental conservation alongside economic and social well-being (Thinley & Hartz-Karp, 2019). This philosophy reflects Bhutan's deep respect for nature, a tradition nurtured by its visionary monarchs and a strong cultural foundation (Ura et al., 2009).

Bhutan's ecological significance lies at the crossroads of two major biogeographic realms: the Indo-Malayan and the Palearctic. As part of the Eastern Himalayan global ecoregion, Bhutan is recognized for its exceptional biodiversity (Olson & Dinerstein, 1998). The dramatic rise in elevation, from subtropical forests at 95 meters above sea level to snow-capped peaks exceeding 7,500 meters within a mere 170 kilometres (DoFPS, 2023),

creates a remarkable range of habitats. Temperatures vary widely across the country, with annual averages ranging from 15°C to 30°C, and precipitation levels spanning from 300 to 7,800 millimetres (NSB, 2023). This diversity fosters distinct ecosystems, broadly classified into six vegetation zones, ranging from tropical to arctic (Wangda & Ohsawa, 2006).

Bhutan's impressive 70% forest cover (FMID, 2023) serves as a refuge for over 11,000 species of terrestrial and aquatic life (NBC, 2022). More than 52% of the country is protected in the form of national parks, wildlife sanctuaries, strict nature reserve and biological corridors (Fig. 3). The country is a critical haven for a multitude of globally threatened mammals, including tiger *Panthera tigris*, snow leopard *Panthera uncia*, common leopard *Panthera pardus*, Asiatic elephant *Elephas maximus*, Greater one-horned rhinoceroses *Rhinoceros unicornis*, Asiatic black bear *Ursus thibetanus*, and red pandas (Dorji et al., 2018; Dendup et al., 2023; Dorji et al., 2012; Letro et al., 2022). Bhutan's commitment to conservation ensures the continued survival of these magnificent creatures within its breathtaking mountain realm.

2.1.2. Survey Area and Study Design

Bhutan's protected area network comprises five national parks, four wildlife sanctuaries, one strict nature reserve, and a botanical park, interconnected by a mosaic of nine biological corridors. While each protected area has independent administrative management, the biological corridors fall under the jurisdiction of different Divisional Forest Offices (DFOs) based on their location within the country's fourteen DFOs.

A key distinction between PAs and DFOs lies in their management zones. PAs have stricter conservation regulations that prohibit major destructive activities like mining and commercial logging (NCD, 2023). However, species distribution and forest coverages between PAs and DFOs are not vastly different, with some DFO areas even exhibiting higher species richness (Penjor et al., 2021).

The red panda survey encompassed both the ten protected areas and the fourteen DFOs. Red pandas are habitat specialists, meaning their distribution is influenced by various factors like bamboo availability, forest type, slope, aspect, elevation, and human disturbances (Dendup et al., 2020; Dorji et al., 2011). Consequently, they are not found throughout Bhutan.

To tailor the survey area selection for the red panda population survey, researchers first gathered all point locations with confirmed red panda presence from various sources: a nationwide camera trapping survey conducted between 2014 and 2015 (DoFPS, 2015), wildlife surveys in various protected areas for management planning and opportunistic encounters with live red pandas or their evidence during ranger patrols. Using this red panda presence data, researchers employed a maximum entropy algorithm (MaxEnt 3.3.3k) (Phillips et al., 2006) to map potential red panda habitats across Bhutan. This analysis considered nine habitat and climate variables known to influence red panda habitat use (Letro et al., 2022). The MaxEnt predictive probability index (0.30-1 based on a 10-percentile threshold) was then used to generate a map of potential red panda habitat in Bhutan, which served as the base layer for overlaying sampling grids (Fig. 4).

Variables	Variable type	Per cent contribution to Predictive mapping	Permutation importance
Mean annual temperature	Continuous	35.9	32
Mean annual precipitation	Continuous	30.6	31.2
Forest types	Categorical	8.5	4.4
Elevation	Continuous	7.3	18.2
Distance from settlement	Continuous	6.9	4.3
Aspect	Continuous	5.1	5.6
Slope	Continuous	3.8	1.6
Distance from road	Continuous	1.4	2.2
Hill shade	Continuous	0.6	0.5

Table 1. Variables influencing red panda habitat use as per MaxEnt modelling

Bhutan utilizes a national biodiversity monitoring grid system with 4km x 4 km grids that can be further subdivided into 2x2 km sub-grids (DoFPS, 2020). These 2km x 2 km sub-grids were overlaid onto the potential red panda habitat map using QGIS ver. 3.28.5 (QGIS, 2023) to optimize the selection of survey sites. This process considered the median red panda home range size of 1.41 km^2 in similar habitats of Nepal (Bista et al., 2021). Due to resource constraints, the survey area underwent a final optimization step. The respective PAs and DFOs conducted a thorough validation of the selected grids to ensure that the prime red panda habitat was not missed and areas with a low probability of red panda occurrence were excluded from fecal sampling. This meticulous process resulted in the identification of 666 final red panda survey sites distributed across the 20 participating field divisions and parks (Fig. 5, Table 3).

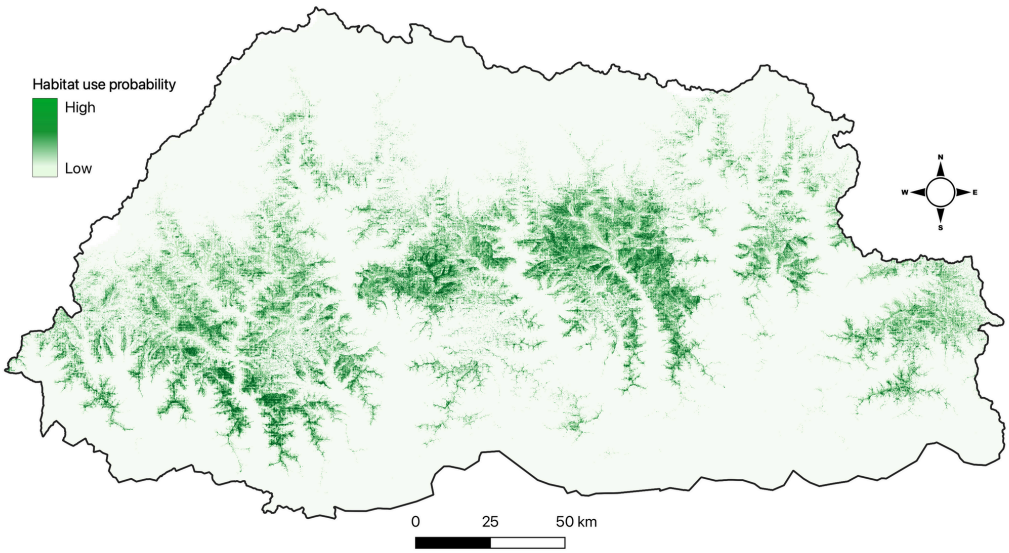

Figure 4. Red Panda habitat suitability map as obtained from MaxEnt modelling

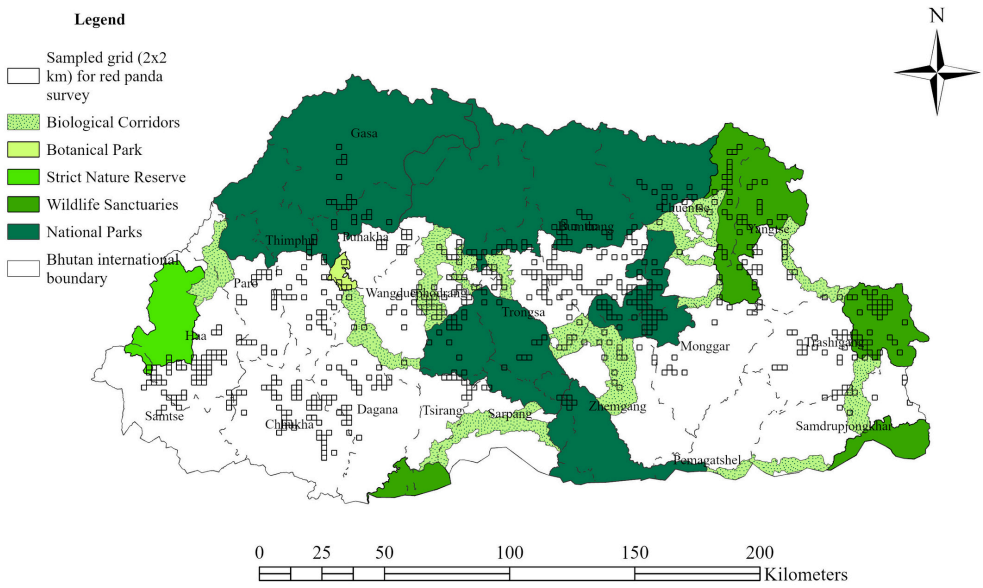

Figure 5. 666 grids each measuring 2km x 2km were selected for red panda fecal sampling after a rigorous expert reviews and consultations.

2.2. Red Panda Faecal Sample Collection

Within each sampled grid, researchers established transects ranging from 3 to 5 kilometres in length, depending on accessibility (Dendup et al., 2020; Panthi et al., 2017). These transects served as search paths for collecting red panda fecal samples (dungs/scats).

The rugged Himalayan terrain presented challenges for laying out systematic transects. To address this, researchers employed a "trail transect" approach, utilizing existing trails such as traditional human paths, game trails, livestock trails, and any other accessible routes. When existing trails within a sampled grid were insufficient to reach the desired 3–5 km length, researchers conducted at least three shorter trail transects, each measuring at least one kilometre and spaced no less than 300 metres apart. Transect survey forms were filled out at the start, end, and every 500 metres along the transect during the survey.

Figure 6: A ranger examines a tree branch for red panda samples.

While traversing the designated trail transects, surveyors scanned the surrounding areas for red panda droppings. Red pandas use various locations as latrines, including tree branches, fallen logs, rocks, and the ground (Bhatta et al., 2014) (Fig. 6). These locations can easily conceal droppings, so surveyors received training on effectively searching these areas. Latrine sites might contain piles of droppings of varying age, indicating resting areas. Alternatively, red pandas may leave behind a few pellets (1-16) during other activities, or these droppings may fall from trees where the animals reside. However, red pandas typically defecate repeatedly at the same location, which can accumulate over 100 pellets (Yonzon, 1989).

Fresh red panda droppings are spindle-shaped, soft, moist, and green in color, with size variations depending on whether they are from adults or cubs/sub-adults (Table 2). Heavy rain can alter the droppings' shape, causing them to lose their spindle form and shrink to half their normal size (Fig. 7).

Due to the similarity between red panda droppings and porcupine scats, surveyors received training to differentiate between the two. Porcupine scats are typically darker in color.

Dimension	Adult	Sub-adult/cub
Length (mm)	41.6 ± 6	34.7 ± 7.1
Diameter (mm)	19.2 ± 2.3	14.9 ± 2.6

Table 2. The size range of spindle-shaped pellets of adult and sub-adult/cub red panda (Yonzon, 1989).

Upon locating red panda droppings, surveyors collected 1-2 fresh pellets with pointed ends. These were then stored in a plastic vial containing silica gel desiccant (Fig. 7). If the droppings were found at a latrine site with a mix of old and fresh pellets, only the fresh ones were collected. However, in the absence of fresh pellets, old pellets were collected as well. To minimize contamination, surveyors used new gloves and a clean twig for each collection. After placing the pellets in the vial, they secured the lid tightly to ensure an airtight seal.

Data collection using the Epicollect5 app accompanied each sample. This information included the date of collection, geo-referenced location coordinates, and the condition of the droppings.

Figure 7: Red panda scats in the wild (left) and samples collected and stored in vials filled with silica desiccants to keep them dry(right)

Following the field survey, samples were transported to a cool, dry location at the station for temporary storage. Subsequently, they were shipped to the central repository unit at the Nature Conservation Division. Here, the samples were initially stored in a deep freezer (-4°C) before being transferred to a laboratory for analysis. Finally, at the lab, the samples were submerged in 70% ethanol for long-term storage and to inactivate any potential viruses, parasites, or bacteria.

2.3. Laboratory Analyses

2.3.1. DNA Extraction

Of the 482 fecal samples of red panda collected, 462 samples qualified to be analyzed further for DNA analysis based on the quality of sample. To obtain the gDNA, the outermost layers of each sample were scraped with a scapula and collected in 2 µL micro centrifuge tubes, and genomic DNA extraction was carried out using QIAamp Fast DNA Stool Mini Kit (QIAGEN Germany) following the manufacturer's instruction. The quality of extracted g-DNA was checked on 1 % agarose gel (Fig. 8).

2.3.2. PCR Amplification and Sequencing

All the extracted samples were then subjected to amplification with partial segment (approximately 440 bp) mitochondrial control region using specific primers ThrL (5' -CCT TGGTCTTTGTAAACC-3') and DLH (5' -CCTGAGGTAAGAACCAGATG-3'), following Su et al. (2001) and Li et al. (2005). PCRs were carried out in 10 µL reaction volume comprising of 5 to 10 ng template DNA, 1U Taq polymerase (Takara), 5X PCR buffer, 1 mM MgCl2, 2.5 mM dNTP Mixture, 0.1 µM of each primer, 0.1 µg/µL BSA.

Thermal Cycle: The reactions were performed with an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 30 seconds, 53°C for 45 seconds, initial extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The amplification was checked in 2% agarose gel (Figure 2B).

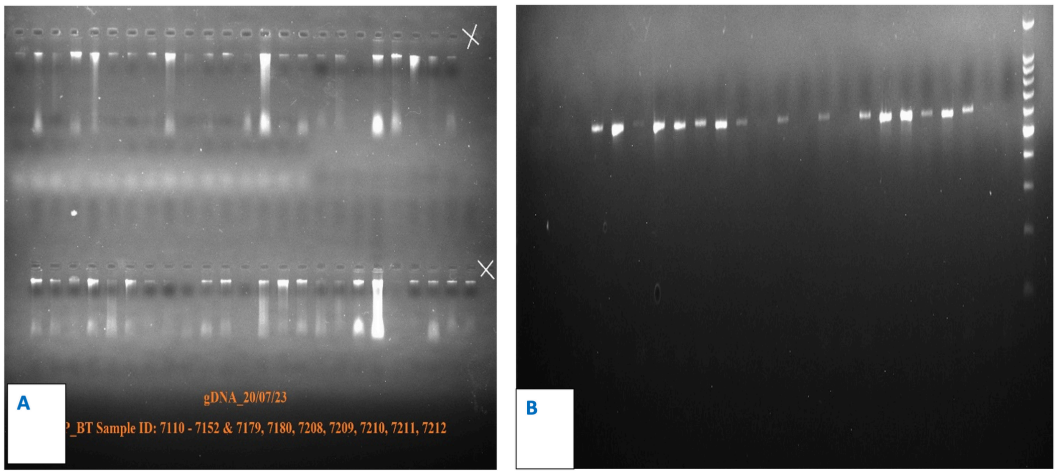

Figure 8 A) Gel image of DNA extracted from red panda samples **B)** D-Loop PCR amplified on 2% agarose gel

The PCR products were cleaned using Exo-SAP treatment. Exo-SAP possesses exonuclease activity and it is used to remove any residual PCR oligonucleotide primers and dNTPs that may interfere with the sequencing process. The cleaned PCR products were then processed for sequencing. Sanger sequencing was performed on Genetic Analyzer ABI 3730 (Applied Biosystems, USA) using the Big Dye terminator cycle sequencing kit v 3.1 (Thermo Scientific, USA).

All sequences generated were cleaned manually using Sequencher version 5.4.6 (Gene Codes Corporation, USA) (https://www.genecodes.com) and aligned using CLUSTAL W (Thompson et al., 2003) algorithm in BioEdit v.7.2.5 (Hall et al., 2011) and trimmed to generate similar length of sequences for further analysis.

All the generated sequences were subjected to nucleotide BLAST to confirm the species identity. Consensus sequences of the control region of red panda available in the public domain were also retrieved from the online database/GenBank and aligned along with the generated sequences using MUSCLE algorithm (Edgar, 2004) in MEGA X software (Kumar et al., 2018). All these sequences were then used for the phylogenetic analysis.

2.3.3. Selection of Microsatellite Markers

A total of 22 microsatellites were selected from previous studies that were already tested on red panda (Liang et al., 2007; Yang et al., 2019). The microsatellites markers selected for current study were as follows: *CRP385*, *CRP335*, *CRP41*, *CRP409*, *CRP429*, *CRP381*, *CRP240*, *CRP 357*, *CRP367*, *Aifu01*, *Aifu05*, *Aifu23*, *Aifu24*, *CRP261*, *CRP50*, *CRP84*, *CRP260*, *CRP404*, *CRP442*, *CRP391*, *Aifu09*, *Aifu25* (Table 2). Forward primer of each marker was labelled with one of the dye colors i.e., FAM, VIC, PET, NED at 5'end and therefore multiplexing of loci was attempted based on the fluorescent dye chemistry.

Markers	Repeat Motif	Sequence(F)	Sequence(R)	Allele Size Range	References
CRP385	(TAAA)9	CATCCCAGGAGACCAAAG	ATAAACTGACAAGAAGTCCTCC	370-420	Yang et al 2019
CRP335	(AAACA)9	GACTCTTGGTTTCAGGGTTC	TCTACAGTGGACCATTTTTGATA	220-270	Yang et al 2019
CRP41	(TCTA)13	AACCTCTGTCCTCCTCCATC	GCACAATGGTTTAGGCTTTT	140-190	Yang et al 2019
CRP409	(TAT)13	CCACCATCTGTTAGGGAGTAG	CCTTGATTTGTTGGAGCATT	270-320	Yang et al 2019
CRP429	(TCTA)11	TAGGGTTCAAGCTCTTACTTAGTC	AGCAAACTGTTCTACCACTTCTC	200-250	Yang et al 2019
CRP381	(TCTA)12	AATGTCAAGGAAGAACCCAA	TTCACTGCTCACCGTTTCTA	240-300	Yang et al 2019
CRP240	(AAAC)11	TGATTCAAGGTTCCCTATGT	AAGAAAGTGGTTAGTTTAAATGTT	160-210	Yang et al 2019
CRP357	(TGA)12	TCCAAAATAATGGTAAAGCC	CAACTCAACTACATCGCCTC	320-450	Yang et al 2019
CRP367	(TAGA)11	TCCATGTAAGCCTCCAAACT	AGAACCAAATGTCTCCACGT	240-290	Yang et al 2019
Aifu24	(TATC)10	AGAGGGAAGTCTGCTTGAA	CATGGCATAGTGAGCTGGT	225-243	Liang et al 2007
Aifu01	(ATAG)12	CCTGCATCAGACTCAGCA	GGTATCAGACGTGGGAACTA	126-178	Liang et al 2007
Aifu05	(GATA)13	GAATAATGAGCTTGCCTTCC	TTGACATTGGCTATGTGAACA	328-348	Liang et al 2007

CRP261	(TGA)12	TGCCGGATTTCA GATTATTG	ACGGGTCAGAGTC AGAGGAG	310-360	Yang et al 2019
CRP50	(TTTA)10	AGGGTCTGAGG GCGTTTC	TCTTGACTCCAACCT CTTTCG	190-240	Yang et al 2019
CRP84	(GTAA)10	AAGTTGAAATGCC CGAGTTG	TTGCTGGTAGTTGTA GTAATGGTG	350-400	Yang et al 2019
CRP260	(GAAT)9	GGGGCCTTGTCT AATTCTGT	TCACTCCTGGTGCT GGTCT	200-250	Yang et al 2019
Aifu23	(TATC)13	GGACCCAACAA GCCCTTTACT	TTCTCTCTCAGCCTC TCACCCT	133-161	Liang et al2007
CRP442	(TCAT)11	TTCTGTGCCTGGC TTTA	CCTCTTTTACCTCTG CCTCC	256-272	Yang et al 2019
CRP391	(AAAC)9	GAAGGGAGGAA CCATAGTCAG	TTGATTGAGGTGCT GTAGTGTTA	445-453	Yang et al 2019
CRP404	(ATTC)9	GTTCCCTGTAGTTT CATCCC	TTTGTTTGCCATTGTA TTCC	380-392	Yang et al 2019
Aifu09	(GATA)9	CAAAGTGCCTGG TGAAGT	AAATGAACCCACC GTTGA	173-201	Liang et al 2007
Aifu25	(CTAT)15	AATTGCATGAGC CAGTTC	GAGACCATTTTGGG ACCG	154-190	Liang et al 2007

Table 3. Microsatellites and their characteristics selected for red panda analysis

2.3.4. Microsatellite Genotyping

Initial screenings were carried out with two reference tissue samples from India and four scat sample collected from Bhutan with all 22-microsatellite markers. The PCRs were carried-out in 10µl reaction volume following Dalui et al., 2020 using different temperature gradients.

Only 15 microsatellites markers were successfully amplified in the sample collected from Bhutan. Amplified markers were then categorized into three multiplexes based on the allele size, dye and annealing temperatures (Table 3). Genetically identified samples of the red pandas were then amplified with the three multiplexes. The thermal cycle profile was: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of PCR and a final step of 72° C for 30 minutes. The annealing temperature (Ta) for multiplex 1 was 53 °C, multiplex 2 was 55 °C and multiplex 2 was 57 °C (Table 3). The PCR products were resolved on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and allele scoring was done using Gene Mapper 4.1 (Applied Biosystems, Foster City, CA, USA) (Figure 9).

S. No.	Locus	Repeat Motif	Size Range	Ta (°C)	Multiplex
1	Aifu01	(ATAG)12	126-178	53	MP1
2	RP240	(AAAC)11	160-210		
3	RP357	(TGA)12	320-450		
4	RP381	(TCTA)12	240-300		
5	RP409	(TAT)13	270-320		
6	Aifu05	(GATA)13	328-348	55	MP2
7	Aifu23	(TATC)13	133-161		
8	Aifu24	(TATC)10	225-243		
9	RP367	(TAGA)11	240-290		
10	RP429	(TCTA)11	200-250		
11	RP260	(GAAT)9	200-250	57	MP3
12	RP385	(TAAA)9	370-420		
13	RP41	(TCTA)13	140-190		
14	RP50	(TTTA)10	190-240		
15	RP84	(GTAA)10	350-400		

Table 4. Details of three multiplex panels designed for the multilocus genotyping of red panda

2.4. Analysis of Genetic Data

2.4.1. Mitochondrial D-loop Region

Cleaned sequences were matched for sequence similarity using National Centre for Biotechnology Information (NCBI) BLAST (Basic Local Alignment Search Tool). Reference mitochondrial genomes of red panda were downloaded from NCBI Database and aligned using MEGA (Kumar et al., 2018). All the generated sequences were validated using the NCBI BLAST search. Once the sequences were verified as red panda, sequence data were used for studying demographic history and population genetics.

2.4.2. Diversity Indices, Phylogenetic Analysis and Demographic History

Mitochondrial genetic diversity estimates i.e., haplotype diversity (Hd), number of polymorphic sites (P), mean number of pairwise nucleotide differences (k) and nucleotide diversity (π), were estimated using DnaSP version 6 (Rozas et al., 2017). The Haplotype file was generated using DnaSP and median-joining networks (Bandelt et al., 1999) were obtained using NETWORK 4.5.1.0 (www.fluxus-engineering.com) to study relationships among haplotypes and their geographic distribution.

DnaSP v6 was utilized to evaluate the demographic parameters such as the mismatch distribution test (Rogers & Harpending, 1992), neutrality tests i.e., Tajima's D (Tajima, 1989), Fu's Fs (Y. Fu, 1996), and Fu and Li's F and D (Y.-X. Fu & Li, 1993).

2.4.3. Microsatellite Markers

2.4.3.1. Genotyping error and individual identification

Each sample was genotyped three times to minimize the genotyping errors and a heterozygote was ascertained only if there were different alleles in at least three independent attempts. The genotyping errors arising due to null allele and the presence of stutters, scoring error were assessed and validated using MICRO CHECKER 2.2.2 (Van Oosterhout et al., 2004). Maximum likelihood allele dropout (ADO) and false allele (FA) error rates were quantified using PEDANT version 1.0 involving 10,000 search steps for enumeration of per allele error rates (Johnson & Haydon, 2007). Since, individualization is predominantly dependent on the adequacy of the select loci in the panel used and estimates (unique genotypes) may not be realistic if the select panel of loci exhibit a high proportion of missing values for a few individuals or show a modest level of genotyping error. To avoid such ambiguity in ascertaining unique genotypes, the number of loci used was limited based on their high success rate (>75 %), presence of no or minimum genotyping errors and exhibiting an informative PID value (probability of obtaining identical genotypes between two samples by chance). The locus wise and cumulative probability of identity for unrelated individuals (PID) and siblings (PIDsibs) were calculated following identity analysis module in GenAlEx version (Peakall & Smouse, 2012).

2.4.3.2. *Genetic diversity and inbreeding*

Genetic diversity was quantified by estimating the numbers of observed (Na) and effective alleles (Ne), observed (Ho) and expected (He) heterozygosity and any deviation from Hardy-Weinberg Equilibrium (HWE) using GenAlEx version 6.5 (Peakall & Smouse, 2012). FIS (Wright's inbreeding coefficient) was assessed using FSTAT 2.9.4 (Goudet, 2003).

2.4.3.3. *Population genetic structure*

Bayesian and non-Bayesian clustering methods were used to capture the most possible population genetic structure in red panda of Bhutan. Among different Bayesian clustering methods, individuals were assigned exclusively on the basis of their multi-locus genotypes (STRUCTURE). Non-Bayesian multivariate ordination analyses, i.e., discriminant analysis of principle components (DAPC) and spatial principal component analysis (sPCA) were also used, to compare population assignment with the Bayesian clustering outputs since they are not based on any model assumptions. We used STRUCTURE 2.3.4 software (Pritchard et al., 2003) to determine the number of genetic clusters (K) following 20 iterations (20,000 burn-in; 200,000 Markov chain Monte Carlo replicates in each run) with NOPRIOR and PRIOR both options and with no admix and correlated allele frequencies. K number of populations were considered (K- between 1 and 10), with

repeating each analysis for 10 times at each K value. The most probable cluster was calculated by evaluating the likelihood curves and checking the distribution of Delta K (Evanno et al., 2005) using STRUCTURE HARVESTER v.0.68 (Earl & VonHoldt, 2012). GENELAND v 4.0.3 (Guillot et al., 2008) was run through an extension of R v.3.0.1 with the correlated allele frequency and spatial uncertainty model. K was allowed to vary between 1 and 10 following 20 independent runs, each with 100000 iterations, and a thinning of 1000. DAPC and sPCA, were run in Adegenet v1.3.4 package of R (Jombart, 2008). Isolation by distance (IBD) across the study area was generated using Mantal test in Allele In Space (AIS). Genetic landscape surfaces (GLS) were also implemented in AIS (Miller, 2005) to visually check for geographical barriers.

CHAPTER 3
Results

3.1. Mitochondrial Markers

3.1.1. DNA Extraction and PCR Amplification Success

Out of 482 collected scat samples, 462 (96%) yielded usable DNA. We assessed DNA quality and quantity using agarose gel electrophoresis. A total of 458 samples (99% of those with DNA) showed good results and were suitable for further analysis.

These 458 samples underwent PCR amplification to generate DNA sequences. Following validation through NCBI BLAST searches, 432 samples (94% of successfully amplified samples) were confirmed as red panda DNA with 99-100% sequence similarity (Fig. 9).

Figure 9: Number of red panda scat samples that successfully went through each stage, from DNA extraction to genotyping.

3.1.2. Diversity Indices and Population Demography

Analysis of the mitochondrial DNA (mtDNA) control region revealed 22 unique haplotypes defined by 14 polymorphic sites. Substitution sites were identified by comparing the representative haplotype to the reference sequence of red panda (MK886830.1, *Ailurus fulgens*) (Table 5).

Fourteen haplotypes (H1, H4, H8, and H10-H20) were geographically restricted and unique to specific locations (Table 5, Fig. 10). These findings suggest the potential isolation of red panda subpopulations in these areas. Conversely, seven haplotypes (H2, H3, H5, H6, H7, H9, and H21) were widespread across multiple regions, indicating historical gene flow and genetic exchange within the Bhutanese red panda population.

Haa district exhibited the highest haplotype diversity with eight unique haplotypes (H2, H3, H10, H13-H18). The number of haplotypes varied across other regions, ranging from five in Dagana and Paro to three in Gasa and Monggar. Notably, Lhuentse and Trashi Yangtse districts harbored only the common haplotype H3.

Analysis of haplotype sharing between Chinese and Himalayan red panda populations revealed distinct clusters. The Chinese population possessed 38 unique haplotypes, while the Himalayan population harbored 25 (Figure 11a). Three haplotypes (H1, H7, and H6) were shared between the Bhutan and Indian populations, suggesting historical gene flow between the two countries (Fig. 11 b). Interestingly, one haplotype from Tibet (H28) also clustered with the Himalayan red pandas (Fig 11c).

Sl.	Districts	Haplotypes
1	Bumthang	H3, H9
2	Chhukha	H3, H5, H7
3	Dagana	H3, H6, H7, H8, H11
4	Gasa	H5, H13
5	Haa	H2, H3, H10, H13, H14, H15, H17, H18
6	Lhuentse	H3
7	Monggar	H3, H21
8	Paro	H1, H3, H9, H19
9	Punakha	H2, H3, H6, H16
10	Samtse	H2, H5
11	Thimphu	H4, H3, H5
12	Trashigang	H3, H6, H12
13	Trongsa	H3, H6, H7, H21
14	Wangdue Phodrang	H2, H3, H6, H21
15	Yangtse	H3

Table 5. Observed haplotypes in red panda across 15 districts of Bhutan

Overall haplotype and nucleotide diversity were moderate at 0.770 (± 0.046) and 0.011 (± 0.003), respectively (Table 6). These values were slightly lower compared to studies from China (Su et al., 2001; Li et al., 2005; Hu et al., 2011). However, the Bhutan red panda population which is the Himalayan red panda species still exhibited a moderate level of genetic diversity.

Tajima's D neutrality test results were positive and non-significant ($P < 0.5$), suggesting an abundance of rare alleles and a population under equilibrium. Fu and Li's D and F neutrality tests also indicated no significant deviations from neutrality ($P > 0.10$) (Table 6).

Diversity indices						Neutrality tests		
N	P	H	K	Hd	π	Tajima's D	Fu & Li's F	Fu & Li's D
285	14	21	3.216	0.77±0.046	0.01113±0.003	0.431*	0.948*	1.0002*

Table 6. Summary of genetic diversity indices and neutrality tests of demographic expansions of Himalayan red panda. N- Number of samples; P- Polymorphic sites; H- Number of Haplotypes; K- Average number of nucleotide differences; Hd-Haplotypes diversity; π- Nucleotide diversity; * not significant

The observed multimodal mismatch distribution curve suggested a population in demographic equilibrium (Fig. 12). However, the Bayesian skyline plot revealed a more complex history (Fig. 13). While the plot indicated a population under equilibrium currently, it also suggested a sharp decline in effective population size around 5,000 years ago, followed by a recent expansion. These findings, along with the observed moderate genetic diversity, suggest a possible historical population decline followed by recovery.

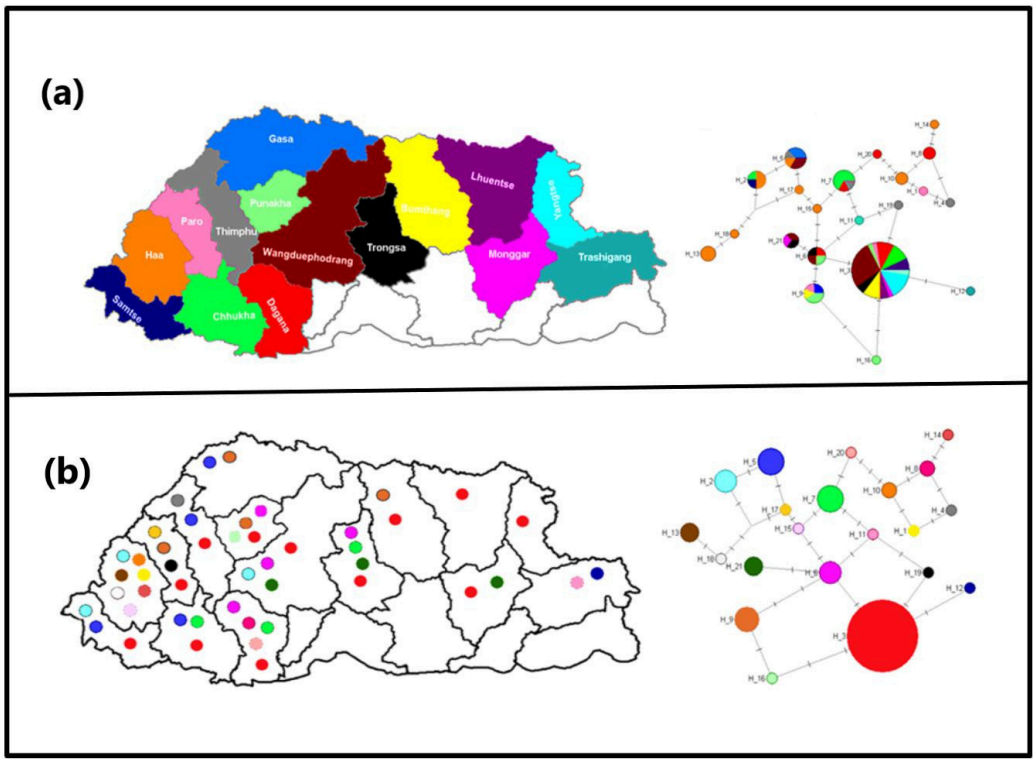

Figure 10. Haplotype network created using d-loop data. (a) various colours depicted the different geographic locations, and a corresponding geographical map is provided for reference. (b) distinct colours depicted the distinguish haplotype, and a corresponding geographical map to illustrate the distribution of the 14 haplotypes in 15 districts of Bhutan.

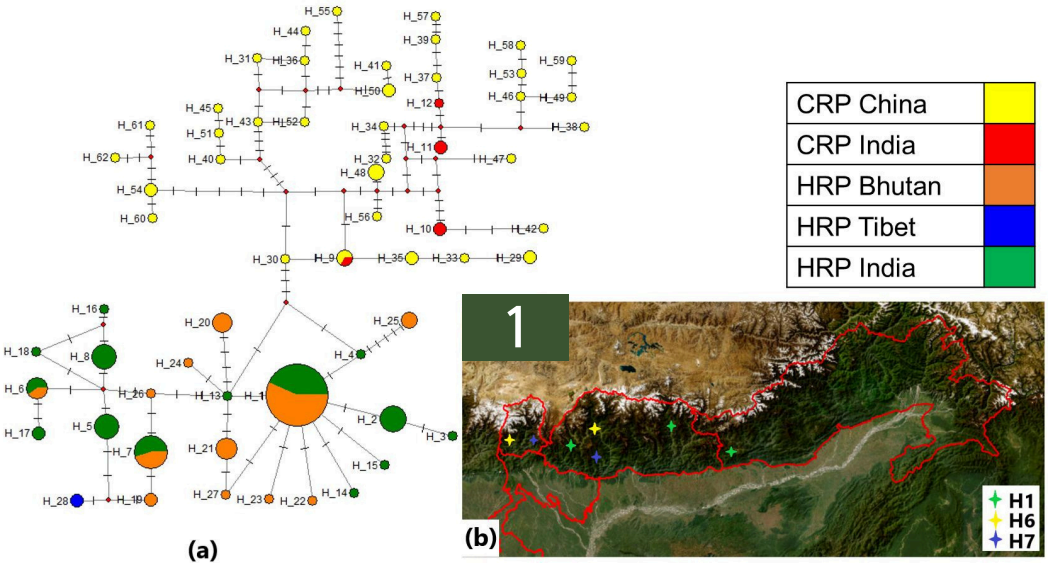

Figure 11. (a) Haplotypes of the control region in red pandas are represented by different colours. Yellow dots correspond to Chinese red pandas from China, red dots represent those from India, orange dots denote Himalayan red pandas from Bhutan (current study), blue dots represent Himalayan red pandas from Tibet, and green dots signify Himalayan red panda from India. (b) Map showing the geographic distribution of shared haplotypes of the Himalayan red panda in India and Bhutan.

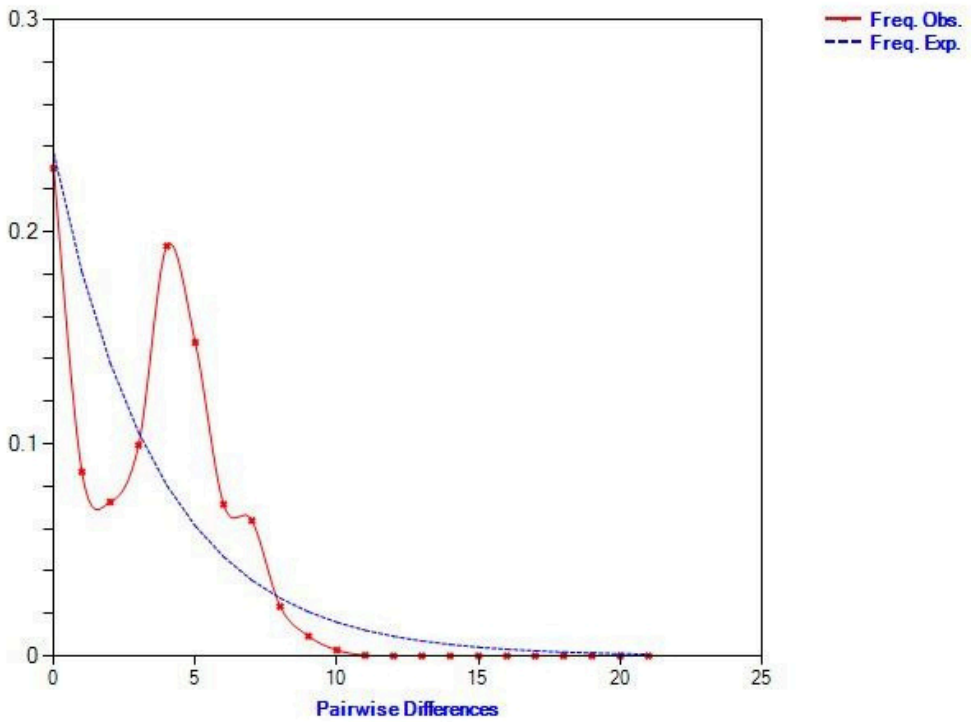

Figure 12. Mismatch distribution modal using the D-Loop in DNA analysis through software DNASP

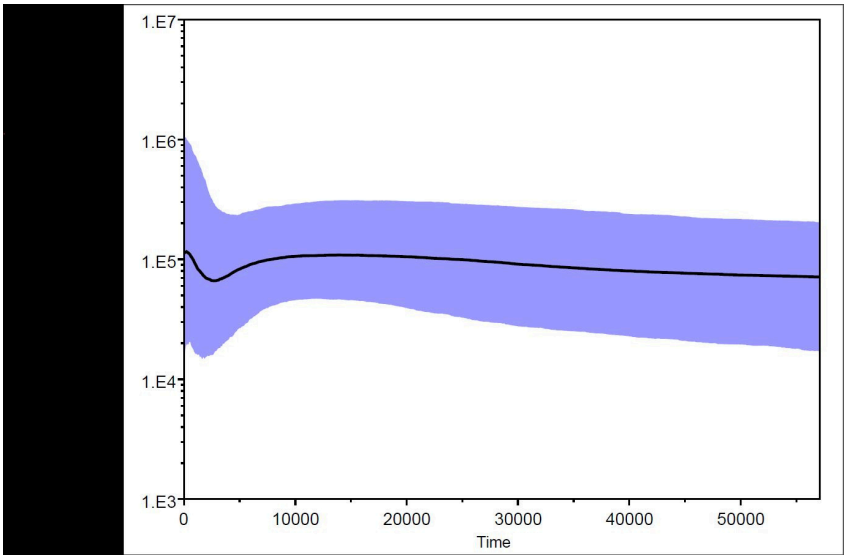

Figure 13. Bayesian skyline plot (BSP) of the control region sequence constructed in the BEAST

3.2. Microsatellite Analysis

3.2.1. Success Rate

All red panda samples were processed for genotyping using a panel of 15 microsatellite loci. Nine markers exhibited a genotyping success rate exceeding 75% across 425 samples (Fig. 14). Due to low success rates, the remaining six loci were excluded from further analyses.

The nine successfully amplified microsatellite markers were all polymorphic, with the number of alleles (Na) ranging from 4 to 14 per locus.

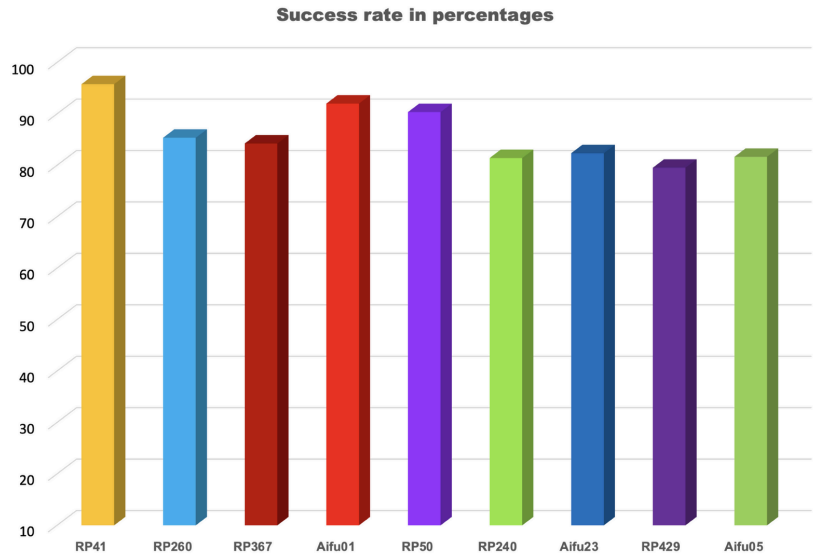

Figure 14. Microsatellite amplification success rate of nine microsatellites with red panda samples

3.2.2. Individual Identification and Distribution

Analysis of allele dropout (ADO) and false allele (FA) rates across loci revealed minimal error. ADO ranged from 0 to 0.4, and FA ranged from 0 to 0.28, respectively (Table 8). These values indicate no significant occurrence of these genotyping errors. Null allele frequencies, however, showed slight variation, ranging from 0.01 to 0.04; however, were not significant and did not influence the population genetics parameters.

We employed a panel of seven microsatellite loci to identify individual red pandas. These genetic markers allowed us to calculate the probability of identity (PID) and probability of sibling identity (PIDsibs). A high PIDsibs value, in this case 2.10 x 10^-3 (indicating a very low chance of siblings having identical genotypes), signifies a strong discriminatory power for individual identification. Using this method, we were able to successfully distinguish 302 unique red pandas within the samples.

Red pandas were detected in all sampling areas except for Tsirang Forest Division and Royal Manas National Park (Fig. 15). The highest capture rate occurred within Jigme Khesar Strict Nature Reserve with 42 individuals followed by Jigme Dorji National Park with 39 individuals (Table 7). The sex of red pandas could not be determined for approximately 30% of the samples. For the remaining 70% where sex was identified with confidence, the observed male-to-female ratio was 1.2 males: 2 females. Red panda capture locations ranged in elevation from 2,143 m asl to 4,374 m asl, with an average elevation of 3,210 m asl.

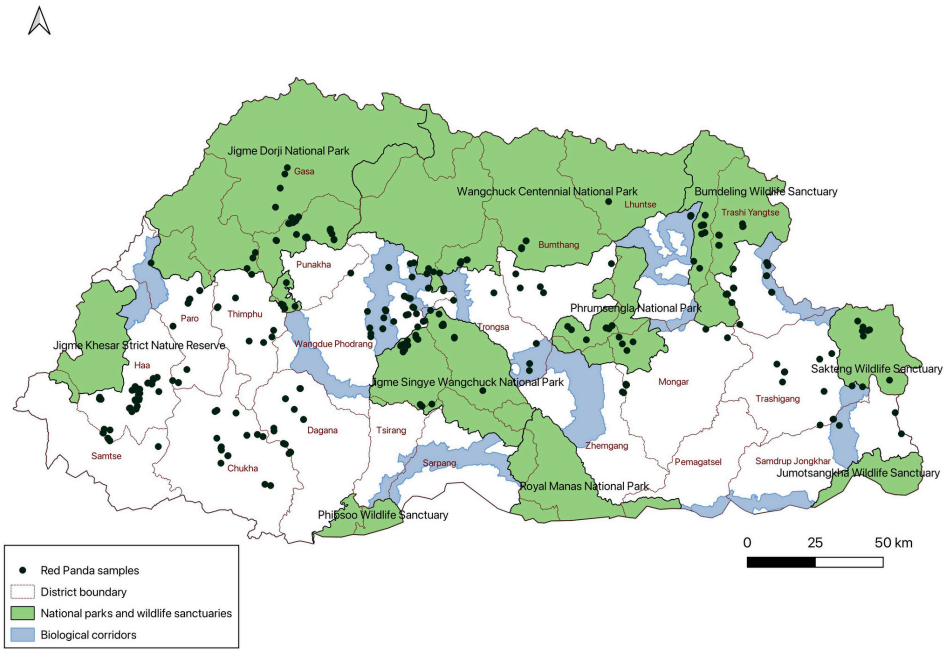

Figure 15. Map showing the locations from where red pandas were recorded through scat sampling during the survey

To estimate the red panda's distribution range, we utilized GeoCAT tool, known for its use in rapid geospatial analysis during Red List assessments (Bachman et al. 2011). We employed the recommended grid cell size of 2km^2 and incorporated recent red panda records, including those from the current survey. The Area of Occupancy (AOO) is estimated at 1436 km^2, representing the total area where red panda presence is confirmed based on suitable habitat patches. This metric provides a focused picture of currently utilized habitat. In addition, the Extent of Occurrence (EOO) encompasses a broader area of 28651.364 km^2, reflecting the total geographical area across which the red panda potentially occurs. This broader measure highlights the potential range of the species and areas for further investigation (Fig. 16).

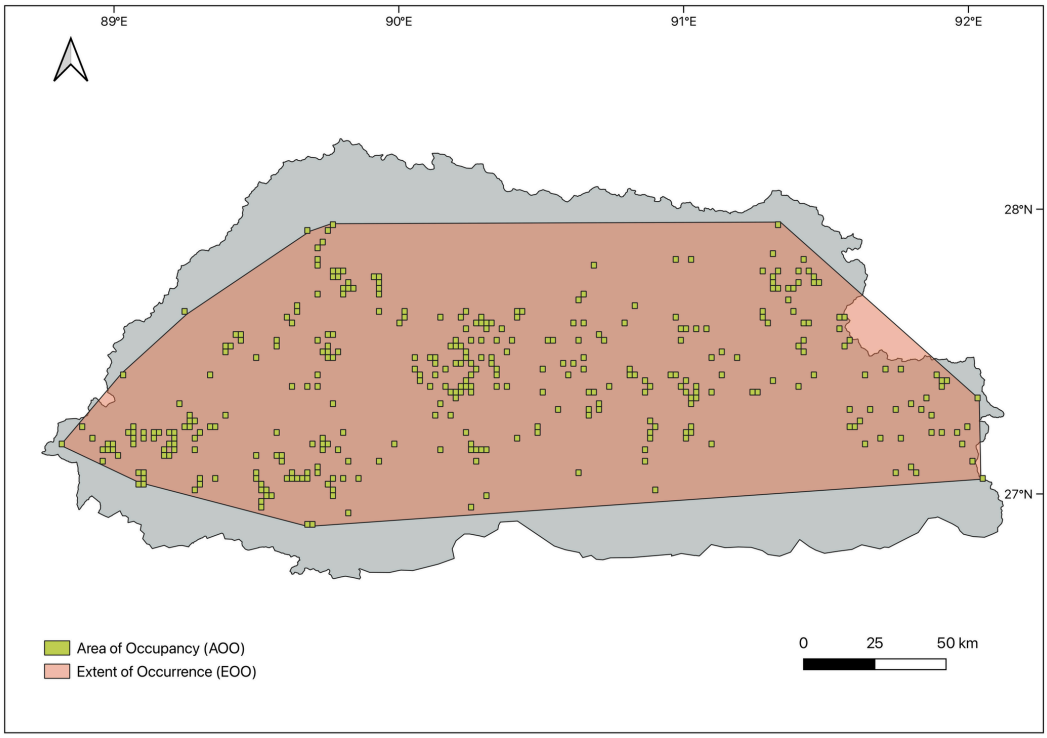

Figure 16. Bhutan map showing the Area of Occupancy and Extent of Occurrence of red panda in Bhutan prepared using GeoCAT

Field Office	No of grids	No. of scat samples collected	No. of minimum individuals counted
Jigme Khesar Strict Nature Reserve	45	91	42
Wangchuck Centennial National Park	50	30	22
Jigme Dorji National Park	25	55	39
Phrumsengla National Park	57	17	14
Jigme Singye Wangchuck National Park	23	32	18
Royal Manas National Park	7	0	0
Jumotsangkha Wildlife Sanctuary	6	3	2
Sakteng Wildlife Sanctuary	39	14	11
Bumdeling Wildlife Sanctuary	44	28	22
Paro Forest Division	27	25	17
Samtse Forest Division	14	1	1
Gedu Forest Division	46	26	15
Thimphu Forest Division	27	14	9
Wangdue Forest Division	66	43	33
Dagana Forest Division	31	26	20
Bumthang Forest Division	64	18	10
Zhemgang Forest Division	27	5	3
Mongar Forest Division	16	11	4
Trashigang Forest Division	40	42	26
Tsirang Forest Division	12	0	0
Total	**666**	**482**	**308***

Table 7. Number of grids, total number of samples collected and number of individuals (*includes recaptures from other sites)

3.2.3. Genetic Variability Assessment

Using 9 microsatellites, we were able to ascertain 302 unique individuals with a success rate exceeding 75%. The average number of alleles per locus was 8.8 ± 0.8 (mean ± standard deviation). Observed heterozygosity (HO) ranged from 0.115 to 0.573 with a mean of 0.352, while expected heterozygosity (HE) ranged from 0.64 to 0.854 with a mean of 0.782 (Table 8).

The inbreeding coefficient (Fis), a measure of genetic inbreeding within a population, ranged from 0.329 to 0.76 with a mean of 0.55±0.044. This value indicates a significant level of inbreeding within the red panda population.

Figure 17. Red Panda camera trapped in the snow

3.2.4. Population Genetic Structure

Both Bayesian and non-Bayesian cluster analyses were performed to assess population genetic structure in the sampled red panda population. In the STRUCTURE analysis, we used both NOPRIOR and LocPRIOR options. Based on the Delta K value (results shown only for NOPRIOR) (Fig. 18b), three clusters were observed. The STRUCTURE analysis indicated a clear clustering pattern of three populations (K=3, Fig. 18a) corresponding to their geographic origin. Individuals originating from western Bhutan were grouped into a separate cluster (Cluster I). Similarly, the majority of samples collected from eastern Bhutan formed a distinct cluster (Cluster III) with some individuals remaining unassigned. The central Bhutan red panda population formed Cluster II and exhibited an admixed clustering pattern. This suggests significant gene flow from neighbouring populations into the central Bhutan population.

The Discriminant Analysis of Principal Components (DAPC) analysis, consistent with the STRUCTURE analysis, also identified three clusters (Fig. 18c). Cluster I (blue) consisted of individuals from eastern Bhutan along with some from the central population. Cluster II (yellow) was characterized by the majority of samples from central Bhutan. Finally, Cluster III (red) contained samples from western Bhutan. The spatial Principal Component Analysis (sPCA) concurred with the STRUCTURE and DAPC analyses and also supported the presence of at least three red panda populations in Bhutan (Fig. 19a).

The Isolation by Distance (IBD) test revealed no significant relationship between genetic distances and geographical distances (observed r = 0.0163) within Bhutan (Figure 19b), and this was quite reasonable considering the limited movement and gene flow in red pandas. The Analysis of Molecular Variance (AMOVA) detected a moderate geographic barrier between eastern and central Bhutan; however, no major geographical barrier was detected (Fig. 19c).

Locus	Na	Ho	He	uHe	Fis	PIC	PID	PIsibs	PID by Locus	PISibs by Locus	HWE	F(Null)	ADO	FA
RP41	10.0	0.573	0.854	0.855	0.329	0.863	3.9E-02	3.3E-01	3.9E-02	3.3E-01	***	0.123	0	0
RP260	9.0	0.286	0.829	0.831	0.655	0.822	1.9E-03	1.2E-01	5.0E-02	3.5E-01	***	0.142	0.01	0.02
RP367	9.0	0.361	0.848	0.850	0.575	0.815	7.9E-05	3.9E-02	4.1E-02	3.4E-01	***	0.21	0.03	0.28
Aifu01	9.0	0.323	0.741	0.743	0.565	0.779	7.1E-06	1.6E-02	8.9E-02	4.0E-01	***	0.383	0.01	0.02
RP50	7.0	0.460	0.756	0.757	0.392	0.778	6.9E-07	6.2E-03	9.7E-02	4.0E-01	***	0.157	0.2	0.26
RP240	14.0	0.323	0.844	0.846	0.617	0.769	2.9E-08	2.1E-03	4.2E-02	3.4E-01	***	0.364	0.04	0.04
Aifu23	8.0	0.323	0.768	0.770	0.580	0.717	2.4E-09	8.1E-04	8.4E-02	3.9E-01	***	0.033	0.01	0.03
RP429	8.0	0.361	0.731	0.732	0.506	0.714	2.9E-10	3.4E-04	1.2E-01	4.2E-01	***	0.347	0.3	0.22
Aifu05	5.0	0.155	0.649	0.651	0.761	0.595	5.1E-11	1.6E-04	1.7E-01	4.7E-01	***	0.468	0.1	0.2
Mean	8.8	0.352	0.780	0.782	0.553									
SE	0.8	0.038	0.023	0.023	0.044									

Table 8. Genetic diversity estimates of sampled red panda population in Bhutan (Na- number of alleles; Ne- Effective number of alleles; Ho- Observed heterozygosity; He- Expected heterozygosity; Fis-Inbreeding coefficient)

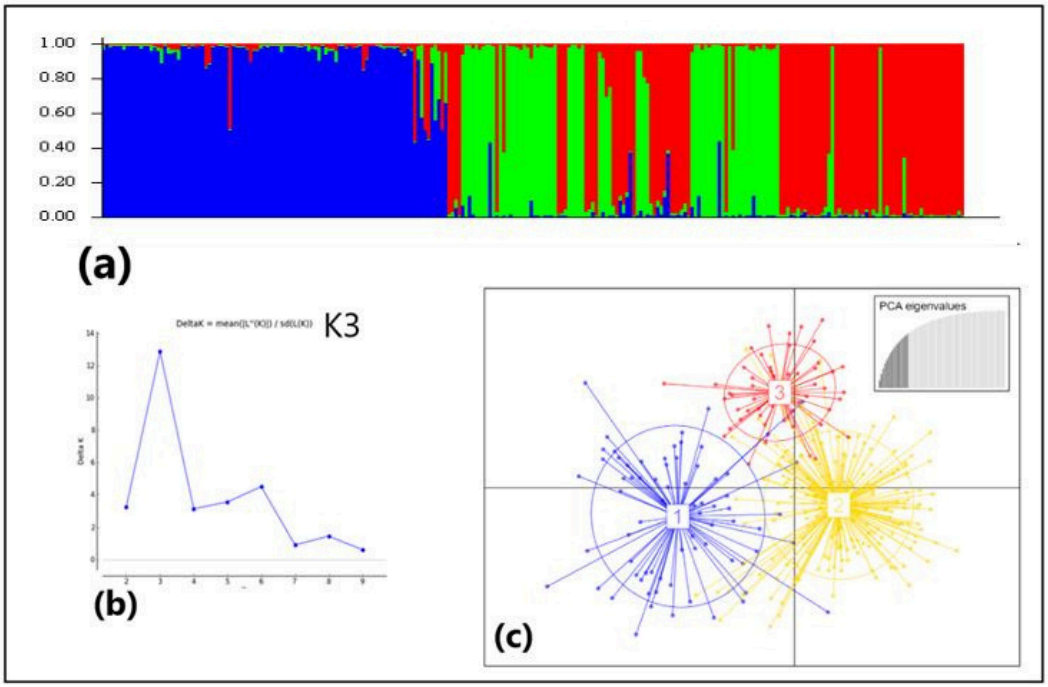

Figure 18. (a) Bayesian STRUCTURE clustering results based on nine microsatellite genotypes among the red panda populations of Bhutan, (b) the ad hoc quantity (delta k) values k3, indicating the most likely number of three genetic clusters, (c) Discriminant Analysis of Principal Components (DAPC) of red panda population in Bhutan. Each circle represents a cluster and each dot represents an individual.

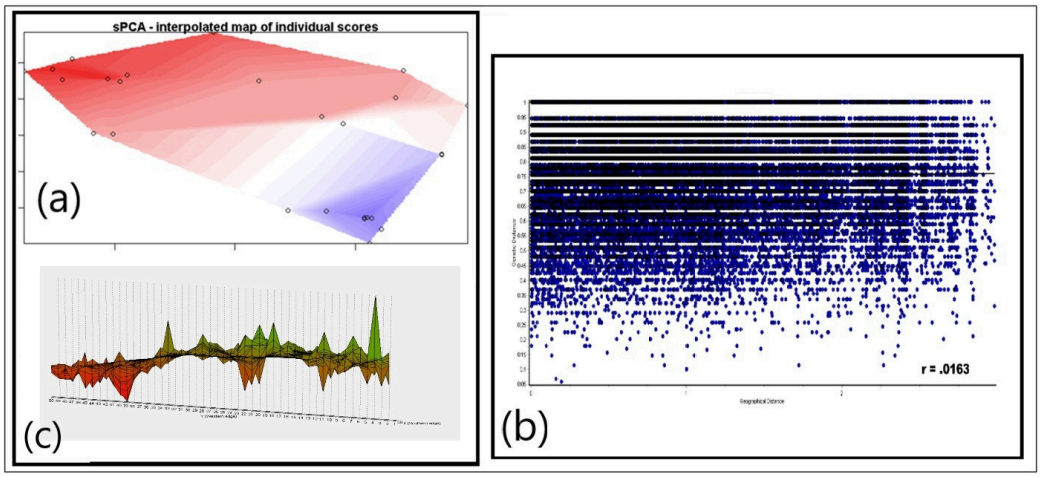

Figure 19. (a) Spatial PCA showing clusters in spatially distributed populations, (b) Scatterplot showing the result of mantel test for presence of IBD (isolation by distance) between significance of geographical distance on the genetic distance (r = 0.0163), (c) Detection of genetic barrier and landscape interpolation using Allele In Space (AIS).

CHAPTER 4
Discussion and Management Recommendations

4.1. Red Panda Numbers and Distribution

Despite surveying only a small fraction of Bhutan's potential red panda habitat, the results offer a glimpse into a potentially thriving population. The survey identified a surprisingly high number of red pandas - **302** unique individuals - within a limited area covering just 6% of the country's total area. This suggests a potentially much larger red panda population distributed across Bhutan. Bolstering this idea is the fact that nearly 28.58% of Bhutan's land area is estimated to be suitable red panda habitat, offering ample space for these elusive creatures to roam (Letro et al. 2022).

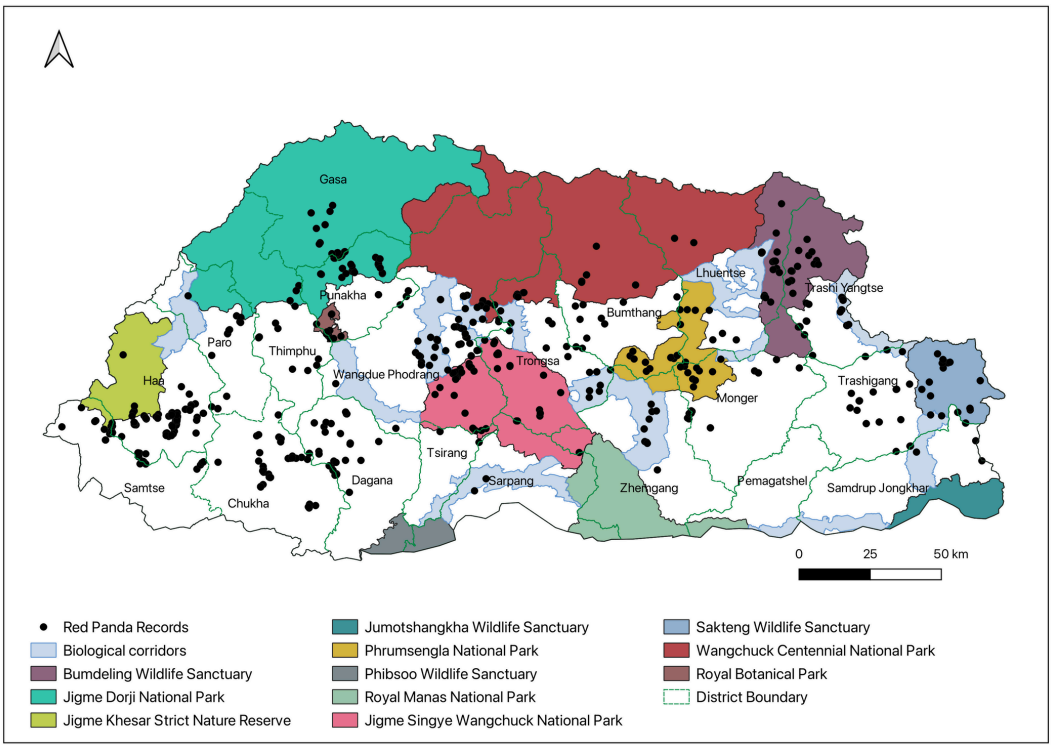

Figure 20. Updated Red Panda distribution map with recent records from the nationwide tiger camera trap surveys in the last 10 years and the current scat survey of 2023.

This nationwide scat survey, combined with camera trap data, revealed a red panda distribution range of 1436 km^2 with confirmed presence and a potential range of 28651 km^2. However, the absence of records in areas with historical sightings, like Tsirang district and Royal Manas National Park, necessitates further investigation. Focused surveys in these areas could uncover new populations or illuminate factors influencing their current distribution and abundance. Additionally, a broader nationwide survey is recommended to get a more accurate picture of the red panda population. This information, along with the Area of Occupancy (AOO) and Extent of Occurrence (EOO) data, can guide conservation efforts. The AOO highlights areas for immediate protection, while the EOO suggests potential for habitat restoration and population expansion. Long-term monitoring remains crucial for tracking population trends and ensuring the long-term health of Bhutan's red pandas.

4.2. Genetic Diversity and Connectivity

This study employed a combination of mitochondrial DNA (mtDNA) analysis for species confirmation and microsatellite genotyping to investigate the genetic diversity and population structure of red pandas in Bhutan. The analysis yielded a genotyping success rate exceeding 75% using nine microsatellites, providing a robust dataset for further investigation.

Analysis of mtDNA revealed moderate genetic diversity within Bhutan's red panda population. However, microsatellite analysis indicated a significant difference between observed and expected heterozygosity. This deficiency in heterozygotes, further supported by the high inbreeding coefficient (Fis = 0.55), suggests a potential reduction in genetic variation. Inbreeding can have detrimental consequences, including decreased fitness, increased susceptibility to diseases, and reduced adaptability to environmental changes (Mills 2014). Future studies could investigate the potential fitness consequences of inbreeding in this red panda population.

Both mtDNA and microsatellite analyses provided evidence for subpopulation structure within the red panda population. The mtDNA data suggested some geographically restricted haplotypes, indicating limited historical or ongoing gene flow between these regions. Microsatellite-based clustering analyses (STRUCTURE, DAPC, sPCA) consistently identified three distinct clusters corresponding to western, central, and eastern Bhutan. This finding aligns with previous studies suggesting that habitat fragmentation, particularly the presence of medium-sized rivers, could be acting as barriers to gene flow between subpopulations.

Interestingly, the central Bhutan population exhibited an admixed clustering pattern in both STRUCTURE and DAPC analyses. This suggests historical or ongoing gene flow from neighboring western and eastern populations in the recent past. This might explain the observed moderate level of genetic diversity and the central region could act as an intermediate mixing zone facilitating some level of connectivity between otherwise isolated subpopulations.

The lack of a significant correlation between genetic and geographic distances (IBD test) suggests that geographical distances between individuals merely have any role in population differentiation, and this is quite reasonable for an arboreal species like red panda which have very limited and confined home ranges. Habitat fragmentation, specific geographic barriers like rivers, or behavioral factors could be at play. This is where a well-designed corridor system comes in. Corridors can act as vital lifelines, connecting fragmented habitats and allowing red pandas safe passage between subpopulations. Increased movement across corridors would lead to a significant increase in gene flow. This, in turn, would help reduce inbreeding and bolster the overall genetic diversity of the red panda population in Bhutan.

Bhutan's existing network of nine biological corridors is a commendable step towards achieving this goal, and protecting the PAs along with corridors seems effective as red pandas of Central Bhutan showed shared ancestry with the red pandas of Central and eastern Bhutan, supporting gene flow in the landscape. However, further research is needed to assess the effectiveness of these corridors in facilitating red panda movement. Are these corridors functional, allowing red pandas to traverse them comfortably? Additionally, the placement of these corridors needs evaluation. Do they connect the most critical areas for red panda subpopulations, or are there gaps that need to be addressed?

By strategically expanding the existing corridor system and ensuring their functionality, Bhutan can create a network of connected habitats. This will allow red pandas to move freely, promoting gene flow and ultimately leading to a healthier, more resilient red panda population throughout the country. It's a collaborative effort that requires researchers, conservationists, and policymakers to work together, identifying priority areas for corridor construction and minimizing disruption to existing habitats. By investing in a well-designed corridor system, Bhutan can ensure the long-term survival and prosperity of its red panda population, a vital part of the country's rich biodiversity.

4.3. Insights into Population History

The study provides evidence for historical gene flow within Bhutan’s red panda population. Shared haplotypes identified across different regions suggest past connectivity and exchange of genetic material between subpopulations. Additionally, neutrality tests and the mismatch distribution curve, which analyze the patterns of mutations within a population, indicate relative stability in population size. This implies the absence of any major recent population expansions.

However, the observed genetic diversity in this study appears to be lower compared to previous studies on red panda populations in other parts of the Himalayas and China (Dalui et al., 2020; Dueck & Steffens, 2022; Li et al., 2005). This discrepancy, coupled with the findings from the Bayesian skyline plot, suggests a possible historical decline in the effective size (breeding population size) of the red panda population in Bhutan. The Bayesian skyline plot is a statistical method that estimates population size fluctuations over time.

While the neutrality tests indicate no recent population expansion, the lower genetic diversity compared to other populations and the Bayesian skyline plot hint at a potential historical bottleneck event. This could have been a period of significant population decline, possibly around 5,000 years ago as suggested by the skyline plot. Following this decline, the population might have undergone a recovery in recent centuries, leading to the observed stability in size but with a reduction in overall genetic diversity.

Further research is needed to explore this hypothesis. Studies employing ancient DNA analysis of historical samples could provide more direct evidence of past population fluctuations. Additionally, investigating environmental and climatic changes that might have coincided with the inferred historical decline could shed light on potential causes of this bottleneck event. Understanding these historical dynamics is crucial for a comprehensive understanding of the current genetic health and future conservation needs of the red panda population in Bhutan.

4.4. Scat Sampling: A Powerful Tool for Red Panda Monitoring

Non-invasive genetic analysis of scat offers a powerful tool for long-term monitoring of red panda populations. This method is particularly effective for assessing abundance, distribution trends, and even genetic health.

Camera traps, a mainstay in wildlife monitoring, have limitations when it comes to detecting small and cryptic mammals like red pandas. Their arboreal habits further reduce their chances of being captured on camera. A recent study in western Bhutan using camera traps at 60 stations over 11 months only photographed red pandas in 1% of locations (NCD 2022). In contrast, our scat sampling approach yielded significantly more data. We collected 482 samples from 666 grids (2km x 2km), with red panda presence confirmed in 158 grids (350 positive samples). An additional 82 red panda samples were collected outside the grids but within close proximity.

Beyond presence/absence data, scat DNA analysis can also provide valuable insights into population abundance and genetic health. Robust study designs can even enable population estimates using spatially-explicit capture-recapture frameworks.

4.5. Conservation Implications for Red Pandas

These findings paint a picture of a red panda population in Bhutan that requires a multi-pronged conservation approach:

1. **Habitat Protection:** Red pandas are habitat specialists, relying heavily on bamboo forests in the mountainous regions. Protecting these existing habitats is paramount for their survival. This can be achieved through protection of the existing red panda habitats and promoting sustainable forestry practices that minimize deforestation and fragmentation of these crucial ecosystems.

2. **Connectivity Corridors:** Given the potential for isolation in some regions and the importance of gene flow for maintaining genetic diversity and population health in the long term, creating habitat corridors between identified red panda clusters is essential. These corridors would allow for movement and interbreeding between populations, preventing genetic isolation and potential inbreeding issues.

3. **Long-Term Monitoring:** Implementing long-term monitoring programs using non-invasive genetic methods like those employed in this study allows researchers to track population trends effectively. This will enable early detection of any future declines, allowing conservation efforts to be adapted and implemented promptly.

4. **Understanding Past Decline:** Further research is needed to delve deeper into the potential causes of the historical population decline hinted at in the study. Investigating past land-use changes, climate fluctuations, or even potential disease outbreaks from this period can provide valuable insights. Understanding these historical threats can inform future conservation strategies to mitigate similar risks.

5. **Transboundary Collaboration:** The shared haplotypes with neighbouring countries highlight the red panda population's transboundary nature. Collaborating with these countries on red panda conservation efforts is crucial. Sharing best practices, research findings, and exploring the feasibility of establishing transboundary protected areas can significantly benefit red panda conservation across the Himalayas.

By implementing these comprehensive conservation strategies based on the genetic insights from this study, Bhutan can play a pivotal role in securing the future of red pandas not only within its borders but also across the wider Himalayan range. The study underscores the importance of utilizing genetic tools to understand population health and guide conservation efforts for this ecologically valuable species. By deciphering the genetic code of red pandas, we can unlock the key to their long-term survival in the face of ever-present threats.

In conclusion, the nationwide red panda survey in Bhutan offers a fascinating glimpse into the past and present of red pandas in the landscape, shedding light on their genetic diversity, population structure, and potential historical trends. This knowledge will be crucial for developing effective conservation strategies for this iconic species.

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Published by:

Nature Conservation Division
Department of Forests and Park Services
Ministry of Energy and Natural Resources
Royal Government of Bhutan