Conservation and distribution of leopard (*Panthera pardus*) in northern Pakistan

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Muhammad Asad

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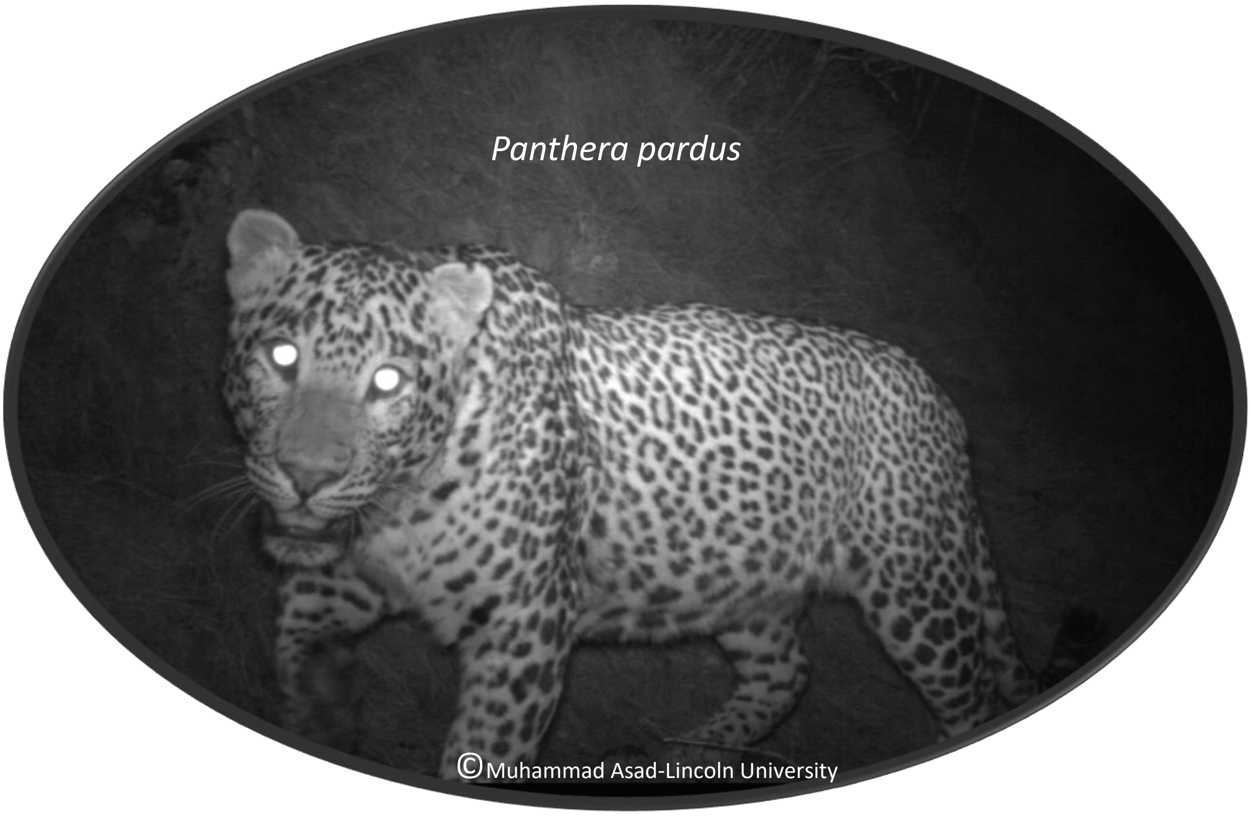

An adult male leopard (01) photographed during our camera-trap survey at Gallies Forest Division Khyber Pakhtunkhwa Pakistan.

وَٱلْأَرْضَ وَضَعَهَا لِلْأَنَامِ

"And the earth, He has assigned it to all living creatures" *(Al Quran 55:10).*

فَمَنِ ٱهۡتَدَىٰ فَإِنَّمَا يَهۡتَدِي لِنَفۡسِهِۦۖ

"**Whoever is guided, then surely he is guided for (the good) of his own**" *(Al Quran 27:92)*

وَمَا مِن دَآبَّةٖ فِي ٱلۡأَرۡضِ وَلَا طَٰٓئِرٖ يَطِيرُ بِجَنَاحَيۡهِ إِلَّآ أُمَمٌ أَمۡثَالُكُمۚ مَّا فَرَّطۡنَا فِي ٱلۡكِتَٰبِ
مِن شَيۡءٖۚ ثُمَّ إِلَىٰ رَبِّهِمۡ يُحۡشَرُونَ

“There is not an animal that lives on the earth, nor a being that flies on its wings, but they form communities like you. Nothing have we omitted from the Book, and they all shall be gathered to their Lord in the end” *(AL Quran 6:38)*

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of philosophy.

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by

Muhammad Asad

Common leopard (*Panthera pardus* Linnaeus, 1758) populations are in serious decline throughout the range of the species. The leopard is considered a vulnerable species globally and is critically endangered in Pakistan, although there is little scientific information available from this area.

The leopard shows morphological and genetic variation across its species range, which has resulted in the classification of nine different subspecies globally. Despite extensive research to clarify its genetic structure across the geographic range, the leopard in South Asia, particularly in Pakistan, has had very little sampling carried out, resulting in a limited understanding of the local subspecies in this region. The knowledge gap of the leopard's genetic structure and its current geographical distribution has impeded the conservation of leopards in Pakistan.

This study was carried out between 2016-2019 to better understand the species status, ecology, and the conservation of leopards in northern Pakistan. We investigated the presence, distribution and abundance of leopards in northern Pakistan. Leopard presence was confirmed in the Swat, Dir and Margalla Hills regions, despite local wildlife departments considering them extinct in these areas. Leopard abundance in Ayubia National Park, the Guzara Forest, and the Murree Forest Divisions were assessed via camera-trapping and track surveys. Camera-trapping identified 18 individual leopards

(eleven males and seven females). The estimated leopard density from capture-recapture analysis was 9.5 individuals/100 km^2, in 2017, and 4.5 individuals/100 km^2, in 2018.

The Pakistani leopard subspecies status was confirmed using mitochondrial NADH-5 gene sequence variation. Three distinct haplotypes were identified: haplotype A, from 23 samples, was identical to two sequences previously identified as *Panthera pardus fusca* (Accession number: AY035274.1) and *P. p. orientalis* (Accession number: HQ185550.1); haplotype B, from eleven samples, had identical sequences to *P. p. saxicolor*; and haplotype C, from a single Pakistani sample, was not the same as any previously identified haplotype. Haplotype diversity among the Pakistani samples was based on three segregation sites with nucleotide variations ranging between 0.003 and 0.006 (the average numbers of nucleotide differences per site). Leopard haplotypes were found in sympatry in northern Pakistan.

Morphological traits were used in 3D geometric morphometrics to characterise variations in leopard skulls, particularly with regard to subspecies status. Procrustes shape analysis indicated that variation among individual skulls, crania, and mandibles were not determined by gender, diet or collection location. Multivariate analysis confirmed that gender (P = 0.6) and location (P = 0.6) did not account for any morphological differences. We also observed patterns with slight variation at the tip of the coronoid process on mandibles, after 3D morphing and species averaging by combining the two surfaces using IDAV landmark. This variation did not match the putative subspecies.

This study is a useful addition to knowledge about leopard distributions and status in a region (Pakistan) with little current data. Pakistan hosts a contact zone for subspecies *P. p. fusca* (near threatened) and *P. p. saxicolor* (endangered). There is, potentially, an undescribed leopard population in the region. The overlapping distribution of these two subspecies provides the impetus to extend full protection to leopards beyond the limits of regional parks and reserves, to the whole Pakistani territory.

Keywords: Camera-trapping, craniomandibular variation, distribution, genetic variation, geometric morphometric, haplotypes, individual recognition, Leopard, Northern Pakistan, *Panthera pardus saxicolor*, *P. p. fusca,* presence-absence.

Acknowledgments

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I would like to express my deep gratitude to my mother, wife, and other family members, for their love and support.

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Abbreviations

AEC	Animal ethics committee
ANP	Ayubia National Park
BIC	Bayesian Information criterion
BRC	Bioresource research centre
CMR	Capture Mark Re-capture
DICOM	Digital Imaging and Communication in Medicine
ESA	Estimated Survey Area
IDAV	Institute of Data Analysis and Visualization
IDC	Islamabad Diagnostic Centre
IUCN	International Union for Conservation of Nature
KPK	Khyber Pakhtunkhwa
ML	Maximum likelihood
MMDM	Mean maximum distance moved
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
WWF-P	World Wide Fund for Nature -Pakistan

An adult male leopard (03) photographed during our camera-trap survey at Murree Forest Division Pakistan

1. General Introduction

Large carnivores are experiencing a substantial population decline throughout the world (Ripple et al., 2014). Different countries possess different threats but, in most cases, they are imperilled because of their bold nature and their natural behaviour involved in killing prey. These carnivores are opportunistic and depend on small to large vertebrate prey (Carbone et al., 2007) and expansive habitats (Ripple et al., 2014). Their fearless nature and opportunistic behaviour often leads them into conflicts with humans and, in these conflicts, carnivores are the ultimate losers. Human modifications of habitats, retaliation, depletion of prey and limited management capacity in many countries, have created stressful situations for large carnivores. Carnivore ecology largely depends on the availability of prey, so a knowledge of their feeding behaviour and prey base is essential for initiating any conservation planning and management of carnivores (Swanepoel, 2009).

The leopard (*Panthera pardus*) is one of the most widespread territorial species among the big cats (Jacobson et al., 2016). They are adapted to a wide range of habitats from tropical, humid forests to savannah, scrub and deserts (Athreya et al., 2016; Shehzad et al., 2014; Nowell and Jackson, 1996). The range of leopards extends from Africa to the Middle East and, in Asia, northwards to the far north of Russia, and eastwards to Southeast Asia (IUCN 2019; Stein and Hayssen, 2013; Marker and Dickman 2005; Nowell and Jackson 1996). Despite all their flexible characteristics, the leopard population is declining throughout their species range. As a result, leopards are now absent from 63 - 75% of their historical ranges, with the highest rate of decline in Asia, where several subspecies are characterised as endangered (Stein and Hayssen 2013). Jacobson et al., (2016) have shown that leopards have disappeared from 83–87% of their former range in Asia, while the distribution in Africa has declined to around 48 - 67% of their former range.

Leopards can co-exist with humans in modified habitats (Athreya et al., 2016) but the increase of human populations and the needs and behaviour of leopards produces conflicts between the species countries throughout the range of the species (Jacobson et al., 2016).

Due to the huge losses in range and the varying threats among regions, the International Union for the Conservation of Nature (IUCN 2016) has re-categorised leopards from “near threatened” to “vulnerable.”

Understanding the ecology of a species negates the chances of duplicating inappropriate resources allocation (Pokheral, 2013). Several factors, from human modification of their habitats, to the lack of compensation, illegal hunting, prey depletion and limited management capacity, are likely to have an impact on leopard populations and contributed to their decline. The common leopard is “critically endangered” in Pakistan. Loss of forests and excessive hunting in densely populated human habitats further threaten the species within the remaining ranges.

The ongoing leopard attacks on livestock and humans, as well as the lack of compensation, causes people to have negative perceptions toward leopards. Community-based conservation has been recognised as an effective method of species conservation (Robinson and Sasu, 2013). Lethal encounters and livestock losses have developed hostility among people toward leopards (Constant, 2015). The lack of suitable compensation for the local community in response to livestock depredation by leopards or human injury has made the relations between the local community and the government Departments concerned increasingly strained and increases the negative perception towards leopard protection. Understanding the local perceptions and factors influencing them are essential for addressing human-leopard conflicts (Dar et al., 2009).

For any conservation effort to succeed knowledge of a species presence-absence, abundance and density are vital at both the species and population levels (Williams et al., 2002; Royle and Dorazio 2008). Knowledge about the status of leopard distribution and its density is vital for successful conservation and management programmes (Borah et al., 2013; Jacobson et al., 2016).

There are currently nine recognised subspecies of the common leopard (Uphyrkina et al., 2001) and two of these, the African *(P. p. pardus)* and Indian (*P. p. fusca*), are considered “near threatened”, while the rest are classified as “critically endangered”, “endangered” and “near-threatened to endangered” (Sheikh & Molur 2004; Stein et al., 2016). Globally, leopard subspecies recognition is based on genetic, morphological and geographical information (Jacobson et al., 2016). However, leopards show high genetic and morphological variations across their range and, in many cases, genetic patterns do not align with the geographical variation recorded for previously defined subspecies (Uphyrkina et al., 2001). Therefore, the study of the genetic structure of leopard populations is considered vital for a better understanding of both the subspecies and population subdivisions and, consequently, for their conservation (Sugimoto et al., 2013; Khorozyan et al., 2006).

With the broad geographic distribution, and the range of leopard diets, their skull morphology may vary across the range and, even, within the same area. The mass of leopards in most of Africa is smaller than those of Iran and India (Stein and Hayssen, 2013). The body mass of an African leopard is 20 - 45 kg while those in the Middle East and Asia range from 30 - 70 kg, and up to 90 kg in the Middle East (Stein and Hayssen, 2013).

In Pakistan, while leopards are known to be present, there is no information on their current distribution, actual population numbers or subspecies status. Based on the severe rate of decline of leopard populations in Asia, the Pakistani population is likely to be fragmented and with depleted genetic variation. The conservation of leopards is hampered due to insufficient information on their genetic structure, distribution, and abundance. Additionally, local students and the local community throughout the country have a naive perception about leopards as they seldom distinguish between leopards, cheetahs, tigers and other big cats (pers obs, confirmed by showing different big cat images to the locals and children).

1.1 Research questions

In consideration of the above knowledge gaps, our study questions were as follows:

1. Which particular leopard subspecies is present in our Pakistani study areas?

2. Are leopard subspecies morphologies different?

3. Have leopards been extirpated from previously reported habitats?

4. Do some areas need urgent attention or priority for leopard conservation?

1.2 Research objectives and thesis outline

In this thesis, I attempt to answer these research questions using a variety of approaches (genes, morphometrics, camera-traps and surveys) I assess the genetic variation of the leopards of Pakistan using mitochondrial NADH-5 gene sequence variations. Morphological variation will be assessed using 3D geometric morphometric analysis of leopard skulls and landmark analysis. This study examines the link between morphological traits and genetic variation to understand the value of current morphologically determined subspecies. In addition, the demographic structure, current leopard density and habitat will be assessed using data collected from extensive camera-trapping that is analysed in MARK™ and PRESENCE®, to delineate the existing range of leopards in northern Pakistan.

The species assessment at both the national and international level will allow line departments (Government Wildlife Departments) to develop comprehensive species conservation management plans, including habitat management, their reintroduction and translocation, and a trans-boundary conservation initiative programme.

This thesis consists of general introduction, four data chapters, conservation and management implications, conclusions and recommendations. The data chapters are presented as stand-alone manuscripts for publication in international journals; each data chapter has a distinct aim and study approach and details of the contributions by each author for the final paper. The contents of the chapters are self-explanatory for their aims and objectives. Because of this format, there is some duplication in the general introduction and the introduction for each chapter.

Chapter 1. General Introduction

In Chapter 1, I present a general overview of leopard and highlight the threats that have caused massive declines across the species range.

Chapter 2. The un-common leopard: Presence, distribution and abundance in Gallies and Murree Forest Division, northern Pakistan

In Chapter 2, I determine leopard presence-absence, distribution and abundance by conducting camera-trap surveys in the Galyat, Murree, Swat and Dir Districts and rely on transect sampling data from the Margalla Hills. One important outcome from this research is to prioritise a focus on leopard high activity areas. The camera-trap images taken will be examined to identify individual leopards using their rosette patterns. I used closed population and capture-recapture frameworks to estimate the leopard abundance in Galyat and Murree regions.

Chapter 3. Presence-absence and occupancy in a northern Pakistan leopard population

In Chapter 3, I investigate site occupancy and current distribution of leopards, using presence-absence and occupancy modelling from randomly placed camera-traps along the trails in northern Pakistan. I used data collected for density estimation in chapter 2 and analysed it with the focus on site occupancy as opposed to estimating population size. I highlight the problems with occupancy estimations using PRESENCE® software for designing sampling method and suggest solutions to the problems encountered.

Chapter 4. Assessing subspecies status of leopards (*Panthera pardus*) of northern Pakistan using mitochondrial DNA

In Chapter 4, I investigate the sequence variation in the subunit 5 of the mitochondrial gene NADH from 43 leopard tissue samples and compare the results with the previously established 238 sequences available from online databases. I used the software MEGA X (Kumar et al., 2018) to identify the best model of nucleotide substitution based on the Bayesian Information Criterion (BIC). A maximum likelihood (ML) NADH 5 gene tree was generated using MEGA X, setting the bootstrap to 10000 replicates. A haplotype network analysis was performed using PopArt (Leigh and Bryant 2015) to construct a Median Joining haplotype network ($\varepsilon = 0$) of all the sequences from Asian leopards.

Chapter 5. Craniomandibular variation of sympatric leopard (*Panthera pardus)* subspecies from Pakistan

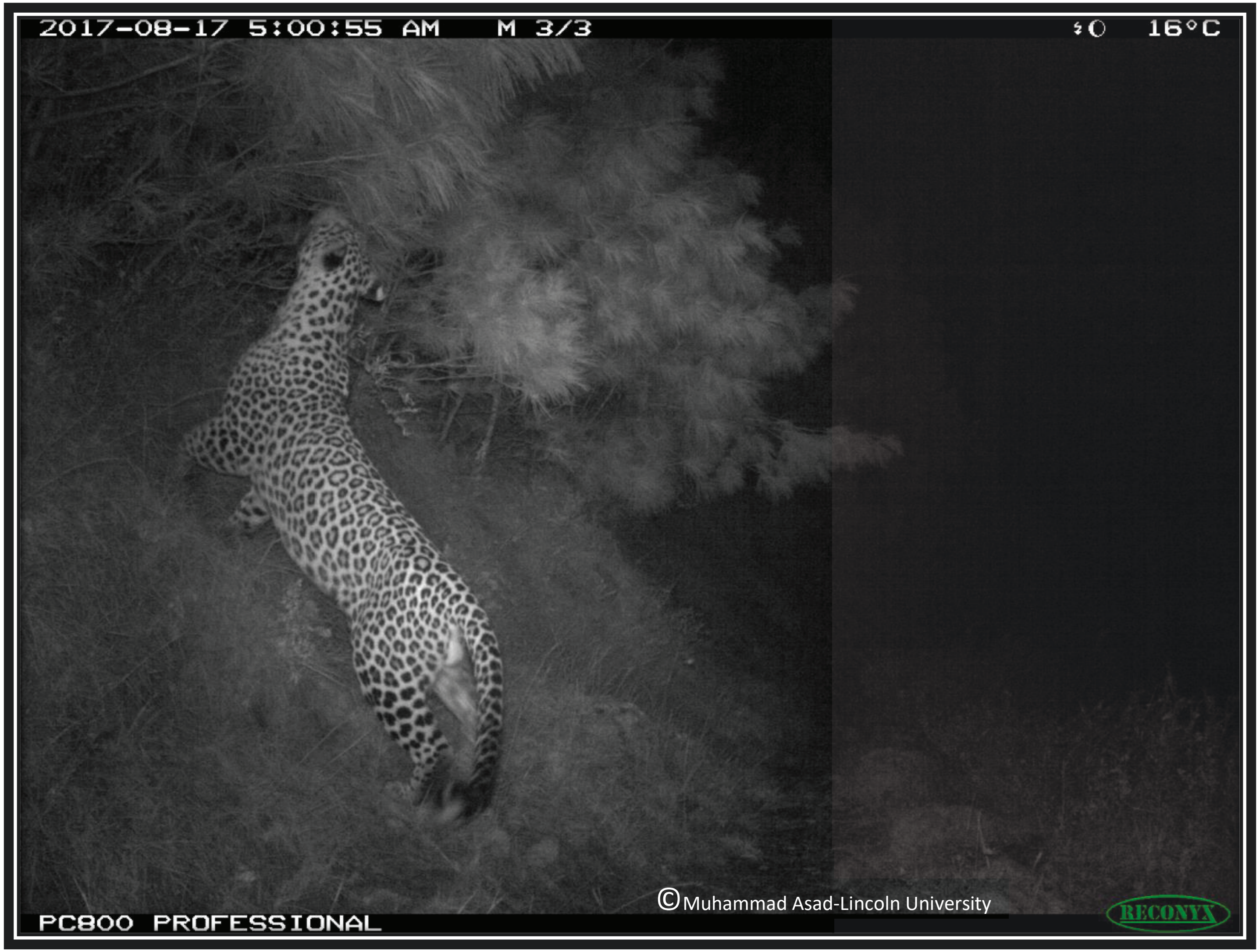
In Chapter 5, I will determine whether morphological variation in skull shape matches the leopard subspecies (*P. p. fusca* and *P. p. saxicolor*), found in sympatry in Pakistan. I will use 3D geometric morphometrics and linear measurements to characterise variation in the crania and mandibles. I will use Procrustes shape analysis to determine the variation among individuals in the over all skull. I will use 3D morphing and species average by combining two surfaces using IDAV landmark. I will also use multivariate analysis also confirm whether gender accounts for any morphological differences.

Chapter 6. Conservation and management of leopards in Pakistan

In Chapter 6, I highlight some of the complex issues raised by this research and briefly touch on the potential implications of this work for the management of leopards in Pakistan. I conclude that it is important to establish data for future leopard research in other habitats previously reported in Pakistan. I highlight the lack of knowledge and importance of understanding leopard ecology and the involvement of locals in achieving conservation goal.

Chapter 7. Conclusion and Recommendation

In Chapter 7, I summarise the key conclusions of the study and provide recommendations for improving leopard conservation. The information generated from this study will contribute to the development of conservation plan for the endangered Persian leopard; thus, aiding in the long-term conservation and future monitoring of the leopard in Pakistan.



An adult male leopard (06) was photographed during our camera-trap survey at Gallies Forest Division Khyber Pakhtunkhwa Pakistan.

2. The un-common leopard: Presence, distribution and abundance in the Gallies and Murree Forest Division, northern Pakistan

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Contribution

1. Conceived and designed the experiment

Muhammad Asad, Adrian Paterson, James Ross

2. Performed the experiment

Muhammad Asad (Muhammad Waseem helped with the extensive fieldwork)

3. Analysed the data

Muhammad Asad, Adrian Paterson, James Ross

4. Contributed to reagents/material/analysis tools

Muhammad Asad, James Ross, Darryl Mackenzie

5. Drafted the work

Muhammad Asad

Adrian Paterson and James Ross provided constructive feedback on the draft revisions of the manuscript.

This chapter has been submitted to the Nature Conservation Journal and is currently in the review process.

2.1 Abstract

The leopard, *Panthera pardus* is thought to be sparsely distributed across Pakistan and there is limited understanding of the demographic structure and distribution of the species in this country. We conducted a study, from April to July 2017, and, from March to June 2018, in the northern Pakistan region where we investigated the presence, and distribution together with a preliminary abundance estimates in the northern Pakistan. Leopard presence was confirmed in the Swat, Dir and Margalla Hills regions. Leopard abundance in Gallies and the Murree Forest Divisions were preliminary assessed via camera-trapping. As a result, a total of 69 potential areas of high leopard use (hotspots), were identified through a preliminary survey. Leopard were detected at 28 locations with 54 capture events yielding 195 images over 3,022 active trap-nights. Camera-trap images were examined to identify leopard individuals using their rosette patterns on both the left and right flanks and the dorsal side of the tail. Ultimately, 15 leopard individuals were identified during the first survey period of the study and four individuals were recaptured in the second survey period, together with three new individuals. The detection probability of individual leopards from MARK™ varied from 0.10 and 0.20 with a population size estimated as 16 - 25 (SE = 3.18), in 2107, and 7 - 13 (SE = 1.87), in 2018. This gave an estimated density of 4.5 to 9.5 leopards/100 km^2, respectively. Home ranges of various individual leopards were found to extend from the Gallies Reserved Forest to the extended corridors of Guzara Forest. These corridors were likely a key for the long-term survival of the leopard population, as it allows them to extended movement while searching for food and mates.

2.2 Introduction

The leopard is one of the most widespread territorial mammalian carnivores on earth (Nowell and Jackson 1996; Hunter et al., 2013; Ripple et al., 2014). The solitary and opportunistic nature of this species enables it to be highly adaptable to different environmental conditions, which range from Africa to the Middle East, in Asia, northwards to far north Russia, and then eastwards to Southeast Asia (IUCN 2019; Stein and Hayssen, 2013; Marker and Dickman 2005; Nowell and Jackson 1996). Leopards are found in a wide range of habitat types from tropical, subtropical and humid forests, mountain, savannah and scrub to deserts (Athreyaet al., 2016; Shehzad et al., 2014; Stein and Hayssen 2013; Sanei et al., 2011a; Nowell and Jackson 1996). They feed on a broad range of prey species, such as ungulates, birds, rodents and reptiles (Kshettry et al., 2018; Mondal et al., 2012; Sanei et al., 2011b; Hayward et al., 2006). Leopards also persist in high 'human-use' areas (Kshettry et al., 2017; Athreya et al., 2016). However, despite all these flexible characteristics, the leopard population is declining throughout their species range. As a result, leopards are now absent from 63 - 75% of their historical range, with the highest rate of decline in Asia, where several subspecies are characterised as endangered (Stein and Hayssen 2013). Jacobson et al., (2016) demonstrated that leopards have disappeared from 83 - 87% of their former range in Asia, while the distribution in Africa has declined to around 48 - 67% of their former distribution (Sanei et al., 2016; Laguardia et al., 2015; Ghalib et al.,2007; Sheikh and Molur 2004).

The main threats to leopards include habitat loss and degradation, developments close to protected areas, rapid depletion of the natural prey base, poaching, and conflicts with livestock causing revenge killing by the livestock owners (Qi et al., 2015; Kabir et al., 2013; Sanei et al., 2012; Sanei and Zakaria 2011c; Mondol et al., 2009; Athreya and Belsare 2007; Fahrig 2003). Isolation and fragmentation of habitats are further threatening the leopards within their remaining ranges by negatively influencing the genetic health of the populations (Quaglietta et al., 2013; O'Brien and Johnson 2005; Spong et al., 2000).

In this study, we aim to establish baseline information for long-term monitoring of leopards to further improve the management and conservation of the species in the Gallies and Murree Forest Division in northern Pakistan. The findings will also provide a general understanding about leopard habitats in Pakistan. The objective of this current study is to establish baseline information for the long-term monitoring and effective management of leopard conservation in the Gallies and Murree Forest Division. The approach will provide a model for other leopard habitats in Pakistan. This approach also provides additional information on the presence of prey species for leopards in the area.

2.2.1 *Leopards in Pakistan*

In Pakistan, leopards were once widely distributed across the country in a variety of habitats and regions, such as Punjab Baluchistan, Khyber Pakhtunkhwa, Sindh, Azad Jammu and Kashmir (Robert 1977). Leopards are known to be sparsely distributed across the country (WWF-Pakistan 2014; Henschel et al., 2008; Sheikh and Molur 2004). A principle reason for this is deforestation (only about 2.5% of the original forests remain) with an annual rate of decline of forest cover of 2.1% (FAO 2007). Erosion of land and landslides, mainly due to high deforestation, private land ownership in the surrounding buffer zones of protected areas, and the right of local communities to collect fuelwood within these zones, are further threatening these habitats (Ashraf et al., 2014).

In Pakistan, the leopard is classified as critically endangered in the Conservation Assessment and Management Plan developed by the IUCN (Henschel et al., 2008; Sheikh and Molur 2004). As habitats in Pakistan are increasingly populated by humans, there has been decline in the local leopard populations and ranges, as well as their prey species, and livestock-leopard conflicts have increased (Ripple et al., 2014; Mondol et al., 2009; O'Brien and Johnson 2005; Uphyrkina and O'Brien 2003). Leopard attacks on humans occur occasionally across northern Pakistan (Lodhi 2007) and several leopards are poisoned or shot annually in reprisal (Muhammad Asad, pers. obs as Conservation Officer (2013 - 2014). Six leopard mortalities were reported in Ayubia National Park and the surrounding buffer zone between November 2011 and December 2012 after two children were attacked and killed (Khyber-Pakhtunkhwa Wildlife Department and WWF-Pakistan). It is likely that many leopard mortalities are not reported (Muhammad Asad, pers. obs.). For example, we found two leopard bodies with their skin removed in the Gallies Forest Division during our data collection for this study that had not been reported to Khyber-Pakhtunkhwa Wildlife Department.

Other threats to leopards include poaching, trapping cubs for animal dealers, and the trade of body parts and skins. Skin, claws, and teeth are available for sale in markets in northern Pakistan (Muhammad Asad pers. obs., Ripple et al., 2014; Theile 2003). Leopards are protected under the Wildlife Act (1975) but the rules and regulations are poorly implemented on the ground, as there are no checks and balances within the limited management capacity of local wildlife departments.

Lack of a compensation programme to recompense livestock owners for the relative losses, e.g. in Swat and Dir regions, or slow compensation procedures, e.g. in Gallies Forest, affect leopard conservation by causing resentment of local people toward leopards (Shehzad et al., 2014).

Leopard vulnerability to extinction is higher than for other cats due to their wide-ranging natural movements for food and mates as this exposes them to higher risks (Cardillo et al., 2004). Stein et al., (2016) suggested a sparse distribution of leopards in Waziristan, Sindh, Punjab, and Ayubia National Park. However, knowledge of species abundance and density is vital for conservation efforts at both the species and population levels (Royle and Dorazio 2008; Williams et al., 2002). Knowledge about the status of leopard distribution and density is vital for successful conservation and management programmes (Jacobson et al., 2016; Borah et al., 2013). This allows the allocation of appropriate resources, the development of effective mitigation measures, adaptive responses by wildlife managers in case of conflict, and to prioritise conservation efforts in high-value habitats.

In Pakistan, there are no robust estimates for the current distribution and density of the leopards. The leopard genetic structure across Pakistan is also based on very limited sampling. Few studies on diet analysis and conservation have been conducted that address human-leopard conflicts (Shehzad et al., 2014; Kabir et al., 2013; Lodhi, 2007), although these studies at least indicate the presence of leopards in different areas (e.g. Galyat, Azad Kashmir and Sindh). Khan et al., (2013) assessed the current distribution and status of mammals, including leopards, in the Khirthar protected area. Anecdotally, the leopard is assumed to have a stable population in northern Pakistan, including the Swat, Dir, and Margala Hills regions (Shehzad et al., 2014). However, the local people and the Wildlife Department in Khyber Pakhtunkhwa (Province) believe that the species had disappeared from the Swat and Dir districts. Recently, the presence of leopards has been confirmed in the Ayubia National Park and the surrounding Forest Reserve of Khyber Pakhtunkhwa and Murree following the detection of leopard scats (Shehzad et al., 2014). Observations of human-leopard conflicts have been made in the Machiara National Park, Azad Jammu and Kashmir, and the Ayubia National Park by Kabir et al., (2013) and Lodhi (2007).

Estimating the presence of leopard populations is difficult as they have large ranges in their natural habitats and usually occur at low densities (Tobler and Powell, 2013). Observations of human–leopard conflicts have been made in Machiara National Park, Azad Jammu and Kashmir, and Ayubia National Park, by Lodhi (2007), Kabir et al. (2013) and Shehzad et al., (2014), analysed the diet of leopards in Ayubia National Park. Many of these also act as conservation studies and so are important for understanding the ecology and behaviour of the top predators in specific ecosystems and for the effective conservation of the species (Brodie, 2009). Meetings and semi-structured interviews have been used to ascertain leopard presence-absence from regions (Abdollahi, 2015). Semi-structured questionnaires were used to collect presence-absence information from villagers in Swat (Ahmad et al., 2014) and their presence was confirmed by installing camera-traps to collect spatial and temporal information and develop a picture of species distribution (Mondal et al., 2012). The presence-absence data of a species is vital for researchers in conservation-related efforts, particularly for cryptic carnivores (MacKenzie, 2005). Therefore, sampling with multiple procedures and combining sampling processes is often required to construct reliable presence-absence datasets (MacKenzie, 2005; Manly et al., 2002).

Obtaining information about abundance, predictability, and site occupancy is challenging over the range of habitats that leopards inhabit, as they have a wide range and occur in low densities (Kery et al., 2010; Balme et al., 2009). Closed populations and capture-recapture frameworks have been used to estimate the abundance of many elusive carnivores, such as tigers (*Panthera tigris)* and leopards

(Wang and Macdonald, 2009; Wegge et al., 2009; Karanth, 1995), jaguars (Sollmann et al., 2013; Tobler and Powell, 2013), ocelots (Trolle and Kery, 2003), clouded leopards (Borah et al., 2013), and snow leopards (Alexander et al., 2016). These frameworks require all individuals to be identifiable and to have reasonably high capture probabilities, with a random sampling of individuals (Harmsen et al., 2011).

Estimates of felid presence include abundance and density estimates for the common leopard and the clouded leopard in Manas National Park (Borah et al., 2013), estimates of a tiger population from camera-trap data (Wang and Macdonald, 2009; Karanth, 1995), estimates of jaguar density with camera-traps (Tobler and Powell, 2013), and estimates of grazing mammal densities using camera-traps (Rowcliffe et al., 2008).

Camera-trapping has recently emerged as a promising method for estimating the abundance of elusive carnivore species in ecological sciences by identifying individuals through their unique pelage patterns with minimal disturbance (Alonso et al., 2015; Heilbrun et al., 2003; Henschel and Ray, 2003; Karanth, 1995). Camera-traps are a useful technique for observing and assessing animal information *in situ* (Bashir et al., 2014; Tobler and Powell, 2013; Balme et al., 2009; Khorozyan et al., 2008). As such, camera-traps have been adopted widely for detecting the presence, abundance, and the proportion of sites occupied by a species (Kéry et al., 2010). Camera-traps and field-based surveys obtain sufficient spatial and temporal information, for the species that are present within the sample unit, to estimate the total population for an entire area (Bino et al., 2014; Karki et al., 2013; Sollmann et al., 2013; Sundaresan et al., 2011; Wang and Macdonald, 2009; Maffei and Noss, 2008; Rowcliffe et al., 2008; Jackson et al., 2006; Silver et al., 2004; Henschel and Ray, 2003). As a territorial animal, a leopard generally does not leave a territory unless removed by humans or killed. Males usually show less variation in movement patterns than females, although the distance travelled by both sexes is similar, except around birth (Morten and Per, 2005). We assumed that any differences in movement patterns between the sexes would not affect our closed population assumption because we sampled over the same season. Encounter histories were constructed from individual leopards observed in each sampling period (Alonso et al., 2015; Wang and Macdonald, 2009; Silver et al., 2004).

Capture-recapture methods have been widely used to estimate abundance and density from camera photos in many carnivores, such as snow leopard (P*anthera uncia*), tiger (*Panthera tigris) (*Sharma et al., 2010*)*, bobcat *(Lynx rufus), (*Alonso et al., 2015*),* black bear (*Ursus americanus) (*Fusaro et al., 2017*)*, jaguar (*Panthera onca), (*Silver et al., 2004*)* and common leopard (*Panthera pardus)* (Harihar et al., 2009). MARK capture-recapture (CMR) has also helped quantify distribution and abundance of prey (Alonso et al., 2015; Balme et al., 2009; Adam et al., 2008, 2007; Jackson et al., 2006; Soisalo and Cavalcanti, 2006; Karanth, 1995; Otis et al., 1978). The CMR framework presents an advantage if individual animals are able to be identified from their unique pelage patterns as this allows individual encounter histories to be constructed on different occasions using this framework (Alonso et al., 2015; Soisalo and Cavalcanti, 2006). Density and capture probabilities of the target species may be estimated (Alonso et al., 2015) and produce robust population estimates for many elusive carnivores (Rozhnov et al., 2015; Thornton and Pekins, 2015; Balme et al., 2009; Jackson et al., 2006; Heilbrun et al., 2003;).

The same robust approach was used in this study to estimate the abundance of common leopards in the Gallies and Murree Forest Division of Pakistan. A CMR model combined with Arc GIS mapping was used. CMR analysis is used to estimate abundance for closed and open populations (Alonso et al., 2015; Borah et al., 2013; Karanth, 1995). A closed population allows a more robust estimate of population size than an open model, assuming there are no births, deaths or migration during the study period (Balme et al., 2009; Jackson et al., 2006; White and Burnham, 1999; Otis et al., 1978;). Several studies have used the average of mean maximum distance moved MMDM or ½ MMDM for individuals captured by more than one camera-trap. A buffer is then estimated around each camera-trap to calculate the estimated sample area (ESA) (Sollmann et al., 2013; Tobler and Powell, 2013; Karanth and Nichols, 1998). The ESA is considered more accurate if calculated with the buffer of a full MMDM, which is believed to be larger for species with large home ranges (Sollmann et al., 2013; Soisalo and Cavalcanti, 2006; Silver et al., 2004). A small sample area could mislead and allow overestimation of a population size (Thornton and Pekins, 2015; Tobler and Powell, 2013; Harmsen et al., 2011).

2.3 Materials and Methods

2.3.1 *Study area*

The study was conducted in northern Pakistan, across the Gallies Forest Division (34°04'07'N-73°41'03''E), Murree Forest Division (33°52'26.34"N-73°23'42.21"E), Swat (35°01'10.70"N-72°08'50.93"E), and Dir districts (35°51'11.19"N -72°50'30.46"E and the Margalla Hills region (33°44'23.99"N-73° 2'18.00"E).

The area of the Gallies Forest Division comprises a 15,716 ha Reserve Forest and a 8,224 ha Guzara Forest, which is also managed by the Khyber Pakhtunkhwa Forest Department. The Ayubia National Park is located in the Forest Reserve of Gallies Forest Division with a total area of 3,312 ha surrounded by other Reserved Forest. The Guzara Forest of the Gallies Forest Division is linked to the Reserved Forest that is surrounded by five villages within the buffer zone. The boundaries of the Reserve Forest and Guzara Forest are disputed (Lodhi, 2007). Guzara Forest acts as a buffer zone that allows species to extend their movement into an area with low human densities while searching for food. Buffers may also help support a viable leopard population in the area (Figure 2.1). The main source of income for local people is seasonal tourism and livestock (Lodhi, 2007). The total area of the Murree Forest Division is 19,000 ha and comprises Reserve Forest, Protected Forest, and Guzara Forest. The Murree Forest Division manage 22 Protected and 23 Reserve Forest patches (Ashraf et al., 2014; Ahmed and Mahmood 1998). Most of the available knowledge on Pakistani leopard presence-absence, diet and human leopard conflicts are only available for these places, so it is assumed that they have relatively stable leopard populations (Shehzad et al., 2014). The total area of the Margalla Hills is 17,386 ha. The Margalla Hills is an extension of the Islamabad Wildlife sanctuary that includes Shakar Parian Hills and Rawal Lake (WWF-Pakistan).

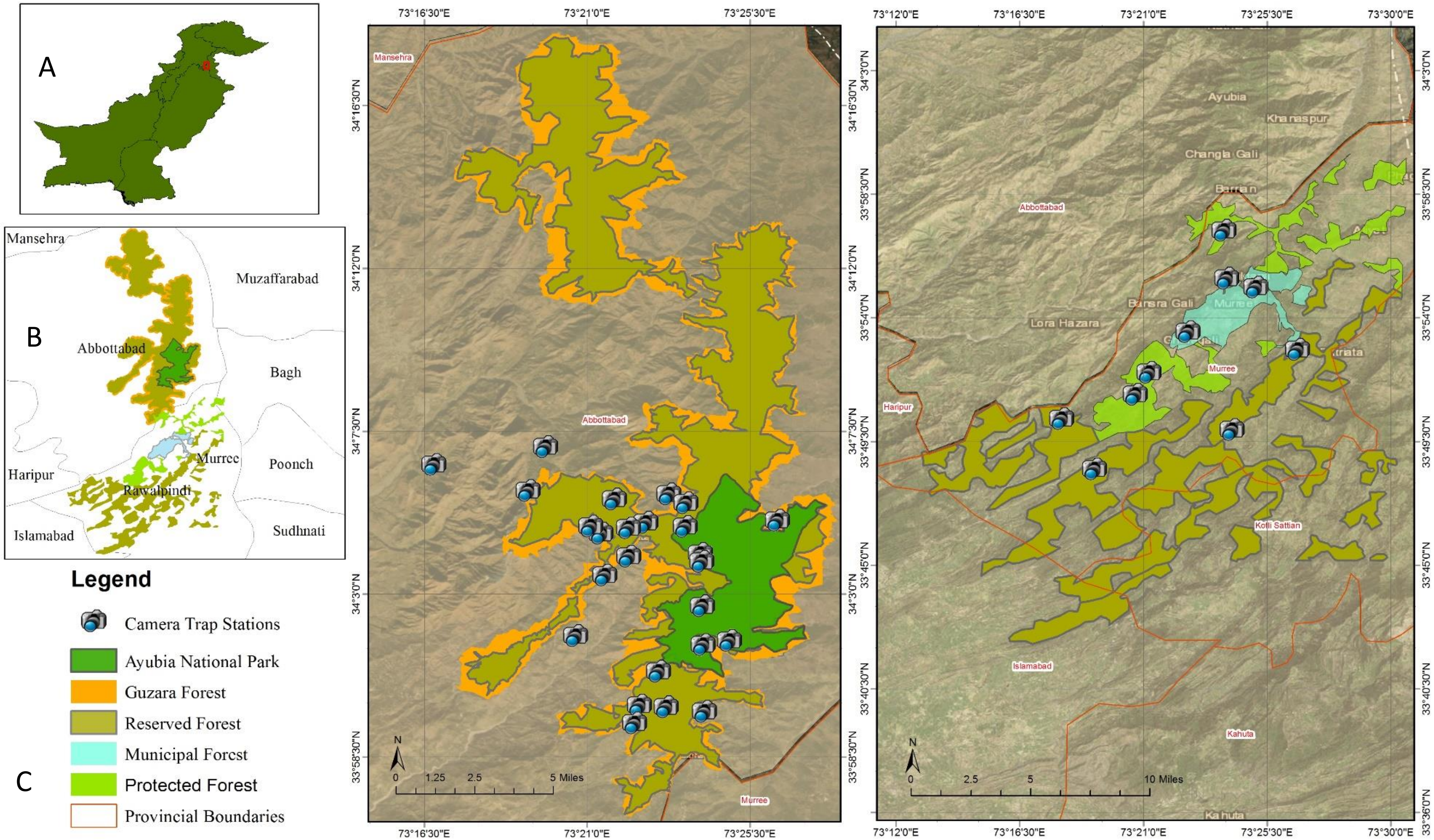

Figure 2.1 Sampling locations of the camera-traps survey in Gallies Forest (Ayubuia National Park, surrounding Reserved Forest and Guzara Forest), and Murree Forest (Protected, Reserved and Municipal Forest). (A) Country map (top left - green) followed by (B) study area showing different city boundaries. (C) showing legend.

Our study area lies in the outer Himalayas in the sub-tropical continental highlands and encompasses two distinct ecological zones, 'moist temperate coniferous forests' and 'chir pine subtropical forests' (Ashraf et al., 2014). Mammals within the sampled area include leopard cat, *Prionailurus bengalensis,* jackal, *Canis aureus indicus,* rhesus monkey, *Macaca mulatta,* marten, *Martes flavigula,* Indian palm civet, *Paradoxurus hermaphroditus,* Himalaya palm civet *Paguma larvata* and red fox, *Vulpes vulpes* (Shehzad et al., 2014). There are no recent forest maps in Pakistan that include buffer zones, except for the Murree Forest Division, and the Ayubia National Park that were developed by WWF-Pakistan during delineation of the forest boundaries (Ashraf et al., 2014; Abbas et al.,2010). We delineated the boundary between Reserved Forest and Guzara Forest with the help of ArcGIS from the old maps provided by the Khyber-Pakhtunkhwa Wildlife Department (Figure. 2.1).

2.3.2 Questionnaire survey

Between April 2017 and March 2018, we conducted questionnaire surveys (*n* = 1028, see appendix A) among local communities living close to the study areas of Galyat, Murree, Margalla Hills, Swat and Dir where they were asked to identify potential sites for detecting leopard presence. Around 30 questionnaires were completed at each village; in total, there were 35 villages. These villages were randomly selected from the union council map and within each village people were randomly interviewed. The following information was collected: livestock depredation, time of attack, and type of injury, e.g. bite marks on neck or missing dogs or human causalities. Where possible, we validated the collected data by visiting each site as well as interviewing local Khyber Pakhtunkhwa Wildlife Department staff and nomads.

A total of 69 leopard records were identified from the questionnaire surveys. Out of these, 39 records were from Galyat and Murree, 24 from Swat and Dir, and 6 from Margalla Hills National Park. Six locations were later discarded from Galyat region due to doubling of location with different names, giving a final count of 63 sampling sites. Data were used to choose sites for future tracking surveys and camera-trapping (Figure 2.1).

2.3.3 *Tracking survey*

We conducted surveys in selected sites where leopards had been reported. Each trail surveyed was between 4-10 km in length and was completed between 0700 and 1700 hr. We searched trails for signs of scats, territorial markings, and tree scratches that implied leopard presence-absence. Signs of leopard were recorded and photographed. Areas were identified on the trails that had frequent leopard movements (Figure 2.2). Such locations were used as sites for the camera-traps. We were not able to sample from the Margalla Hills Region due to insufficient number of camera-traps available and relied on transect sampling data for confirmation of leopard presence-absence.

Secondary data on leopard attacks on humans that resulted in injuries or deaths, cases of revenge killing, as well as leopard natural deaths when detected, were collected from Khyber Pakhtunkhwa Wildlife Department and WWF –Pakistan for the years 2005–2018 for the district of Abbottabad.

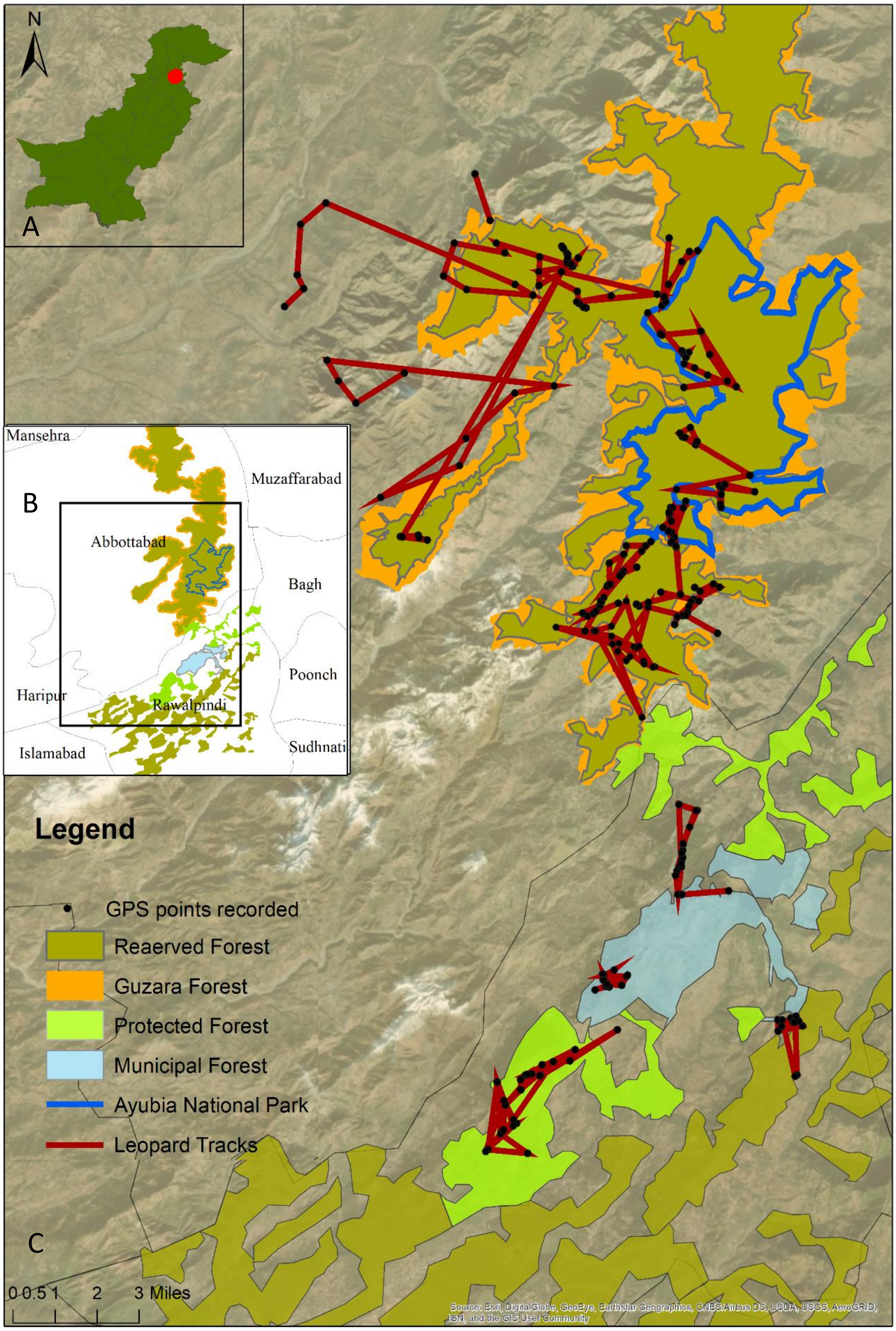

Figure 2.2 Sampling locations of the tracks survey in Gallies Forest (Ayubuia National Park, surrounding Reserved Forest and Guzara Forest), and Murree Forest (Protected, Reserved and Municipal Forest). (A) Country map (top left - green) followed by (B) study area showing different city boundaries and (C) showing leopard trails and legend.

2.3.4 Camera-trapping

We conducted camera-trap surveys along the tracking trails in 63 locations across Galyat and Muree, Swat and Dir. Camera-trapping was conducted, from April to July 2017 and from March to June 2018. The survey was carried out in summer because of heavy snowfalls in winter. The study area was divided into three sections: Galyat and Murree, Swat and Dir, and the Margalla Hills. We deployed 14 camera-traps in two sections (total of 63 locations) for a period of 14 days at 20-40 cm above the ground (Balme et al., 2009).

Leopards are generally nocturnal and most active during dawn and dusk. Although male and female leopards have different activity patterns over a 24-hour period (Ray et al., 2018). Nomadic farmers and their grazing livestock were generally active during the daytime. Thus to extend the battery life, the camera-traps were only active between 6 pm - 8 am. The camera-traps were checked every two weeks to replace the batteries and re-position the cameras, if required. Camera angles, trigger speed, detection zones and time-lapse between the triggers were set according to the previous literature about installation considerations (see Rovero et al., 2013; Trolliet et al., 2014 and details below).

Two cameras, one on either side of the trail, were placed at each site facing toward each other, to capture both flanks of a passing leopard.

This allowed the identification of individual leopards from their unique rosette of spots (Silver et al., 2004; Heilbrun et al., 2003; Karanth 1995). Cameras were set with a high trigger speed (0.2 - 0.8 seconds) with at recovery time of 1 and 0.5 seconds, respectively, for the two cameras. Three photos per trigger were taken to capture leopards as they move faster on trails than in other topography types (Scheibe et al., 2008). The photographs were then manually examined for leopard detections. The photos of individuals were verified by the wildlife expert from WWF-Pakistan. We relied on transect sampling data in the Margalla Hills as we did not have a sufficient number of camera-traps or time to complete the standard method.

2.3.5 A preliminary abundance estimate

To estimate the abundance in Galyat and Murree, where the leopard population is considered stable (Shehzad et al., 2014), we repeated the camera-trapping for a second season, from March–June 2018. Camera-traps were also placed on the five connecting trails identified, earlier, through the preliminary survey, but where no leopard signs were found after scoping. This was to satisfy the assumption that no animal have zero capture probability (Balme et al., 2009) as there are chances for leopard to be photograph on trails with no signs (Muhammad Asad personal obs.).

The spacing of camera-traps was based on the minimum home range recorded for an adult female leopard with cubs, 5.2 - 6.6 km^2 in Nepal (Morten and Per 2005) and 8 km^2 in north-central Namibia (Marker and Dickman 2005). Most studies show that the home range size varies in leopard females around birthing time (Rozhnov et al., 2015) while the distribution of prey and the location of females most affects the home range size in males (Odden and Wegge 2005; Bailey 1993). Since the width of trails varies from 0.5 - 3 metres it was not practical to obtain detailed images of both flanks on trails with less than 1 m width. On wider trails, over 1 m, the cameras were set at 90^o from the direction of the trail to obtain images of both flanks of every passing leopard. Cameras were placed 20 - 40 cm above ground level (Balme et al., 2009). It was difficult to obtain detailed images of both flanks on trails less than 1 m in width. Cameras were set at a 45^o angle on trails less than one metre in width to better obtain clear flanks for individual identification.

Cameras that were non-active for various reasons, such as spent batteries and malfunctions, were excluded from further analysis. The sex of individuals was identified by their distinctive morphological features (Balme et al., 2012), and each identified individual was given a name based on the track that they were recorded in. Camera-traps were moved frequently from one location to another after 14 days, as we discovered new trails with leopard sign. Individual leopards were identified from their unique rosette patterns, based on guidelines from Heilbrun et al., (2003) and Jackson et al., (2006). The independence of events was based on guidelines modified from (Heilbrun et al. (2003) and Jackson et al. (2006). We used the image manipulation program GIMP, available online at http://gimp.org, to digitise unique noticeable spots found in different areas of the body. We analysed spot to spot to differentiate individuals from one another.

2.3.6 *Preliminary mark-recapture practice*

The MARK™ Program (Version 8.2; White and Burnham, 1999) was used to estimate the abundance of common leopard (Alonso et al., 2015; White and Burnham, 1999). We assumed the population was closed as leopards live long enough that there should be no natural mortality during the study and no permanent movements into or out of the survey area over this period. We believed that we met this assumption because of the short duration of the study and available suitable resources and historical records showing leopard presence in surrounding areas.

The capture histories were constructed from 14-day sampling periods for each leopard simply by defining each day and night into a single session, resulting in 14 sampling occasions, to provide the maximum number of capture histories. We fitted seven *a priori* models to the data to represent different combinations of factors that may affect capture probabilities: behaviour (probability of recapture different to probability of first capture), individual heterogeneity, year, and survey night. The models are notated with the factors affecting capture probability indicated in parentheses. The data from each year were entered as different 'groups' in the software to enable parameters to be shared between the two years. Models were compared using AIC, and model averaging was used to obtain overall abundance estimates. To estimate density from the abundance data, we determined the effective study area by putting a 6 km buffer (calculated as the average of the maximum distance travelled between capture locations) around each camera-trap, as determined by MMDM. (Sollmann et al., 2013; Tobler and Powell, 2013; Karanth and Nichols, 1998). The density of leopards was calculated from the abundance data generated by Program MARK™ $D = N/A$, where A is the area covered during the sampling period (effective study area) and N is the number of leopards estimated by Program MARK.

2.4 Results

2.4.1 *Presence-absence*

In Galyat and Murree we recorded 192 leopard photos over 1,930 trap-nights, representing a capture success of 9.94 captures/100 trap-nights. We also located 58 territorial markings that included scats, scrapes and tree scratches. Most camera-trap photos (67%) were caused by the movement of local people and mostly from four camera stations Lalazar track, Pipeline track, Baragali track and Nagribala track. Non-target species, such as fox, jackal, porcupine, wild boar, martens, rhesus monkey, and civet, comprised 21% of the images. Domestic livestock (goats and cows) represented 8.7% of the total images and false triggers, where there was no obvious reason for activation, comprised a relatively low 2% (Table 2.1).

Table 2.1 Summary of the camera-trap images from 39 hotspots/trails for common leopard and non-target species showing active trap-nights, total photos and false images in Gallies and Murree Forest Division 2017 and 2018.

Sampling period	Hotspots/trails	Active trap-nights	Total photos	False images	Non target capture			Common leopard			
					Other species	Livestock	Local community	Photos	Captures events	Initial capture	Un-identified
2017	39	950	21,410	281	3,480	1,186	16,342	121	33	15	7
2018	39	980	8,221	328	2,927	1,455	4,040	71	21	**Recaptured-initial capture**	4
										4 3	

In Swat and Dir we recorded one leopard at one camera-trap station from Shangla (Swat) over the 1,092 traps nights and located only two territorial markings in Nehagdara (Upper Dir) while scoping trails for leopard signs. We collected a total of 11,806 photographs, which mainly comprised the movement of livestock (sheep, goats, and horses) by nomads (34%), members of the local community, including hunters, comprised additional 32% of the total images, false triggers contributed 18%, and non-target species, such as fox, jackal, porcupine, dogs, and cat represented 14% of the total images (Table 2.2).

Table 2.2 Summary of the camera-trap images and trail scoping for presence-absence of the common leopard and non-target species at different location sampling periods and sites, showing active trap-nights, total photos and false images in 2017 and 2018.

Location	**Sampling period**	**No of sites**	**Active trap-nights**	**Total photos**	**False images**	**Non target capture**			**Common leopard**	
						Other species	**Livestock**	**Local community**	**Photos event**	**Territorial markings**
Galyat and Murree	April-July 2017 March-July 2018	39	1,930	30,231	609	6,407	2641	20,382	192	58
Swat and Dir	March –June 2018	24	1,092	11,806	2,140	1,677	4,116	3,870	3	2
Margalla Hills	March –June 2018	6	-	-	-	-	-	-		4

In the Margalla Hills, we conducted a walking survey for leopard signs at six locations across these hills along 4 - 10 km long transects, between 0700 and 1700 hr. We found four territorial signs of leopard territorial markings and scats at four survey sites, which confirmed the presence of leopards in the area.

2.4.2 Individual recognition

We examined each individual leopard image for their unique spot pattern. The most distinctive body parts used for identification and comparison were the left and right flanks and the dorsal surface of the tail (Figures 2.3, 2.4, 2.5 and 2.6). Of the 15 individuals identified during the first season, four were recaptured in the second season along with three new individuals. Leopards were recorded from 27 of 39 hotspots (identified in the questionnaires or by previous signs). A total of 18 individuals were identified based on their unique pelage patterns. Leopard ID 01, an adult male, was observed on three separate trails that were adjacent. Four males and three females were observed on two trails with overlapping territories, and the rest of the identified leopards were observed on individual trails. Leopards from eight hotspots were not identified during the second year, as there were no clear images of their distinguishing spots.

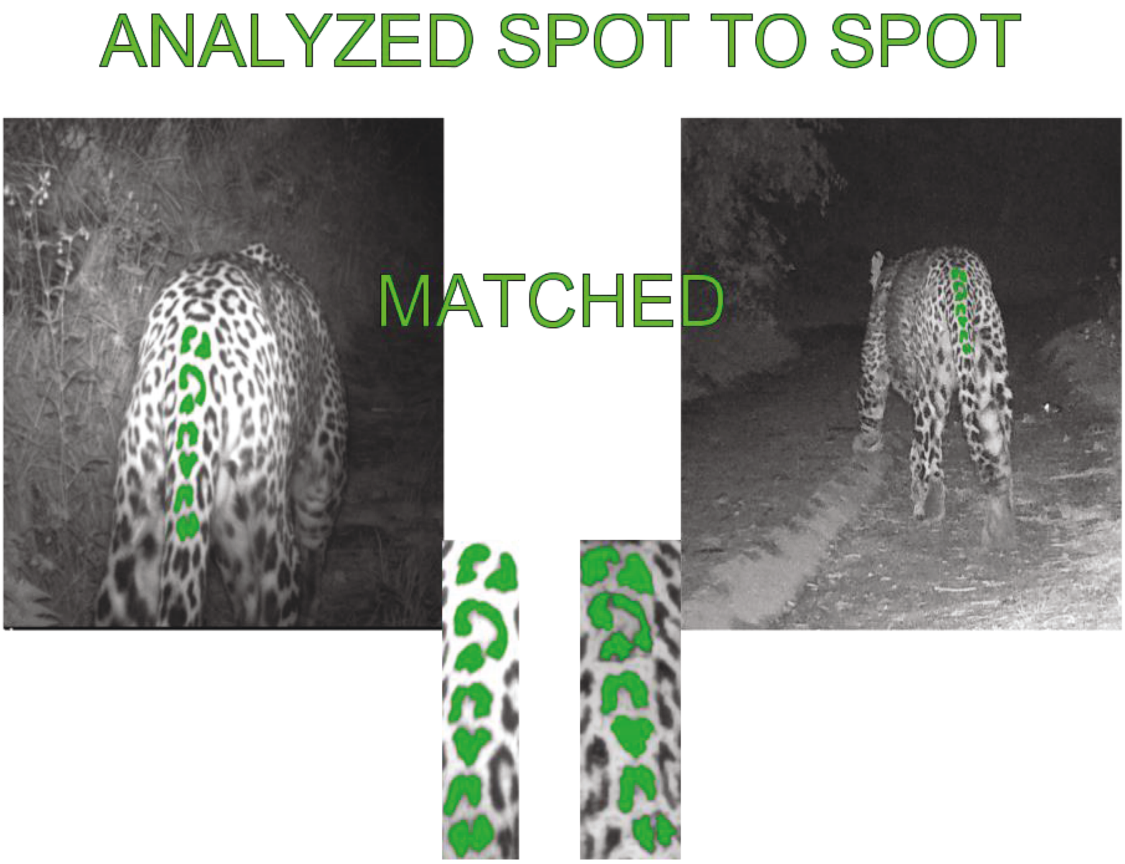

Figure 2.3 Example of Individual identification of the same male leopard based on its unique rosette pattern on the dorsal surface of the tail captured in two different location at Gallies Forest Division Khyber Pakhtunkhwa Pakistan (Muhammad Asad-Lincoln University).

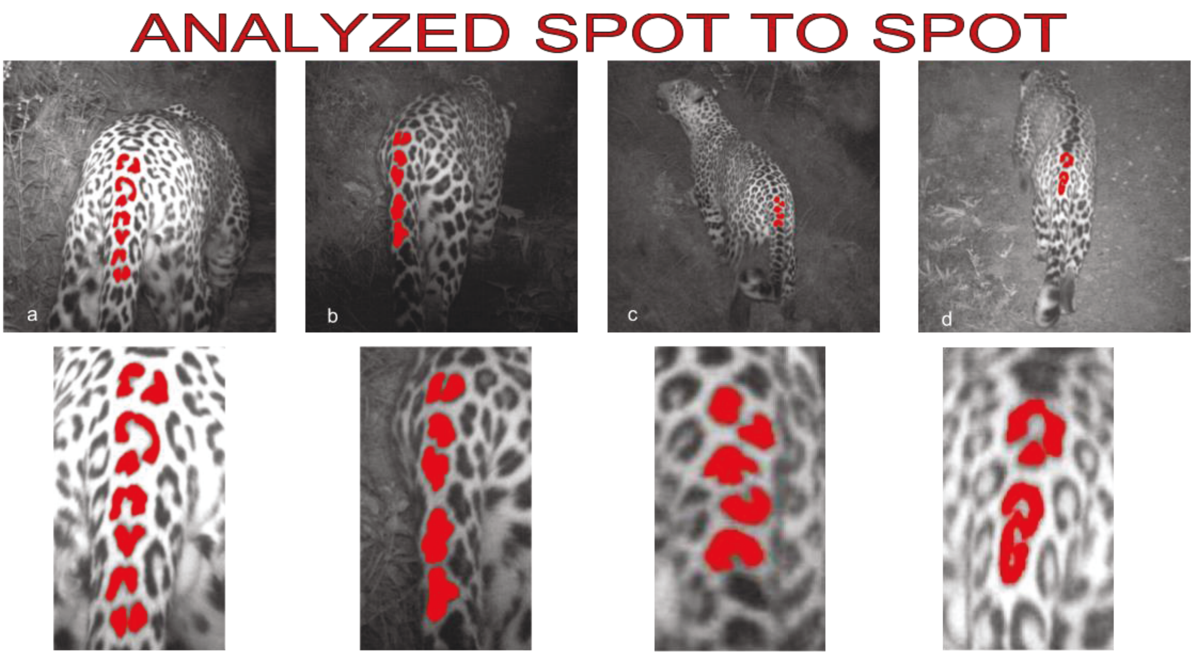

Figure 2.4 Example of individual differences of the leopards based on the prominent dorsal surface of a tail captured in different trails at Gallies and Murree Forest Division (Muhammad Asad-Lincoln University).

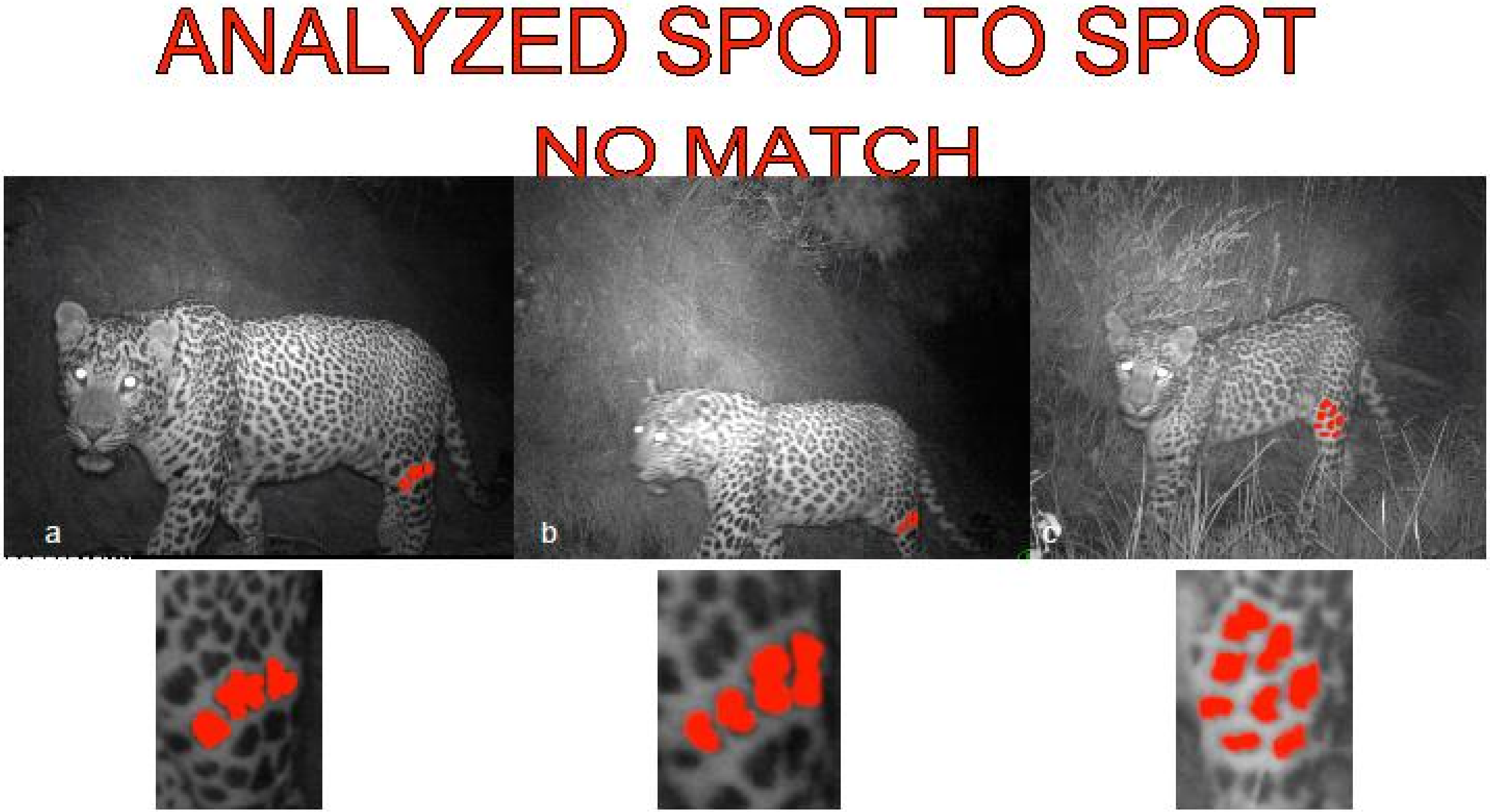

Figure 2.5 Example of individual differences based on the prominent left flanks of three different individuals (a, b, c) captured on different trails in Gallies and Murree Forest Division.

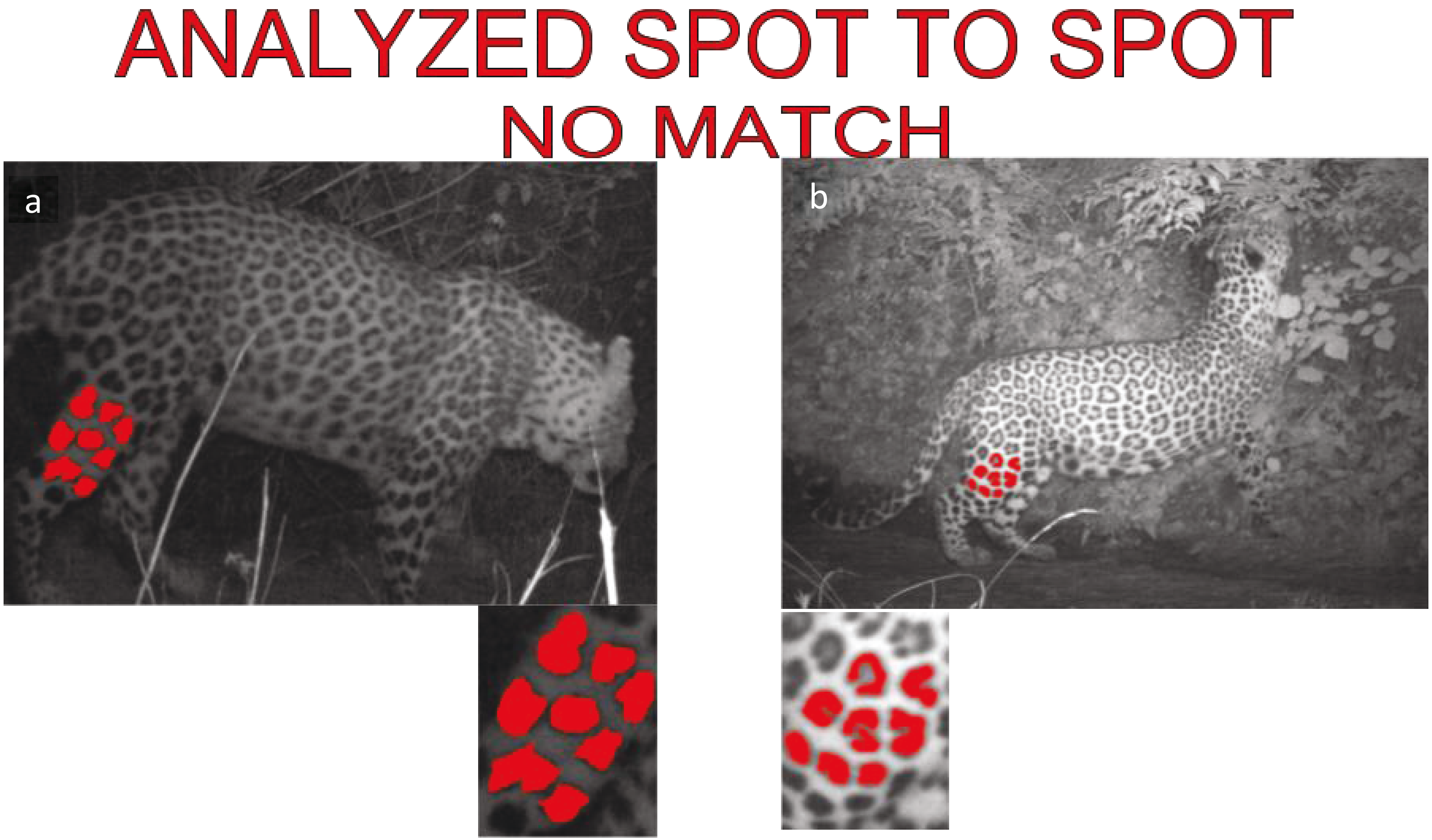

Figure 2.6 Example of individual differences based on the prominent right flanks of two different individuals (a, b) captured on different trails in Gallies and Murree Forest Division

2.4.3 *Model selection*

A summary of the model selection process is given in Table 2.3, where models are ranked according to AICc. The top-ranked model assumes a constant capture probability, while the second-ranked model allows different capture probabilities in each year, although the small difference in -2*log-likelihood, suggests the additional parameter explains little additional variation in the data. All models produced similar estimates of leopard abundance for each year. Models that allowed for capture heterogeneity, *p*(het),*p*i(.), *p*(Year+het),*p*i(.), were not ranked highly by AICc, suggesting little evidence of heterogeneity. The model-averaged abundance estimates for Galyat and Murree are 19, in 2017, and 9, in 2018, with 95% confidence intervals of 16 - 24 and 7 - 12, respectively, in the effective sample area of 200 km^2 as derived from MMDM.

Table 2.3 Model selection results from MARK analysis for common leopard population estimates in Gallies and Murree Forest Division Pakistan 2017 and 2018.

Without behaviour models					**2017**		**2018**	
Model[a]	**Delta AICc**	**AICc Weights**	***K***	**-2*log-likelihood**	**Estimate**	**SE**	**Estimate**	**SE**
p(.)	0.00	0.62	1	139.19	18.97	2.82	8.86	1.73
p(Year)	1.96	0.23	2	139.09	19.36	3.30	8.53	1.80
p(het),*p*i(.)	4.13	0.08	3	139.19	18.97	2.82	8.85	1.73
p(het),pi(Year)	5.70	0.04	4	138.65	19.91	3.59	8.88	1.82
p(Year+het),*p*i(.)	6.15	0.03	4	139.09	19.36	3.30	8.53	1.80
p(Year*t)	20.35	0.00	14	130.54	19.08	3.15	8.35	1.67
Model average					19.10	2.99	8.77	1.76
				lower	**16.77**		**7.61**	
				upper	**24.54**		**12.16**	

[a] Key to model notation: delta AICc = difference between model listed and the model with lowest Akaike information criterion; AICc Weights = Model weight based on model AICc compared to all other model AICc values; *k* = no. of parameters; -2*log-likelihood = twice the negative likelihood;

The mean distances travelled by male and female leopards were 7 km and 4.6 km, respectively. We used a 6 km buffer (Figure 2.7) for each camera-trap, derived from the mean maximum distance moved by four adult males and three adult females with more than one capture event on more than one camera-trap (Figure 2.8). The estimated leopard density at 200 km^2 was 9.5 individuals/100 km^2, in 2017, and 4.5 individuals/100 km^2, in 2018. The total estimated area of the Gallies and Murree Forest Division, is approximately 430 km^2, giving a total population estimate of 40 leopards, in 2017, and 19, in 2018.

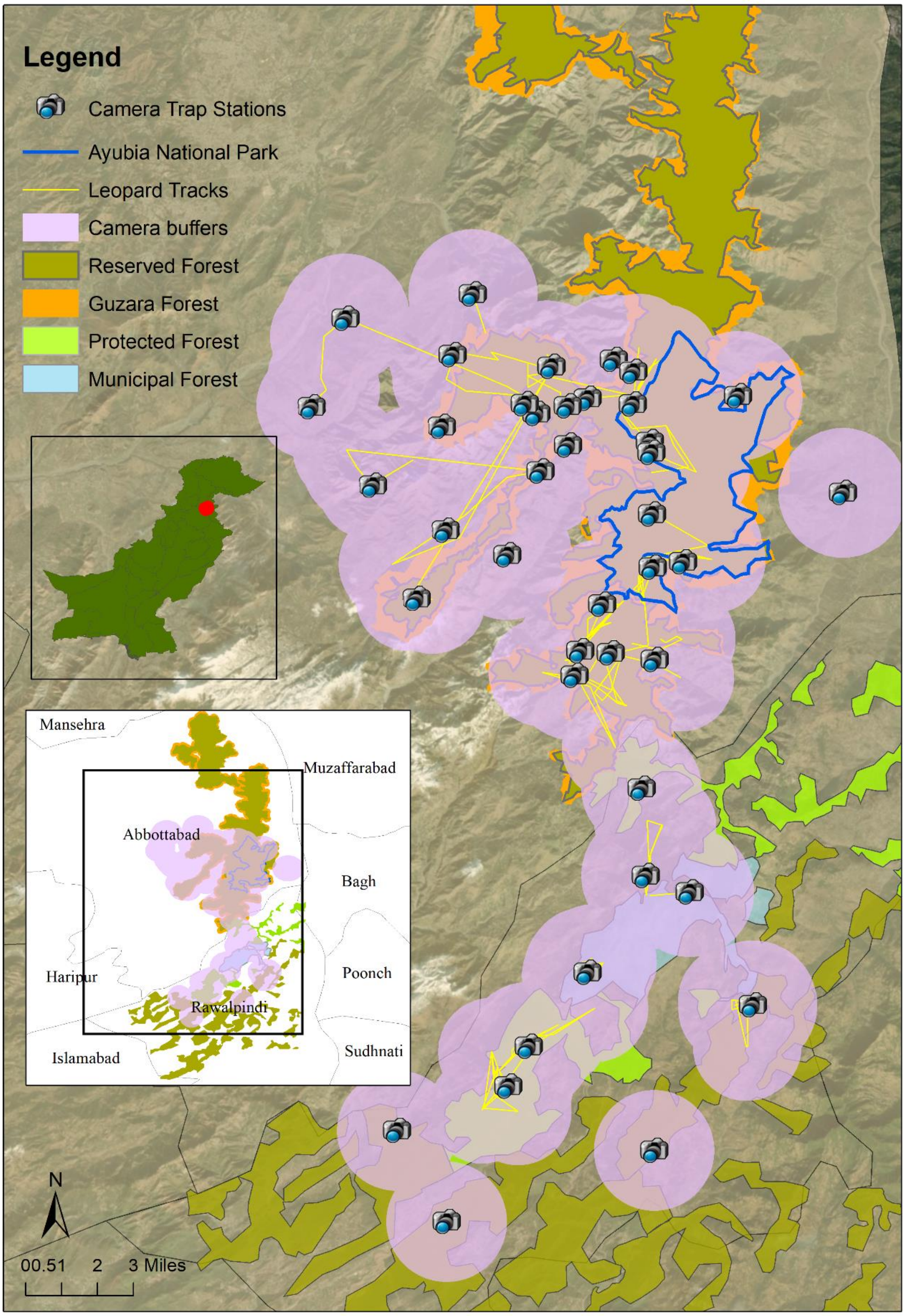

Figure 2.7 Representation of the study area with the effective sample area of the different hotspots identified. The buffer shows the MMDM from the leopards captured more than once from different locations. The hotspots were linked with the number of signs found on each track. The trails were named by the closest village, and the leopards were named according to the track they were photographed.

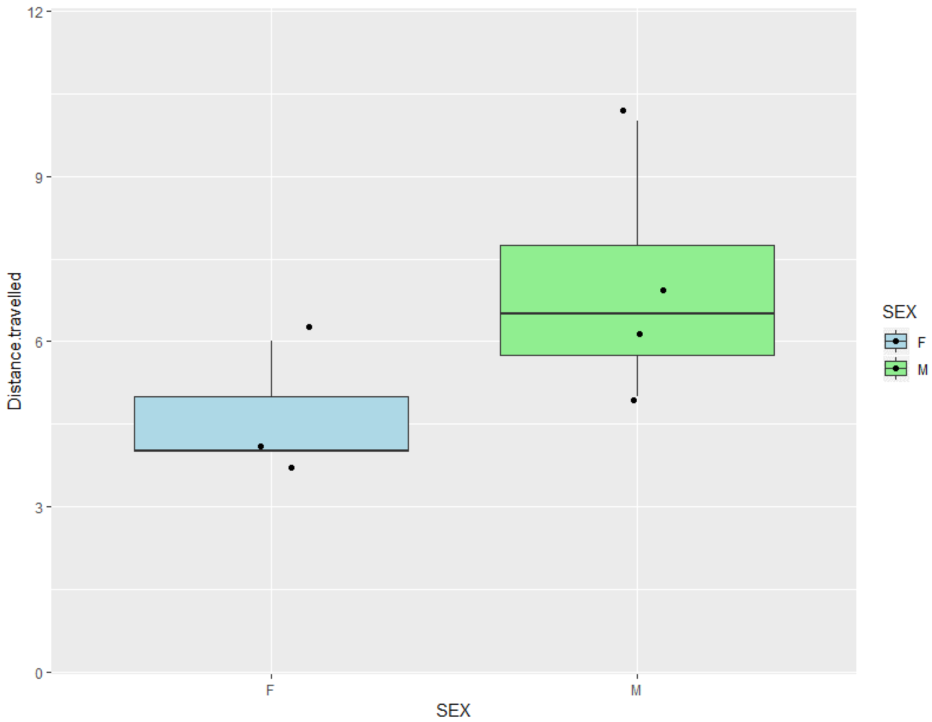

Figure 2.8 Box plot showing distance travelled by identifiable male and female leopards captured at multiple locations in our study area.

Individual sexes were identified from their external morphological characters. Eleven males and seven female leopards were individually identified. The sex ratio of male to female leopards in the study area from camera-traps, was 1.5:1.0. The mean encounter rate of females was higher than males on individual trails, perhaps indicating that males have larger home ranges than females (Morten and Per, 2005). The number of identified hotspots and leopard encounters was high in Guzara Forest. The Cantonment and Municipal Forest provided the least number of hotspots and encounters (see Figure 2.9).

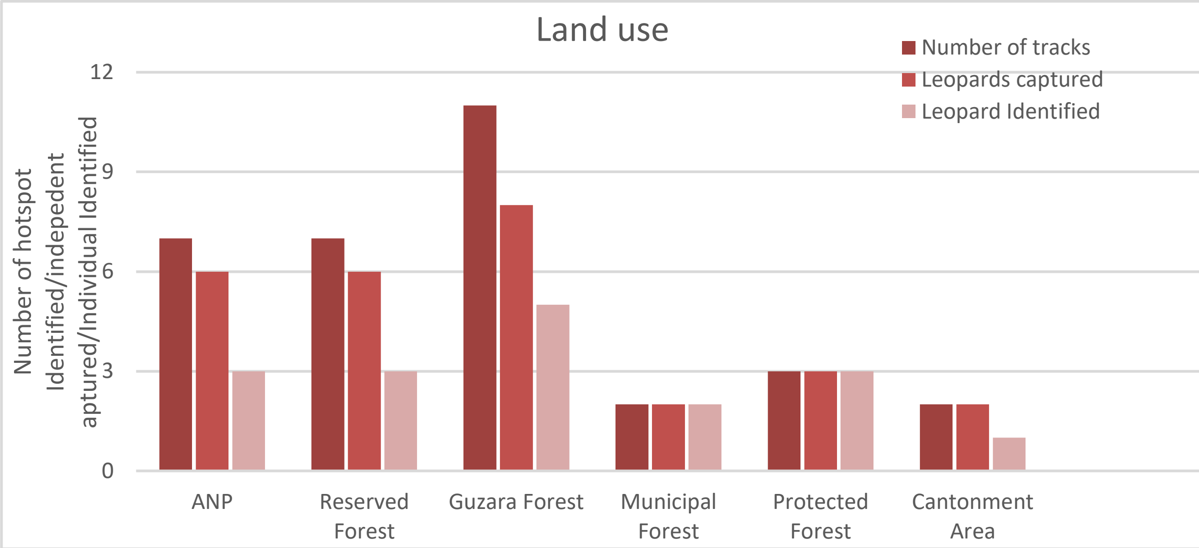

Figure 2.9 Number of hotspots identified in different land uses in the Galyat and Murree Forest, independently captured through camera-traps and individuals identified on each trail; Protected Forest, Reserved Forest, Municipal Forest, Ayubia National Park, Guzara Forest, and Cantonment area

Based on records obtained from the Wildlife Department Khyber Pakhtunkhwa and WWF-Pakistan in Abbottabad District, 21 attacks on humans were registered between 2005 and 2018 and, in response, 40 leopards were killed in retaliation (Figure 2.10).

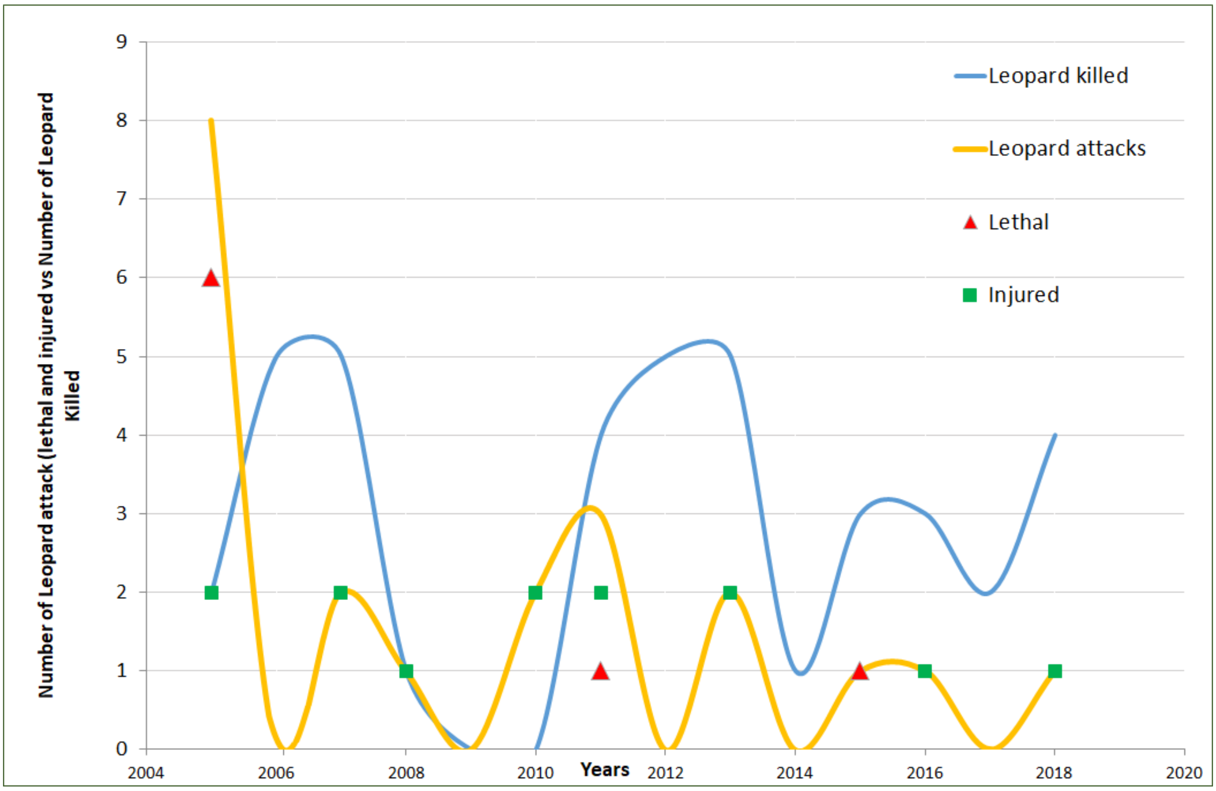

Figure 2.10 Number of leopard attacks on humans (red triangles lethal, green squares injuries) vs. number of leopards killed in the district of Abbottabad from 2005 - 2018.

The mean number of leopard attacks per year was 1.5 ± 0.60. Ten of the attacks were lethal and the other 11 caused severe injuries. The mean number of leopards killed per year was 2.8 ± 0.50. Eight leopards also died in captivity, including two cubs. Two leopard bodies were found during our camera-trap survey, in 2018, that were not reported but that showed signs of illegal hunting (their skins were removed). The overall number of leopards that died in winter was the same as that in summer although more males were killed in summer than in winter, whereas more females were killed in winter than in summer (Figure 2.11).

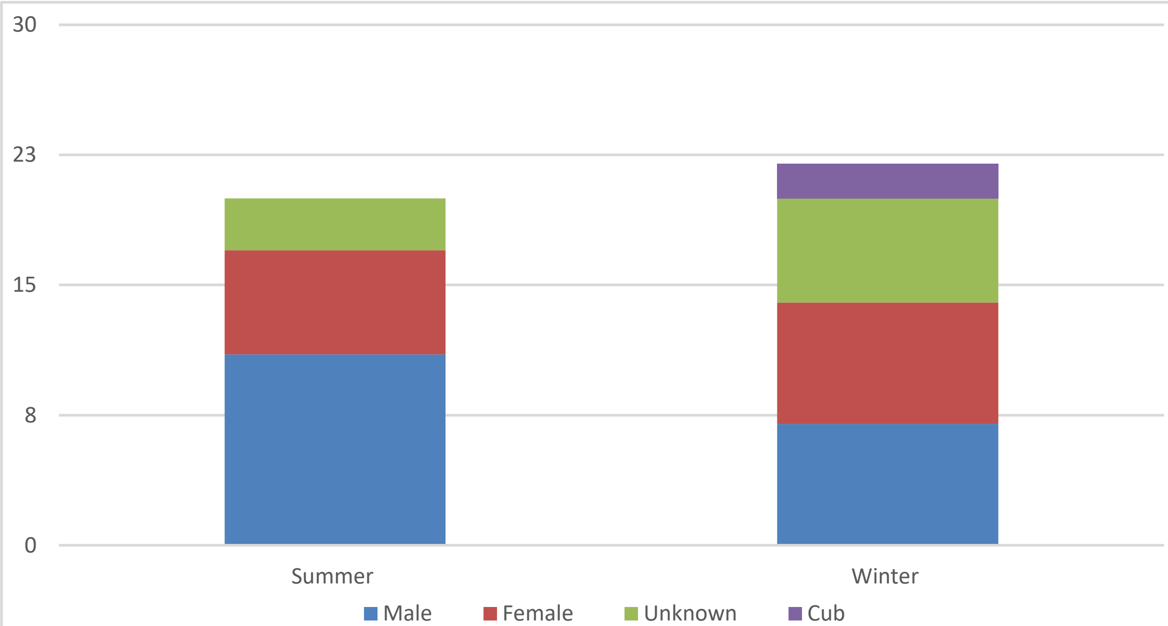

Figure 2.11 Number of male and female leopards that died in two seasons in the district of Abbottabad.

2.5 Discussion

Our Study confirmed leopard existence in the Swat, Dir and Margalla Hills regions of Pakistan. Combined with a MARK-recapture model, our camera data provided a first estimate for the leopard population in the Gallies and Murree Forest Division. The preliminary public survey was useful in identifying hotspot/trail information and this allowed us to cover a large area of rugged terrain, with limited accessibility, to maximise the capture probability and monitor population abundance.

Our study suggests that camera-trapping, combined with the MARK-recapture method, can be useful for estimating density or abundance of leopards in their habitats. The data for abundance was collected over two sampling periods, April – July 2017 and March – June 2018. Many biologists have recommended a shorter duration of 2 - 3 months to minimise the gain/loss that occurs during a study (Jackson et al., 2006; Karanth 1995); however, others have urged for an extended survey in order to capture more data and maximise the accuracy and high confidence for a closed population (Wang and Macdonald 2009; Simcharoen et al., 2007). Our camera-traps were not distributed in a grid pattern as is also recommended to maximise accuracy (Tobler et al., 2008). This was difficult to achieve in our study areas because of accessibility and logistics. Local knowledge and expert opinions maximised the number of captures and increased the chances of individual identification. It is crucial to identify trails that leopards may use before placing the camera-traps, as this will maximise capture probability (Balme et al., 2009; Karanth and Nichols 1998).

It was difficult to obtain quality images of both sides of a leopard's flanks on paths narrower than 2 m in width. We were able to match sharply defined pelages on the hind limbs and dorsal tail surface of each individual, which increased our precision when using CMR analysis in a single estimate for a given population (Alonso et al., 2015). The common leopard also changes the shape of its rosettes with the movement of its body and its orientation to the camera-trap. We found that setting the camera at an angle of 45^o to the track obtained clear spots and rosettes from the hind limbs or dorsal surface of tail, for individual identification. We identified individuals by comparing photos of each individual to other leopards and photos with clearer patterns were considered for comparison for individual recognition.

We are confident in our ability to identify individual leopards from photograph images and the capture histories obtained from camera-traps. We believe that camera-trapping is a viable tool for estimating the common leopard population size by maximising capture probability by placing traps at hotspots located prior to the placement of camera-traps, and our confidence in the identification of an individual leopard. Leopards are territorial (Odden and Wegge 2005), and their capture probability is likely to be heterogeneous. However, mixing probability estimates suggested little evidence of heterogeneity as the estimate for pi is essentially 1, which we suggest was due to the small sample size.

The abundance estimates obtained from our analysis of the two years of camera-trapping data are 16-24 leopards in 2017, and 7-12 in 2018. Rather than a large decline in population between years, we think it is likely that the population has remained the same. While there were fewer captures in 2018 (71 vs 121) there were also far fewer clear images that allowed individual patterns to be identified (46% vs 71%). If, for example, the identification rate in 2018 had also been 71% then this would have estimated a population of 12-18, which falls within the range of 2017.

We are not sure why there was an absolute change in leopard images captured. The difference may have been a result of a change in individual movements around their territory or possibly due to weather. There were drought conditions in 2018 compared to 2017 (Pakistan Metrological Department). Drought conditions may have affected the prey density in the study areas forcing leopards to extend their home range in search of prey and, therefore lowering the detection probability for the camera traps. Typically, studies have found little difference in the overall travelling of leopards in different seasons (Marker and Dickman 2005; Mizutani and Jewell, 1998), but there has been no research on the effects of atypical seasons.

The frequency of capturing a leopard on the same track may be different depending on the size of the home range (Rozhnov et al., 2015; Silver et al., 2004; Smith 1978). For example, a leopard captured twice a week on a track may have a smaller territory than a leopard captured once a fortnight. There is a possibility that the decline in population was real and may be due to the killing of leopards by poachers or unreported retaliatory killings. The total number of unreported killings may skew the number from 2.8 per year to a much higher number, as we did find signs of hunting from our field surveys and camera-trap data.

The density of leopards in our study area, at 8 - 12 and 3.5 - 6.5 leopards/100 km^2, is more or less similar to other CMR: 13 - 14/100 km^2 India (Harihar et al., 2009), 3 - 4 leopards/100 km^2 in Mondulkiri Protected Forest Cambodia, and Manas National Park Assam, India (Borah et al., 2013; Balme et al., 2009). This study confirmed the presence of leopards in the Swat and Dir and Margalla Hills despite the local people and the local wildlife department believing that the species had been eradicated from the area. There is no record of livestock depredation and retaliation by villagers in close vicinity to the forest area; however, the nomads that were interviewed confirmed depredation in the summer seasons although they were unsure as to the actual cause of those deaths. The attack events were described as like that of leopard predation (e.g. bite marks, dogs missing, etc.). The nomads travelled from the plains to the hills during summer and kept the livestock under open skies overnight. Leopards from one camera-trap station and two sets of territorial markings on one track were close to these grazing areas. We did record higher capture probabilities, 0.10 to 0.20, according to the model selected based on AICc weight p(.), than reported for leopards in other studies, e.g. 0.04 (Wang and Macdonald 2009) and 0.02 - 0.10 (Borah et al., 2013) for similar habitats and this might be due to the identification of areas of high leopard use with the questionnaire survey.

Another approach used for estimating leopard density by Balme et al. (2009) was to try to fit trail count data (*n* = 39 sites) to the single-season models where each pair of rows represented a unique trail where the camera-trap was later installed. Unfortunately, we did not have enough replicates for each trail, and we often observed the same leopard on several trails (using their unique rosette patterns), which would overestimate the density of leopards in the study area. Therefore, we did not rely on the trail count data in our capture analysis. Camera-trapping is more effective if the cameras are equally distributed, cover a large area and identify each individual captured, but this is hard to achieve in rugged terrain and with animals from large home ranges (Tobler et al., 2008).

Leopards in Pakistan are sparsely distributed throughout the country and, hence, require extensive sampling efforts and financial resources to cover the whole area to obtain sufficient information about their populations. One of the important outcomes from this research is to prioritise focusing on leopard spot patterns from camera-trap surveys, as they are a cost-effective method for CMR analyses. To date, the abundance of leopards in Pakistan (Gallies, Swat, Dir, and Murree) has been anecdotal. The information about movement patterns and their dispersal behaviour is a conservation concern for many carnivores (Kanagaraj et al., 2013).

We observed that leopards use the Guzara Forest around reserve areas extensively as part of their home range. Based on information obtained from the Wildlife Department Khyber Pakhtunkhwa and WWF-Pakistan, 70 % of the leopards that died were due to retaliation by humans outside the reserve area (Guzara Forest i.e the buffer zone) and near the villages, mostly during the winter season (Muhammad Asad personal obs.). The Guzara Forest received comparatively less snow to the protected areas, which may be a reason that leopards were more likely to be found there in winter season and close to human settlements (Muhammad personal obs.).

Conservation efforts should focus more on hotspots identified in the Guzara Forest surrounding the Reserved Forest as this may reduce human-leopard conflicts. These forests allow leopards to extend their movements in search of food and help to support viable leopard populations. This extension also occasionally led to livestock depredation by leopards and resulted in the death of leopards. Immediate compensation for livestock owner losses and a comprehensive awareness program for school children and the local community may significantly reduce the conflict between humans and leopards.

The methods used in this work can be replicated in other parts of Pakistan to obtain census populations and to set priorities for the conservation efforts for endangered leopards. Locating the remaining leopard population needs to be a high priority as this will, then, allow a focus on other conservation issues for this species.

The ultimate threat to leopards and their occurrence in this habitat is extensive legal and illegal hunting practices. The Wildlife Department has issued many licenses to locals for bird hunting as part of their revenue generation. If a person is reported for illegal hunting, they are first fined a small amount of money and then re-issued with a license. Once the license is issued, a person can effectively hunt whatever they want, as there are no checks and balances. Illegal hunting has created a traumatic situation for leopards that drives them away from particular habitats. The other threats to leopards in this area are habitat degradation, unplanned development, encroachment by humans, and an alarming depletion of their natural prey base.

This camera-trap study provides the first evidence of the species presence in this area; however, to develop a sound conservation plan additional camera-traps studies on natural densities, habitat and the associated threats are needed.

In conclusion, this research provides baseline information for leopard conservation and the mitigation of human and leopard conflicts for the Gallies and Murree Forest Division. The hotspots can be used to monitor population trends and any demographic changes through time. We also recommend future studies in different seasons and for longer periods. Our study suggests that the most productive conservation efforts may be beyond the protected area with special consideration given to buffer zones to ensure the long-term viability of leopard populations.

An adult male leopard (04) photographed during our camera-trap survey at Gallies Forest Division Khyber Pakhtunkhwa Pakistan.

3. Estimating occupancy for leopards and scaling diversity using camera-traps in northern Pakistan.

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Contribution

1. Conceived and designed the experiment

Muhammad Asad, Adrian Paterson, James Ross

2. Performed the experiment

Muhammad Asad (Muhammad Waseem helped with the extensive fieldwork)

3. Analysed the data

Muhammad Asad, Adrian Paterson, James Ross

4. Contributed to reagents/material/analysis tools

Muhammad Asad, James Ross, Darryl Mackenzie

5. Drafted the work

Muhammad Asad

Adrian Paterson and James Ross provided constructive feedback on the draft revisions of the manuscript.

3.1 Abstract

The common leopard (*Panthera pardus*) previously occurred throughout Pakistan and appears to have experienced a huge decline in numbers and prime habitat in many regions. To determine site occupancy and current distribution, we used presence-absence and occupancy modelling from randomly-placed camera-traps along the trails in northern Pakistan. We used the data collected for density estimation in chapter 2 and analysed it with the focus on site occupancy as opposed to estimating population size. Pre-determined target sites for camera-trap placement maximised the detection probability of the initial survey; however, the movement of cameras to adjacent sites appeared to have reduced detection rates in a second survey. Detecting leopards over a large home range requires a longer period of survey, which might improve the estimates produced from the statistical occupancy model.

Several issues affected the analysis. The data was too sparse and detection was very low in the second survey (<0.1 photos per camera per day), suggesting that occupancy modelling was not appropriate for that situation. We suggest that random placement of cameras in predetermined target sites can help to increase the detection probability of obtaining reliable inferences on population estimations for capture-recapture analysis, providing there are a sufficient number of camera-traps and identifiable individuals. We suggest that cameras should be active for at least one month, in an attempt to increase detections by allowing leopards to completely patrol their home ranges. We suggest a future comprehensive assessment for a longer period throughout the country to clarify the distribution status and priorities for conservation actions.

3.2 Introduction

Presence-absence data are often key for wildlife managers in conserving specific species within a large landscape, particularly in prioritising prime habitats for protection (MacKenzie et al., 2003). The habitat use by species is key for their survival (Abdollahi, 2015; Gavashelishvili and Lukarevskiy, 2008). Presence-absence data are also very useful for managing rare and elusive species in wildlife conservation (MacKenzie, 2005). Occupancy of rare and cryptic fauna in a high-value habitat also allow adaptive responses by wildlife managers for conservation and management. Researchers often use analytical programs, such as PRESENCE® (Hines 2006) and MARK™ Version 8.2 (White and Burnham, 1999), to make reliable inferences about the habitat surveyed (MacKenzie, 2005). Obtaining spatial information regarding presence-absence, predictability, and site occupancy, are challenging for a wide range of habitats (Kéry et al., 2010). Single season occupancy models have been developed in PRESENCE® software to investigate species occurrence in specific sites with-or-without explanatory covariates, such as habitat type, size within the sampled area, and the extrapolation of occupancy rates for whole areas (MacKenzie, 2003). The occupancy approach helps deal with imperfect detections that could otherwise signal misleading conclusions (MacKenzie, 2005).

Occupancy of specific species in a given habitat requires an appropriate sampling technique (Barata et al., 2017). A recent emerging method in ecological sciences for species detection is the use of passive camera-traps (Henschel and Ray, 2003). Camera-traps have been adopted widely for detecting species and the proportion of survey sites occupied (Kery et al., 2010). Camera-trap data are useful for observing and assessing spatial and temporal information *in situ* (Bashir et al., 2014; Khorozyan et al., 2008). However, researchers designing a study to collect presence-absence data must first decide how to appropriately employ camera-traps (i.e. pre-targeted sites or random locations) to maximise capture histories over a defined time frame for the type of analysis performed (O'Connor et al., 2017). Detection probabilities are substantially higher for some species on trails, and this benefits density estimations for capture-recapture modelling. However, this sampling method is not considered appropriate for occupancy and species richness studies (Burton et al., 2015). The placement of cameras on pre-determined grids or in random locations, has significant consequences on the applicability and reliability of the resulting ancillary information (Sollmann et al., 2013). However, targeted sampling is debatable in any situation and depends on the overall goal of the research. In our case, we randomly-placed cameras along known trails in a region to meet the assumption of equal probability of detection and also to partially satisfy the assumption of an unbiased sampling design. The abundance of species in a given space and the rate of detection probability of camera-traps is greatly influenced by many behavioural and biological factors, such as home range size and species size (Tobler et al., 2008; Kelly and, Holub 2008).

A closed-population modelling approach is considered more accurate, and with higher confidence across repeated surveys, as it meets the assumption of no gain or loss of individuals from the study area during the course of the study (MacKenzie, 2006). This assumption is hard to achieve for leopards in practice. For example, leopards travel long distances, e.g. 194 km (A straight-line distance cover in one year; Fattebert et al., 2013) and the home range size of an adult male was estimated to be at 670 km^2 (Hunter, 2011) in central Iran. However, in occupancy modelling, an observed absence of a species from only one survey on a site can still be interpreted as a site in use (MacKenzie, 2005). Occupancy modelling is an effective method for monitoring the spatial distribution of target species when the presence-absence information is available (MacKenzie, 2003).

The site occupancy model has been widely adopted for estimating the proportion of sites occupied by a species (Kery et al., 2010). Distribution, occupancy, and population size are important for the effective conservation and management of rare wildlife that are present over a large area and with a low density (MacKenzie, 2005). Assessing the size of leopard populations is difficult as they have large ranges in their natural habitat and usually occur in low densities (Tobler ad Powell, 2013). Sampling with multiple procedures and combining sampling processes are often required to elucidate reliable inferences (Manly et al., 2002). In Pakistan, there has been no systematic documentation or model created for the current distribution status of leopards. There has been some limited diet analysis where human-leopard conflicts have provided an indirect indication of their presence. For example, Khan et al., (2013) assessed the current habitat, distribution, and status of mammals in the Khirthar protected area complex in Sindh and suggested that leopards were extirpated from the area. Similar observations of human-leopard conflicts have been made in the Machiara National Park, Azad Jammu and Kashmir, and the Ayubia National Park by Kabir et al., (2013) and Lodhi (2007). Shehzad et al., (2014) analysed the diet of leopards in Machiara National Park. These conservation-related studies are important for understanding the behaviour and importance of the top predators in ecosystems and for the effective conservation of the species (Brodie, 2009). For example, a study in 2010 assessed the status of the common leopard in Iran (Ghoddousi et al., 2010). Researchers have used meetings and semi-structured interviews to ascertain the presence-absence of leopards from regions (Abdollahi, 2015).

Information has been previously collected from leopards using GPS points from local villagers to map the distribution of the species using GIS-based modelling (Mondal et al., 2012). Researchers often use a semi-structured questionnaire to collect data on carnivores presence from villagers and then confirmed the data by installing camera-traps (Engel et al., 2017; de Carvalho and Morato, 2013; Thorn et al., 2011). A questionnaire survey often has inadequate spatial and temporal information, which makes it difficult to form even a basic picture of species distribution (Ghoddousi et al., 2016)). Habitat modelling has been used in the assessment of wildlife habitats and mapping (Lee eta al., 2015; Xiaofeng et al., 2011). To obtain more reliable information about species presence and distribution, camera-traps and field-based surveys are combined to obtain sufficient spatial and temporal information for the species present within the sample unit (Bino, et al., 2014).

For confirmation of the presence-absence of leopards in African forests, several multiple detection methods have been used, including track surveys and interviews with the local community (Long et al., 2011; Henschel and Ray, 2003). The information from these interviews was used to identify the best places for camera-traps installation. We assumed that the leopards should have a detection probability close to one if a site is occupied; however, the frequency at which the leopard uses a trail still depends on the size of its home range.

In our study, different leopard populations in northern Pakistan were subjected to Presence® analysis using four a *priori* single-season models (MacKenzie et al., 2003). The data for the construction of detection histories were established from our camera-trap survey and after surveying local people. Based on the survey information there are plausibly three leopard strongholds remaining in this part of Pakistan, Gallies Forest, Murree Forest, and the Azad Kashmir region. Due to the huge range loss and varying threats among regions, the International Union for the Conservation of Nature (IUCN 2016) has re-categorised leopards from near threatened to vulnerable.

Our initial study was primarily aimed at estimating leopard density using capture-recapture analysis (Chapter 2); however, we also tested our data to establish baseline information for leopard occupancy using occupancy modelling in the program PRESENCE® to estimate the proportion of area occupied. Using these results, we provide recommendations and general guidelines that can be used in future studies by the wildlife conservationist. The findings will also provide a general understanding of leopard habitats in Pakistan.

3.3 Material and Methods

3.3.1 Study area

The study area and data collection is the same as Chapter 2.

These unique geographic landscapes mostly comprise mountains that provide diverse habitats for a wide range of flora and fauna. Northern Pakistan is considered the last stronghold of the common leopard population and stretches from the Margalla Hills to Murree, to Ayubia, and from Thandani into parts of Azad Jammu and Kashmir (Kabir et al., 2013; Sheikh and Molur, 2004). These areas consist of dense forests, mountains, caves, plain scrub zones of the sub-tropical continental highlands and encompasses two distinct ecological zones, ‘moist temperate coniferous forests’ and ‘chir pine subtropical forests’ (Ashraf et al., 2014). Like many other countries, leopards in Pakistan also share their space with humans (Dar et al., 2009). Increasing human population pressure and expanding areas of land-use, such as converting natural habitats into settlements, are major threats to leopard conservation. Protected areas, such as the Ayubia National Park, are surrounded by villages where livestock graze freely. This has occasionally led to livestock depredation by leopards and resulted in the death of leopards (Lodhi, 2007).

Pakistan shares its borders with countries that have populations of two different subspecies of leopard. To the east, India has a stable population of the Indian leopard *P. p. fusca.* To the west, Iran has the endangered Persian leopard *P. p. saxicolor* population (Ghoddousi, et al., 2010). Afghanistan also has the Persian leopard, although the full status of their subspecies distribution is not clear (Stevens et al., 2011). This research was conducted mostly in protected areas of different ecological landscapes in northern Pakistan.

3.3.2 Data collection

We initially conducted a questionnaire survey among local communities to pre-determine target sites for camera-trapping. Data about leopard presence were collected before camera deployment and included direct leopard observations, livestock depredation points, human-leopard conflicts, and revenge killings by local people. Where possible, we validated the information from the local Wildlife Department with interviews of the local people who had been injured and visits to the location where a direct leopard sighting was observed.

The study area was then divided into three sections: Galyat and Murree, Swat and Dir, and Margalla Hills. The initial survey in 2017 was conducted in the Galyat and Murree Region, where 39 leopard observations were obtained. During the second survey in 2018, we also conducted camera-trapping in the Swat and Dir district, where 24 leopard observations were obtained, and we relied only on transect sampling data from the Margalla Hills, due to the unavailability of camera-traps. For the analyses, we treated the data separately for each section. A total of 69 leopard sightings were identified from the questionnaire surveys. Out of these, 39 of the records were from Galyat and Murree, 24 from Swat and Dir and 6 from Margala Hills National Park. Six locations were later discarded from the Galyat and Murree Regions due to incorrect identification giving a final count of 63 sampling sites.

We were also interested in the presences of other species, e.g. other carnivores that might compete with leopards and prey species. Wild ungulates were reported absent from this area except wild boar and other potential prey species are available, such as domestic dogs, livestock (goat, sheep, and cattle), rhesus monkeys, rodents and pheasants (Shehzad et al., 2014).

3.3.3 Camera-trap survey

We placed camera-traps on leopard travel routes in 63 locations across the Galyat and Murree, and Swat and Dir districts. The study was conducted from April to July 2017, and, from March to July 2018. Initially, the camera-trap study in 2017 was conducted in the Galyat and Murree region. During the second survey we repeated camera-trapping in Galyat and Murree from March–July 2018 and also covered the Swat and Dir districts. We were not able to sample from the Margalla Hills Region due to an insufficient number of camera-traps being available. Instead, we relied on transect sampling data for confirmation of leopards' presence-absence. All camera-traps were active between 6 pm and 8 am, when leopards are most active (Ray-Brambach et al., 2018). The camera-traps were active for a period of 14 days at each site. Camera-traps were moved frequently from one location to another to cover the whole area over 14-days. This allowed us to scope other trails with leopard signs for future camera locations. Leopards are conspicuous and will be detected provided there are a sufficient number of camera days. The assumption of equal species detection probabilities for leopards is unlikely to be met on trails with and without leopard signs; however, to satisfy the assumption of an unbiased sampling design we also installed camera-traps on known trials with no leopard signs. This method was adapted from Alonso et al. (2015).

Given that the leopard has large home ranges and the logistical constraints of adequately sampling these ranges, we use pre-determined targeted sites for camera placement as a sampling technique to maximise the chances of recording leopards (Brassine and Parker, 2015). Territorial leopards often use the same trails to defend their territory and monitor their mate. The probability of detection is substantially higher for leopards on trails rather than off trails and this benefited the density estimations when using MARK capture-recapture software in Chapter 2 (as well as Asad et al., 2019).

Given the behaviour and ecology of leopards, we attempted to maximise detection histories by placing camera-traps on trails previously identified from a scoping visit. Camera-traps were used, followed (Janecka et al., 2011) along with field surveys in different habitats to provide data on the presence-absence of leopards in previously confirmed habitats. The total number of camera detections in each district depended on the number of tracks identified.

3.3.4 *Occupancy analysis*

A model-based approach was used to investigate species occurrence and site occupancy following the robust design of MacKenzie et al. (2005). Data were collected from predefined areas and subjected to single season models to determine the predictability and occupancy of leopards in Galyat and Murree. As a territorial animal, there may be slight seasonal variation in the movement patterns of leopards on trails; for example, due to heavy snow (Muhammad, pers. obs.). We assume that this difference has no effect on the model as we collected our data in summer where we observed more frequent visits by leopards to trails. If this is the case, there will be no changes in occupancy given by the time or seasonal occupancy, and we assume the detection probabilities will be same in the area.

Assuming the detection probability of the leopard is $p = 1$ on active trails, we used single-season models to observe whether a site was occupied or not occupied by leopards in a given time, t. The detection histories and probabilities of the detection model were combined with a likelihood model and the explanatory variables of land use selected, as defined by location (i.e. Reserved Forest, Guzara Forest, Ayubia National Park, Cantonment area, Municipal Forest and Protected Forest). The likelihood method was run through the program PRESENCE® 2.1 (Hines 2006) and the predictability and occupancy with different variables on the site were determined.

Data were arranged as one row per camera-trap station and one column per survey, giving encounter periods of 28 nights for both surveys. Leopards were recorded as '0' if the species was absent, '1' if the species was detected and '-' if the cameras were not active for that night. We fitted three predefined models to the data, such as: (1) One group constant p (same occupancy probability for all transects) and that detection probability (*p*) is constant across both transects and survey occasions (i.e., only two parameters will be estimated); (2) two groups with constant *p* (all transects have the same probability of occupancy, but *p* can vary between the two surveys although at each survey occasion, *p* is the same at all transects); and (3) one group, survey - specific *p*. Sites were considered closed to change in the state of occupancy for the sampling duration. The data from each section were treated separately when estimating occupancy.

3.4 Results

Overall, there were 3,022 trap nights (1,930 in Galyat and Murree and 1,092 in the Swat and Dir district) from 63 locations. We collected 43,118 photo images. Of the total, the majority (76%) were caused by movement of the local people, mostly from four camera stations in Galyat. At the Galyat and Murree 39 sites we surveyed and recorded 192 leopard images. At the Swat and Dir district, of the 24 sites surveyed, we recorded leopards at one camera-trap site (three photos; Shangla, Swat district) and two leopard territorial markings in Nehag Dara (upper Dir). In the Margalla Hills, we found four territorial markings (Table 3.1).

Other non-target species captured in Galyat and Murree during the survey were mammals: including leopard cat (*Prionailurus bengalensis,* 35 photos), red fox (*Vulpes vulpes,* 1,934), golden jackal (*Canis* aureus, 1,459), Indian-crested porcupine (*Hystrix indica,* 142), wild boar (*Sus scrofa,* 2,541), rhesus monkey (*Macaca mulatta,* 115), yellow-throated marten (*Martes flavigula,* 25), Indian-palm civets (*Paradoxurus hermaphroditus,* 85), Himalaya-palm civet *Paguma larvata,* 54), and red giant flying squirrel (*Petaurista petaurista,* 4). Bird species captured were Khalij pheasant (*Leucomelanos hamiltoni,* 4), Koklass pheasant (*Pucrasia macrolopha,* 3) and blue-whistling thrush (*Myophonus caeruleus,* 6).

Species occurrence, as well as the photo capture rates, were fewer in Swat and Dir than Galyat and Murree. We recorded red fox (732 photos), porcupine (44), rhesus monkey (26), jackal (653), domestic cat (*Felis catus,* 92), domestic dog (*Canis lupus familiaris*, 127) and blue-whistling thrush (3). Most of these photos came from two locations: Shangla in Swat and Nehag Dara in Dir.

Table 3.1 Summary of the camera-trap images and trail scoping for presence-absence of the common leopard and non-target species at different location sampling periods and sites, showing active trap-nights, total photos and false images in 2017 and 2018.

Location	Sampling period	Sites	Active trap-nights	Total photos	False images	Non-target capture			Common leopard	
						Other species	Livestock	Local community	Photos	Territorial markings
Galyat and Murree	April-July 2017-2018	39	1,930	30,231	609	6,407	2,641	20,382	192	58
Swat and Dir	March –June 2018	24	1,092	11,806	2,140	1,677	4,116	3,870	3	2
Margalla Hills	March –June 2018	6	-	-	-	-	-	-		4

In Galyat and Murree, the estimate of occupancy produced by the predefined model was 1, 100% occupancy with zero standard error (Table 3.2). This implied that a leopard found in every cell. Our assessment for Galyat and Murree Forest was that the data were too sparse with not many repeats for the model to accurately converge, although the 'One group constant *p*' model was able to be ranked highest (Table 3.3). In addition, in the Swat and Dir District, we obtained only one capture event with three photo images (camera-trap station from Shangla, Swat District) over 1,092 traps nights and found two territorial markings in Nehagdara (Upper Dir) while scoping trails for leopard signs. This sample size was insufficient to robustly estimate occupancy using any modelling technique. Given our findings on leopard movement, we suggests that cameras should be recording for at least one month to allow individuals leopards to complete their patrolling circuit, which would increase detection probability (to above <0.1 per camera site per day).

Table 3.2 Overall estimates of the fractions of sites occupied by leopards in Galyat and Murree and the associated standard error over two seasons with three predefined models: (1) one group constant *p,* (2) two group constant *p*, and (3) one group survey-specific *p.*

Model	Occupancy estimate (psi)	Std. error	95% conf. int
One group constant *p*	1.00	0.0000	1.0000
Two group constant *p*	1.00	0.0000	1.0000
	0.500	2.5093	0.0000
One group survey specific *p*	0.9422	0.1043	0.2764

Given the problems, above, with the modelling, we were unable to include land-use as a covariate given the low numbers of detections. For our data, one or both of the estimates for occupancy were often 1.0. Even when an estimate was not close to one, they had large standard errors meaning a large degree of variation in the estimated values of *p* and, therefore, we were unable to produce reliable results from the occupancy analysis.

Table 3.3 AIC values, difference between AIC and delta AIC, and number of parameters for the three *priori* models (one group constant, *p,* two group constants, *p,* and one group survey-specific, *p*) in the model set used to investigate site occupancy in Galyat and Muree.

Model	AIC	Delta AIC	AIC wgt	Model likelihood	No. parameter	-2*LogLike
One group constant *p*	394.91	0.00	0.4649	1.0000	2	390.91
Two groups constant *p*	398.91	4.00	0.0629	0.1353	4	390.91
One group survey-specific *p*	404.60	9.69	0.0037	0.0079	29	346.60

3.5 Discussion

In the present study, the estimated occupancy from PRESENCE® was inadequate, most likely because of the small sample size and limited recorded events. Therefore, the weighted average of *p* and occupancy estimations (ψ) in the models were ill-conditioned. Camera-trapping data allows for presence-absence modelling using the Presence® program even when the individuals are not recognised from pictures. However, the noted multiple detections of the same individual in different trails (see Chapter 2) may also overestimate occupancy. We assessed that data were too sparse, and the cameras probably needed to be out for at least one month because the detection probability appears to be low (<0.1 per camera site per day).

When conducting occupancy modelling there are several key assumptions that must also be met. First, leopard occupancy must remain constant over the survey period (i.e. sites remain either occupied or unoccupied, Mackenzie et al. (2002)). To achieve this, the survey period was brief to minimise any chances of changes occurring, such as the removal of an animal through natural death, or illegal hunting; however, in the Galyat and Murree Forest, the number of sites with camera detections actually decreased from 15 to seven in the second survey. We are not convinced that this represents a real decline in leopard population. The difference in number may be due to many potential factors. First, the movement of camera-traps from one location to another can affect the detection probability.

We may have been fortunate to the detect maximum leopards on the 1st survey but were unlucky in the 2nd occasion. The brief sampling occasions may influence the detection probability. Home range size may also play a role for different individuals. The frequency of recapturing a leopard on the same track may be different with time, depending on the size of its home range (Rozhnov et al., 2015). For example, a leopard captured twice a week on a track may have a smaller territory than a leopard captured once a fortnight. The home range of an individual leopard depends on the availability of food and the presence of their mates. The capture probability can be influenced by the amount of time spent near a kill within its home range. We did not use any baits or lures to increase capture probability, nor did we find any traumatic experiences that might have caused them to avoid an area after the first capture. The killing of leopards by poachers (Kabir et al., 2013) or unreported retaliatory killing observed during the study period is likely to be another reason for the low detection in the second survey, which also violates the closed population assumption. Five species have been reported locally extinct from the Galyat and Murree Regions and this is assumed to be due to hunting practices (Lodhi, 2007): black bear (S*elenarctos thibetanus)*, musk deer (*Moschus moschiferus)*, grey goral (*Naemorhedus goral)*, barking deer (*Muntiacus muntjak)* and monal pheasant (*Lophophorus impejanus)*.

Leopards should be detected during a survey provided there are sufficient trap nights for a leopard to complete its patrolling circle. However, we observed more people in the sample area during the second season, and they were present until later in the night because of fasting. Local people often move into the hills during the month of Ramadan to spend the fasting month in a cool environment. They mostly sleep during day because of the summer temperatures and long day hours, and are active throughout the night, as the temperatures are cooler then. During this time, local people take walks and make noise, which might discourage a leopard from such areas. For trail count data, an individual's detection at multiple sites should be considered as either one detection or multiple detections. In the case of multiple detections of the same individuals, this approach may overestimate occupancy. Increasing sampling bout lengths can increase detection probabilities and produce more precise estimates (Mackenzie and Royle, 2005).

In addition, our analysis of 63 hotspots required a total of 114 camera locations, which could only be achieved by moving a set of camera-traps to the adjacent location within a larger study area over a shorter time frame, which means we did not always detect a leopard in that area. The detection probability is unlikely to be met over short survey periods with species that have a large home range. The detection probability likely varies temporally due to the amount of time spent at the kill site. Another issue with our data was the manner in which the camera-traps were relocated. Our camera-traps were placed randomly on identified trails to maximise detection and, for the Galyat and Murree Regions, most trails had a leopard detected but generally only once. To overcome this, we tried to rearrange the capture histories data in several different formats for PRESENCE®. We constructed capture histories data up until the first detection. This was to compensate for the sparseness of the detection data but, resulted in not using the re-detection data when available. We also tried arranging the data into 14-day sampling periods for each survey, by simply defining each day into a single night session, resulting in a final count of 28 occasions for both surveys. Another approach was to construct capture histories by simply merging two days into a single event and that resulted in a total of 14 sampling occasions. This allowed us to provide the maximum number of capture histories.

The design method, above, may enable density estimations in capture-recapture analysis for cryptic carnivores (see Chapter 2); however, we suggest caution for estimating occupancy if the probability of detection is low, using short sampling occasions, and the monitored species have large home ranges. In such circumstances, when the detection probability changes from one survey to another, it is difficult for the model to produce reliable inferences particularly when occupancy does not remain constant over the survey period. There are other approaches that could be used to estimate leopard occupancy. For example, a simulation approach could have been used to estimate site occupancy. Such a set-up allows a design to accommodate the more frequently-surveyed sites to the total number of sites, and estimate the expected value for occupancy. However, in our case, we were not convinced that it would provide us with useful information due to the low numbers of detections given the movement of camera-traps from one location to another.

Finally, we also tried fitting trail count data (n = 39 sites) to the single-season model where each pair of rows represented a unique trail where the camera-trap was installed, following Balme et al. (2009). However, we did not have enough replications for each trail and we often observed the same leopard on several different trails (using their unique rosette patterns), which would overestimate our estimates of leopards in the study area.

In addition to the leopard detection, a total of 12 mammalian species and three bird species were recorded in the study area, which belonged to 15 genera from 10 families. Wild boar (*Sus scrofa)* made up the majority of occurrences in the camera-traps among other wild species in Galyat and Murree (Figure 3.1, 3.2 and 3.3). They were followed by red fox (*Vulpes vulpes)* and golden jackal (*Canis* aureus). In Swat and Dir the red fox and golden jackal made up the majority, although at a comparatively lower rate than in Murree and Galyat. Carnivore ecology largely depends on the availability of prey (Ripple et al., 2014). However, hunting success depends to a great extent on dense cover. Prey who prefer dense vegetation are easier to capture than those prefer an open habitat (Balme et al., 2007). Lodhi (2007) suggested that rhesus macaques constitute the major portion of leopard diets in Pakistan. However, Shehzad et al.'s (2014) findings on diet analysis suggest that leopard rarely preyed on rhesus macaques in the study area. In our camera-trap study we detected rhesus macaques, but the capture rate was lower than expected, being 1.79% of the total species detected. Local shopkeepers feed the monkeys to attract tourists (Lodhi, 2007). Tourists bring food to feed the monkeys to bring them closer so they can take photos. The increase in tourism in recent years, and the continued feeding of monkeys on the roadside by tourists and locals, has changed their behaviour and resulted in the disproportionate use of the landscape by monkeys. Monkeys perceive the roadside as being for food and now extensively use it for foraging. Large troops of monkeys, approximately 20 - 100, spend most of their time near roadsides and towns waiting to feed

Figure 3.1 Photos of other mammal recorded during the leopard camera-trap survey in Galyat and Murree Forest Division: A) wild boar; B) rhesus monkey; C) yellow-throated marten; D) golden jackal.

and that has reduced their risk from leopard predation because of the presence of humans. This also reduces the availability of prey in the forest and increases the chance of leopards forging more widely, which lead to increase human-leopard conflicts. The leopard kill rate varies from 3 - 5 days (Bothma and Le Riche, 1984) depending on the size of the kill. Our findings indicate that Galyat and Murree have a lower number of prey species and diversity to sustain a viable leopard population. The prey density and diversity is also much lower in the Swat and Dir District and would not support the leopard population.

Feral domestic cats (*Felis catus)* and dogs (*Canis lupus familiaris)* were in high numbers in Swat and Dir. In our study area, prey species for leopards are threatened due to legal and illegal hunting, poaching, and habitat loss. Grey goral (*Naemorhedus goral),* and musk deer (*Moschus chrysogaster)* were only reported in the Kashmir region (Kabir et al., 2013). Gray goral (*Naemorhedus goral)* were categorised as ‘vulnerable’ in the CAMP assessment conducted by the IUCN Red List (Shiekh and Molour 2004). Wild boar was previously recorded only in the Murree region (Chughtai et al., 2018); however, we detected this species in our camera-traps from the Galyat region but not in the Swat and Dir Districts. We did not detect civets in Swat and Dir.

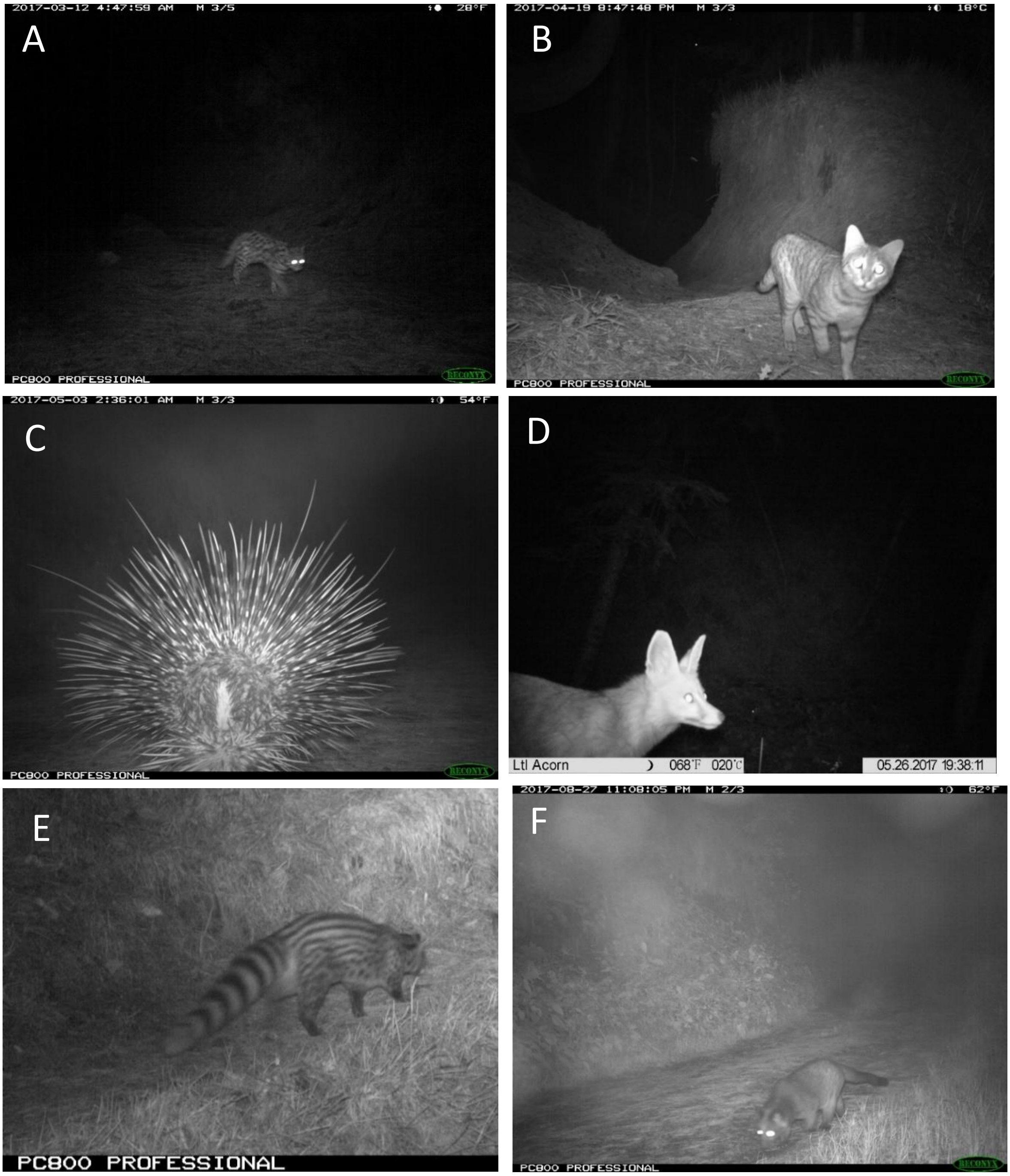

Figure 3.2 Photos of other species recorded during leopard camera-trap survey in Galyat and Murree Forest Division: A) leopard cat; B) domestic cat; C) porcupine; D) red fox; E) Indian palm civet F) Himalaya palm civet.

Low prey detections are also linked to forest exploitation, the growth of human populations, and the intensive use of habitats. Local people depend on forest resources, such as fuel wood, medicinal plants, fodder, mushrooms and other non-timber forest products (Begum et al., 2014). Our camera-trap data allowed us to detect that red foxes followed leopards on the trails, appearing 4-5 minutes after a leopard had been detected and moved on. The foxes are most probably scavenging on leopard kills. The presence of red fox in leopard habitats could be used as an indicator of the presence of leopards. Similarly, the absence of dog detections at Galyat, compared with the high rate of detection of dogs in the Swat and Dir Districts could explain the rareness of leopards in these areas. Shehzad et al., (2014), analysed the diet of leopard in Ayubia National Park and suggested that domestic goats, *Capra hircus,* made up the majority of leopard diets, followed by domestic dogs.

This study shows that the wild prey density and diversity was low in our study area, bringing challenges for the management in the area. The low density, as well as the low diversity of prey species, is a substantial threat for leopard population survival as, ultimately, leopards will engage in more risky sites for predation. The reduced prey levels may increase the likelihood of human and leopard confrontations as well as livestock depredation and could increase the chances of lethal human encounters. We suggest that legal and illegal hunting practices should be examined in the short term to reduce the loss of further diversity, particularly in the Swat and Dir District. Re-introduction and legal protection of wild ungulates into the region could be the long-term solution to reduce losses that, in turn, will help to stabilise local leopard populations. Immediate compensation for livestock owners' losses due to leopards should be urgently actioned to encourage locals to keep domestic livestock until a sound scheme for the re-introduction of prey species is implemented.

Undoubtedly, the development of a conservation programme, for a species depends on the presence and distribution of species in the area. The current knowledge of the distribution and status of leopards

Figure 3.3 Bird species recorded during leopard camera-trap survey in Galyat and Murree Forest Division: A and B) blue-whistling thrush; C) Koklass pheasant; D) Khalij pheasant.

in Pakistan is lacking throughout their ranges. Our study does show, however, where leopards are present, which adds to the current knowledge. Unfortunately, occupancy estimates, which would have added more detailed knowledge, were unable to be robustly calculated but our analysis does provide some guidance for future survey design and analysis.

In conclusion, we suggest that repeating survey intervals at each site for a longer duration may produce more reliable occupancy estimates. We also suggest repeating survey efforts in the same season to obtain estimates that are more precise. In addition, we suggest a comprehensive leopard-monitoring programme to establish baseline information that includes standardised monitoring. We suggest that it is imperative to established one database for leopard monitoring throughout the country. This will also help negate the chances of duplicate and inappropriate resource allocation to research and future monitoring.

An adult male leopard (01) photographed during daytime in our camera-trap survey at Gallies Forest Division Khyber Pakhtunkhwa Pakistan.

4. Assessing subspecies status of leopards (*Panthera pardus*) of northern Pakistan using mitochondrial DNA

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Contribution

1. Conceived and designed the experiment

Muhammad Asad, Adrian Paterson

2. Performed the experiment

Muhammad Asad (Muhammad Waseem and Fakhar-I-Abbas helped with the collection of samples)

3. Analysed the data

Muhammad Asad, Adrian Paterson, Francesco Martoni

4. Contributed to reagents/material/analysis tools

Muhammad Asad, Adrian Paterson, Francesco Martoni

5. Drafted the work

Muhammad Asad

Adrian Paterson and James Ross provide constructive feedback on the draft revisions of the manuscript.

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4.1 Abstract

Despite being classified as critically endangered, little work has been done on leopard protection in Pakistan. Once widely present throughout this region, leopards are now sparsely distributed, and possibly extinct, from much of their previously recorded habitat. While leopards show morphological and genetic variation across their species range worldwide, resulting in the classification of nine different subspecies, the leopard genetic structure across Pakistan is unknown, with previous studies including only very limited sampling. To clarify the genetic status of leopards in Pakistan, we investigated the sequence variation in the subunit 5 of the mitochondrial gene NADH from 43 tissue samples and compared it with 238 sequences available from online databases. Phylogenetic analysis clearly separates the Pakistani leopards from the African and Arabian clades, confirming that leopards from Pakistan are members of the Asian clade. Furthermore, we identified two separate subspecies haplotypes within our dataset: *P. p. fusca* (*n* = 23) and *P. p. saxicolor* (*n* = 12).

4.2 Introduction

Leopards (*Panthera pardus*) are classified as critically endangered in many habitats where they are present (Uphyrkina et al., 2001), including Pakistan (Sheikh and Molur 2004). There are currently nine recognised subspecies of the common leopard (Uphyrkina et al., 2001) and two out of these, African *(P. p. pardus)* and Indian (*P. p. fusca*), are considered “near threatened”, while the rest are classified as “critically endangered”, “endangered” and “near-threatened to endangered” (Stein et al., 2016; Sheikh and Molur 2004).

Globally, leopard subspecies recognition is based on genetic, morphological, and geographical information (Jacobson et al., 2016). However, leopards show high genetic and morphological variation across their range, and in many cases, genetic patterns do not align with the geographical variation recorded for previously defined subspecies (Uphyrkina et al., 2001). Therefore, the study of the genetic structure of leopard populations is considered vital for a better understanding of both subspecies and population subdivision and, consequently, for their conservation (Sugimoto et al., 2013; Khorozyan et al., 2006). Molecular studies have contributed significantly in the field of conservation for many other elusive cat species, such as snow leopards, lions, and tigers (Bhavanishankar et al., 2013; Wei et al., 2008; Dubach et al., 2005). Similarly, in the case of leopards, genetic analyses increasingly provide taxonomic guidance for subspecies identification (Sugimoto et al., 2014; Arif et al., 2011; Uphyrkina et al., 2001).

Conservation and management of leopards are difficult tasks made even harder by a general lack of understanding of the broad geographic ranges and adaptability of this species, factors that make leopards more detectable than their actual numbers would warrant (Jacobson et al., 2016). These detections increase the misconception that leopards are not as severely endangered as they actually are (Jacobson et al., 2016). In Pakistan, while leopards are known to be present, there is no information on their distribution, numbers or subspecies status. Based on the severe rate of decline of leopard populations in Asia, the Pakistani population is likely to be fragmented and with depleted genetic variation (Jacobson et al., 2016; Dutta et al., 2013). Asian leopards have lost around 83–87% of their former range, compared with a 48–67% decline in Africa (Jacobson et al., 2016; Laguardia et al., 2015; Khorozyan et al., 2006; Sheikh and Molur 2004). It is, therefore, essential to identify the subspecies present in Asia to prevent further loss of biodiversity (Arif et al., 2011).

Pakistan provides a major disjunction in the fauna and flora of southern Asia, divided along the western edge of the Indus Basin and the upper Indus valley of Kohistan (Frodin 1984). In the past, this biogeographic disjunction has served as a geographical limit to two of the main standard floras for Southwest and South Asia: Boissier's Flora Orientalis (1867-1888) and Hooker's Flora of British India (1872-1897).

Four leopard subspecies have been suggested as being present in Pakistan: *Panthera pardus fusca, P. p. saxicolor, P. p. sindica,* and *P. p. millardi.* These subspecies were identified based solely on morphological characters, such as unique coat pattern, colouration, fur length, body size and skull size (Roberts et al., 1977; Pocock 1930a and b). However, these characters vary in different environmental conditions and may lead to subspecies misidentification (Uphyrkina et al., 2001). A morphological analysis of Pakistani leopard skulls (Khorozyan et al., 2006) suggested the presence of only two subspecies: *P. p. sindica* from Baluchistan, similar to the subspecies population in southern Iran, and *P. p. millardi* from Kashmir, similar to the population present in India. A recent study based on the global geographic distribution patterns suggested that the subspecies present in the region were *P. p. saxicolor* and *P. p. fusca* (Jacobson et al., 2016). The only genetic sample collected from a Pakistani leopard in the wild was from a single individual in the region of Baluchistan, and identified as the subspecies *P. p. saxicolor* (Uphyrkina et al., 2001). This study aims to better understand the genetic structure of the leopard in Pakistan, identify the subspecies present, and establish baseline information for future monitoring. We present results based on more than 40 leopard samples obtained from Pakistan, and we investigate genetic variation in the mtDNA, targeting the subunit 5 of the NADH gene (NADH-5), following the approaches of previous studies (Anco et al., 2018; Farhadinia et al., 2015; Uphyrkina et al., 2001). The choice of mtDNA was based on the good performances and wide adoption for felids (Havird and Sloan 2016; Barnett et al., 2006; Luo et al., 2004; Jae-Heup et al., 2001). In particular, the choice of NADH-5 gene was based on the good performance of this gene in subspecies delimitation for carnivores, showing a high mutation rate for this group (e.g., Lopez et al., 1997). Furthermore, considering the general use of NADH-5 in leopard studies (Anco et al., 2018; Farhadinia et al., 2015; Uphyrkina et al., 2001), we focused on this gene also for continuity with previous works, by generating sequences that can be added to previous datasets. More specifically, our samples are mostly sourced from the northern Pakistan (Galyat, Murree, and Aazad Kashmir) with a single specimen from Baluchistan. This research will add to the scarce existing scientific knowledge of species assessment in Pakistan, helping prioritize the conservation efforts for leopards, and will consequently contribute to the international works on felids.

4.3 Materials and Methods

4.3.1 *Sample collection*

Lincoln University Animal ethics committee approval AEC2017-02 was obtained prior to the experiment. A total of 49 samples of leopard skin tissue was initially obtained for this study. Most of these samples were from the northern region of Pakistan, where the leopard population is considered stable (Shehzad et al., 2014) (Figure.4.1). Of these, 22 leopards were killed by communities in retaliation for attacks, including seven killed during the period of this study. Two leopards had died of natural causes; the rest of the leopard mortality is unknown. Eighteen samples were provided by the Bioresource Research Centre of Islamabad (Pakistan) and had been used in a previous study (Bebi et al., 2015). Nine tissue samples were provided by Wildlife Department Khyber Pakhtunkhwa and 15 samples from the World Wide Fund for Nature Pakistan (Figure.4.1). The majority of the samples were collected from two different areas: the Azad Jammu Kashmir and the Galyat region. Additionally, two samples were provided by BRC from Baluchistan and Sukkur.

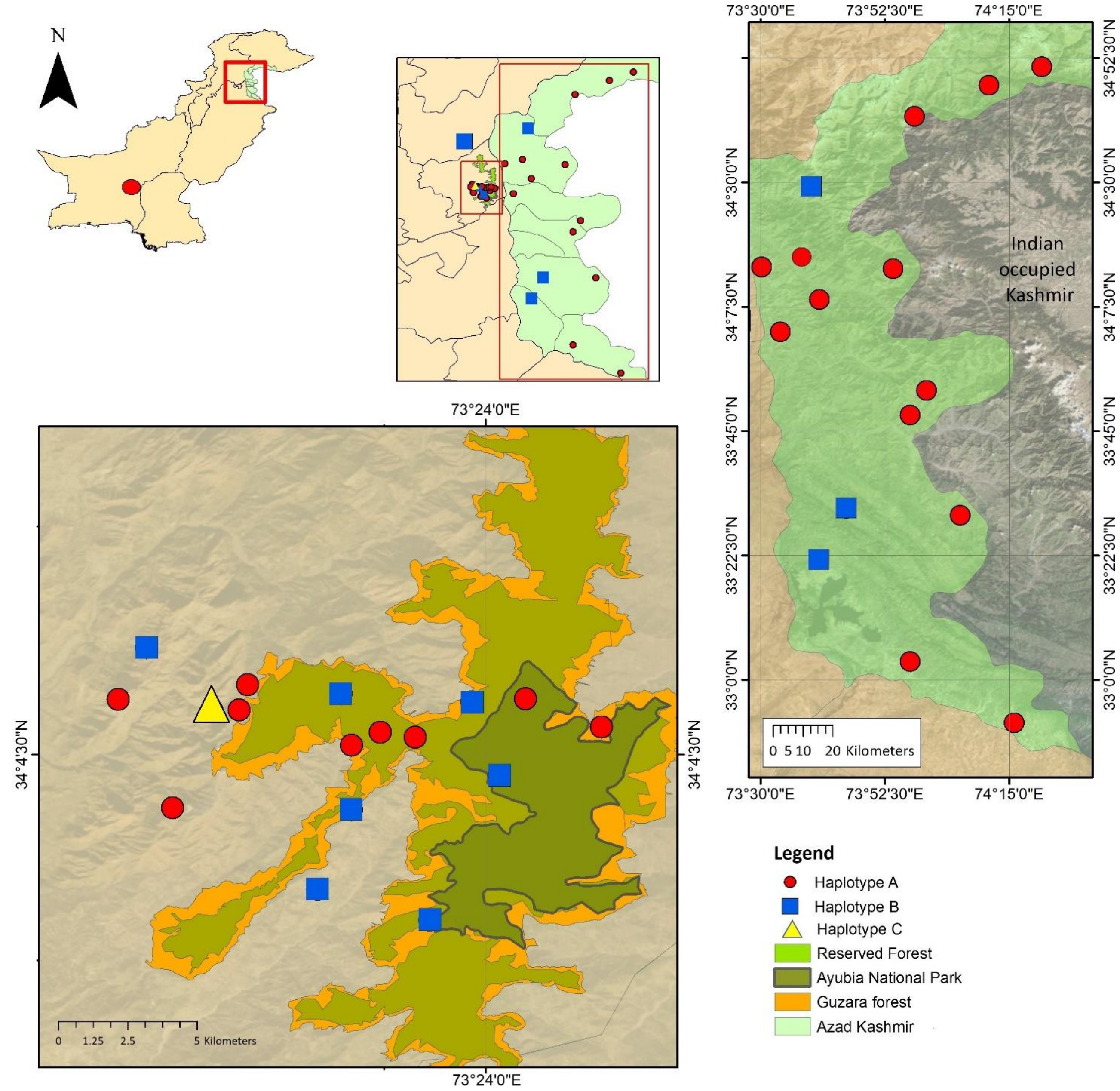

Figure 4.1 Sampling locations of *Panthera pardus* in the northern region of Pakistan (Galyat and Azad Kashmir) for the 35 samples included in the haplotype analysis. Red dots indicate samples belonging to haplotype A; Blue squares are samples from haplotype B, and the single Yellow triangle indicates haplotype C.

4.3.2 *DNA extraction and amplification*

All tissues were preserved in 70% ethanol until DNA extraction and amplification.

In a preliminary analysis conducted at the Bio Resource Research Centre facilities of Islamabad (Pakistan), DNA was extracted from three samples using the QIA amp DNA Mini Kit (QIAGEN) following the manufacturer's instructions while the remaining samples were sent to Macrogen Inc. (Seoul, Korea) for DNA extraction and polymerase chain reaction (PCR) optimisation. Four samples were discarded because of a lack of detailed information on their origin, while two samples were not sequenced due to possible contamination during the transport.

Using the full mitochondrial sequence of leopard available on the National Center for Biotechnology Information (NCBI) database (accession number EF551002.1); two set of primers pairs (F/RL2 and FL2/RL4; Table 4.1) were designed to target two overlapping regions of the subunit 5 of the NADH mitochondrial gene.

Table 4.1 Two set of primers pairs (F/RL2 and FL2/RL4) were designed to target two overlapping regions of the subunit 5 of the NADH mitochondrial gene.

Primer Name	**Direction**	**Sequence (5' to 3')**
F	Forward	GTGCAACTCCAAATAAAAG
RL2	Reverse	TAAACAGTTGGAACAGGTT
FL2	Forward	CGTTACATGATCGATCATAG
RL4	Reverse	TTAGGTTTTCGTGTTGGGT

These corresponded to nucleotide positions 12632-13242 of the mitochondrial DNA of leopards. Primer design, PCR amplification, PCR purifications, and sequencing were outsourced to Macrogen Inc. (Seoul, Korea) and were carried out following the methods of previous studies (Uphyrkina et al., 2001; Farhadinia et al., 2015). Sample preparation and DNA extraction were conducted in a laminar flow hood in an area isolated from other samples to prevent any contamination. Genomic DNA was isolated using InstaGene Matrix (Bio-Rad Laboratories; USA) and MG Tissue SV (Doctor protein inc, Korea). PCR was run for 35 cycles, with 5 min pre-denaturation at 95 °C followed by denaturation at 94 °C for 30 sec, and 30-sec annealing at 50 °C, and by 1 min extension at 72 °C. Products were then checked in 1.5% agarose gel, running for 20 min at 300V, 200A. Purification was carried out with multiscreen filter plate (Millipore Sigma; USA). Each PCR product obtained was sequenced (both forward and reverse) by Macrogen Inc. (Seoul, Korea) using Sanger sequencing technologies.

4.3.3 *Data analysis*

A total of 43 samples produced viable sequences. Sequencing data is available at NCBI GenBank via accession numbers MK425702-MK425744. This data is also available as a Supplemental File. The software MEGA 7 (Kumar et al., 2016) was used to visually inspect the electropherograms, to align the sequences and generate a pairwise distance matrix that was then used to identify the different haplotypes.

In order to compare the haplotypes obtained here with other sequences belonging to the species *Panthera pardus,* all 238 sequences present on the GenBank database (to the month of August 2018) were obtained (Table 4.2) and aligned with those collected in this study together with sequences from snow leopards, tigers and lions as outgroups (Table 4.2). We used the software MEGA X (Kumar et al., 2018) to identify the best model of nucleotide substitution based on the Bayesian Information Criterion (BIC). This reported the Hasegawa-Kishino-Yaano + G model of nucleotide substitution (gamma distribution with five rate categories) as the best model, as previously found elsewhere (Farhadinia et al., 2015). Additionally, the Kimura-2-parameters (K2P) substitution model was also tested here. A maximum likelihood (ML) NADH 5 gene tree was generated using MEGA X, setting the bootstrap to 10000 replicates and using both the substitution models. PopArt (Leigh and Bryant 2015) was used to construct a Median Joining haplotype network (ε = 0) of all the sequences from Asian leopards.

Table 4.2 Leopard sequences obtained from GenBank along with outgroups used in our study.

Accession numbers	Sequences	Gene	Species	Subspecies	Sample Area	Reference
KY292222.1 - KY292277.1	56	NADH5	*Panthera pardus*	Africans	Africa	Anco et al. 2018
JX559073.1 - JX559076.1	4	NADH5	*P. pardus*	Saxicolor	Iran	Farhadinia et al. (Unpublished)
JF720187.1 - JF720319.1	133	NADH5	*P. pardus*	LEO(African)	Africa	Ropiquet et al. 2015
HQ185549.1 - HQ185550.1	2	NADH5	*P. pardus*	orientalis	Caucasus	Rozhnov et al. 2011
HQ185544.1 - HQ185548.1	5	NADH5	*P. pardus*	saxicolor	Caucasus	Rozhnov et al. 2011
EF551002.1	1	Mitochondrion complete	*P. pardus*	Not specified	China	Lei et al. 2011
EF056501.1	1	NADH5	*P. pardus*	Not specified	India	Shouche (Unpublished)
AY035292.1	1	NADH5	*P. pardus*	Melas	Java (Indonesia)	Uphyrkina et al. 2001
AY035280.1 - AY035291.1	12	NADH5	*P. pardus*	Shortridgei	Central Africa (Namibia, Botswana, Kruger & Zimbabwe)	Uphyrkina et al. 2001
AY035279.1	1	NADH5	*P. pardus*	Nimr	South Arabia (wild animal, exact location not specified)	Uphyrkina et al. 2001
AY035277.1 - AY035278.1	2	NADH5	*P. pardus*	Saxicolor	Central Asia (Captive)	Uphyrkina et al. 2001

AY035276.1	1	NADH5	*P. pardus*	Sindica	Central Asia (Baluchistan)	Uphyrkina et al. 2001
AY035270.1 - AY035275.1	6	NADH5	*P. pardus*	Fusca	India	Uphyrkina et al. 2001
AY035267.1 - AY035269.1	3	NADH5	*P. pardus*	Kotiya	Srilanka	Uphyrkina et al. 2001
AY035264.1 - AY035266.1	3	NADH5	*P. pardus*	Delacouri	East Asia (South China)	Uphyrkina et al. 2001
AY035262.1 - AY035263.1	2	NADH5	*P. pardus*	Japonensis	East Asia (North China)	Uphyrkina et al. 2001
AY035260.1 - AY035261.1	2	NADH5	*P. pardus*	Orientalis	Russia	Uphyrkina et al. 2001
KF768352-KF768354	3	NADH5	P. pardus	Saxicolor	Iran	Farhadinia et al. 2015
EF551004.1	1	Mitochondrion complete	*Uncia uncia*	Out Group	China	Wei et al. 2008
EF551003.1	1	Mitochondrion complete	*Panthera tigris*	Out Group	China	Lei et al. 2011
AF385614.1	1	Mitochondrion complete	*P. tigris*	Out Group	Africa	Dubach et al. 2005
HM589215.1	1	Mitochondrion complete	*P. tigris*	Out Group	China	Zhang et al. 2011
AF385613.1	1	Mitochondrion complete	*P. leo*	Out Group	Brookfield zoo (Exact location unknown)	Dubach et al. 2005

4.4 Results

We used 43 NADH 5 sequences for the final stage of the analysis. When these were aligned to the 238 leopard sequences available on GenBank, the ML analysis grouped all Pakistan sequences within the Asian leopards, well separated (with a bootstrap of 85% using a K2P model and 52% using a HKY+G model) from both African and Arabian leopards (Figure.4.2). While the new sequences obtained in this study could be separated from those of African leopards and attributed to the Asian leopards, the ML analysis showed low genetic variation within the two groups, highlighted by very low bootstrap values throughout the tree (Figure.4.2).

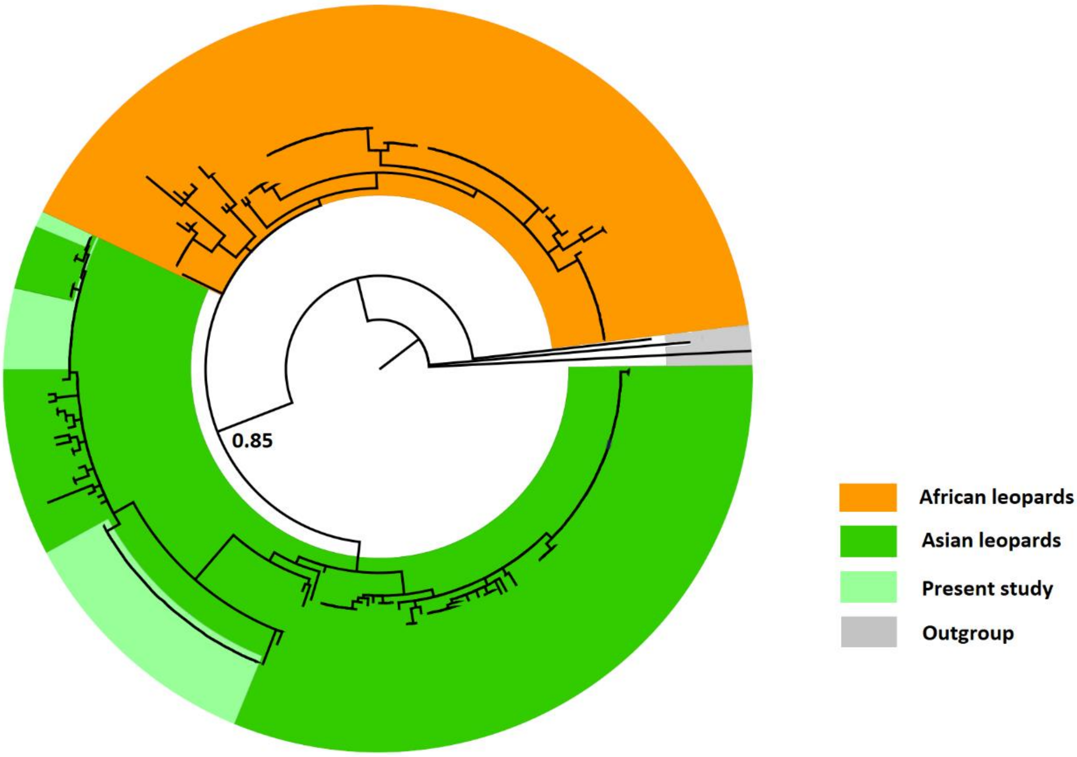

Figure 4.2 ML tree of 273 sequences from across the globe separating Asian leopards from Africans, with a bootstrap of 85%. Green colours represent all Asian samples, while the orange colour represents African leopards.

After confirming that all our sequences belonged to Asian leopards, further analysis aimed to understand the relationships of our samples to the different subspecies of this group. In order to measure genetic variation between our sequences and within the Asian leopards, a haplotype network analysis was performed on a total of 35 sequences (Accession numbers MK425702-MK425736). For this analysis, eight samples were discarded due to shorter length of the sequence. Hence, this analysis included 35 samples from this dataset, all the sequences of Asian leopards available on GenBank, a subset of 13 sequences of African leopards and one sequence from an Arabian leopard (Figure.4.3). The sequences presented here identified three distinct haplotypes (Figure.4.1, Figure.4.3). The first haplotype (A) included 23 samples and was identical to two sequences previously identified as *Panthera pardus fusca* (Accession number: AY035274.1) and *Panthera pardus orientalis* (Accession number: HQ185550.1) (Figure.4.3). The second haplotype (B) included 11 samples with identical sequences to *Panthera pardus saxicolor* (Figure.4.3). The third haplotype (C) was represented by a single Pakistani sample (Figure.4.3) and was not the same as any previously identified haplotype. The haplotype diversity amongst the Pakistani samples was based on three segregating sites with a nucleotide variation ranging between 0.003 and 0.006 (Table 4.3).

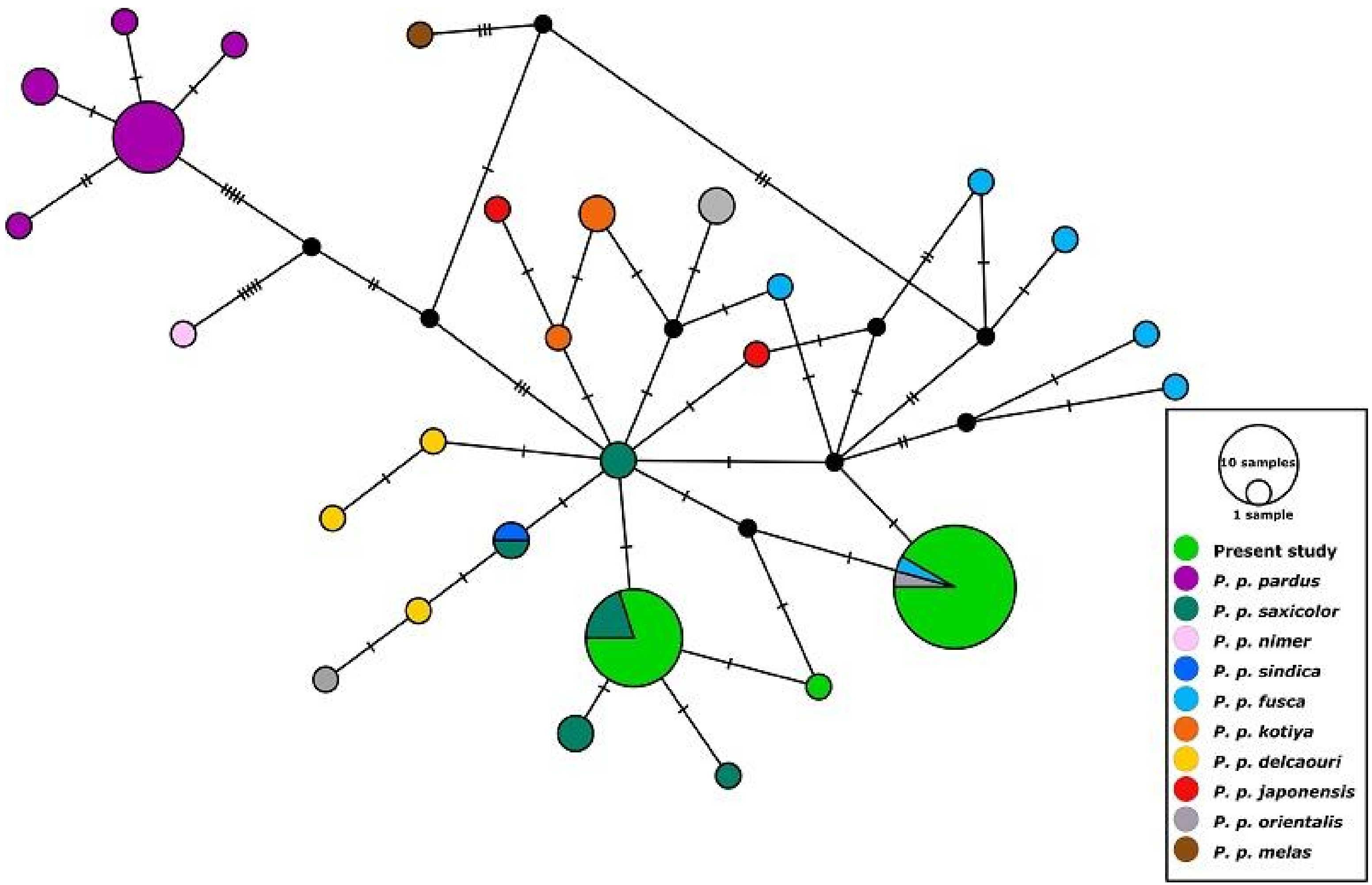

Figure 4.3 Median-joining network including 35 sequences of Pakistani leopards together with all the sequences from Asian leopards, 13 sequences from African leopards and a single sequence from a Persian leopard. Haplotypes are colour-coded according to similarities in sequences to the various leopard subspecies. Size of the circle represents the number of individuals that have similar sequences.

Table 4.3 Genetic variation among leopard mtDNA from different geographical locations. Number of samples and number of haplotypes are reported. Haplotype diversity is calculated based on the number of different nucleotides, while nucleotide diversity was obtained from the pairwise distance matrix. Number of segregating sites are reported specifying the number of substitution and if they were transitions or transversions.

Subspecies	Geographic location	Accession Number	No of samples	Haplotypes	Haplotype diversity (nt)	Nucleotide diversity	Segregating sites	Transitions	Transversions	Substitutions
This study	Pakistan	MK425702-MK425744	43	3	1-3	0.003-0.006	3	3	0	3
P. p. sindica	Pakistan	AY035276.1	1	1	NA	NA	NA	NA	NA	NA
P. p. saxicolor	Iran	JX559073.1 - JX559076.1 HQ185544.1 - HQ185548.1 AY035277.1 - AY035278.1 KF768352-KF768354	14	4	1-3	0.002-0.004	6	6	0	6
P. p. fusca	India	AY035270.1 - AY035275.1	6	6	2/3-6/9	0.003-0.01	15	15	0	15
P. p. kotiya	Srilanka	AY035267.1 - AY035269.1	3	2	1	0.002	1	1	0	1
P. p. orientalis	Russia	HQ185549.1 - HQ185550.1 AY035260.1 - AY035261.1	4	3	3-6	0.01	7	7	0	7
P. p. nimer	Arabia	AY035279.1	1	1	NA	NA	NA	NA	NA	NA
P. p. leo ***P. p. shortridgei***	Africa	KY292222.1 - KY292277.1 JF720187.1 - JF720319.1 AY035280.1 - AY035291.1	201	30	1-20/23	0.002-0.038	77	75	2	77
P. p. melas	Indonesia	AY035292.1	1	1	NA	NA	NA	NA	NA	NA
P. p. delacouri	South Chinese	AY035264.1 - AY035266.1	3	3	2-4	0.003-0.01	6	6	0	6
P. p. japonensis	North Chinese	AY035262.1 - AY035263.1	2	2	3/5	0.005	3-5	3-5	0	3-5

4.5 Discussion

Despite the limitations of our sample size, this study generated the first genetic datasets for leopard populations in Pakistan. The genetic diversity of leopards from the northern region of Pakistan was determined here based on 43 specimens. As expected, all the samples included in this study clustered within the Asian leopard clade. However, the haplotype network analysis highlighted the presence of three different haplotypes. We recorded a similar nucleotide diversity, ranging between 0.003 and 0.006, to that reported for different leopard subspecies in other studies (Farhadinia et al., 2015; Uphyrkina et al., 2001).

The genetic variation of the Pakistan leopard samples was not consistent with there being a single subspecies in Pakistan, whether existing or novel. Eleven samples (Haplotype B) grouped with sequences of *Panthera pardus saxicolor* and are here attributed to this subspecies. Similarly, the haplotype represented by a single specimen (Haplotype C) grouped close to the other *P. p. saxicolor* haplotypes and is here attributed to this subspecies, as well. The main Pakistan haplotype (Haplotype A), including 23 samples, grouped with two separate sequences previously recorded from two studies in Caucasus (Rozhnov et al., 2011; Uphyrkina et al., 2001). These sequences (HQ185550.1 and AY035274.1) are identical to each other although they were named differently: respectively *P. p. orientalis, and P. p. fusca.* Based on the similarities between our sequences and the other haplotypes of *P. p. fusca*, however, we are inclined to consider them as belonging to this subspecies. Similarly, considering the genetic distance between this single sequence of *P.p. orientalis* obtained from GenBank and the other sequences of the same subspecies, we consider its attribution to *orientalis* an incorrect identification. However, it is possible that the subspecies *P. p. fusca* has a large amount of genetic variation, to the point that it could subsume some of the other subspecies. In order to confirm this hypothesis, additional samples from this subspecies are required, together with additional samples of *P. p. orientalis,* together the analysis of additional gene regions.

This analysis is consistent with Uphyrkina and colleagues who grouped the subspecies *P. pardus sindica* and *P. pardus millardi* with the Persian and Indian leopards (Uphyrkina et al., 2001). These two subspecies, first described by Pocock (1930) based on morphometric characters only, were retained by Khorozyan and colleagues (2006). On the other hand, all the samples collected for this study likely belong to two subspecies of *Panthera pardus: P. p. fusca* (Indian) and *P. p. saxicolor* (Persian), highlighting a higher than expected subspecies diversity for the area that we examined. In fact, our findings are not consistent with the assumption that the river Indus separates the *saxicolor* and *fusca* subspecies, as was hypothesised in previous studies (Jacobson et al., 2016; Khorozyan et al., 2006). The vast majority of our samples (except those from Baluchistan, Nizampur, and Sakher) belonged to the northern region of the Indus, reporting the presence of both *P. p. saxicolor* and *P. p. fusca* in this region, where only latter was hypothesised to live.

The limited number of samples used for this study (especially from the regions of Baluchistan, and Nizampur) most certainly does not fully reflect the subpopulation of these regions. Therefore, further studies are likely to discover an even higher diversity in subspecies than reported here, as already confirmed from Baluchistan, where subpopulation closely related to the Persian leopard are known to be present (Jacobson et al., 2016).

Consequently, our study suggests that the leopards in Pakistan do not appear to have an endemic haplotype or subspecies (Figure.4.1). Instead, the Pakistani landscape plays a key role in the ecological overlap of the two leopard subspecies recorded here and it provides suitable conditions for a high level of gene flow between them. Random mating in the expanded region may support the hypothesis that overall, the Asian population is panmictic and it presents a moderate level of genetic variation (Figure. 4.2). Panmixis assumes that there are no mating restrictions and, for this to happen, gene flow between leopard sub-populations and sub-species has to happen across all their habitat in Asia. Leopards are known to travel long distances, e.g. 194 km (Fattebert et al., 2013), and have the largest home ranges recorded of large cats, e.g. 670 km^2 (Hunter, 2011) in central Iran.

Pakistan assumes a role in leopard conservation by providing a contact zone for the subspecies *P. p. fusca* and *P. p. saxicolor*, subspecies considered endangered (*P. p. saxicolor*) or near threatened (*P. p. fusca*). The overlapping distribution of these two subspecies provides an impetus to extend full protection to leopards beyond the limits of regional parks and reserves, overall the Pakistani territory.

The primary constraint in the conservation of leopards in Pakistan is a lack of detailed information, such as presence-absence of each subspecies within the highly fragmented habitat. While the information obtained in this study enables a better understanding of leopard genetic diversity in the areas monitored, further work is required for the more remote regions. Unfortunately, the political instability of some of these regions, together with a widespread lack of financial resources remain a key challenge for sustainable management and conservation of leopards. In addition, illegal hunting of leopards and limited management capacity of local wildlife department in many areas have created an ongoing stressful situation for leopards. This results in leopards moving away from many habitats, such as the Kirthar National Park where they have been reported as a locally extinct (Khan et al., 2013).

4.6 Conclusions

With the present work, we have highlighted the co-existence of multiple subspecies in the same area in the north of Pakistan. In order to compare these results with other areas of the country, we suggest that further studies focusing on the presence-absence of leopards in Baluchistan, Sindh and in the areas of Punjab and Khyber Pakhtunkhwa are urgently required. In fact, obtaining genetic information from these areas will complement the knowledge now available and enable a better understanding of distribution and ecology of leopards, not only in Pakistan but also worldwide.

4.7 Leopard subspecies and what they mean

Carnivora, particularly the Felidae family, contains some of the most threatened taxa within the mammals. Most endangered species are endangered by anthropogenic activity, such as human population, expansion, loss of habitat, illegal trade, retaliation, and populations are likely to be fragmented and with depleted genetic variation (Jacobson et al., 2016, Dutta et al., 2013, Uphyrkina et al. 2001).

The concept of subspecies has a long history in taxonomy. It was called “variety” in the Linnaean period for ordinary species, where no distinction was made between individual and geographical varieties. The term subspecies was first used by Esper to designate geographical varieties and this has continued to be the meaning of subspecies (Mayer 1942). By the end of the 19th century, the majority of authors defined subspecies as any distinct natural population that was not considered sufficiently different to be called a separate species and that became a standard for later scholars (Wilson and Brown 1953, Mayer 1942).

Defining a subspecies is a difficult task when it comes to the biological, morphological and phylogenetic species concepts, where each species definition leads to a different subspecies definition. These species concept does not have specific levels of separation that mark species and subspecies and therefore give rise to different classification. All those concepts have wide acceptance and critiques (Mishler and Brandon 1987, Thomoson 1969, Amadon 1949, Mayer 1942). The most widely acceptable definition of subspecies is "*an aggregate of phenotypically similar population of a species inhibiting a geographic subdivision of the range of that species*" (Mayer and Ashlock 1991).

The morphology of the leopard *Panthera pardus* ssp. has been studied extensively for taxonomic clarification. In particular, most studies have focused on measuring cranial variables of head shape to designate subspecies. These morphological characters are often confounded by sexual dimorphism, diet and geographic location. The designation of subspecies using morphological characters has always been debated (Farhadinia et al., 2015; Khorozyan, 2014; Stein and Hayssen, 2013; Yamaguchi et al., 2004; Meijaard, 2004; Pocock, 1930).

These different definitions lead to many problems in recognition of different subspecies depending on which concept is adopted. How much interbreeding is enough to consider two groups part of the same subspecies? Which morphological characteristics are included in the diagnostic suite, and how different do they have to be? How much genetic divergence is enough to warrant subspecies designation? These are the questions that need to be settled when designating and naming subspecies.

In the study of leopard populations, the term subspecies, *Felis fusca,* was first used by Friedrich Albrecht Anton Meyer in 1794. Leopards have been classified into 27 subspecies, between 1794 and 1956, based on coat thickness, colouration, body size, eye colour, skull size, and geographic distribution (Pocock, 1930). With the emergence of molecular technology, biologists incorporated genetic differentiation in their definition of subspecies (O'Brien and Mayr 1991, Avise and Ball 1990). Most of those scientific names for subspecies have little scientific evidence and are based on one or a few specimens with no comparison to others and, therefore, are invalid (Kitchener et al., 2017).

Genetic structure of leopard populations is considered vital for a better understanding of inbreeding behaviour, hybridization, genetic and morphological variation of both subspecies and population subdivision and, consequently, for their conservation (Sugimoto et al., 2013, Khorozyan et al., 2006). The leopard subspecies is defined in the revised taxonomy of Felidae "A *group of individuals within a species that most share morphological and molecular characters that distinguish them from most other individuals within a species and that occupy a distinct part of the geographical range of the species. These distinguish characters are not expected to be 100% diagnostic and gene flow is also expected between subspecies where ranges are contiguous"* (Kitchener et al., 2017).

Molecular studies have contributed significantly in the field of conservation for many elusive cat species, such as snow leopards, lions, and tigers (Bhavanishankar et al., 2013; Wei et al., 2008; Dubach et al., 2005). Similarly, in the case of leopards, genetic analyses increasingly provide taxonomic guidance for subspecies identification (Sugimoto et al., 2014; Arif et al., 2011; Uphyrkina et al., 2001). The identification of subspecies also helps policy makers and the biologist put in place measures that prevent further decline in the wild by highlighting areas where urgent research is needed. Subspecies can be seen as a marker in the progress of ongoing speciation. In the case of geographic distribution, where the boundaries are less prominent, subspecies can offer information about colonisation, gene flow and particular phenotypes. Protecting leopard subspecies is important in the field of conservation, as it ensures the preservation of a wide range of genetic and morphological variation within the species.

Recently, the leopard taxonomic system Cat Classification Task Force CCTF was developed to indicate certainty of each taxon based on at least three of the correlated evidences of the following 1- Morphological, 2- Genetic, 3- Biogeographical, 4- Behavioural, 5- Ecological and 6- Reproductive (Kitchener et al., 2017).

In this study, we examined the genetic structure and morphometric geometric to analyze leopard skull in northern Pakistan. It was assumed that the river Indus separates the *saxicolor* and *fusca* subspecies, as was hypothesised in previous studies (Jacobson et al., 2016; Khorozyan et al., 2006).

We followed the methods of previous studies (Farhadinia et al., 2015, Uphyrkina et al., 2001) using mtDNA to investigate the genetic diversity in this area. Our findings were not consistent with the assumption that the river Indus separates the *saxicolor* and *fusca* subspecies. Both subspecies overlap in their distributions, and leopards in this hybrid zone have genes from one or other of the parent populations. This analysis, therefore raises a question about the distinctiveness of these species as valid subspecies. Although mtDNA markers are useful and allowed the confirmation of the presence of both previously defined subspecies. However, nuclear genes might be useful in determining the distinctiveness of these populations. If the nDNA also indicated two distinct subspecies then this would support the idea of the two subspecies being able to share the same area. If the nDNA is indistinguishable for these individuals then it would support a hybrid scenario, where high levels of gene flow and moderate level of genetic variation it is not warranted to classify these populations as distinctive subspecies.

The detailed geometric information also does not distinguish putative subspecies indicates that the subspecies hypothesis is dubious; low genetic variation, high genetic mixing, lack of morphological variation consistent with neutral genetic markers all suggest normal population variation and may reflects genetic drift at a neutral locus resulting in a (partial) pattern of spatial structure.

Subspecies are complex and often difficult to define and delimit only using mtDNA segments. In our study the populations are not conclusively distinct from other Asian subspecies of leopards. We suggest that future leopard subspecies re-assessment should be performed with nuclear markers and genome-wide assessment of SNPs (single nucleotide polymorphisms), or even complete genomes, with increased geographic sampling to more conclusively assess population distinctiveness and gene flow among areas to confirm and delimit subspecies.

Sub adult female leopard (07) photographed during our camera-trap survey at Gallies Forest Division Khyber Pakhtunkhwa Pakistan.

5. Craniomandibular variation of sympatric leopard (*Panthera pardus)* subspecies from Pakistan

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Contribution

6. Conceived and designed the experiment

Muhammad Asad, Adrian Paterson

7. Performed the experiment

Muhammad Asad (Muhammad Asif provided samples)

8. Analysed the data

Muhammad Asad, Adrian Paterson, Godsoe, William

9. Contributed to reagents/material/analysis tools

Muhammad Asad, Adrian Paterson, James Ross

10. Drafted the work

Muhammad Asad
Adrian Paterson and James Ross provide constructive feedback on the draft revisions of the manuscript.

This chapter has been submitted to the Mammalian Biology Journal and is currently in the review process.

5.1 Abstract

The morphology of the leopard *Panthera pardus* ssp. has been studied extensively for taxonomic clarification. In particular, most studies have focused on measuring cranial variables of head shape to designate subspecies. These morphological characters are often confounded by sexual dimorphism, diet and geographic location. Here we identify whether morphological variation in skull shape matches leopard subspecies (*P. p. fusca* and *P. p. saxicolor*), found in sympatry in Pakistan. We used 3D geometric morphometrics and linear measurements to characterise variation in the crania and mandibles. Procrustes shape analysis indicated that variation among an individuals' overall skull, cranium (86%). and mandible (82%) is not determined by gender, diet or location for both datasets. Multivariate analysis (MANOVA) confirmed that gender (P = 0.6) and location (P = 0.6) did not account for any morphological differences. We did observe slight variations at the tip of the coronoid process of the mandibles, after 3D morphing and the species average was computed by combining the two surfaces using IDAV landmark, although these differences did not match with the nominal subspecies identification that the samples came from. Additional studies on leopard morphometrics, in combination with genetic data, are needed to better understand these morphological traits.

5.2 Introduction

Leopards are one of the most widespread territorial species on earth (O'Brien and Johnson, 2005) Their solitary and opportunistic nature make them highly adaptable to different environmental conditions and their ranges extend from Africa, through the Middle East, to South east Asia and north into Russia (Nowell and Jackson, 1996). Leopards are found in a wide range of habitats from tropical and humid forests, to savannah, scrub and deserts (Athreya, et al., 2016; Shehzad et al., 2014; Nowell and Jackson, 1996). Leopards can also co-exist with humans in modified habitats (Athreya et al., 2016). Leopards feed on a broad range of prey, such as ungulates, birds, rodents and livestock (Henschel et al., 2011; Hayward et al., 2006).

Leopard taxonomy, historically based on morphology, has been updated by recent molecular studies (Anco et al., 2018; Farhadinia et al., 2015; Sugimoto et al., 2014; Uphyrkina et al., 2001). The designation of subspecies using morphological characters has always been debated (Farhadinia et al., 2015; Khorozyan, 2014; Stein and Hayssen, 2013; Yamaguchi et al., 2004; Meijaard, 2004; Pocock, 1930). Female skull size is typically used to designate subspecies because of their small home range compared to males. Female leopards are considered less mobile and habitat fragmentation may likely force females to retain any variation in the local population (Khorozyan, 2014).

The morphological features of leopards vary throughout their geographic range. Leopards have been differentiated into 27 subspecies, based on morphology (Miththapala et al., 1996) and nine subspecies, based on genes (Uphyrkina et al., 2001). Typically, leopards have been classified based on coat thickness, colouration, body size, eye colour, skull size, and geography across their geographic distribution (Jacobson et al., 2016, Khorozyan, 2014, Brodie, 2009; Pocock, 1930). For example, in the Middle East *P.p saxicolor* (Persian leopard) is described as having a large body, pale, long-haired, yellowish fur, medium-sized rosettes and a short tail, *P.p nimr h*as a smaller body size, pale colour, short coat, long tail, and small thin rosettes; *P. p. sindica* is described as possessing large widely spaced rosettes of a darker greyish-yellow; while *P.p millardi* possesses a furry coat with more closed-up rosettes. These traits often vary with the external environment (Christiansen and Harris, 2012). For example, in colder climates leopards typically have paler and longer fur than in warmer climates (Stein and Hayssen, 2013) although this relationship has never explicitly been tested.

With the broad geographic distribution and the breadth of diet of the leopard, skull morphology may vary across their range and even within the same area. The mass of leopards in most of Africa is smaller than those of Iran and India (Stein and Hayssen, 2013). The body mass of an African leopard is 20 - 45 kg while those in the Middle East and Asia range from 30 - 70 kg, and up to 90 kg in the Middle East (Stein and Hayssen, 2013). Leopard body mass is also correlated with prey body mass (Hayward et al., 2006). For example, leopard predation on large prey in northern Iran appears to be linked to the larger leopard body size (Farhadinia et al., 2015). A difference in diet and habitat use has also impacted the shape of other species, such as lions and tigers (Fabre et al., 2016; Hartstone et al., 2014). Khorozyan (2014) suggests that the skull size of the Persian leopard increases in size with latitude. Size differences between males and females are common in most carnivores (Christiansen and Harris, 2012; Yamaguchi et al., 2004). Morphology is often confounded by sexual dimorphism, as the male leopard body mass and skull size are comparatively larger than in females (Farhadinia et al., 2014). Meijaard (2004) established a link between phenotype and geography by analysing 121 skulls from Malaysia, Indochina, Java, Sri Lanka, India, Nepal, Kashmir and Africa.

Roberts (1977) recognised four subspecies from Pakistan based on morphology (e.g., unique coat pattern, colouration, fur length, and body size); however, Khorozyan et al. (2006) and Pocock (1930a and b) suggested only two subspecies based on skull size. Recently, Jacobson et al. (2016) used historic and current leopard ranges, based on geographical features and habitat distribution to suggest two subspecies in Pakistan. More recently, Asad et al. (2019) used genetic variation to identify *Panthera pardus fusca* and *Panthera pardus saxicolor* as the two likely subspecies in Pakistan (see Chapter 4).

Molecular studies (Anco et al., 2018; Farhadinia et al., 2015; Sugimoto et al., 2014; Uphyrkina et al., 2001) have addressed wider leopard taxonomy. Their findings allow us to ask whether morphological variation covaries with genetic variation. Landmark-based geometric morphometrics is used to define shape based on landmark points on the surface (Wiley et al., 2005). Landmark-based geometric morphometrics have been used in quantifying morphological variation for many species in recent years. For example, phenotypic disparity and morphological evolution in carnivores (Michaud et al., 2018; Hartstone- Rose et al., 2014).

The main purpose of this study is to see if there is significant variation across leopard skulls from northern Pakistan and whether this variation might be useful for identifying sex and subspecies using morphology. We test the geometric morphometrics of head shape, especially the cranium and mandibular of leopards as a marker for leopard subspecies *P. p fusca* and *P. p saxicolor*. We use a three-dimensional geometric morphometric approach to determine the characters, which can be effective for species differentiation within the population. Concurrently, we also examined other morphological data: weight, length, and height, from leopards killed in Pakistan between 2008 - 2018. In this study, our samples are mostly sourced from Galyat and Azad Kashmir, where the two-population subspecies overlap (Asad et al., 2019).

5.3 Materials and Methods

5.3.1 Sampling area

Our sampling area included the Galyat and Kashmir regions of Pakistan, in the Western Himalayas. The two-leopard subspecies found in Pakistan, *P.p.saxicolor and P.p fusca,* are found in sympatry in this area. The area includes Ayubia National Park, Machiara National Park and the surrounding Reserved and Protected Forests. The forest-type encompasses temperate, tropical and subtropical pine and subalpine meadows. Ashraf et al. (2014) characterised the main vegetation as including blue pine (*Pinus wallichiana)*, Chir pine (*Pinus roxburghii)*, fir (*Abies pindrow)*, deodar (*Cedrus deodara),* Himalayan yew or barmi (*Taxus wallichiana)*, morinda spruce (*Picea smithiana)*, horse chestnut or banker (*Aesculus indica)*, oaks (*Quercus incana* and *Quercus dilatata)*, and *Rhododendron arboreum*. Shehzad et al (2014) listed the main mammals of this region, including leopard cat (*Prionailurus bengalensis),* golden jackal (*Canis aureus indicus),* Indian-crested porcupine (*Hystrix indica),* rhesus monkey (*Macaca mulatta),* wild boar (*Sus scrofa),* yellow-throated marten (*Martes flavigula),* Indian palm civet (*Paradoxurus hermaphroditus),* Himalaya-palm civet (*Paguma larvata),* flying squirrel, and red fox (*Vulpes vulpes)*. A few wild ungulate and carnivore species are located only in the Kashmir region only, i.e., Himalayan ibex (*Capra ibex)*, grey goral (*Naemorhedus goral*), musk deer (*Moschus chrysogaster)*, snow leopard (*Uncia uncia)*, grey langur (*Semnopithecus entellus),* Himalayan black bear (*Ursus thibetanus),* Himalayan griffon vulture (*Gyps himalayensis)* and galliform bird species.

5.3.2 Data acquisition

We quantified 14 crania and 17 mandibles from dead leopards from throughout the sample area. The Wildlife Department Khyber Pakhtunkhwa provided ten samples, the Pakistan Museum of Natural History (PMNH) provided five samples and two samples were provided by the World Wide Fund for Nature Pakistan (WWF-P). The majority of those leopards were killed in retaliation for attacks on local people and their livestock, and were from throughout the Galyat and Kashmir regions. Some of the leopard mortality was unknown due to the lack of detailed information of their exact geographic origin within the region. Both regions have similar habitats and environments. There was gender information and the exact provenance for eight samples. The age of each animal was determined from skull characteristics, e.g. eruption of basilar suture fusing (Meijaard, 2004) and dental attributes (Stander, 1997). All our specimens were adults aged between seven and 10 years.

Each specimen was scanned with a Toshiba 16-Slice CT tomographic scanner at the Islamabad Diagnostic Centre. DICOM images were obtained for further analysis. The DICOM images were processed using the open-source software OsiriX lite 64-bit (www.osirix-viewer.com), Dicom Scanner (http://ngavrilov.ru/invols/) and 3D Slicer (http:// www.slicer.org) image manipulation software for volume rendering, cleaning and surface rendering (Rosset et al., 2004). The images were processed and saved in ply file format for further processing in IDAV landmark (Wiley et al., 2005). A total of 83 anatomical landmarks (Tables 5.1 and 5.2) were recorded from the cranium (55) and mandible (28) (Figures 5.1 and 5.2) using "IDAV landmark" software (Wiley et al., 2005). An atlas surface was created and the landmarks were transferred to the atlas. Each point was adjusted manually to fix any error created, by any floating points.

Table 5.1 Description of the anatomical landmarks on the cranium used in the analysis.

Landmark	Description
1	Most anterior point in the premaxilla above the middle of the incisor
2	Cranio-dorsal point of the premaxilla
3	Most anterior (dorsal) part of the nasal aperture
4	Naso-frontal suture
5	Anterior point of the frontal bone
6	Dorsal point of the cranial vault
7	Tip of the occipital crest
8	Most lateral point of the incisors row end
9	Most lateral contact point of the canine and the maxillary
10	Most caudal contact point of the canine and the maxillary
11	Most caudo-lateral contact point of the last premolar and the maxillary
12	Most caudo-lateral contact point of the last molar and the maxillary
13	Most caudal point between the maxillary and the palatine
14	Most cranio-lateral point of the infraorbital foramen
15	Most cranio-medial point of the infraorbital foramen
16	Most cranio-dorsal point of the infraorbital foramen
17	Dorsal tip of the lachrymal foramen
18	Point of the maximum of concavity between the zygomatic process and the jugal
19	Tip of the postorbital processes
20	Point of maximum of curvature between the post-orbital process and the lachrymal foramen
21	Most dorsal contact point between the jugal and the squamosal
22	Lateral point of the frontal bone
23	Lateral point of the parietal bone
24	Most dorsal point of the maximum of convexity of squamosal suture
25	Point at the maximum of convexity at the dorsal part of the squamosal
26	Cranio-lateral tip of the glenoid cavity
27	Most caudal point of insertion of the zygomatic process on the braincase
28	Most dorso-lateral point of the mastoid process
29	Ventral point of the middle of mastoid process
30	Ventral tip of the mastoid process

31	Most mediodorsal point of the paracondylar process
32	Most ventral point of the occipital protuberance
33	Dorso-lateral point of the maximum of convexity of the nuchal crest
34	Most dorsal point on the foramen magnum,
35	Point of maximum of curvature between the most dorsal point of the foramen magnum and the occipital condyle
36	Point of maximum of curvature between the most dorsal point of the foramen magnum and the occipital condyle
37	Most ventral point of the junction of the occipital condyle and the foramen magnum
38	Most ventral point of the foramen magnum
39	Most caudal point in the middle of the incisive row
40	Most caudal point of the incisive foramen
41	Most ventral point of the incisive foramen toward canine
42	Most caudal point between the maxillary and the palatine
43	Point of maximum of curvature of the palatine
44	Point at the palatine-pterygoid suture
45	Most posterior point in the middle of the palatine, middle
46	Insertion point of the pterygoid process in the basisphenoid
47	Tip of the pterygoid process
48	Contact point between the pterygoid and the oval foramen
49	Cranio-medial point of the glenoid cavity
50	Cranio-lateral tip of the glenoid cavity
51	Most cranial point of the tympanic bulla on the basisphenoid
52	Point of contact between the basioccipital and the basisphenoid,
53	Caudal tip of the tympanic bulla
54	Most cranial point of the paracondylar process
55	Most lateral point of maximal curvature of the occipital condyle

Table 5.2 Description of the anatomical landmarks on the mandibles used in the analysis.

Landmarks	Description
1	Point of medial condyle
2	Tip of condyle toward midsagittal plan
3	Point of lateral condyle
4	Most posterior point of angular process
5	Point of mandibular notch
6	Caudal posterior tip of the coronoid process
7	Uppermost posterior tip of the coronoid process
8	Dorsal apex of coronoid process
9	Anterior lateral point of coronoid process
10	Anterior upper extent of masseteric fossa
11	Anterior lower extent of masseteric fossa
12	Most caudal point of angular process
13	Anterior edge of the masseteric ridge
14	Most prominent point on middle protrusion of mental process
15	Posterior edge of M1
16	Anterior edge of M1
17	Ventral edge of mandibular symphysis
18	Anterior edge of P1
19	Point at middle mental foramen
20	Point at caudal mental foramen
21	Most posterior contact point of C1 and alveolar process
22	Most lateral contact point of C1 and alveolar process
23	Most Anterior contact mandibular symphysis
24	Most Anterior contact point of C1 and alveolar process (toward incisors)
25	Anterior middle contact point at the base of Incisors
26	Most prominent point on inferior margin of angular
27	Most caudal contact point of the mandibular foramen
28	Antero-inferior point on projection of pre-maxilla between central incisors

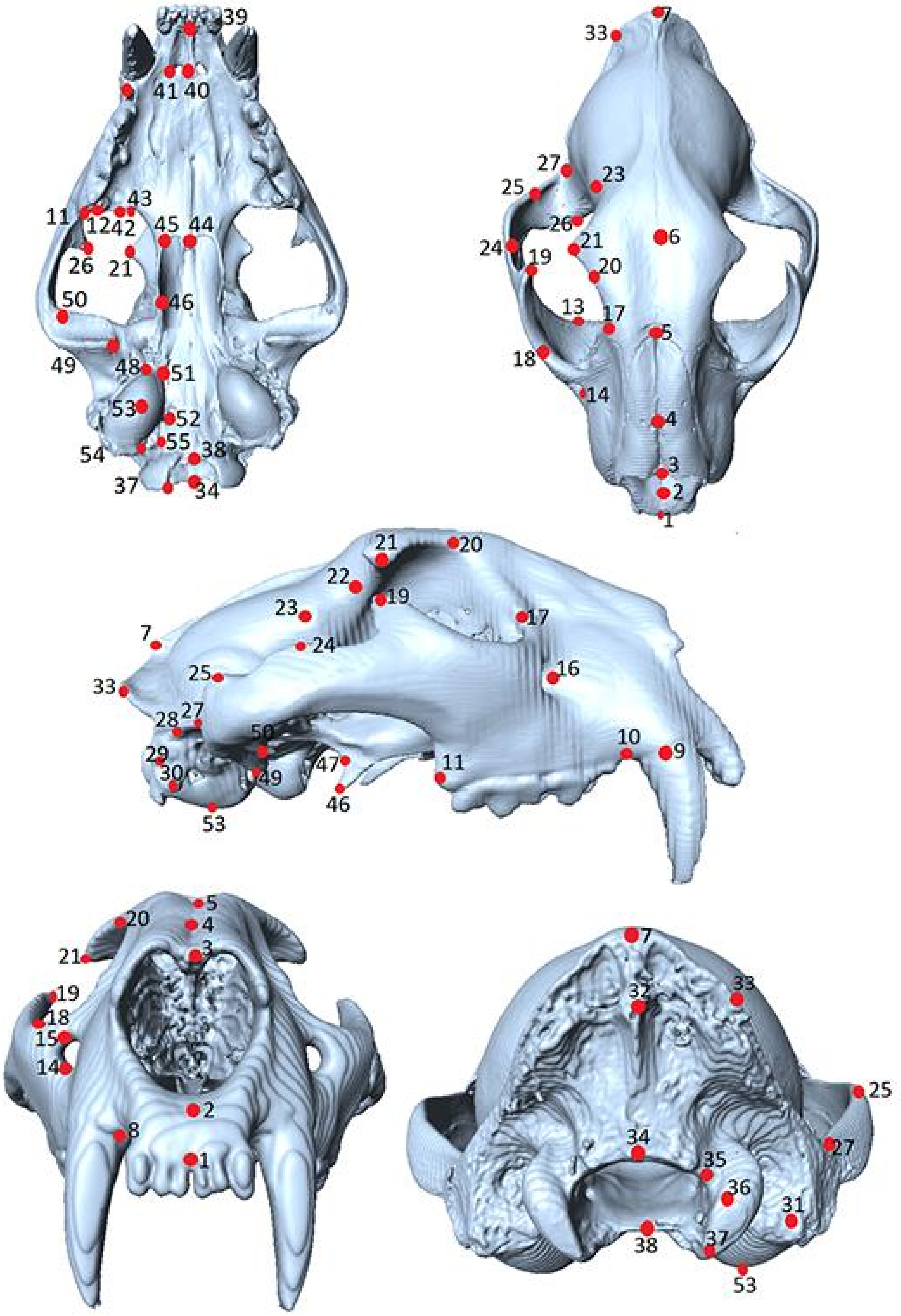

Figure 5.1 Position of the landmarks taken to quantify shape variation on the crania (55 landmarks represented by red dots on the dorsal view, ventral view, lateral view, anterior view, and posterior view).

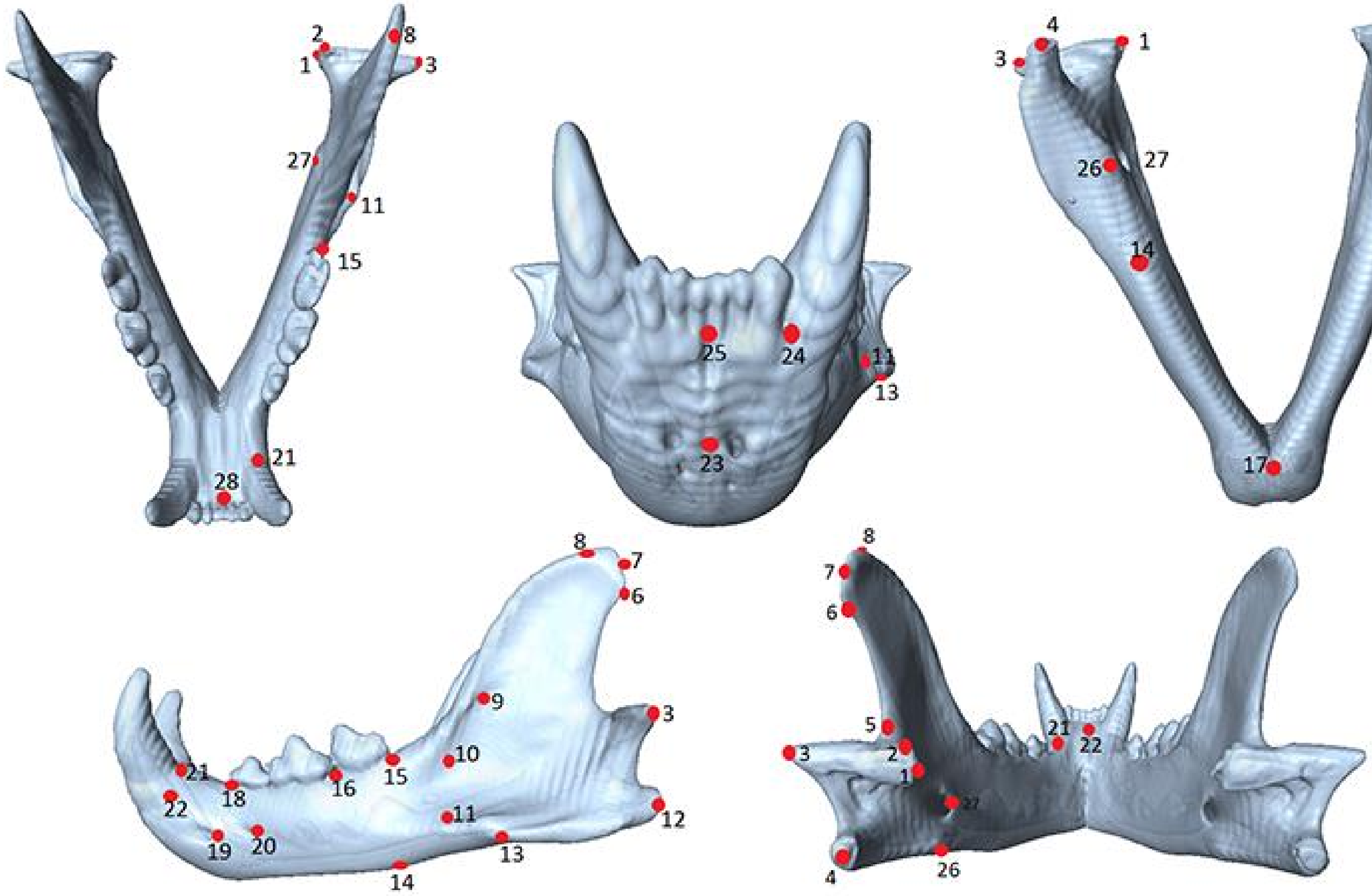

Figure 5.2 Position of the landmarks taken to quantify shape variation on the mandibles (28 landmarks represented by red dots on dorsal view, ventral view, lateral view, anterior view, and posterior view).

The 3D surface scan data were processed in a 3D slicer to obtain the measurement of (a) cranium: (1) greatest length, (2) width of cranium of the skull, (3) zygomatic breadth, (4) frontal breadth, (5) breadth between infra-orbital foramina, (6) rostrum breadth, alveolo included, (7) length of upper tooth row, (8) greatest palatal breadth posterior to P4, alveoli included, (9) condylobasal length, (10) greatest breadth of occipital condyles, (11) basal length of the cranium, (12) skull height at the supraorbital process; and (b) mandibles: (1) length of the mandible, (2) width of the mandibles, (3) coronoid height from the angular process of the mandible, (4) length between lower canines, alveoli included, (5) length of lower tooth row C-M1, (6) breadth between coronoid process (Figure 5.3). In addition, we obtained other morphometric data for whole animal length, height and weight from 24 different leopards that died in retaliation killings during 2008 - 2018 and from a radio-tagged animal 2013 from WWF-Pakistan. We removed sub adults (*n* = 5) from the dataset.

5.3.3 Statistical analysis

The data were subjected to multivariate analysis (MANOVA) and generalised Procrustes analysis (GPA) (Rohlf and Slice, 1990; Gower, 1975). The data were imported to R using the package Geomorph (Adams et al., 2013) to determine shape differences. A Procrustes fit was performed to eliminate the size effect on the data set.

Principal component analysis (PCA) of Procrustes residuals was performed independently for the two datasets crania vs mandibles, to evaluate the shape and size variance among leopard head shapes. A multivariate ordination was then performed on the first two principal components of the cranium and mandible data sets to explain the shape variation. Sex and geographic location were considered as explanatory variables. To visualise patterns, variation in size and shape 3D morphing and the species average were computed by combining these two surfaces using IDAV landmark. The difference in body size was also tested using two-sided sample t-tests to investigate sexual dimorphism between genders.

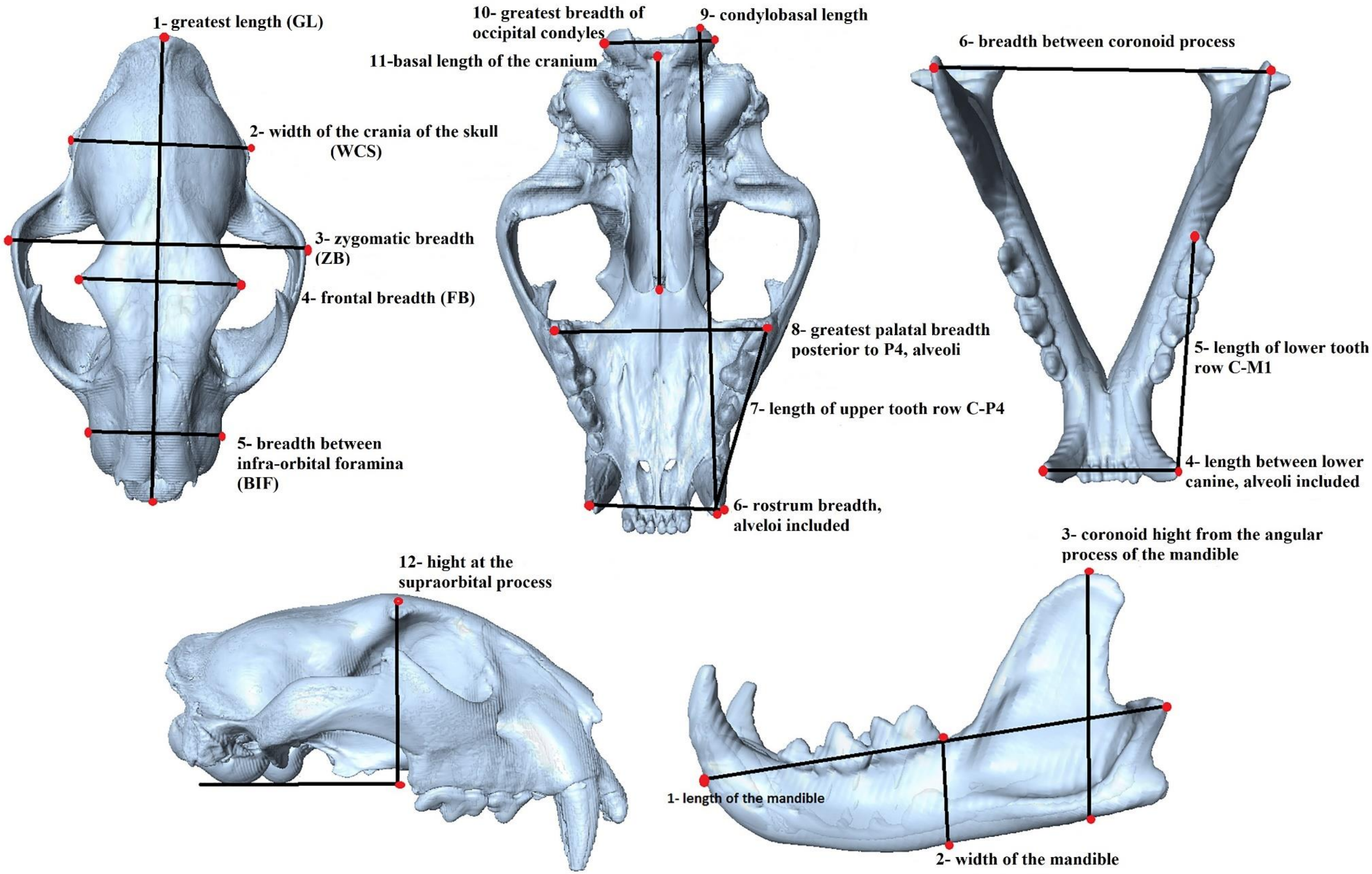

Figure 5.3 Carniomandibular variables measured (dorsal view, ventral view, lateral view, of the cranium and dorsal and lateral view of mandible).

5.4 Results

5.4.1 Craniomandibular shape analyses

The Procrustes shape analysis presented variation among individuals regardless of gender, diet, and location for both datasets. The first two principal components (PCs) accounted for 86% of the total variance of the cranium. PC1 accounted for 50%, while PC2 accounted for 36%, of the cranial shape differences (Figure 5.4). Multivariate analysis shows no significant differences in cranium shape between males and females (p = 0.3) and location (p = 0.7).

For mandibles, the first two principal components (PCs) accounted for 82% of the total variance. The score from PC1 was 53% while from PC2 it was 29% (Figure 5.5). Procrustes analysis revealed shape differences in the mandibles among individuals. The multivariate analysis (MANOVA) performed on the PCs with regard to gender (p = 0.6) and location (p = 0.6) also did not show any significant morphological differences.

A two-sided sample *t*-test performed on the morphological variations of body length and weight were significant for both sexes for overall length (p = 0.005) and weight (p = 0.002), suggesting clear sexual dimorphism. The samples from both regions were not discriminated from each other and showed no significant effect of region or diet differentiation.

The 3D morphing and species averaging revealed visual size and shape changes in the crania and mandibles (Figures 5.6 and 5.7). The changes were more obvious in the coronoid process of the mandibles where the apex of the coronoid process were rounded in some samples and elongated upward making a triangular shape (Figure 5.6). The prominence of the coronoid shape may be useful as a distinguishing character among the leopard subspecies but this requires further investigation and confirmation with genetic data.

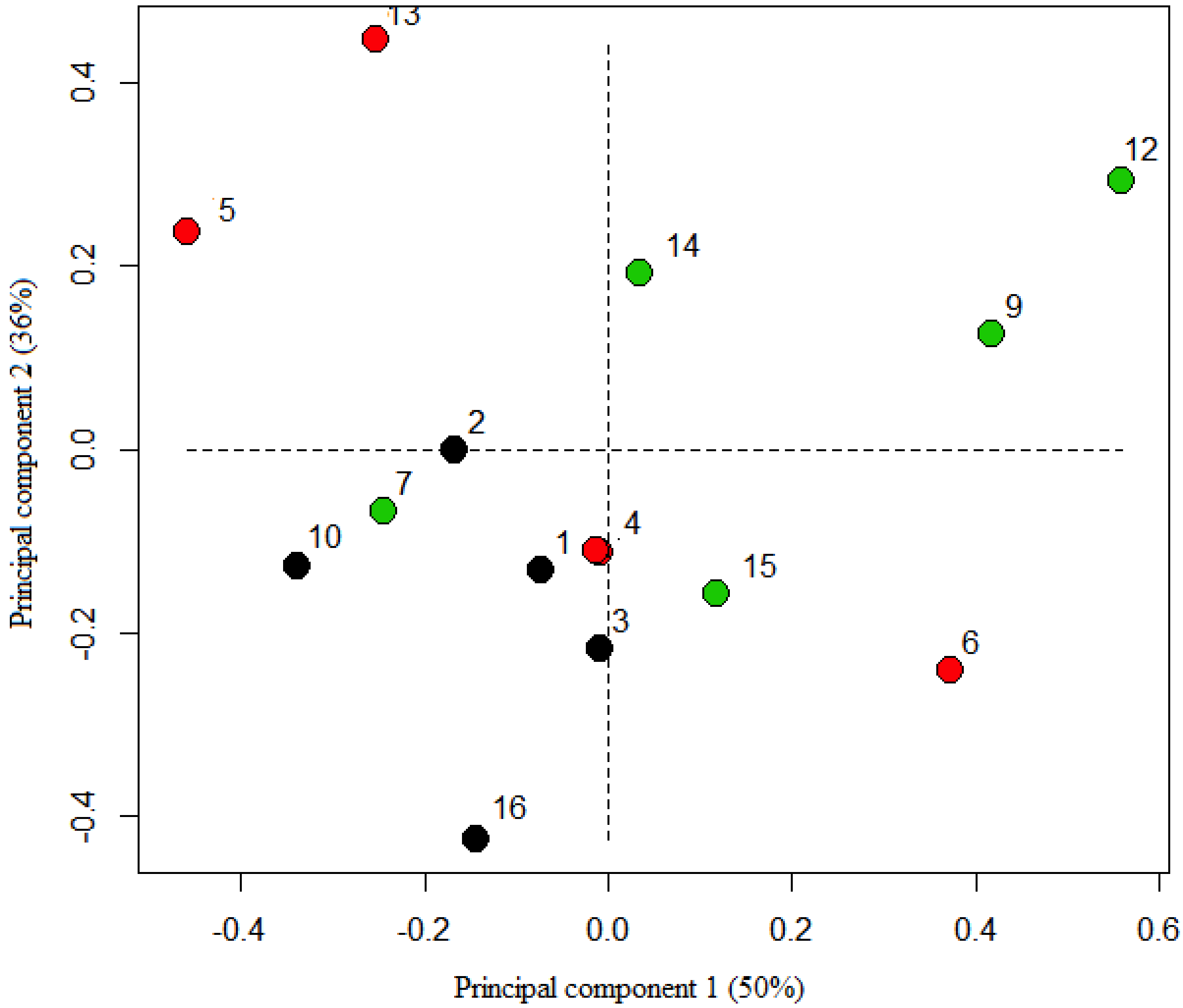

Figure 5.4 Procrustes PCA on size independent shape variables for cranium different coloured circles represents sexes (red: males, black: females and green unknown).

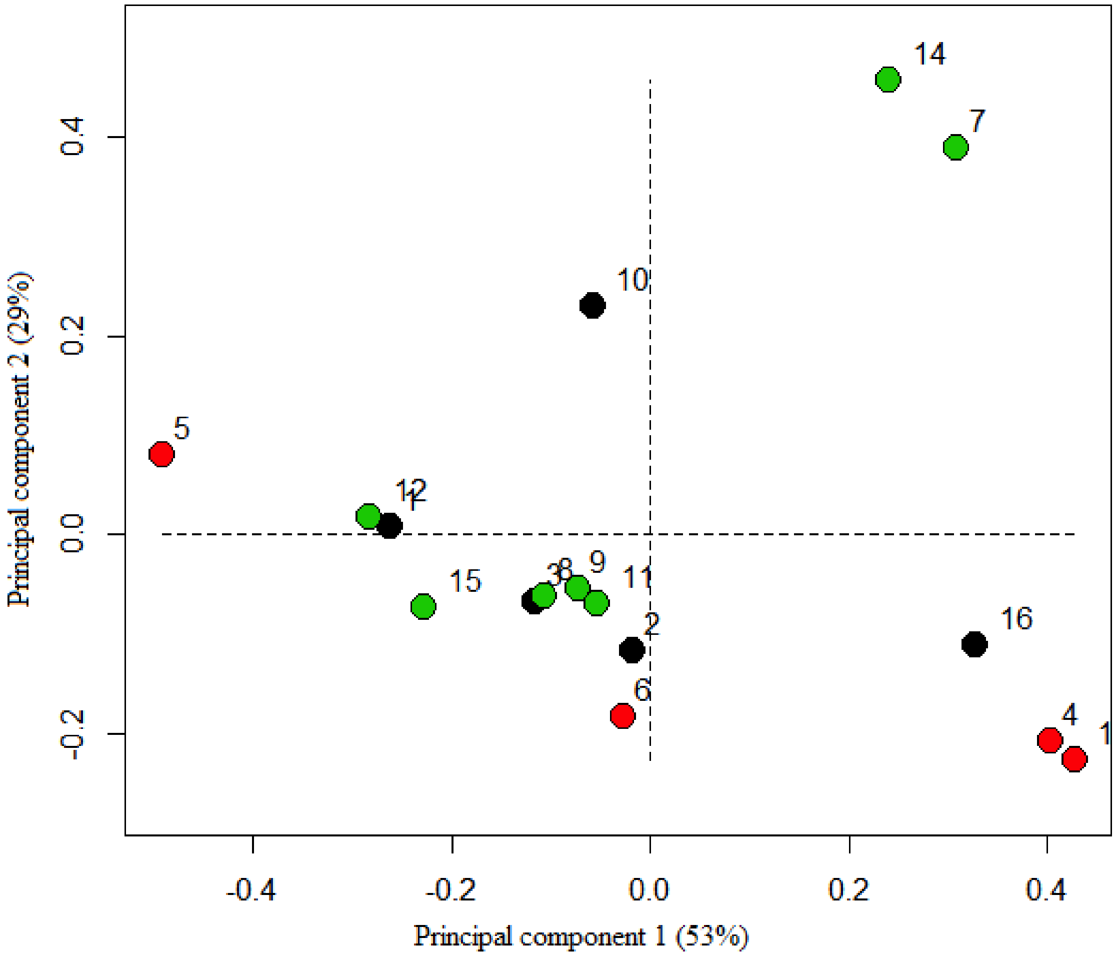

Figure 5.5 Procrustes PCA on size independent shape variables for mandibles different coloured circles represents sexes (red: males, black: females and green unknown).

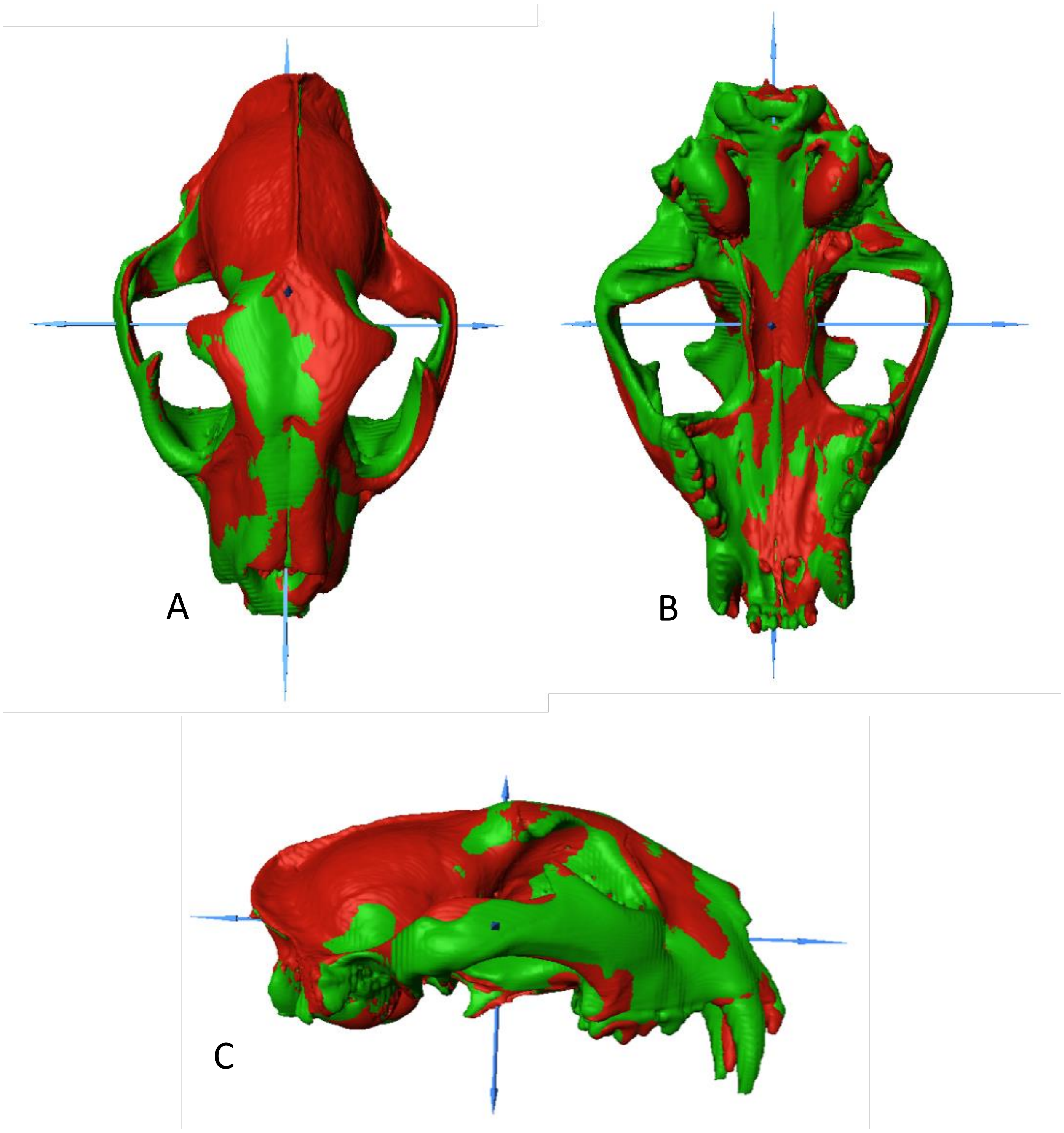

Figure 5.6 A crania from combining surfaces from two leopards (red and green) on corresponding landmarks. The colour represents which of the two individuals is larger in that region. Arrows are used for orientation only, (A) dorsal view, (B) ventral view and (C) lateral view of the cranium.

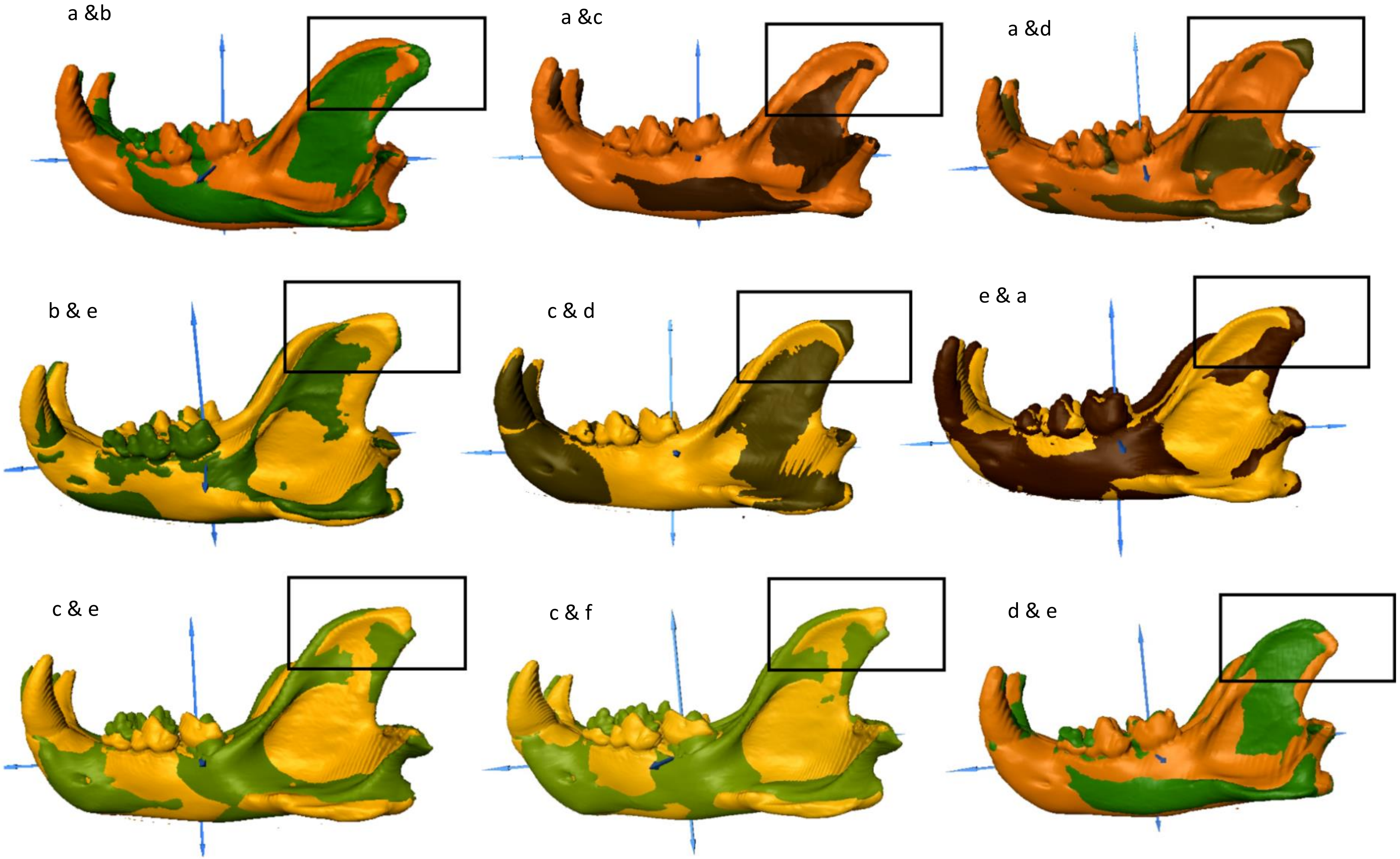

Figure 5.7 Variation on the mandibles from combining surfaces from two leopards (two colour represent two different leopards) based on the corresponding landmarks on the atlas surface. The colour represents size variation between individuals. Arrows are used for orientation only, coronoid process representing by the box shows differences in shape.

5.5 Discussion

The results of our analysis demonstrated significant shape and size gradients among individual leopard skulls from northern Pakistan. Procrustes PCA showed variation in the shape of both the mandible and cranium for both sexes.

An anatomical feature related to the diet in leopards is the general morphology and size of the skull. This variation may be due to diet/prey differences in the two locations. For example, Wild ungulates are only located in the Kashmir region, e.g. Himalayan ibex (*Capra ibex)*, grey goral (*Naemorhedus goral)* and musk deer (*Moschus chrysogaster)*. Differences in diet have strong associations with size (Caumul and Polly, 2005), however, the presence of domesticated animal i.e., goats and cattle may not support this hypothesis. Large leopard sizes in northern Iran are associated with the larger prey, e.g., Maral red deer *(Cervus elaphus maral;* Farhadinia et al., 2015). However, leopards travel long distances and their opportunistic nature makes it difficult to absolutely link size variation to diet preferences. Mandibular traits are often influenced by the biting force required for processing different prey (Van Heteren and Figueirido, 2018). Leopard cranial morphology is directly linked with prey biomass (Farhadinia et al., 2015). Males with thicker canines and a stronger bite force can hunt larger prey than females in *P. leo* (Christian and Harris, 2012) However, our results suggest that the shape morphology of the skull is not influenced by location or diet (Figure 5.4).

Our results from skull measurements (Figure 5.3) are consistent with previous analyses for leopard sexual dimorphism (Farhadinia et al., 2014; Stein and Hayssen, 2013). Our data on leopard body length and weight suggest that males are proportionally larger than females; however, there was no significant variation associated with location and diet. The variation in mandibles is more prominent when compared to that within the crania when 3D morphing and species average were performed. The coronoid process is characterised by a round shape or an elongated tip that creates a triangular shape (Figure 5.6, 5.7). These structural differences can be used to determine the subspecies, diet differences or sex of individuals. The variation in coronoid processes requires further investigation to explore other factors that may be associated with the variation, e.g. sex, diet, or bite force or subspecies.

One of the limitations of our study is that the small sample sizes may not represent the entire population across Pakistan. Due to the lack of information about the presence of leopard and the number of available samples, e.g. skulls in other potential habitats, it was difficult to definitively link ecological status and morphological differences, but the results did demonstrate the importance of a fully integrated holistic approach to take into account the morphometric data. Consequently, additional studies on leopard morphometrics in combination with genetic data are needed to better understand whether these morphological variations match the subspecies.

The results of this study are suggestive for two reasons. First, the shape data show significant differences among individuals for both sexes. Second, the variation in the coronoid process may indicate a difference in diet or, possibly, of a different subspecies. We treat these results as preliminary due to the lack of genetic information from these samples and the small sample size. It appears that diet defined by location does not alter leopard skull morphology, at least at the scale we measured at, but how and why the shape changes occur in the mandible(s) merits further investigation. In addition, this study has shown that location, as a factor, does not have any influence on the skull morphology at the scale we sampled, despite diet and habitat differences at these sites.

The functional traits of the coronoid process could be explained in future morphological research, particularly if associated with diet and habitat use. Another possible explanation for these shape changes could be associated with age. We strongly recommend that any new leopard skull samples are stored and preserved, allowing for future collection of genetic samples and other morphometric data. Our primary aim was to look for any morphological functional traits in the crania and mandibles using 3D geometric data that could be used in differentiation of species present in the area, with regards to location and diet, and therefore did not focused on the genetic data of those samples. The results demonstrate the importance of a fully integrated approach to take into account the morphometric data alongside with genetic data of the same individuals to understand the value of current morphologically determined subspecies.

Leopard skulls are a very valuable resource. Skull information could be useful as a guide to comparing genetically different subspecies. 3D geometric morphometric analysis is a practical way of standardising these morphological traits. A Procrustes fit allowed us to eliminate the size effect on the data set and focus only at the shape differences. Future studies on larger datasets on leopard morphometrics in combination with genetic data, are needed to better understand these morphological traits.

The subspecies recognition dilemma between major population of leopards based on relationships between morphometrics, genetics and geographic still controversial. Subspecies designation based on a combined effect is important for the effective conservation and management for large carnivores. For example Mazak, (2010) suggested that subspecies designation is important for appropriate genetic reinforcement from other closely related geographical populations of Barbary lions (*Panthera leo)*. So far, research on subspecies has generally focused on a single functional trait, with no comprehensive analysis. A multi analysis research intended to retain or repudiate the nominal subspecies and to establish global leopard recognition protocol. This may lead to an effective approach passively recognising subspecies, globally.

In conclusion, we suggest that repeating the same method with more samples and with exact provenance along with genetic data may further clarify the taxonomic status of the nominal subspecies in Pakistan.

An adult female leopard (08) photographed during daytime in our camera-trap survey at Gallies Forest Division Khyber Pakhtunkhwa Pakistan.

6. Conservation and management of leopard in Pakistan

6.1 Management application

Extensive legal and illegal hunting, retaliatory killing, lack of trained and motivated personal, lack of a standardised monitoring protocol and a general lack of understanding of the geographic ranges and distribution of this species are some of the complex issues for management of leopards in Pakistan. This is especially so in areas with challenging law and order situations. This study revealed the current status of leopards in north Pakistan and illustrates the need for obtaining systematic data on leopard presence-absence, distribution, genetic structure, and morphology using a modified version of our protocol in other areas in Pakistan. This study also suggests the development of a standarised monitoring protocol for leopard monitoring across the country.

The findings of this study confirmed the presence of leopards at low densities in northern Pakistan. However, there are numerous threats to the survival of the leopard population. Given the various stresses from different issues, the leopard population is likely to continue to decrease without some intervention. Enhancing knowledge, with local monitoring by local staff members of the Khyber Pakhtunkhwa Wildlife Department using advanced monitoring technology together with a comprehensive awareness programme, are important steps for Pakistani leopard conservation.

6.2 Data establishment

This study generated the first genetic datasets for leopard populations in Pakistan. The genetic diversity of leopards from northern Pakistan was determined and it confirmed the presence of two major haplotypes *Panthera pardus fusca,* and *P. p. saxicolor.* The study suggests that more samples from other regions are likely to discover an even higher haplotype diversity and, potentially, subspecies. This research established a protocol to determine leopard presence-absence in northern Pakistan. Leopard presence was confirmed in the Swat and Dir Region where it was considered extinct locally. However, the relatively low number of detections (one station, three photos) from the total survey effort of 1,092 traps night, available reports on livestock depredation, direct sighting records by the community, and scoping the trails for leopard signs, suggest that the presence of a non-viable, or at least very cryptic, leopard population. Similar studies using camera-trap surveys have yielded relatively more photos (81 images of leopard over 1,110 trap nights) in India (Athreya et., al 2013), 71 images over 1,012 trap nights, in Iran (Ghoddousi et al., 2010), and 82 images from 2,639 camera-trap nights in Bhutan (Goldberg et al., 2015). Leopards are likely to be critically endangered or near-extinct in other habitats previously reported in Pakistan.

6.3 Individual recognition and identification of leopard hotspots

During this project, 37 leopard hot spots were identified with the help of interviews (with the local community and Wildlife Department), followed by trail surveys and camera-trapping. We conducted camera-trap surveys along tracking trails in 63 locations across the Galyat, Murree, Swat, and Dir. We identified individuals from these trails by their unique rosette patterns and named them according to trail name. These data will allow wildlife departments to continue to monitor individual leopards, including their movement and other life-history traits relevant to conservation, such as resource selection, for longer periods and over different seasons. Leopards chose habitats for various reasons, such as selecting sites where prey are easy to catch, (Strampeli et al., 2018), there are few disturbances (Ngoprasert et al., 2007), good prey abundance (Balme et al., 2007), or for locating partners (Odden and Wegge, 2005). The protection of these habitats will increase their survival and reproductive success. In addition, understanding habitat utilization by leopards near human settlements and the frequent use of trails will help managers set priorities for high activity areas. Identifying these areas will allow for a fast response in cases of livestock depredation or human casualties by leopards and allow the allocation of appropriate resources to the nearest schools and villages. This knowledge will also allow wildlife departments to implement effective livestock protection measures in villages during the day. A comprehensive awareness programme, which builds awareness and enthusiasm among local schoolchildren and the local community for protecting their resident leopard, may change the perceptions of the local people. Such interactions with the local people may also assist with mitigation measures to avoid human-leopard conflicts, for the future long-term survival of local leopard populations.

6.4 Capacity building

A total of 14 days of training for the Khyber Pakhtunkhwa Wildlife Department staff and selected community members was conducted during this project (2017). This training was led by Muhammad Asad. Twenty-one staff members and 25 local community members were trained and are now capable of monitoring their local resident leopards using camera-traps. They also now have information to raise awareness in their local community. These trained members will work with Khyber Pakhtunkhwa Wildlife Department staff to achieve conservation goals and to improve the co-existence of humans and leopards in their local areas. The one-hour session began with differentiating different cat species, highlighting their importance in the ecosystem, followed by mitigation measures and a quiz to create interest in readers and build enthusiasm among the schoolchildren to know their resident leopards. Mitigation was undertaken using the following methods.

1). We notified local people about leopard movements, particularly local women involved in fuelwood collection (a risky activity), and suggested mitigation measures, such as avoiding specific trails at specific times (dawn and dusk) and moving through some areas in groups.

2). We notified schoolchildren in close proximity to leopard trials about leopard behaviour.

3). We organised special local emergency sessions if there was a leopard attack on humans or livestock.

6.5 Educational material

We received very positive responses from schoolteachers and community members about the clarity of the awareness material for understanding leopard ecology and importance. Other schools have since contacted our trained staff and community members and asked to participate in the sessions and obtain awareness material for their schools. We will seek to distribute these materials to other parts of the country where the leopard is present.

Local community members, particularly school students, were the main target for information in our project. We aimed to build enthusiasm among the students to develop 'ownership' and to conserve their resident leopards by performing a series of activities, such as spot recognition of individual leopards. We showed camera-trap images of the local resident leopard(s) to the schoolchildren and helped them identify individuals using their unique rosette patterns. Currently, local students and the local community throughout the country have a naive perception about leopards as they seldom distinguish between leopards, cheetahs, tigers and other big cats (pers obs, confirmed by showing different big cat images to the locals and children). The project team members have already designed material that will be used in other regions with slight modifications in the threats section. (Appendix A).

An adult male leopard (01) photographed during our camera-trap survey at Gallies Forest Division Khyber Pakhtunkhwa Pakistan.

7. Conclusions and recommendations

7.1 Conclusions

In Pakistan the conservation of leopards is hampered due to insufficient information on their genetic structure, distribution, and abundance. This study was the first to obtain systematic data on leopard presence-absence, distribution, genetic structure, and morphology from northern Pakistan. This study confirmed the presence of leopards in the Swat, Dir and Margalla Hills Regions of Pakistan, although their presence is rare, based on at camera-trap effort over 1000 trap-nights. Several factors, from human modifications of the habitat, to a lack of compensation and Illegal hunting, are likely to have had an impact on leopard populations and contribute to their decline. This research provides the first quantitative estimates of the population size of the common leopard in the Gallies and Murree Forest Division. The home ranges of various leopard individuals were found to extend from the Gallies Reserved Forest into the surrounding buffer zone of Guzara Forest. These buffer zones act as corridors and are likely a key for the long-term survival of the leopard population, as the corridors allow leopards to extend their range while searching for food and mates, with minimal interactions with humans.

One important outcome from this research is to prioritise a focus on leopard rosette patterns in high activity areas (hotspots) from camera-trap surveys, as they are a cost-effective method for MARK-recapture analyses. We identified 39 high activity areas and 18 individually recognisable leopards in the local population, based on their unique rosette patterns. These data will allow wildlife departments to continue to monitor individual leopards, their movements, survivability, and other life-history traits relevant to conservation. These data will also help managers develop a comprehensive local awareness programme that builds knowledge and enthusiasm among the local schoolchildren and community for living with, and protecting, leopards in their region.

Leopard presence, distribution, and genetic structure also require immediate attention in other parts of Pakistan. This study is consistent with the findings of Jacobson et al, (2016). Although this increases the known range of this subspecies, they are likely at very low densities and their full range still needs to be identified. A second haplotype was identical to two sequences previously identified as *Panthera pardus fusca* and *Panthera pardus orientalis*. We also detected a new haplotype that needs further investigation. The new haplotype may be the result of random mating of the two subspecies, *P.p saxicolor* and *P.p. fusca*. This haplotype could possibly be restricted to the Pakistan area and, in fact, could add important variation to the local population. More samples of this rare haplotype would further clarify the variation and perhaps link it to other subspecies.

The 3D geometric morphometric analysis of leopard skulls showed shape variation. However, when using multivariate analysis this variation was not influenced by gender, diet or location for both datasets. There is variation at the tip of the coronoid process on the mandible but how and why the shape changes occur in the mandible merits further investigation. This variation may be an adaptation to diet/prey differences at the two locations. For example, wild ungulates are only located in the Kashmir region, e.g., Himalayan ibex *Capra ibex*, grey goral *Naemorhedus goral* and musk deer *Moschus chrysogaster*. Skull information could be useful as a guide to compare genetically different subspecies. The functional traits of the coronoid process could be explained in future morphological research, particularly if associated with diet and habitat use. Another possible explanation for these shape changes could be associated with age. Future studies on larger datasets of leopard morphometrics in combination with genetic data, are needed to better understand these morphological traits.

Pakistan should assume a role in leopard conservation as it provides a contact zone for the near-threatened *P. p. fusca* and endangered *P. p. saxicolor* subspecies. The overlapping distribution of these two subspecies provides an impetus to extend full protection to leopards beyond the limits of regional parks and reserves, and throughout all Pakistani territory.

7.2 Recommendations

(i) In Pakistan, leopards are possibly extinct from many of their previously recorded habitats, for example, Kirther National Park. To determine their presence-absence scientifically and map the current existing range throughout the country in a different habitat, we strongly recommend using a modified version of our protocol, in areas where the presence of leopard populations is ambiguous. Our study suggests that camera-trapping, combined with a MARK-recapture method, can be useful for estimating the density or abundance in leopard habitat over time.

(ii) A substantial threat to leopards in the Swat and Dir regions is the extensive legal and illegal hunting (Figure 7.1). The Wildlife Department has issued a huge number of licenses to locals for bird hunting as part of their revenue generation. If a person is reported for illegal hunting, they are first fined a small amount of money and then issued with the license. Once the license is issued, a person can hunt whatever they want, as there are no checks or balances. Illegal hunting has created a traumatic situation for leopards that drive them away from this habitat. The other threats to leopards in this area are habitat degradation, unplanned development, encroachment by humans, and the alarming depletion of their natural prey base (Shehzad et al., 2014; Lodhi 2007).

An understanding of what is occurring in the Swat and Dir Region is required in regard to illegal hunting and shooting as a serious problem for removing or driving leopards away from that region (Figure 7.2). Therefore, we would strongly recommend to the Khyber Pakhtunkhwa Wildlife Department to address this issue and instruct watchers (rangers) to regularly visit their compartments. Most of the watchers we met during our surveys have never visited their compartments and were unknown to the local people.

(iii) Habitat, distribution, and prey-based studies are lacking in many parts of the country. Addressing this should be considered of central importance for the conservation of leopards throughout Pakistan.

Figure 7.1 Illegal hunting practices recorded during scoping the trails for leopard and camera-trap survey in Swat and Dir district.

(iv) The Swat and Dir regional forests are potential leopard habitats. We only had two photo captures in this area, so it would be impractical to make decisions on habitat suitability without further surveys. This area is linked to Galyat and Kashmir with connecting corridor patches where the leopard population is stable. Further extensive investigations are required, to evaluate information about the prey base and the effect of human disturbances on leopard behaviour, to reach conclusions that are more reliable.

(v) A lack of trained and motivated personnel, and advanced equipment, constrains the conservation of leopards in Pakistan. A comprehensive species conservation management plan is required, which includes habitat management, enhancing knowledge of local staff members from the Wildlife Department and community members using advanced monitoring technology. Ultimately, raising awareness of leopard biology will increase interest in leopards, in general, as well as the value of conservation.

Camera-trapping, as a method, would have been more effective if the cameras were equally distributed and covered a larger area. However, this is hard to achieve in rugged terrain and with animals that have large home ranges (Tobler et al., 2008). To collect representative samples, we selected sample sites using help from the questionnaire survey that had been

Figure 7.2 Leopard carcasses found with their skin removed in Galyat during trail surveys.

undertaken previously. GPS telemetry studies can provide information on the spatial patterns of leopards in those landscapes. However, large carnivores often show adverse implications when being handled (Dembiec et al., 2004; Levine et al., 1990) that may result in many negative consequences, such as loss of human life and increased livestock depredation (Athreya et al., 2011). Logistical constraints and time limit the wider applicability of the method. It requires huge effort and time to produce results that are more accurate. Our technique suggests that, if applied correctly, camera-trapping can produce reliable estimates. For the leopard, the current view among management agencies is that they are too difficult or costly to count (IUCN 2007). At the very least, our method can detect the presence of leopards in an area, which is at least a good start to understanding leopard conservation as leopards are often assumed wrongly to be absent from areas. We also recommend future studies in different seasons and for longer periods. Our study suggests that the most productive conservation efforts may be beyond the protected area with special consideration given to buffer zones to ensure the long-term viability of leopard populations.

(vi) Our camera-trap effort combined with MARK-recapture analysis for leopard presence and abundance ensured that the leopard population was detected in those regions. However, our chief objective was to evaluate the abundance of our study population (Galyat and Murree Forests). This analysis method provides a reasonable way of estimating *p* (capture probability in this case), and we maximised the probability by first identifying likely hotspots. The MARK-recapture framework presented an advantage as the individuals were often identifiable from their unique pelage patterns. Although our method cannot provide absolute certainty about abundance, we believe that the estimate we obtained from MARK™ provided a reasonable degree of confidence. We could have estimated the density from track count data (Balme et al., 2009) but we found that individuals overlapped their territories, so that would have overestimated the density of leopards in the study area. However, obtaining an estimate from track count data and knowing that it has limitations can still be useful and can only emphasise the conservation issues.

(vii) For the genetic study, the limited number of samples used (especially from the regions of Baluchistan and Nizampur) most certainly do not fully reflect the subpopulations of these regions. Therefore, further studies are likely to discover an even higher diversity in subspecies than reported here, as already confirmed from Baluchistan, where the subpopulation is closely related to the Persian leopard that is known to be present (Jacobson et al., 2016). Obtaining genetic information from these areas will complement the knowledge now available and enable a better understanding of the distribution and ecology of leopards, not only in Pakistan but also worldwide.

(viii) The results from geometric morphometrics data showed differences in head shape among individuals for both sexes. However, we treat these results as preliminary due to the lack of genetic information to identify the haplotype of the individual skulls and the small sample size.

It appears that diet does not alter leopard skull morphology. We strongly recommend that new leopard skull samples are preserved and stored, allowing the future collection of genetic samples and other morphometrics data. Leopard skulls are a very valuable resource.

In conclusion, our study suggests a comprehensive species conservation management plan is required for other parts of Pakistan:

1. A pragmatic awareness education programme should be an important step for leopard conservation across the country, which will build knowledge and enthusiasm among local schoolchildren and the community for living with, and protecting leopards in their region.

2. Intensive camera-trapping is viable tool and can provide detailed information of the presence-absence status in other parts of Pakistan where the presence of leopard populations is uncertain. However, this requires a large effort, time, and a large number of camera-traps to systematically cover an entire study area.

3. Further genetic analysis from other parts of Pakistan is likely to discover higher diversity. Obtaining more samples of haplotype (C) might be useful for classification of a third subspecies and to enable a better understanding of the distribution and ecology of leopards, not only in Pakistan but also worldwide. There is also the possibility of detecting other rare haplotypes.

4. Additional studies on the leopard morphometrics, in combination with genetic data, are needed to better understand whether these morphological variations match subspecies. We strongly suggest that wildlife departments from across the country agree on a policy to use the same conservation package and address the converging issues related to leopard conservation.

5. Knowing that individual leopards inhabit particular areas, we suggest further research, using a modified version of our protocol in the identified hotspots, will reduce delays in detection, in particular, and establish more accurate abundance estimates.

6. We suggest regular visits to the forest compartments by rangers from the Wildlife Department and Forest Department would be an important step to deter the illegal hunting and keep check on other legal activities.

7. We suggest that the area of identified hotspots should be recognised by managers as highly important areas for leopard conservation, and warrant increased protection from anthropogenic activities.

8. We propose a standardised monitoring protocol to be developed and implemented across the country through wildlife departments for future leopard conservation in Pakistan. This will enable wildlife departments concerned to store and pool spatial data for comparative analysis and to coordinate local, national and international research as well as generate a species-specific distribution map, addressing key conservation issues about leopards.

9. Effective community measures for livestock, such as keeping them in enclosures for some of the time, as well as immediate financial compensation for livestock owners after leopard depredation, may significantly reduce conflicts between humans and leopards.

10. Sound conservation measures, such as habitat improvement, reintroduction of wild prey and increasing prey diversity, will alleviate the vulnerability of leopards towards extinction.

This research provides a re-assessment the leopard status in the north of Pakistan, yet this pilot study is still incomplete. It is clear from this study that the lack of understanding of the genetic structure, distribution and abundance of leopard populations across the country hinders our ability to protect them. In response to this need, this research contributes to elucidate understanding and promote stewardship; thus, aiding in the long-term conservation of the leopard in Pakistan.

Summary

This study took a multidisciplinary approach, involving genetic structure, 3D geometric morphometrics, and camera-trapping. We used this multidisciplinary approach to answer a number of key disparate but interconnected issues at different scales around leopards in Pakistan as this information is mostly lacking. We were interested in the status of leopards at the local level in terms of identifying where leopards are found and in what numbers (camera trap project). We were interested in which species shared leopard habitat (camera trap diversity survey) and how they interacted with people (questionnaire of locals). We were interested in how leopards were distributed at the landscape level (haplotype diversity and distribution). We were also interested in the identity and distribution of leopard subspecies across northern Pakistan (genes and morphometrics). An understanding at these different scales in important if we are to maintain and restore leopards in their habitats. We were also able to establish baseline information and allow management to formulate strategies for leopard conservation.

Given the severity of the threats leopards face across their range, from habitat loss, prey depletion, and poaching and retaliation, the leopard in Pakistan requires immediate conservation action. A better understanding of the species status, population densities and distribution must be obtained to ensure maintaining and enhancing populations in the region.

Taking a multidisciplinary approach has allowed us to reach a point where we can provide advice for other regions of Pakistan as to how they can assess their leopard populations. It has also made clear where future research can be most productive and which research questions are most pressing. For example, including further genetic samples from other regions using microsatellite analysis, along with the patterns of morphological diversity using 3D morphometrics and Intensive camera-trapping, in other parts of Pakistan where the presence of leopard populations is uncertain, could further improve the conservation of leopards in these region. Although the study confirmed the presence of two major haplotypes *Panthera pardus fusca,* and *P. p. saxicolor,* further genetic analysis in other regions could detect other rare haplotypes, such as obtaining more samples of haplotype (C) that might be useful for classification of a third subspecies and to enable a better understanding of the distribution and ecology of leopards, not only in Pakistan but also worldwide.

One lesson from our research is the engagement of local community in conservation is an effective method of species conservation (Robinson and Sasu, 2013), especially for leopards. We learnt that improving local knowledge on leopard ecology and behaviour could be used as an effective conservation practice in addressing the human-leopard conflict. In turn, the research findings and the output generated from the project bilingual broachers raise awareness and build huge enthusiasm among the local schoolchildren and community to develop 'ownership' for protecting their local resident leopard.

Undertaking multidisciplinary research involves tradeoffs, for example we could have expanded our research in each discipline. Focusing on genes might have allowed us to better understand the subspecies around more of Pakistan and maybe have a better picture of gene flow between populations. Focusing more on morphometrics would have allowed a better understanding on what drives changes in morphology in leopards. Similarly, focusing on camera trapping would have allowed a better focus on estimating population densities and doing so over more season and in different areas. However, we feel the gains in understanding the issues at all of these scales outweighs the losses.

Our study has implications for leopards in other parts of Pakistan that have previously been reported as potential leopard habitat, such as Kirthar National Park (Sindh), Machiara National Park (Azad Kashmir), Kithi Bunder South Wildlife Sanctuary (Sindh), Hingol National Park Baluchistan, Hazarganji-Chiltan National Park and Wildlife Sanctuary (Baluchistan), Dasht National Park (Baluchistan). For example, leopard have been reported as locally extinct (Khan et al., 2013), however these results were based on field observation and not camera trapping. Using camera trapping would be valuable in determining presence-absence and density estimates from this region. Obtaining information about leopard presence-absence and collecting genetic samples from those areas is not an easy task, given that there are many issues with law and order situation and accessibility when working in those regions.

We now have a better understanding of leopards in Pakistan. While challenges remain, the research, as presented in this thesis, has given us baseline information about leopards, genetic structure, distribution, and morphological variation from this region. For the first time, there is relevant data that will improve the understanding of leopards in Pakistan.

We expect this work, in combination with strong education and outreach programme, and improved management practices in other parts of the species ranges. Hopefully this will contribute to the conservation of leopards not only in Pakistan but also worldwide.

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Appendix A
Analysis and information for chapter two

1. Model selection results for seven occasions (two day and night is considered into a single session resulting in 7 occasion) from MARK analysis for common leopard populations in Gallies Forest Division and Murree Forest Division Pakistan 2017 and 2018.

With behaviour models						2017		2018	
Model	Delta AICc	AICc weights	Num. par	-2*log-likelihood		Estimate	SE	Estimate	SE
p(.)	0.00	0.46	1	139.19		18.97	2.82	8.86	1.73
p(behav)	1.78	0.19	2	138.92		17.50	2.94	8.17	1.62
p(Year)	1.96	0.17	2	139.09		19.36	3.30	8.53	1.80
p(Year+behav)	3.86	0.07	3	138.91		17.68	3.70	8.13	1.64
p(het),pi(.)	4.13	0.06	3	139.19		18.97	2.82	8.85	1.73
p(het),pi(Year)	5.70	0.03	4	138.65		19.91	3.59	8.88	1.82
p(Year+het),pi(.)	6.15	0.02	4	139.09		19.36	3.30	8.53	1.80
p(Year*t)	20.35	0.00	14	130.54		19.08	3.15	8.35	1.67
Model average						18.71	3.11	8.61	1.74
					Mt+1	15.00		7.00	
					C	2.57		3.11	
					lower	16.44		7.52	
					upper	24.52		12.03	

Without behaviour models						2017		2018	
Model	Delta AICc	AICc weights	Num. par	-2*log-likelihood		Estimate	SE	Estimate	SE
p(.)	0.00	0.62	1	139.19		18.97	2.82	8.86	1.73
p(Year)	1.96	0.23	2	139.09		19.36	3.30	8.53	1.80
p(het),pi(.)	4.13	0.08	3	139.19		18.97	2.82	8.85	1.73
p(het),pi(Year)	5.70	0.04	4	138.65		19.91	3.59	8.88	1.82
p(Year+het),pi(.)	6.15	0.03	4	139.09		19.36	3.30	8.53	1.80
p(Year*t)	20.35	0.00	14	130.54		19.08	3.15	8.35	1.67
Model average						19.10	2.99	8.77	1.76
					Mt+1	15.00		7.00	
					C	2.33		2.91	
					lower	16.77		7.61	
					upper	24.54		12.16	

2. Model selection results for fourteen occasions (each day and night is considered into a single session resulting in 14 occasion) from MARK analysis for common leopard populations in Gallies Forest Division and Murree Forest Division Pakistan 2017-2018.

With behaviour models						2017		2018	
Model	Delta AICc	AICc weights	Num. par	Deviance		Estimate	SE	Estimate	SE
p(.)	0.00	0.46	1.00	199.16		19.42	3.01	9.08	1.85
p(behav)	1.52	0.21	2.00	198.66		17.34	2.75	8.09	1.52
p(Year)	2.01	0.17	2.00	199.14		19.59	3.36	8.92	2.10
p(Year+behav)	3.51	0.08	3.00	198.60		16.95	2.79	8.18	1.64
p(het),pi(.)	4.07	0.06	3.00	199.16		19.42	3.01	9.06	1.84
p(Year+het),pi(.)	6.10	0.02	4.00	199.14		19.59	3.36	8.91	2.09
p(Year*t)	30.2248	0.00	28	169.577		19.18	3.15	8.53	1.81
Model average						18.81	3.18	8.76	1.87
					Mt+1	15.00		7.00	
					C	2.55		3.06	
					lower	16.49		7.58	
					upper	24.74		12.41	

Without behaviour models						2017		2018	
Model	Delta AICc	AICc weights	Num. par	Deviance		Estimate	SE	Estimate	SE
{p(.)}	0	0.64744	1	199.1599		19.42	3.01	9.08	1.85
{p(Year)}	2.0088	0.23714	2	199.1424		19.59	3.36	8.92	2.10
{p(het),pi(.)}	4.0659	0.08478	3	199.1599		19.42	3.01	9.06	1.84
{p(Year+het),pi(.)}	6.1015	0.03064	4	199.1424		19.59	3.36	8.91	2.09
{p(Year*t)}	30.2248	0	28	169.577		19.18	3.15	8.53	1.81
Model average						19.47	3.11	9.03	1.92
					Mt+1	15.00		7.00	
					C	2.25		2.80	
					lower	16.99		7.72	
					upper	25.06		12.70	

3. Histories of photo capture-recapture of the leopard in the Galyat and Murree Forests for both surveys used in CMR analysis.

Survey 1	**Nights/occasions**													
Leopard ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0	0	1	1	0	0	0	0	0	1	0	0	x	x
2	0	0	1	0	0	1	0	0	1	0	0	0	0	0
3	1	0	0	0	0	0	0	0	0	0	0	0	0	1
4	0	0	0	0	1	0	1	0	0	0	0	0	0	0
5	1	0	0	0	0	0	0	0	0	0	0	0	0	1
6	0	0	0	1	0	0	0	0	0	0	0	0	x	x
7	1	0	0	0	0	0	0	0	0	0	1	0	0	x
9	0	1	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	1	0	1	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	1
11	0	0	1	0	0	0	1	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	1	0	0	0	0	0	0
13	0	0	1	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	1	0	0	0	0	0	0	0	0	0
15	1	0	0	0	0	0	1	0	0	0	0	0	1	0

Survey 2	**Nights/occasions**													
Leopard ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
11	1	0	0	1	0	0	0	1	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	1	0	0	0
New 18	0	0	0	0	0	0	0	0	0	0	0	0	0	1

New 18	0	0	0	0	0	0	0	0	0	0	0	0	0	1
9 (Leopard)	0	0	0	0	0	0	0	0	1	0	0	0	0	0
3	0	0	0	0	1	0	0	0	0	0	0	0	0	1
1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
3	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Leo17 (new)	0	0	0	0	0	0	0	0	0	0	0	0	0	1
11	0	0	0	1	1	0	0	0	0	0	0	0	0	0
NI	0	0	0	0	0	0	1	0	0	0	0	0	0	0
NI	0	0	0	0	0	0	1	0	0	0	0	0	0	0
NI	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Leo 16 (new)	0	0	0	0	0	0	0	0	0	0	1	0	0	0
11	0	0	0	0	0	1	0	0	0	0	0	0	0	0

4. Secondary data of leopard attacks on humans that resulted in injuries or deaths; cases of revenge killings as well as leopard natural deaths, when detected, were collected from Khyber Pakhtunkhwa Wildlife Department and WWF-Pakistan for the years 2005 - 2018 in the district of Abbottabad.

Year	Leopard killed	Leopard attacks
2005	2	9
2006	5	0
2007	5	2
2008	1	1
2009	0	0
2010	0	1
2011	4	3
2012	5	0
2013	5	2
2014	1	0
2015	3	1
2016	3	1
2017	2	0
2018	4	1
	40	21

5. Number of male and female leopards that died in two seasons, for the years 2005-2018, in the district of Abbottabad.

Gender	Male	Female	Unknown	Cub
Summer	11	6	3	0
Winter	7	7	6	2

6. Number of hotspots identified in different land-use of Galyat and Murree Forests; independently captured through camera-traps and individuals identified on each trail; Protected areas, Reserved Forest, Municipal forest, Ayubia National Park, Guzara forest, and Cantonment area.

land use	Number of tracks	Leopard captured	Leopard Identified
ANP	6	5	4
Reserved Forest	7	6	3
Guzara Forest	11	8	5
Muncipal Forest	2	2	2
Protected Forest	3	3	3
Cantonment Area	2	2	1

7. Questionnaire used among the local communities living close to areas across the study area to identify potential sites of leopard presence during the study period.

LEOPARD QUESTIONNAIRE

Recorder name: ______________________Start date: ________ Completion date: _______

Location: __________________ GPS coordinates:_____________ Elevation: _____________

Name of respondent: ____

Age: _________________ Sex: ____

Profession: ____

Habitat ____

Questions:

Leopard sighted in the area? Yes No

Name of place: ________________________________ Exact location: ___________________

Date observed ___________________________Person activity _________________________

Number of leopards sighted in your whole life: _____________________________________

Full-grown _______________________ Young: _____________________________________

Activity of the leopard when sighted walking, resting eating/attacking

GPS reading (sighting place): ____

Livestock depredation:

Time of incident: ---

Kind and age of Livestock: -----------------------------------Killed or Injured.

Where Inside home or out in grazing area

Name of place: _________________________________ Exact location: ___________________

GPS reading (attack site): ____

Type of livestock attacked (goat, cow, and bull, other) ________________________________

Owner details: ____

Details of attack: ____

Any other sign recorded i.e. roaring, marking, and scat: ________________________________

Habitat analysis

How does the habitat look like where the animal was attacked?

Terrain: Steep _______Gradual _______Flat ________Valley________

Type of vegetation ____

Nearest forest from attack site: 0-100m, 100-500m, 500-1000m, >1km

Forest cover: __________________Type: __________________ Coniferous forest/Deciduous forest/Mixed forest

Distance from water _________________ (GPS data will be recorded)

Anthropogenic/disturbance ______________ (Roads, Residential land,Commercial land)

Retaliatory killing:

Any leopard killed in the area:

Poisoned/ shot ____

Name of place: _______________ Exact location: ______________Reason for killing___________

Reported/unreported: ____________________

8. Leopard observation sheet used among the local communities living close to areas across the study area to identify potential sites of leopard presence during the study period.

LEOPARD OBSERVATION SHEET

Recorder name:______________________________ Date: _______________________________

Location: ____________________ GPS coordinates: ________Elevation: ______________

Sex: Male Female

Physical Examination:

Weight: (kg) ____

Age estimation (Baily 1993 estimate from teeth state I.e. colour, worn out, broken etc.) _____

Coat: (colour fades with age, moulting hairs, etc.) _____________________________________

Others: ____

Body measurements (in metres and centimetres):

Body length (nose tip to the base of tail (last vertebra of the body) _______________________

Tail length (from the end of the body (last vertebra) to the tip of the tail) _________________

Total length: _________________________

Shoulder height (highest point on the shoulder to the sole of the foot):__________________

Hind foot (longest toe, sine, and unguis/ without claw):_______________________________

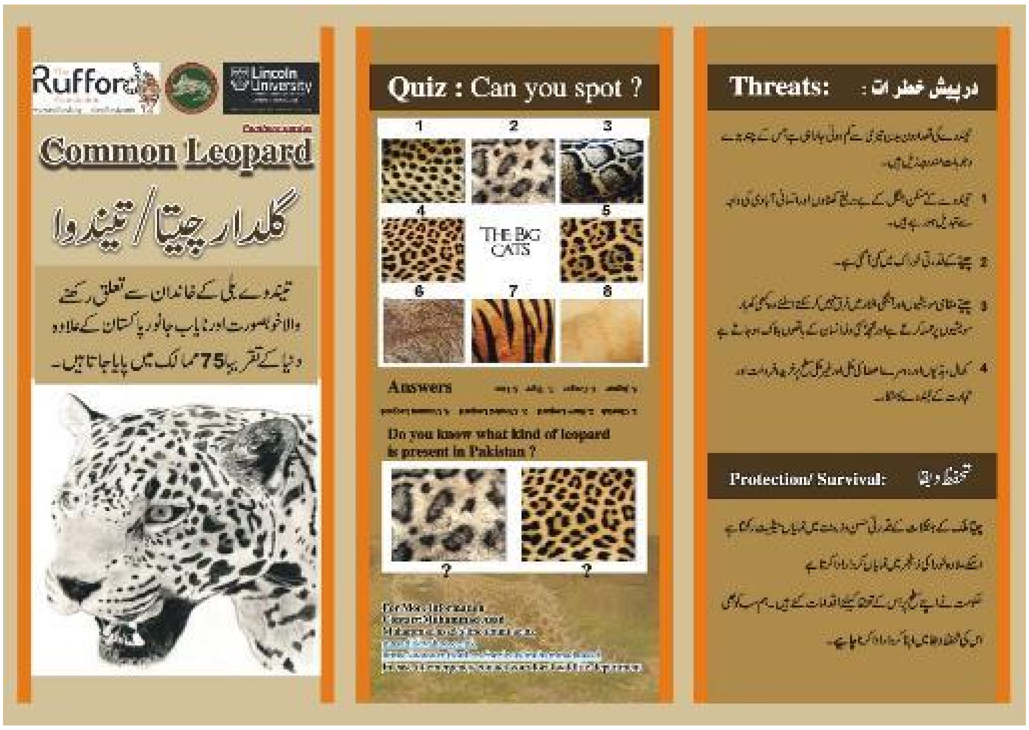
Neck girth (behind occiput and ears):____

Ear length (lower notch to the most extended part) __________________________________

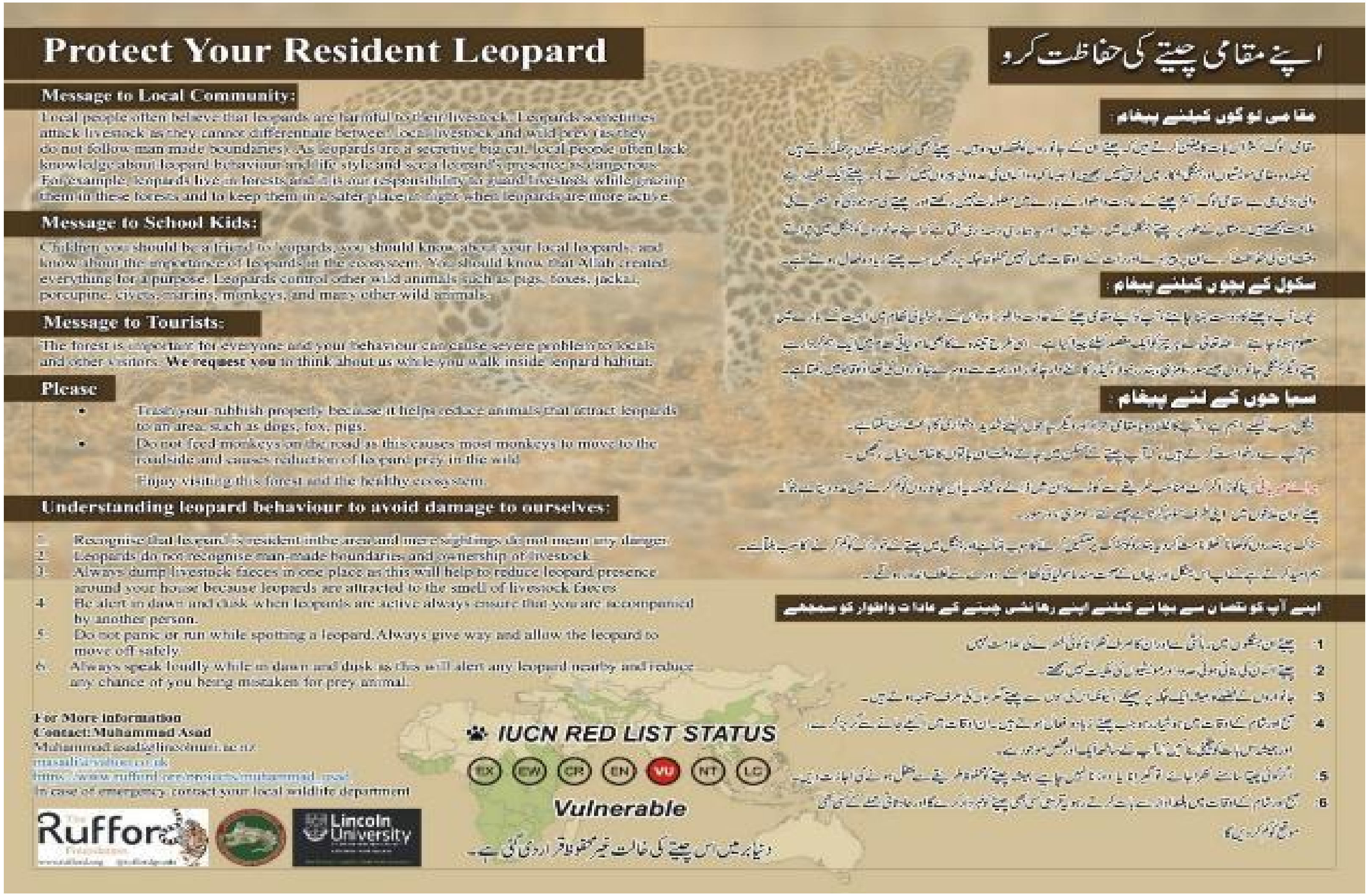
Biological samples collected (Sample ID): 1: Hair 2: Blood 3: Flesh 4:

Any other?____

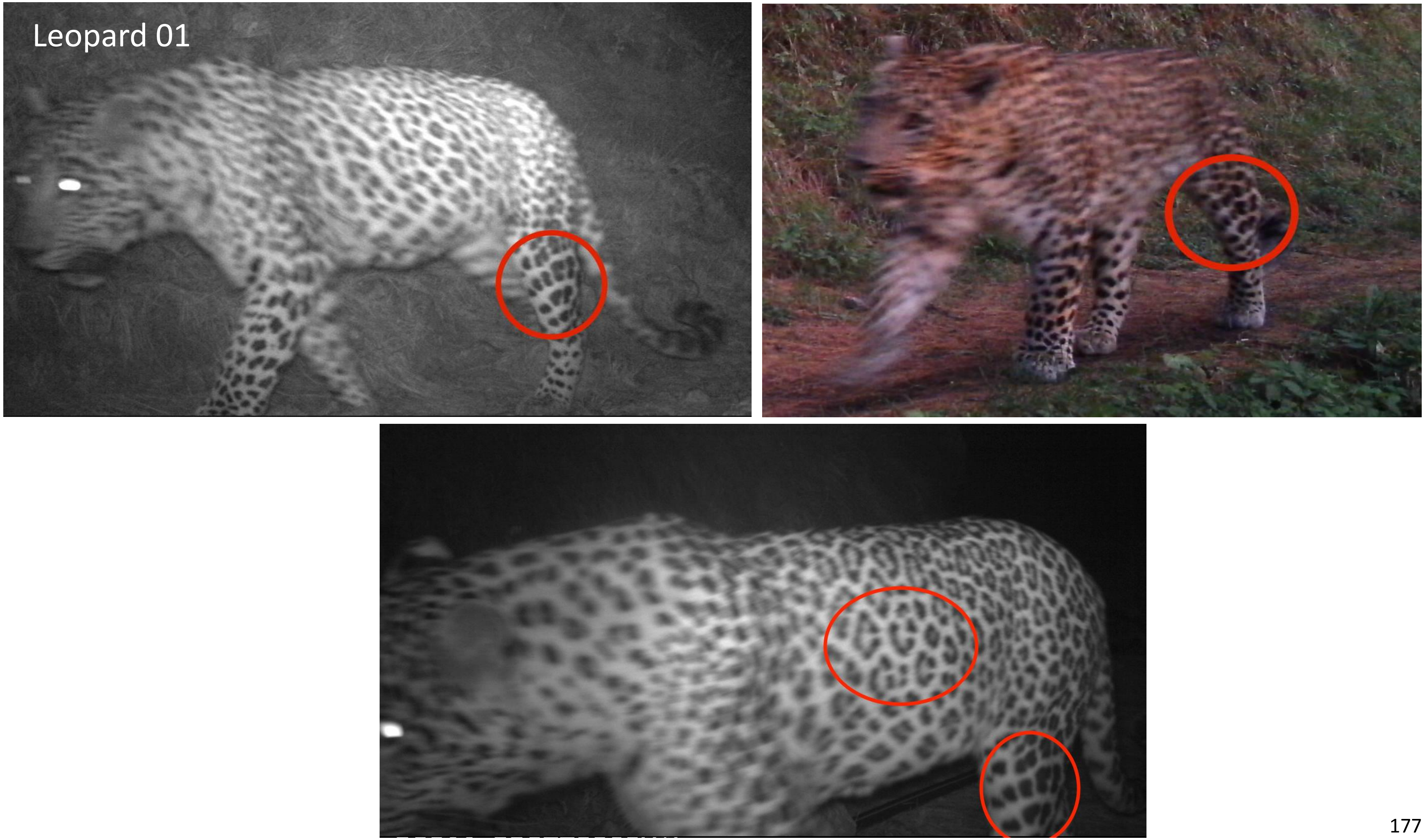
9. A bilingual brochure was designed as a by-product for schoolchildren and the community to recognise and understand leopard behaviour.



10. A bilingual poster was designed for schoolchildren and community members to recognise understand and protect their resident leopard.



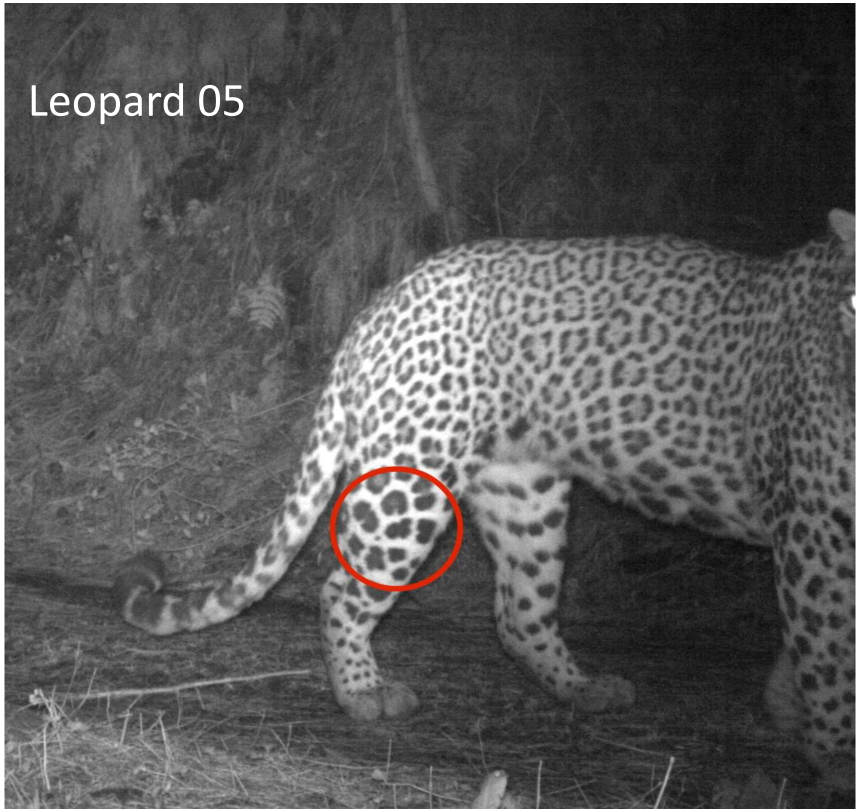
11. Number of individual identified based on the prominent spots captured at different occasions (red circles) showing the spot used for the identification of individuals from Gallies and Murree Forest Division.

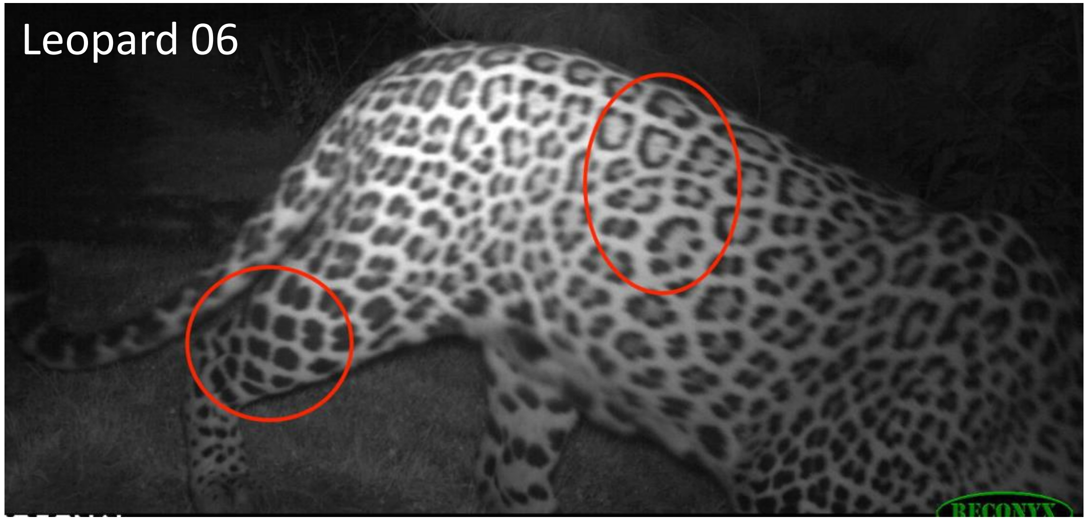



Leopard 02


Leopard 03


Leopard 04


Leopard 05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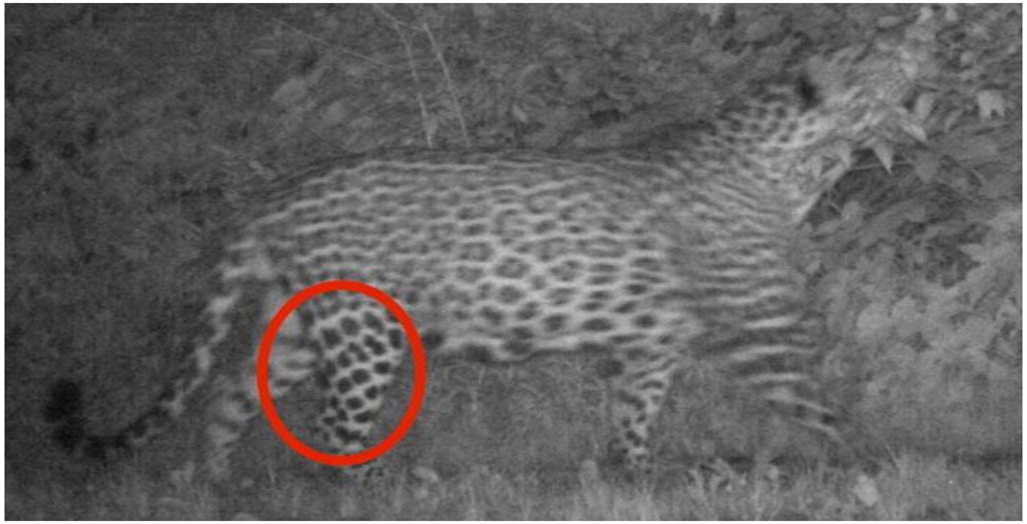
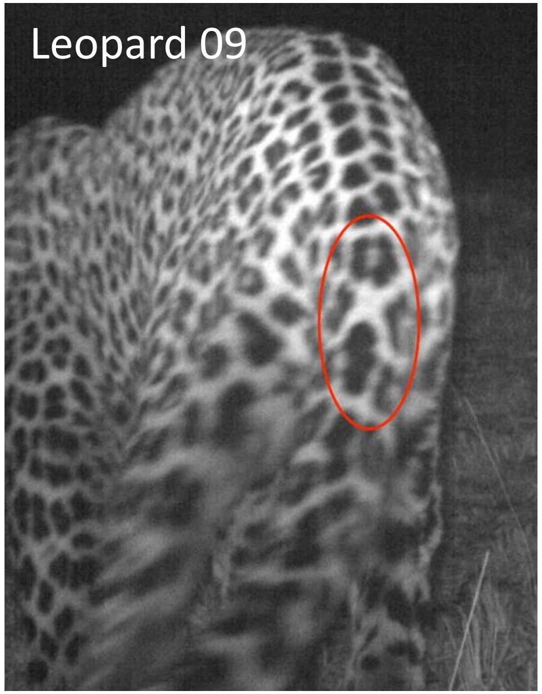



Leopard 07


Leopard 08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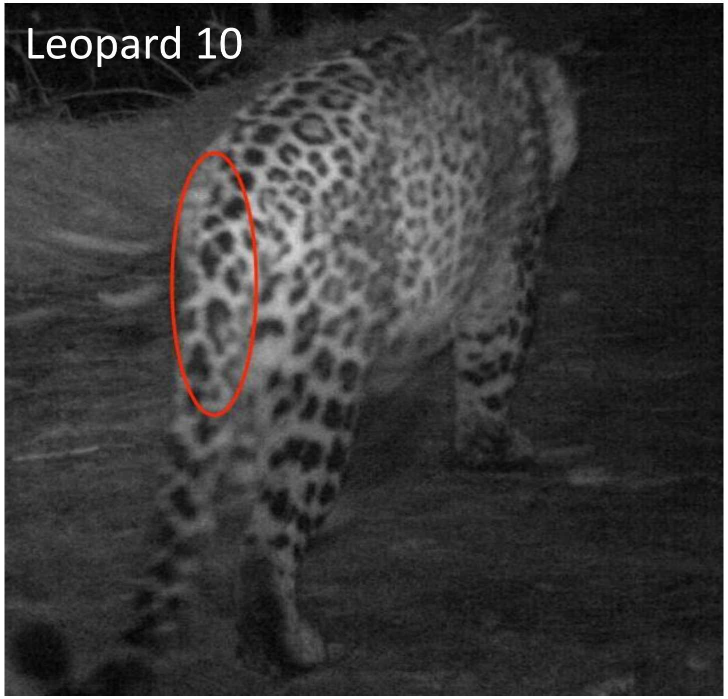
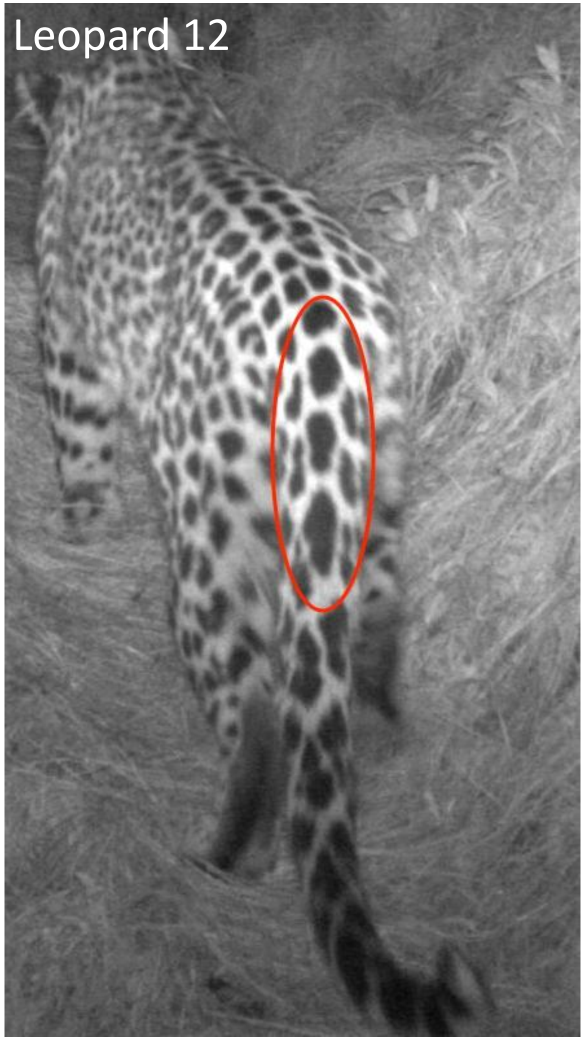
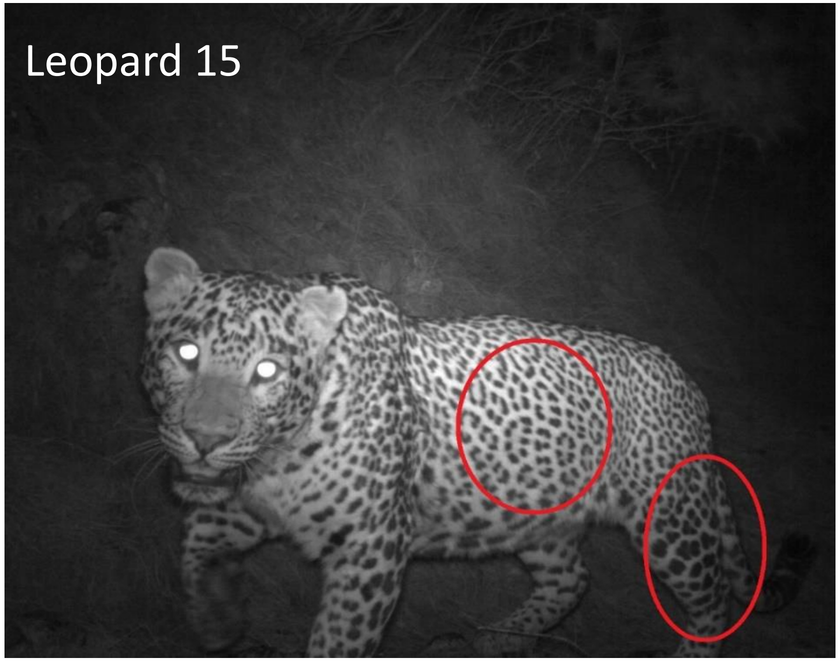

Leopard 10


Leopard 11


Leopard 12


Leopard 13


Leopard 14


Leopard 15


Leopard 16

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Leopard 17


Leopard 18

Appendix B Analysis and information for chapter four

1. Sequences ID, sampling locations and collection dates for the 43 Pakistani leopard specimens used in the genetic analysis for subspecies identification.

Sequence ID	Specimen voucher	Collection date
Seq1	AB1_Fus	2017
Seq2	AB3_FL2/rl4	2018
Seq3	AB4_FL2	2018
Seq4	AB5_FL2	2018
Seq5	AB6-FULL	2017
Seq6	AJK1_FL2	2005-2014
Seq7	AJK10_RL4	2005-2014
Seq8	AJK13_FL2	2005-2014
Seq9	AJK14_FL2	2005-2014
Seq10	AJK15_RL4	2005-2014
Seq11	AJK16_FL2	2005-2014
Seq12	AJK18_FL2	2005-2014
Seq13	AJK2_FL2	2005-2014
Seq14	AJK22_FL2	2005-2014
Seq15	AJK3_FL2	2005-2014
Seq16	AJK4_FL2	2005-2014
Seq17	AJK5_FL2	2005-2014
Seq18	AJK6_FL2	2005-2014
Seq19	AJK7_FL2	2005-2014
Seq20	AJK9_FL2	2005-2014

Seq21	BALOCHISTAN_FL2	2005-2014
Seq22	IND_FL2	2005-2014
Seq23	N1_full	2011
Seq24	N11_FL2	2016
Seq25	N12_FL2	2016
Seq26	N2-1_F	2007
Seq27	N22_FL2	2015
Seq28	N3_FL2	2014
Seq29	N4_FL2	2013
Seq30	N5_p1_F	2013
Seq31	N6_FL2	2013
Seq32	N7_FL2	2013
Seq33	N9-1_RL2	2007
Seq34	Nambal-1_F	2012
Seq35	Nizampur_FL2	2013
Seq36	AJK11_FL2(2)	2009
Seq37	L15_FL2	2017
Seq38	L16_FL2	2017
Seq39	N10_FL2	2008
Seq40	N14_FL2	2007
Seq41	N17_FL2	2015
Seq42	N8_FULL	2007
Seq43	N19_FL2(2)	2006

2. PCR material and method used in this study for the 43 specimen used in the genetic analysis for subspecies identification.

- Sample: 25~100ng/rxn
- Genomic DNA extraction: InstaGene Matrix (BIO-RAD, cat.no.732-6030)
- Genomic DNA extraction-Other: MG Tissue SV (Doctor Protein INC, Korea)
- Taq pol.: Dr. MAX DNA Polymerase (Doctor Protein INC, Korea, cat.no. DR00302)
- PCR machine: DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD)
- PCR product purification: Multiscreen filter plate (Millipore Corp.)
- Sequencing Kit: BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)
- Sequencer: ABI PRISM 3730XL Analyzer (96 capillary type)
- Variant analysis: Variant Reporter Software Version 1.1 (Applied Biosystems)
- Different kit may be used in accordance to the sample characteristics.
- Gel electrophoresis method: 1.5% agarose gel, 20min running at 300V, 200A, 2ul of DNA loaded.
- Ladder: Thermo Scientific GeneRuler DNA Ladder Mix (#SM0331)

3. PCR optimization and pre-denaturation reactions used in PCR program for genetic analysis.

Temperature	Time	Cycles
95 ℃	5 min	
95 ℃	30 sec	
50 ℃	30 sec	35
72 ℃	1 min	
72 ℃	7 min	
4 ℃	Storage	

4. Sequence data of 43 samples obtained in this study (available at NCBI GeneBank via accession numbers MK425702-MK425744).

Sequence ID	**Description**
Seq1	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq2	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq3	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq4	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT

Seq5	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq6	ACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATCTCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTT TCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTTAAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTC GTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACA ACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAA CCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq7	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq8	TATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATCTCTTACGCC TTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTTAAACTAGAT TACTTCTCGATCATCTTCATCCCTGTAGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATT TCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACG CAAACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq9	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT

Seq10	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTAGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq11	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq12	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTAGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq13	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTAGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT

Seq14	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTAGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
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Seq16	TCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATCTCTTACGCCTTCACCATC AGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTTAAACTAGATTACTTCTCG ATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTAATCA CTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAAACAC CGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq17	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT

Seq18	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq19	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTAGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq20	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTAGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq21	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT

Seq22	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq23	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTAGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq24	TATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATCTCTTACGCCTTCA CCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTTAAACTAGATTACT TCTCGATCATCTTCATCCCTGTAGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTA ATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAA ACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq25	TATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATCTCTTACGCCTTCACCATCAGCATAATCCCGGCTAT AATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTTAAACTAGATTACTTCTCGATCATCTTCATCCCTGTG GCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTAATCACTATAATAATCCTAGTAA CCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAAACACCGCCGCCCTACAGGCAAT TCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq26	ATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATCTCTTACGCCT TCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTTAAACTAGATT ACTTCTCGATCATCTTCATCCCTGTAGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTT

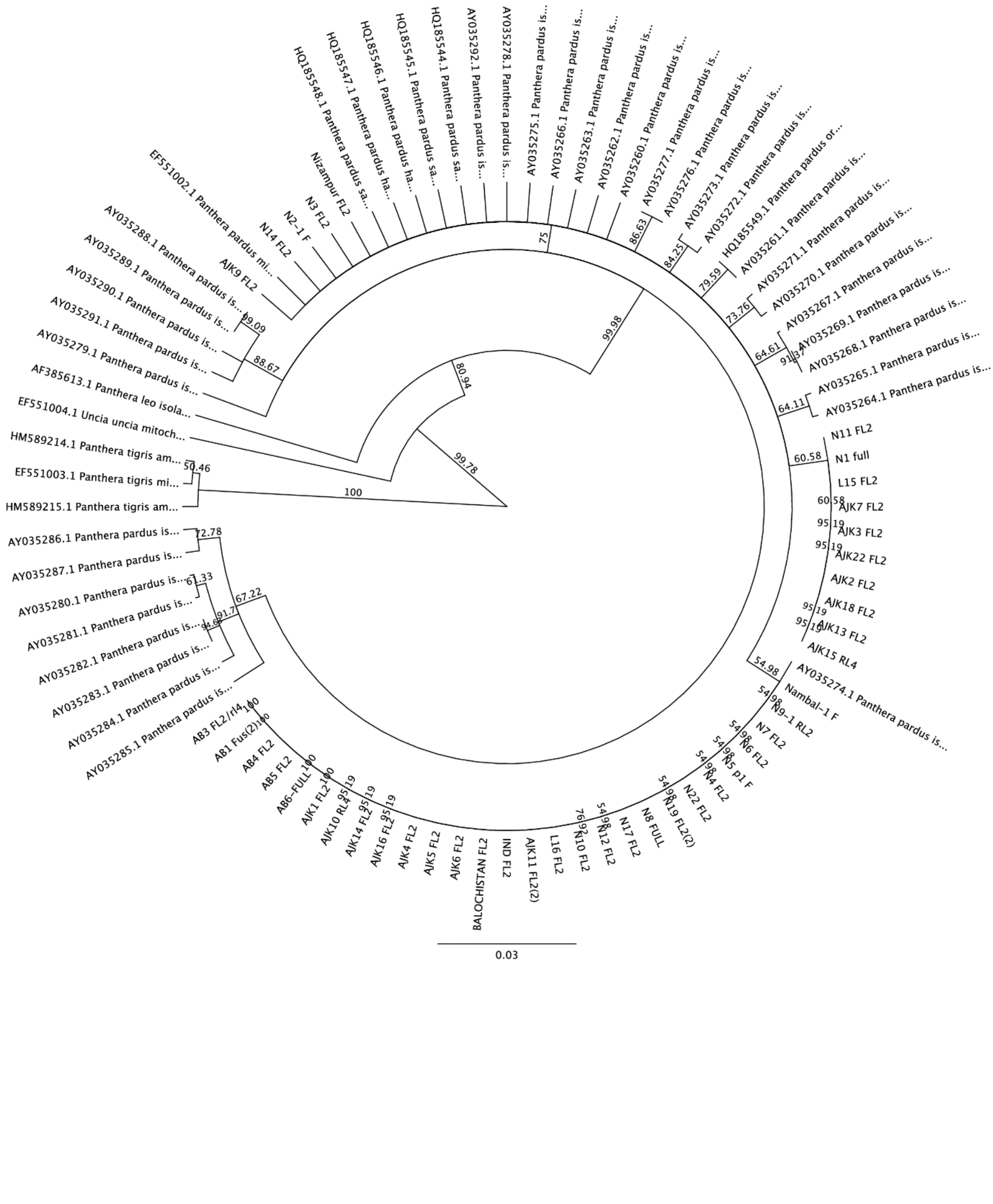
	CTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACG CAAACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq27	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq28	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTTTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTAGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq29	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT

Seq30	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq31	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq32	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq33	TATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATCTCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAA CAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTTAAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCAT AGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTT ATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTA GGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq34	TAATAAACCTATTTATCTTCTTTATACTCACTGCAATATTTATTCTACTCCTACCTATCATTATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATC TCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGAAACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTT AAACTAGATTACTTCTCGATCATCTTCATCCCTGTGGCGCTTTTCGTTACATGATCGATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATC TCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTC

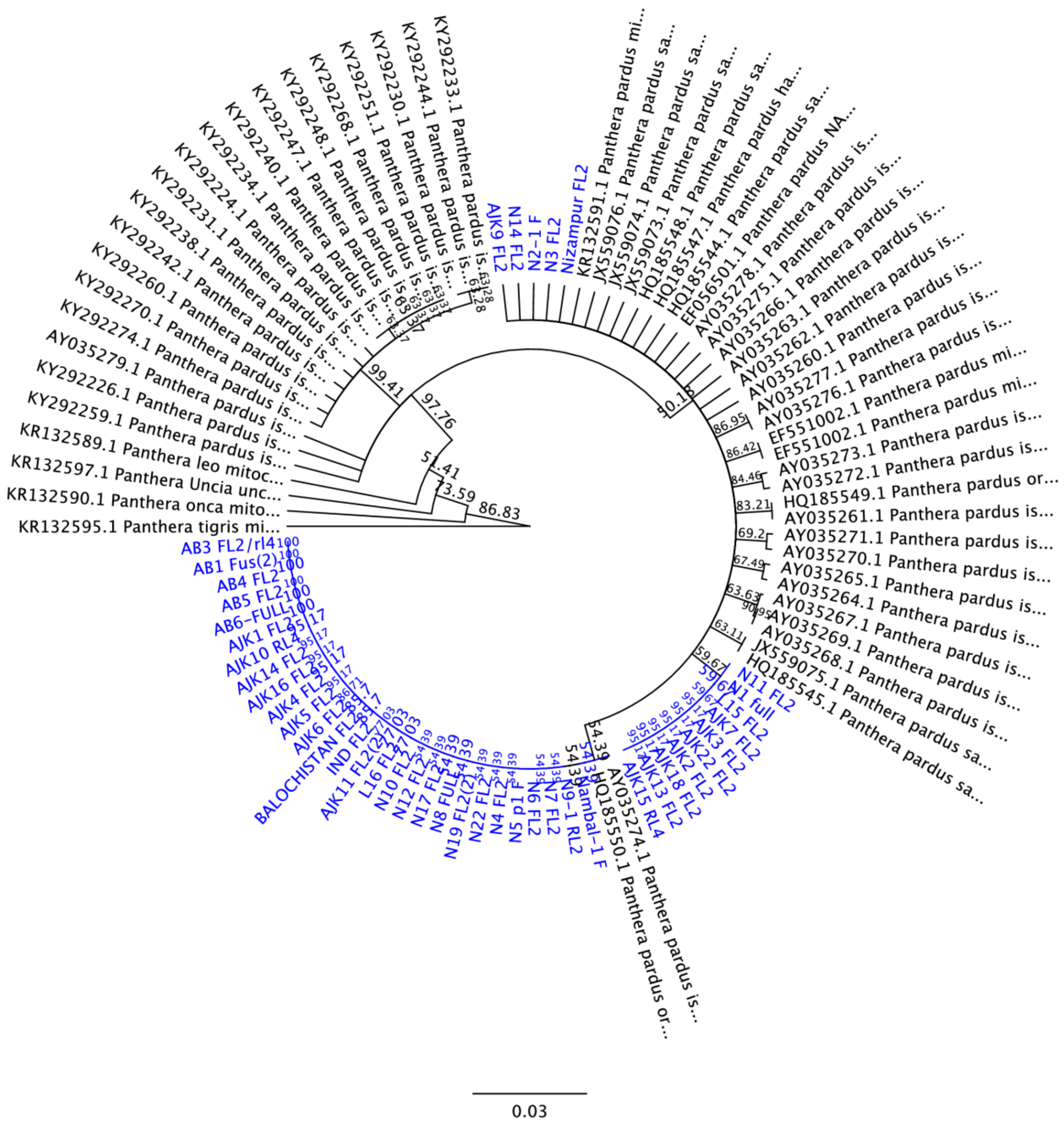
	GAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACA AATCTTTATT
Seq35	TATATCCAACACTCAACTATACAAAAATAACCTATACCCCCACTATGTAAAAACCACAATCTCTTACGCCTTCACCATCAGCATAATCCCGGCTATAATATTCGTTTCCTCCGGACAAGA AACAATTGTCTCAAACTGACATTGACTATCAATTCAAACCCTCAAATTGTCACTAAGCTTTAAACTAGATTACTTCTCGATCATCTTCATCCCTGTAGCGCTTTTCGTTACATGATCGATC ATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGT TTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAAACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGATGT AGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq36	ATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAAC TGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAG ATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq37	ATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAAC TGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAAACACCGCCGCCCTACAAGCAATTCTCTACAACCGCATCGGAGA TGTAGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq38	GATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAA CTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGA GATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq39	GATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAA CTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGA GATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq40	ATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAAC TGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAG ATGTAGGATTTATCACAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT

Seq41	GATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAA CTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGA GATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq42	GATCATAGAATTCTCAATATGATACATACACACAGACCCTTATATCAACCGATTTTTCAAGTATCTCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAA CTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCGGATGATGATATGGTCGAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGA GATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGCATGAGACTTCCAACAAATCTTTATT
Seq43	GATTTTTCAAGTATCTCCTCATATTTCTAATCACTATAATAATCCTAGTAACCGCCAACAACCTGTTCCAACTGTTTATTGGTTGAGAAGGAGTAGGAATCATATCCTTCCTACTCATCG GATGATGATATGGTCGAGCAGACGCAAACACCGCCGCCCTACAGGCAATTCTCTACAACCGCATCGGAGATGTAGGATTTATCATAGCCATAGCATGATTCCTCGCTAATATAAATGC ATGAGACTTCCAACAAATCTTTATT

5. Circle shape maximum parsimony tree generated by comparing 43 samples with the sequences used by Farhadina et al. (2015) from 10,000 bootstrap replicates, number indicates bootstrap percentages.



6. Maximum Likelihood tree generated from comparing 43 samples (highlighted in blue) with the sequences used by Farhadina et al. (2015) from 10,000 bootstrap replicates, number indicates bootstrap percentages.



Appendix C Analysis and information for chapter Five

1. Morphological data: weight, length, height from leopard killed in retaliation or found dead, or obtained from the Wildlife Department between 2008-2018.

Gender	Length	Nick width	Height	Waist	Weight	Date	Location
M	2260	533.4	736.2	711.2	67	3/20/2013	Baragali
M	2257.4	609.6	736.6	711.2	60	5/3/2015	Samandar katha
M	2235.2	609.6	736.6	724.2	65	3/15/2015	Tarnawai
M	2184.4	406.4	736.6	685.8	49	9/2/2013	Haripor
M	2133.6	457.2	711.2	685.8	45	2/13/2013	Qalandeabad
M	2133.6	584.2	736.6	762	54	Mar-17	Haripur
M	2257.4	609.6	762	812.8	58	11/10/2018	Bandi
M	1981.2	406.4	609	685	45	2/27/2008	Balolia
M	2184.4	406.4	736.6	711.2	50	1/2/2013	Haripor
F	2057.4	457.2	736.2	711.2	45	5/8/2015	Samandekata
F	1828.8	457	660.4	762	48	12/8/2017	Haripur
F	2032	406.4	584.2	685.8	48	10/1/2011	Nakar kotwal
F	2057.4	457	609.6	685.8	45	5/13/2016	
F	2057.4	431.8	635	838	50	5/8/2015	NAMBAL
F	1727.2	355.6	584.2	685.8	40	3/12/2015	S kata
F	1828.8	482.6	660.4	685.8	35	10/2/2014	Singlekot
F	2184	482.6	711.2	838.2	38	1/6/2013	Palak
F	1524	355.6	431.8	609.6	32	12/3/2015	

Appendix D   Analysis and information for chapter six

1. Awareness sessions delivered in schools, communities and when visiting students in close proximity to the study area**.**