Phylogeny and Ecology of the Himalayan Wolf

Thesis for the Degree of Doctor of Philosophy in Zoology

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Dedicated to my mother Béatrice Werhahn and my father Peter Werhahn.

Contents

Abstract

This thesis researches the evolutionary history, phylogeny, and ecology of the overlooked Himalayan wolf of the Asian high-altitudes. It explores why this wolf has evolved, what may have been the ultimate causes that led to its speciation, and what proximate mechansism may be responsible for its persistence as a distinct wolf lineage. This work aims to ascertain the appropriate taxonomy of the Himalayan wolf and advance the conservation of this unique high-altitude wolf. Himalayan wolves have been largely overlooked by science and conservation until recently, and their formal taxonomic classification is pending. This thesis presents unprecedented phylogenetic insights into this taxon, its distribution range, and ecology. It draws genetic inferences about its speciation based on samples from across the estimated range, analysed at diverse genetic markers from the mitochondrial to the nuclear DNA including functional genes.

The research was conducted in three study areas in Nepal, from Humla in the far north-western Nepalese Himalayas, to Dolpa in the west, to Kanchenjunga Conservation Area in the far north-eastern Himalayas, and two sites on the Tibetan Plateau of China, Namsai and Zhaqing in the Sanjiangyuan National Nature Reserve in Qinghai province. In addition, genetic samples from a wider range of regions of the Tibetan Plateau of China, the Tian Shan Mountains in Kyrgyzstan, and the southern Pamir Mountains in Tajikistan have been included in the phylogenetic analysis, in collaboration with several research groups.

Wolf faecal samples were analysed with novel tailor-made genetic protocols developed in collaboration with the WildGenes Laboratory at the Royal Zoological Society of Scotland for the non-invasive study of an elusive carnivore. Subsequently, using genetically verified sample locations and the maximum entropy algorithm, the first distribution range approximation to date was modelled for the species.

Himalayan wolves present a phylogenetically distinct *Canis* lineage. The phylogenetic distinction of the Himalayan wolf from the Holarctic grey wolf is supported by mitochondrial DNA (D-loop and cytochrome *b*), the ZF protein gene sequences on the Y and Z sex chromosomes, a microsatellite panel of 17 loci, and four Single Nucleotide Polymorphisms (SNPs) covering three hypoxia-pathway related functional nuclear genes.

Furthermore, a unique genetic adaptation to life in the extreme high-altitudes, where low available oxygen levels challenge all life forms, was revealed for the Himalayan wolf. This hypoxia adaptation was not found in any of the other wolf lineages tested in the study. It seems to give the Himalayan wolf a fitness benefit and selective advantage in the extreme high-altitude habitats compared to the Holarctic grey wolf which is found in the surrounding lower lying habitats. The adaptation is found in diverse functional genes of the nuclear genome presumably involved in the hypoxia pathway, which is the physiological mechanism to cope with low oxygen levels at high-altitudes. The Himalayan wolf is found in habitats above 4,000m elevation in the Himalayas (Nepal and India) and the Tibetan Plateau of Qinghai and the Tibetan Autonomous Region (TAR, China), whereas the surrounding lower elevation regions are inhabited by grey wolves - the Indian wolf *C. l. pallipes* to the south and Holarctic grey wolf *C. lupus* to the west, north, and east. In the distribution boundaries of Himalayan and grey wolves, admixed individuals were found in a geographically stable belt where the elevation is gradually dropping. These admixed individuals showed a consistent pattern of Himalayan wolf mtDNA, and the correlated hypoxia adaptation on most of the SNPs, while their nuclear DNA appeared intermediate between Himalayan and grey wolves. Such admixed individuals at the distribution boundaries of wolf-like *Canis* lineages would be expected, and they may serve an important evolutionary function.

The dietary study of Himalayan wolves presented in-depth insights into their diet by relating what the wolves have eaten to the abundance of respective prey species in the landscape across the study areas. The findings revealed that Himalayan wolves consistently consistently used wild prey species over-proportionally, with a preference for Tibetan gazelle (*Procapra picticaudata*), a small-sized plain-dwelling ungulate. Depredation of livestock, leading to retaliatory killings as a key threat for the Himalayan wolf, was enhanced during the herding season by the high abundance of livestock compared to that of wild prey. This leads to high encounter rates with livestock compared to wild prey and further exacerbates the problem by livestock competing with and displacing wild prey. The herding season temporally overlaps with the wolf pup rearing season, a time when wolves are spatially bound to the denning area, also intensifying conflict. Extensive killing of wolves as retaliation and for illegal wildlife trade was documented.

Conservation actions for the Himalayan wolf are recommended based on the combined results from the phylogenetic research, the dietary habits study and the insights on the local people's perspective of wolves. Conservation must move away from a single species focus to a more holistic conservation approach including the entire carnivore guild. It ought to closely involve local communities and provide them with support from education to financial and material resources. Livestock herding ought to become more sustainable while wild prey populations ought to be restored and safeguarded.

Protecting top predators is increasingly recognized in its importance for maintaining ecosystem function and integrity. The Asian high-altitude ecosystems, home to the Himalayan wolves, present some of the last intact wilderness habitats on our planet and host the water resources for billions of people in southeast Asia. As such, the protection of the Himalayan wolf, sympatric wildlife and its ecosystem, is of global interest.

In conclusion, this phylogenetic research confirms that the Himalayan wolf is an evolutionarily distinct taxon basal to the Holarctic grey wolf, and thus merits taxonomic recognition. It is deeply diversified, to a similar extent as the African wolf (recently recommended to be reclassified as *Canis lupaster*), and more ancient and genetically diversified than any of the presently acknowledged Holarctic grey wolf subspecies. The high-altitude adaptation, unique to the Himalayan wolf, is hypothesized to be a key cause for its speciation and an important mechanism for its persistence by preserving its genetic integrity. This hypothesis is supported by a) the Himalayan wolf's unique genetic adaptation, b) the distributional restriction of the Himalayan wolf to habitats above 4,000m elevation, c) its distribution range being discrete and adjacent to that of the grey wolf, and d) elevation being the main predictor for its distribution.

This thesis informs the Himalayan wolf's taxonomic classification, a critical step required based on the widespread evidence from phylogeny and ecology to its differentiated vocalisation. An appropriate taxonomy is crucial to advance conservation by paving the way for assigning the Himalayan wolf an IUCN Red List Status. These two steps of formal recognition, taxonomic classification and red listing, will provide much needed leverage to raise awareness in the scientific community, governments, and conservation agencies to include this charismatic high-altitude wolf in their conservation and research focus.

Declaration

All contents of this doctorate thesis are the results of my own original research. Any research parts resulting from collaborations are clearly indicated.

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List of original publications

Chapter 2

Geraldine Werhahn, Helen Senn, Jennifer Kaden, Jyoti Joshi, Susmita Bhattarai, Naresh Kusi, Claudio Sillero-Zubiri and David W. Macdonald. **Phylogenetic evidence for the ancient Himalayan wolf: towards a clarification of its taxonomic status based on genetic sampling from western Nepal**. 2017. Royal Society Open Science.

> **Appendix B-1: Werhahn et al. 2017. Supplementary Material**. Phylogenetic evidence for the ancient Himalayan wolf: towards a clarification of its taxonomic status based on genetic sampling from western Nepal. Royal Society Open Science.
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> **Author contributions:** GW has collected the field data, participated in data analysis, carried out sequence alignments, participated in the design of the study, conceived of the study and drafted the manuscript. HS has developed the genetic analysis protocols, participated in data analysis, carried out sequence alignments and participated in drafting the manuscript; JK has participated in developing the genetics protocol and participated in data analysis; JJ and SB carried out the molecular laboratory work and participated in data analysis; NK has collected field data, participated in data analysis and participated in drafting the manuscript; CS-Z and DWM have contributed to conceiving of the study, participated in the design of the study and helped draft the manuscript.

Chapter 3

Geraldine Werhahn, Helen Senn, Muhammad Ghazali, Dibesh Karmacharya, Adarsh Man Sherchan, Jyoti Joshi, Naresh Kusi, José Vincente López-Bao, Tanya Rosen, Shannon Kachel, Claudio Sillero-Zubiri, and David W. Macdonald. **The unique genetic adaptation of the Himalayan wolf to high-altitudes and consequences for conservation.** 2018. Global Ecology and Conservation.

Appendix B-2: Werhahn et al. 2018. Supplementary material. The unique genetic adaptation of the Himalayan wolf to high-altitudes and consequences for conservation. Global Ecology and Conservation.

Author contributions: GW has collected the field data, participated in data analysis, carried out sequence alignments, participated in the design of the study, conceived of the study and drafted the manuscript; HS has developed the genetic analysis protocols, participated in data analysis, carried out sequence alignments, contributed to conceiving the study, and participated in drafting the manuscript; MG, DK, AMS and JJ worked on the laboratory analysis; NK has collected field data, participated in data analysis and participated in drafting the manuscript; JVLB, TR and SK have contributed samples and supported revising the manuscript, CS-Z and DWM have contributed to conceiving of the study, participated in the design of the study and helped draft the manuscript.

Chapter 4

Geraldine Werhahn, Yanjiang Liu, Meng Yao, Chen Cheng, Zhi Lu, Luciano Atzeni, Zhixiong Deng, Kun Shi, Xinning Shao, Qi Lu, Jyoti Joshi, Adarsh Man Sherchan, Dibesh Karmacharya, Hemanta Kumari Chaudhary, Naresh Kusi, Byron Weckworth, Shannon Kachel, Tatjana Rosen, Zairbek Kubanychbekov, Khalil Karimov, Jennifer Kaden, Muhammad Ghazali, David W. Macdonald, Claudio Sillero-Zubiri, and Helen Senn. **Himalayan wolf distribution and admixture based on multiple genetic markers.** 2019. Submitted for publication.

Appendix B-3: Werhahn et al. Supplementary material. 2019. Himalayan wolf distribution and admixture based on multiple genetic markers.

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Chapter 5

Geraldine Werhahn, Naresh Kusi, Xiaoyu Li, Cheng Chen, Lu Zhi, Raquel Lázaro Martín, Helen Senn, Claudio Sillero-Zubiri, and David W. Macdonald. **Himalayan wolf foraging ecology and the importance of wild prey.** 2019. Global Ecology and Conservation.

Appendix C: Werhahn et al. 2019. Himalayan wolf foraging ecology and the importance of wild prey. **Microscopic hair reference collection.**

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Chapter 6

Geraldine Werhahn, Helen Senn, David W. Macdonald, Claudio Sillero-Zubiri **Canids challenge Taxonomy: A Review of Asian Wolves.** In Preparation for submission.

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Chapter 1.

GENERAL INTRODUCTION

General Introduction

This thesis researches the phylogeny and ecology of the overlooked Himalayan wolf, unique to the Asian high-altitudes. The Himalayan wolf, also called Tibetan wolf, presents an evolutionarily distinct wolf lineage that has been overlooked by science and conservation until recently (Aggarwal et al., 2007; Sharma et al., 2004; Shrotryia et al., 2012). When this thesis research was initiated the available studies on these wolves were few: Sharma et al. (2004) and Aggarwal et al. (2007) had used samples from museum specimen and zoo animals to study mitochondrial DNA markers and were the first to illustrate these wolves' phylogenetic distinctness. Sharma et al. (2004) had proposed a divergence time estimate of 0.8Ma before present based on molecular clock estimates of the control region of the mtDNA. Shrotryia et al. (2012) discussed the confusion around wolves in this region and provided a summary of the historical perspective on them. And Zhang et al. (2014) provided insights into the high-altitude adaptation of wolves from Qinghai Tibet (at the time it was not clear that these wolves present the same lineage as the Himalayan wolf). The here presented research builds on these earlier studies to explore the Himalayan wolf's phylogeny and evolution considering the biogeographic history of the Himalayas and the Tibetan Plateau where this wolf occurs. It explores why the Himalayan wolf has evolved and what ultimate causes may have been responsible for its genetic diversification and speciation. And it explores how this wolf is maintained alongside the Holarctic grey wolf *Canis lupus* found in most other parts of the Northern Hemisphere, that is, what proximate mechanisms are responsible for the genetic maintenance of the Himalayan wolf. This is particularly interesting from an evolutionary biology perspective because the Himalayan wolf and the Holarctic grey wolf present parapatric wolf lineages in Asia, and wolves hybridize and disperse over long distances (Ciucci et al., 2009; Geffen et al., 2004; Mech et al., 1995), with both these characteristics facilitating gene flow and thereby counteracting lineage diversification.

This raises questions around why the Himalayan wolf exists and has persisted until today. Is it geographically isolated from the Holarctic grey wolf? Does it have some specific adaptation and fitness benefit? Or is there another explanation for its existence? Seeking answers to these questions is the quest of this thesis, along with providing the scientific data basis for the taxonomic classification of the Himalayan wolf and informing its conservation with a fundamental understanding of its ecology and main conservation threats.

The Himalayan wolf is a top predator of the Asian high-altitudes. These high-altitude wilderness regions are globally significant as they comprise biodiverse and specialized ecosystems that provide the water resources for billions of people in Asia. The protection of a top carnivore can support wider conservation benefits for the entire ecosystem as reflected in the umbrella species concept (Lambeck, 1997).

The Himalayan wolf may present a charismatic umbrella species along with the snow leopard for the conservation of these high-altitudes. Studies around the trophic ecology of carnivores are increasingly illustrating the importance of carnivores for ecosystem functioning and biodiversity maintenance (Estes et al., 2011; Pace et al., 1999; Ripple et al., 2014; Sergio et al., 2005). For example, the presence of wolves can affect the behaviour of their prey and mesopredators. Foraging herbivores respond to predation risk by differentiated habitat use which can enhance structural habitat diversity and biodiversity in ecosystems (Fortin et al., 2005; Ripple et al., 2001; Ripple and Beschta, 2012, 2004).

While the Holarctic grey wolf of Europe and North America is well studied (Boitani et al., 2018), the wolf populations in the Himalayas and the Tibetan Plateau remain understudied. But genetic evidence, including this thesis research, is rapidly accumulating in support of its evolutionary distinction and calling for a taxonomic recognition (Aggarwal et al., 2007; Koepfli et al., 2015; Leonard et al., 2007; Pilot et al., 2010; Sharma et al., 2004; Werhahn et al., 2017a, 2018; Werhahn and et al., 2019). The Himalayan wolf appears to have diverged from the ancestors of the Holarctic grey wolf (Aggarwal et al., 2007; Pilot et al., 2010; Rueness et al., 2011) before the radiation of the grey wolf in the middle Pleistocene approximately 0.7-0.3 Ma ago (Sotnikova and Rook, 2010; Tedford et al., 2009; Vilà et al., 1999). The Holarctic grey wolf comprises different subspecies and forms a relatively recent genetic lineage (Gaubert et al., 2012; Wozencraft, 2005). In the evolutionary history from the ancestors of the wolf-dog clade in the early to middle Pleistocene (Tedford et al., 2009) to the contemporary Holarctic grey wolf, different wolf lineages such as the Himalayan wolf, the African wolf *C. lupaster* (Gaubert et al., 2012; Koepfli et al., 2015; Alvares et al., 2019; Rueness et al., 2011; Viranta et al., 2017), and the Indian grey wolf *C. l. pallipes* (Sykes, 1831) diverged as monophyletic clades (Sharma et al., 2004). The diversification of the Himalayan wolf has been estimated at approximately 0.8 Ma before present by Sharma et al. (2004) and is addressed in more detail in Werhahn et al. (2018)/Chapter 3.

Early explorers noted the morphologically different wolves on the Tibetan Plateau and summarized their observations in historical descriptions, e.g. “Description of the Changu or wolf of Tibet” (*Lupus laniger*) by Hodgson (1847); “ Notice of the Chanco or Golden wolf (*Canis chanco)* in the Chinese Tartary*”* by Gray (1863). Hodgson (1947) notes a “wolf, with long, sharp face, elevated brows, broad head, large pointed ears, thick woolly pelage, and very full brush of medial length. Above dull earthy brown; below with the entire face and limbs yellowish-white. No marks on the limbs. Tail concolours with the body, that is, brown above and yellowish below, and no dark tip. Length four feet. Height two and a half feet. This animal is common all over Tibet, […]. It has the general form of the European wolf; but its colour is very different, and it has more elevated brows, larger ears, and a much fuller brush. Its pelage is also dissimilar and unique. From this last circumstance I derive its specific name, having no doubt that it is a new species […].” And Gray (1863) noted “It is a very showy animal, rather larger than the common European Wolf. […]. Fur fulvous, on the back longer, rigid, with intermixed black and grey hairs; the throat, chest, belly and inside of the legs pure white; head pale grey-brown; forehead grizzled with short black and grey hairs. Hab. Chinese Tartary. Called *Chanco*. The skull is very like, and has the same teeth as, the European Wolf (*C. lupus*). The animal is very like a Common wolf, but rather shorter on the legs; and the ears, the sides of the body and outside of the limbs are covered with short pale fulvous hairs. The length of its head and body is 42 inch; tail 15 inch.”

The morphological dissimilarity of this Asian high-altitude wolf to the European wolf was well noted by these different explorers but they used different names to describe it. The different historical names have led to a variety of names used for this wolf until today, also reflected in recent scientific publications (e.g. Tibetan wolf *C. laniger* (Shrotryia et al., 2012; Hodgson, 1847); Tibetan or Mongolian wolf *C. chanco* (Chetri et al., 2017, 2016; Gray, 1863; Sharma et al., 2004); Tibetan wolf *C. filchneri* (Bocci et al., 2017; Matschie, 1908); and Himalayan wolf *C. himalayensis* (Aggarwal et al., 2007; Werhahn et al., 2017a, 2017b). This name confusion is addressed in more detail in Chapter 2

(Werhahn et al., 2017a) aiming to achieve clarification.

Later Blanford (1898), merged the wolves of the Tibetan Plateau with the taxon *C. lupus,* and describes this "wolf variety" found in Tibet and Ladakh as pale coloured wolf but also noted black individuals. These varied morphological observations match the observations of the Himalayan wolf in the Nepalese Himalayas and Qinghai Tibetan Plateau during the field researches for this thesis (Figure 1.1).

The type specimen and voucher species are held in European museums (e.g. specimen from the collection by B. Hodgson from Nepal (NCBI GenBank accession AY333738; BM58.6.24.61) and a black specimen (NCBI GenBank accession AY333739; BM99.12.29.1) are held in the Natural History Museum in South Kensington (UK) (Sharma et al., 2004). The scientific name for this wolf has recently been recommended to be *C. lupus chanco* until more evidences become available (Alvares et al., 2019).

Figure 1.1. Himalayan wolves show sand-brown to grey pelt morphology (A), but black individuals are also found (B) (Figure from Werhahn et al. 2017a/Chapter 2).

Conservation status and legal protection of wolves across the Himalayan wolf range

Globally, the grey wolf *C. lupus* is classified as ‘Least Concern’ by the IUCN Red List of Threatened Species (Mech and Boitani 2010). In Nepal, the grey wolf *C. lupus*[*] is classified as ‘Critically Endangered’ by the IUCN National Red List Series based on assumed low population numbers and declining population trends (Jnawali et al. 2011). The grey wolf is protected under Nepal’s National Parks and Wildlife Conservation Act 2029 (1973) as a priority species (GoN 1973, 2015). In India, the Tibetan wolf *C. l. chanco*[*] and the Indian wolf *C. l. pallipes* are protected under Schedule I of the Wildlife Protection Act 1972. In China the grey wolf *C. lupus* is listed as second-class protected wildlife, meaning it is strictly protected and hunting is prohibited (Harris 2014). Based on emerging evidences the wolves in western China, specifically the populations in the Tibetan Autonomous Region (TAR) and Qinghai belong to the Himalayan wolf lineage. CITES lists grey wolves in Appendix II except for wolf populations in Nepal, Bhutan, India and Pakistan being listed in Appendix I, which renders all trade of wild specimens or their parts illegal (CITES, 2017).

[*] Based on emerging evidence, the referred wolf populations are assumed to belong to the Himalayan wolf.

Taxonomy today: challenges and consequences

Taxonomy is the science of categorising biological entities into distinct groups based on shared characteristics. The taxonomic system used today categorises organisms with a binomial nomenclature and traces back to the Swedish botanist Carl Linnaeus in the 18th century (Linnaeus, 1758). Nature is continuous (Galtier, 2019) and taxonomy attempts to impose a discrete ordering system upon it (Zachos, 2016). This discrepancy is at the root of the long-standing discourse of how best to define species which, despite being artificial entities, serve an important function for conservation, legislation, society and science. This species debate has received a lot of scientific interest over the years and in the process different species concepts have been developed (Frankham et al., 2017, 2012). Of the currently around 27-30 different species concepts

(Frankham et al., 2012; Zachos, 2016), none seems to conclusively satisfy the scientific community. As a consequence different species concepts are used as foundation for taxonomic decisions in different taxonomic groups, e.g. for birds the Biological species concept (SC) is often used while for mammals it is often the Phylogenetic SC, which leads to taxonomic heterogeneity (even termed anarchy by Garnett and Christidis (2017)) between and often also within groups (Galtier, 2019).

In the following a brief introduction of the more recent and commonly used species concepts that may be useful in the interpretation of the findings of this thesis. The Biological SC (Mayr, 1942) is based on populations that are reproductively isolated, i.e. populations that do not produce fertile offspring. This species concept had to be reconsidered later on with the discovery of the wide occurrence of gene flow and admixture between taxa (Barton and Hewitt, 1985). The Genetic SC focuses on genetic isolation rather than reproductive isolation (Baker and Bradley, 2006), while the Phylogenetic SC defines species as the smallest definable cluster of individuals with shared ancestry (Cracraft, 1983). The Unified SC by Queiroz (2005) argues that all modern species concepts have a common element, namely a species is a “separately evolving metapopulation lineage”. The Differential Fitness SC by Hausdorf (2011) proposes that “species can be defined as groups of individuals that are reciprocally characterized by features that would have negative fitness effects in other groups and that cannot be regularly exchanged between groups upon contact.” There are two common principles that underlie most species concepts, firstly there is some level of reproductive isolation between the species and secondly, there is phylogenetic continuity, i.e. shared ancestry (Medicine et al., 2019).

While decades ago, gene flow and admixture was not generally known to occur among mammalian species, it is now documented in many taxonomic groups, e.g. canids (Gopalakrishnan et al., 2018), humans (Huerta-Sanchez, 2014; Reich et al., 2010), pigs (Ai et al., 2015), etc. Today the complete absence of admixture is no longer a useful criterion for defining species (Medicine et al., 2019). Hybridization, introgression and cross-species exchange of genes during the evolution of species are increasingly documented through genomic methods (Arnold, 2006; Mallet, 2005) and make the interpretation of the data for taxonomic decisions challenging (Medicine et al., 2019).

Ultimately, taxonomy has to determine whether the group of individuals, at the present snapshot in time, is a population in process of speciation or dissolution and wheter it is distinct enought for it to merit taxonomic recognition (Medicine et al., 2019).

Taxonomy uses specific traits and characteristics of the individuals and populations considered. Historically morphological traits have been used, whereas with the rise of phylogenetic molecular methods this field has increasingly gained importance for taxonomy (Chambers et al., 2012; Thiel and Wydeven, 2011). Today multiple lines of evidence should be integrated considering the following: morphologic, behavioural and ecological, molecular (genetic and genomics), and biogeographic traits (e.g. geographic barriers relevant to the taxon or biogeographic events coinciding with the taxon's coalescence times) (Kitchener et al., 2017; Medicine et al., 2019). Often these traits show overlapping distributions among populations within a species and between species (Hey and Pinho, 2012; Nowak, 1995), so it is a question of where to draw the taxonomic boundaries. Morphology may be misguided by convergent evolution of traits given similar environments (e.g. the African wolf *C. l. lupaster* of North Africa considered a Golden Jackal *C. aureaus* (Rueness et al., 2011)). Molecular techniques are getting more sophisticated and with that the data is also becoming more challenging to interpret. Genomics is increasingly being considered important for taxonomic decisions but given the increasing documentation of gene flow and admixture across taxa that parallels the advancement of the method, genomics, like the other traits, can be gradual and may by itself not be conclusive for taxonomic decision making. Further, if the importance of genomics is being overweighed then there is the risk that taxonomic decisions are increasingly being made in only the best equipped genomic laboratories detached from other biological aspects of the described organisms.

A fundamental reason for differing taxonomic interpretations is that they are based on different datasets (such as different molecular markers, different morphological traits, etc.) which are analysed with different statistical methods, and following different species concepts and criteria for nomenclature (Bercovitch et al., 2017). But the consequences for the involved organisms of this taxonomic heterogeneity can be detrimental and hence a best practice for a consistent species delineation is needed (Garnett and Christidis, 2017).

Such a best practice for species delineation in mammals may adopt an approach that incorporates molecular, biogeographical, ecological and behavioural, and morphological traits. In practice this means to combine the molecular, biogeographic, behavioural and ecological data of a sampled specimen with morphological measurements of the same. But to date this approach is not often seen as it can be logistically challenging. Focussing in on standardizing the molecular traits, Hey and Pinho (2012) propose a combination of divergence time estimates and genetic distance to define species. Critically, at least within taxonomic orders the same set of criteria should be applied and ideally within entire taxonomic classes or even kingdoms (Garnett and Christidis, 2017; Isaac et al., 2004). Attempts to revise taxonomies based on consistent and transparent criteria are promising, such as the revised Felidae taxonomy by Kitchener et al. (2017), which is based on a traffic light system evaluating molecular, biogeographic and morphological data per taxon.

Within taxonomy there has been a longstanding debate among taxonomic splitters and lumpers, i.e. those that believe in keeping populations taxonomically separate and those that believe in combining them under the same taxon. This debate has direct biological consequences for the involved organisms as it means combined management possibly risking outbreeding depression but protecting genetic diversity versus separate management risking inbreeding depression (Senn et al., 2014). Wolves are notorious for challenging taxonomy and this is exemplified by the complex discourse around North American wolf lineages (Chambers et al., 2012; Cronin et al., 2015; Fredrickson et al., 2015; Hohenlohe et al., 2017; Nowak, 2002; Rutledge et al., 2015; vonHoldt et al., 2016; Weckworth et al., 2010; Wilson et al., 2000). The matter is further complicated due to the wolves' characteristics of long dispersal distances, ready hybridization, adaptation, flexibility and generalist nature (Adams et al., 2003; Ciucci et al., 2009; Macdonald et al., 2019; Mech and Boitani, 2003). But splitting or lumping is also a point of contention in other groups, e.g. antelopes (Senn et al., 2014), giraffes (Bercovitch et al., 2017; Fennessy et al., 2016), birds (Gill, 2014; Zink and Blackwell, 1998), felids (Culver et al., 2000), chameleons (Prötzel et al., 2017).

To complicate things further the validity and usefulness of subspecies as subcategory of species is also debated (Cronin et al., 2015; Futuyma, 1998; Mayr, 1970; Zink, 2004). Modern concepts of subspecies characterise the respective populations as exhibiting partial restriction of gene flow, and some degree of phylogenetic distinctness while being reproductively compatible (Medicine et al., 2019).

Zachos (2018) argues that an ‘objective species delimitation is impossible making both lumping and splitting equally correct or incorrect’ and consequently delimited species are not necessarily meaningful taxonomic units for conservation.

Given these disagreements around species and subspecies delineation, the concept of Evolutionary Significant Unit (ESU) may be an approach that is more practical for conservation as it can prioritize units for conservation management irrespective of the underlying taxonomy (Moritz, 1994; Ryder, 1986). ESUs recognize populations based on ecological or genetic differences that have adaptive significance and/or a unique evolutionary history (Crandall et al., 2000; Isaac et al., 2004). The ESU approach can help conservation action in the short- and mid- term by circumventing the at times lengthy taxonomic debates which are not conducive to the conservation of the rapidly diminishing biodiversity. But ESUs cannot solve the problems faced by taxonomy because also ESUs require definition and an appropriate taxonomy should be the final aim.

There is an increasing call to empower conservation to fulfil its role of protecting evolutionary unique populations irrespective of taxonomy and related political and legal consequences (Haig et al., 2006; Mace, 2004; vonHoldt et al., 2016).

May it be the designation of ESU, splitting or lumping of lineages or taxonomy as a whole, all of these delineations have drastic consequences for the involved organisms. These consequences go far beyond the biological consequences of combined versus separate management, but affect conservation, legislation, financial, ethical and welfare aspects around the involved organisms (Haig et al., 2006; Isaac et al., 2004; Morrison et al., 2009; Pillon and Chase, 2007; Zachos et al., 2019; Zink, 2004). And there is often a time lag of updating conservation legislation after taxonomic changes occur (Garnett and Christidis, 2017; Zhou et al., 2016).

Mammalian taxonomy must be based on solid and scientifically sound grounds, i.e. based on carefully selected and widely applied criteria including aspects around their morphology, phylogeny, ecology and behaviour, and biogeography of the involved organisms.

This thesis aims to provide to the phylogenetic, ecological and biogeographic database around the contemporary Himalayan wolf population found in the Asian high-altitudes to inform their taxonomy and in the process also reconsiders the consistency within the currently accepted wolf taxonomy.

Thesis objectives

This thesis researches the phylogeny and ecology of the Himalayan wolf to understand its evolution and inform its taxonomy and conservation. Clarifying its phylogeny supports its appropriate taxonomic classification, which is an important basis for conservation, while understanding the dietary habits of the Himalayan wolf can inform conservation action. The research was triggered by the question of what kind of evolutionary and taxonomic group the Himalayan wolf population presents and how it came into existence in evolutionary time considering the global wolf distribution.

Specifically, the research questions are:

1. What caused the reproductive isolation of the Himalayan wolf and when did this genetic divergence occur?
2. Does the phylogenetic difference between the Himalayan wolf and Holarctic grey wolf justify the classification of the Himalayan wolf as a distinct taxon?
3. What ecological, behavioural or geographical mechanism(s) may maintain the genetic persistence of the Himalayan wolf?
4. What is the distribution range of the Himalayan wolf?
5. What are the main prey species and dietary habits of the Himalayan wolf?
6. How do these insights around the Himalayan wolf relate to contemporary global wolf taxonomy and what can be learnt from it?

In the process of attempting to answer these questions, this thesis 1) places the Himalayan wolf in the phylogeny of modern canids, 2) explores the ultimate evolutionary causes for its diversification, 3) explores the proximate mechanisms that may be maintaining its genetic diversification from the Holarctic grey wolf, and 4) provides first insights into the Himalayan wolf's distribution range based on a landscape scale dataset. It then investigates the 5) Himalayan wolf's dietary habits by relating prey species consumed by the wolves to prey abundance in the landscape. And 6) a review of the Asian wolf lineages shall a) provide the canid family's phylogenetic context within which to understand the phylogeny and taxonomic positioning merited by the Himalayan wolf, and b) provide insights for a revision of wolf taxonomy.

Thesis structure

Following this introduction, the data **Chapter 2** presents the first scientific data to date on the status and phylogeny of wolves in the Nepalese north-western Himalayas and this also presents the first in-depth genetic study of contemporary free-ranging Himalayan wolf populations. Data **Chapters 3 and 4** then take the genetics work of Chapter 2 to a larger scale with respect to both the landscape and genomic regions considered. **Chapter 3** includes wolf data from three study sites across the Nepalese Himalayas from Humla in the far northwest to Kanchenjunga Conservation Area in the far north-east of Nepal. **Chapter 4** builds on the findings on Himalayan wolves in Nepal and includes data from across the Tibetan Plateau of China, the Tien Shan Mountains in Kyrgyzstan and Xinjiang Province of China, and Pamir Mountains in Tajikistan. The wolf faecal samples are analysed at a range of genetic markers from the mitochondrial to the nuclear DNA including functional genes. The genetics work is based on a tailor-made suite of genetic protocols specifically developed or adapted for this research in collaboration with the WildGenes Laboratory at the Royal Zoological Society of Scotland and presented as toolkits for future researches of the species. Data **Chapter 5** investigates the dietary habits of the Himalayan wolf. The study reveals important prey species and trends for prey use, and thereby provides conservation relevant insights into depredation conflict.

The review **Chapter 6** summarizes current knowledge on the evolution and taxonomy of wolves in Asia. The General Discussion **Chapter 7** provides a research synthesis, implications for conservation and taxonomy, and outlines future research requirements. The research findings around phylogeny, distribution, and ecology are discussed in an attempt to answer the questions raised around the causes and mechanisms for the diversification and persistence of the Himalayan wolf.

Over the course of this thesis research the debate around wolf taxonomy has advanced and with that the understanding of the most appropriate scientific name to be used for the Himalayan wolf. Specifically, this development shows in this thesis where in Chapter 2 the name *Canis (lupus) himalayensis* is used for the Himalayan wolf to show respect towards Aggarwal et al. (2007) who in addition to Sharma et al. (2004) conducted important first genetic studies on these wolves. The name *himalayensis* seemed appropriate and the least ambiguous at the time. *Lupus* is noted in parentheses as the appropriate taxonomic level for the Himalayan wolf was and remains undecided. Later it emerges that based on taxonomic priority ruling the name *Canis lupus himalayensis* is taxonomically not valid as it is a *nomen nudum*. Experts advise the use of *Canis lupus chanco* (Alvares et al., 2019). But also *Canis lupus laniger* is used in the literature and in data bases such as GenBank. *Canis lupus chanco* has been used ambiguously in the past as it has also been used for wolves in Mongolia which belong to the Holarctic grey wolf complex. In response to these taxonomic developments, Chapter 3 simply presents the different names used for these wolves and Chapter 4 adopts the then newly available recommendation by Alvares et al. (2019) to use *Canis lupus chanco.*

The three genetic data chapters (i.e. Chapter 2-4) build on each other in the sense that with the findings of each study emerging, the research methods were refined for the next study phase, along with the understanding of which geographic areas would be useful to include in the next dataset. When this thesis research was commenced it was not clear that the Himalayan wolf lineage is also found across the Tibetan Plateau, an insight that got revealed with the analysis of Chapter 3 through the inclusion of all possible sequences from the region available on GenBank, although many of those sequences were attributed different Latin names. Based on these novel findings from Chapter 3 around the distribution of the Himalayan wolf being much larger than previously assumed, Chapter 4 then includes samples from as large of a range as possible to refine the understanding of this wolf's distribution and start pinpointing its distribution boundaries.

Study areas

The data for this thesis research was collected in three study areas in the Himalayas of Nepal and two study areas on the Tibetan Plateau of Qinghai Tibet in the Sanjiangyuan National Nature Reserve of China (Figure 1.2 and 1.3, Table 1.1). The study areas Humla and Dolpa of Nepal are situated within the arid zones of the Nepalese Himalayas and comprise alpine grasslands and alpine steppe habitats. Kanchenjunga Conservation Area (KCA) is situated in the eastern Nepalese Himalaya within the Inner Valleys of the Hindu Kush Himalaya (Miehe et al., 2016). The study areas in Sanjiangyuan (Zhaqing and Namsai, China) are situated on the Tibetan Plateau. Livestock species found in the study areas were yak (*Bos grunniens*), jhoppa (yak-cow hybrid, *Bos grunniens-Bos taurus*), horse (*Equus ferus caballus*), goat (*Capra aegagrus*), and occasionally sheep (*Ovis aries*). The following wild ungulate species were observed in the study areas: kiang (*Equus kiang*), naur (*Pseudois nayaur*), Tibetan gazelle (*Procapra picticaudata*), Tibetan argali (*Ovis ammon hodgsoni*), white-lipped deer (*Cervus albirostris*), and the following smaller mammals: Himalayan marmot (*Marmota himalayana*), woolly hare (*Lepus oiostolus*), several species of pika (*Ochotona* spp.), and different rodents.

Figure 1.2. Map of study areas visited (green dots) and locations of samples obtained through research collaborations (white dots). Extensive research expeditions were conducted in three study areas in the Nepalese Himalayas, Humla, Dolpa and Kanchenjunga Conservation Area (KCA), and two study areas on the Tibetan Plateau in Sanjiangyuan National Nature Reserve in Qinghai Province of China, Zhaqing and Namsai (with a total of more than 200 days in the field). The geographic origin of genetic samples from China included through research collaborations are: Yanchiwan (Gansu), Tibet (Tibetan Autonomous Region), Qilianshan and Sanjiangyuan (Qinghai Province), Garzê (Sichuan Province), Bortala (Xinjiang Province). And from Sachyat-Ertash in the Issyk-Kul region in the Tian Shan Mountains of Kyrgyzstan and Zorkul in the southern Pamir Mountains of Tajikistan (insert top right: modified from Google Earth).

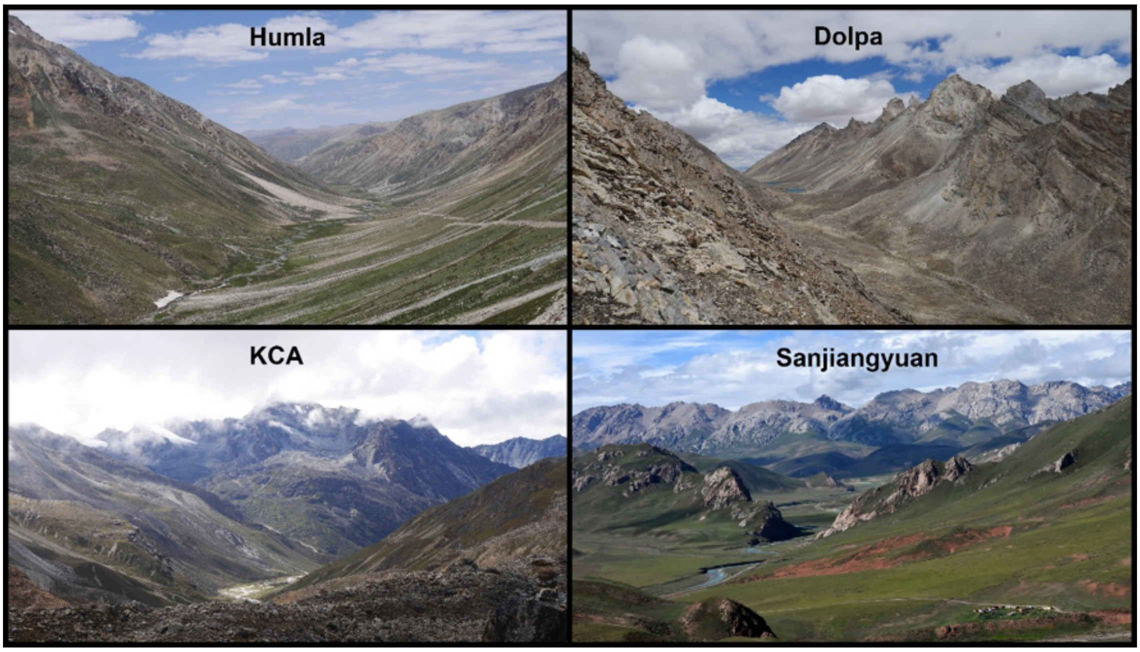

Figure 1.3. Impressions from the study areas visited in Nepal (Humla, Dolpa, KCA) and China (Sanjiangyuan) (© Geraldine Werhahn).

Table 1.1. Study areas visited for wolf sample collection.

Study Area	**Geographic Region**	**Habitat type**	**Elevation [m]**	**Distance walked [km]**	**Study area [km2]**	**Year**
Humla, Nepal	Transhimalayas	Alpine grassland/ steppe	3,700-5,390	605	384	2015#
Dolpa, Nepal	Transhimalayas	Alpine grassland/ steppe	3,510-5,590	1072	1088	2016
Kanchenjunga Conservation Area (KCA), Nepal	Himalayas	Alpine grassland	3,430-5,160	368	368	2016
Zhaqing (Sanjiangyuan, Yushu Prefecture, Qinghai), China	Tibetan Plateau	Alpine grassland	4,380-4,800	158*	900	2017
Namsai (Sanjiangyuan, Yushu Prefecture, Qinghai), China	Tibetan Plateau	Alpine grassland	4,360-4,770		1575	2017

*Vehicles were used to cover large study areas, in contrast to Nepalese study areas where vehicles were not feasible. # Data collected prior to the start of this DPhil.

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Chapter 2.

PHYLOGENETIC EVIDENCE FOR THE ANCIENT HIMALAYAN WOLF: TOWARDS A CLARIFICATION OF ITS TAXONOMIC STATUS BASED ON GENETIC SAMPLING FROM WESTERN NEPAL

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Phylogenetic evidence for the ancient Himalayan wolf: towards a clarification of its taxonomic status based on genetic sampling from western Nepal

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Wolves in the Himalayan region form a monophyletic lineage distinct from the present-day Holarctic grey wolf *Canis lupus* spp. (Linnaeus 1758) found across Eurasia and North America. Here, we analyse phylogenetic relationships and the geographic distribution of mitochondrial DNA haplotypes of the contemporary Himalayan wolf (proposed in previous studies as *Canis himalayensis*) found in Central Asia. We combine genetic data from a living Himalayan wolf population collected in northwestern Nepal in this study with already published genetic data, and confirm the Himalayan wolf lineage based on mitochondrial genomic data (508 bp cytochrome *b* and 242 bp D-loop), and X- and Y-linked zinc-finger protein gene (ZFX and ZFY) sequences. We then compare the genetic profile of the Himalayan wolf lineage found in northwestern Nepal with canid reference sequences from around the globe with maximum likelihood and Bayesian phylogeny building methods to demonstrate that the Himalayan wolf forms a distinct monophyletic clade supported by posterior probabilities/bootstrap for D-loop of greater than 0.92/85 and cytochrome *b* greater than 0.99/93. The Himalayan wolf shows a unique Y-chromosome (ZFY)

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haplotype, and shares an X-chromosome haplotype (ZFX) with the newly postulated African wolf. Our results imply that the Himalayan wolf distribution range extends from the Himalayan range north across the Tibetan Plateau up to the Qinghai Lakes region in Qinghai Province in the People's Republic of China. Based on its phylogenetic distinction and its older age of divergence relative to the Holarctic grey wolf, the Himalayan wolf merits formal classification as a distinct taxon of special conservation concern.

1. Introduction

There are few studies on wolves in Central Asia and the taxonomic status of wolves in this region remains unresolved [1]. The available genetic evidence points towards the presence of two distinct wolf lineages in the region, the Mongolian grey wolf (*Canis lupus chanco*, Gray, 1863) and the Himalayan wolf (table 1) [6–8,11,12]. Aggarwal *et al*. [7] proposed '*Canis himalayensis*' as scientific name for the Himalayan wolf, while Sharma *et al*. [6] refer to it as '*C. l. chanco*-Himalayan haplotype'. A study with 440 bp mitochondrial DNA (mtDNA) cytochrome *b* gene sequences using a molecular clock [6] estimated its time of divergence at more than 800 000 years before present. Recently, Chetri *et al*. [13] have found support for the presence of the Himalayan wolf lineage in Nepal with four scat samples collected in the Annapurna Conservation Area and analysed at the mtDNA control region. Nevertheless, the phylogeny, ecology and conservation status of the Himalayan wolf remains poorly understood [1], and proper recognition of it as a taxon is pending.

The Holarctic grey wolf appeared in the Middle Pleistocene, approximately 800 000–300 000 years before present [14–16]. In the evolutionary history from the ancestors of the wolf-dog clade in the Early to Middle Pleistocene [15] to the contemporary Holarctic grey wolf, different wolf lineages such as the Himalayan wolf, the African wolf [8] and the Indian grey wolf *C. lupus pallipes* (Sykes, 1831) [6] diverged as monophyletic sister clades. The Holarctic grey wolf (*C. lupus* spp.), comprising different subspecies including the domestic dog *C. l. familiaris*, forms a relatively recent genetic lineage [2,9]. More basal is a distinct lineage which has been described as Himalayan wolf and the recently described African (golden) wolf (currently referred to as *Canis aureus lupaster*) [8,9,17]. Based on their phylogenetic reconstruction, Rueness *et al*. [10] propose that the Himalayan and African wolf lineages may have existed before the radiation of the Holarctic grey wolf.

The IUCN recognizes 12 Holarctic grey wolf subspecies with reference to Nowak [4], who historically listed the subspecies *C. l. campestris* for the northern parts of Central Asia, replaced by *C. l. chanco* in southern adjacent regions in Central Asia. These Central Asian subspecies *C. l. campestris*, *C. l. chanco* as well as *C. l. desertorum* were later pooled with *C. l. lupus* by Sillero-Zubiri *et al*. [3].

Holarctic grey wolf taxonomy is the subject of an ongoing debate due to continuing novel genetic insights across the species' range [6–8,10,17–19]. This study identifies the wolves in the Himalayas of northwestern Nepal as belonging to the previously discovered Himalayan wolf clade as described through molecular analysis of mtDNA [6,7]. We confirm the distinction in these wolves with mtDNA D-loop and cytochrome *b* sequences and for the first time using also X- and Y-chromosome sequences, and assess the phylogeny of this wolf lineage by putting it in context with canid molecular data from around the globe. In addition, this study summarizes the currently available genomic evidence around the Himalayan wolf lineage with geographic origin to support clarification of its taxonomy and distribution range.

2. Material and methods

2.1. Study area

The field study was conducted during July–August 2015 in the northwestern district of Humla in Nepal (figure 1). The study area of approximately 384 km^2 (29.97°–30.36° N, 81.50°–82.05° E) is situated in Limi Village Development Committee and covers elevations from 3700 to 5390 m.a.s.l. The study area is situated in arid zones of the Himalayas [20] and characterized by alpine steppe vegetation. The area contains no permanent human settlements, but it is seasonally used by nomadic pastoralists to herd yaks, horses, sheep and goats during summer.

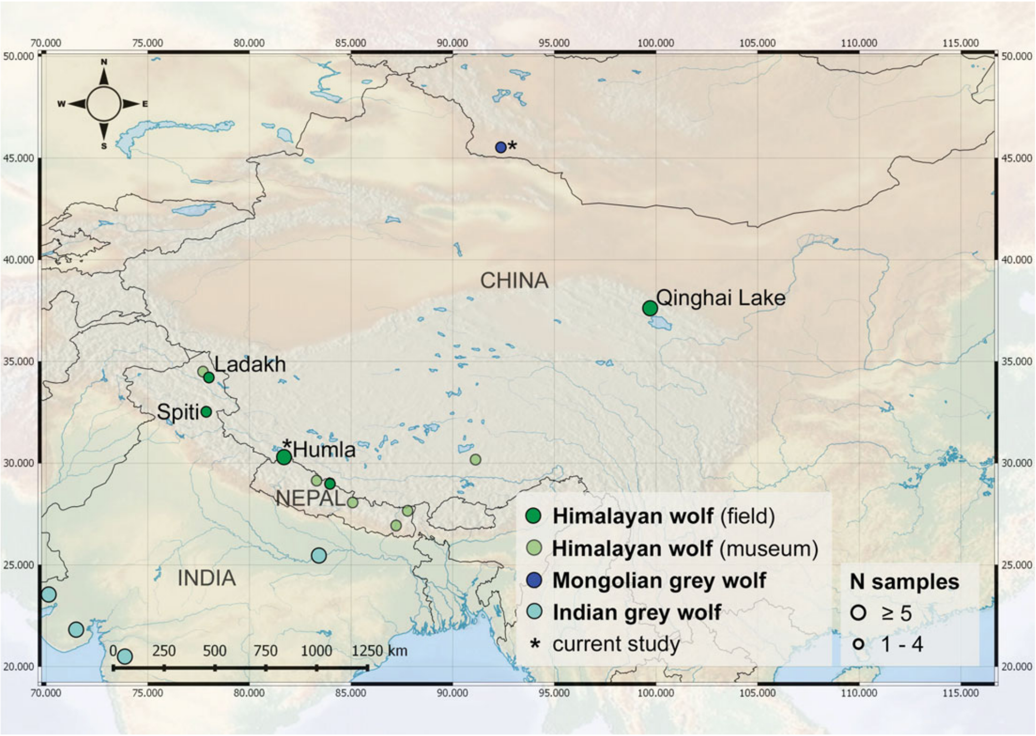

Figure 1. Overview of the current genetic evidence of the Himalayan wolf distribution. The data shown originates from the current and previous studies (Himalayan wolf field collected samples, dark green; Himalayan wolf museum specimens, light green). For overview, samples originating from Holarctic grey wolf lineages found in the region are also shown, i.e. Mongolian grey wolf *Canis lupus chanco* (dark blue) and Indian grey wolf *Canis lupus pallipes* (light blue). This study generated 72 Himalayan wolf sequences from 104 field collected samples in Humla, Nepal. The additional data shown derive from other studies [6,7,13].

Table 1. Overview of discussed canid lineages with names, status and references. The grey wolf subspecies (*Canis lupus* spp.) were to date primarily described on the basis of geographic origin.

scientific name	common name	region	IUCN recognized	references
C. lupus lupus	Eurasian grey wolf	Europe, Asia	yes	[2,3]
C. lupus chanco	Mongolian grey wolf	Tibetan Plateau	no	[2,4,5]
C. himalayensis[a] (*Canis lupus chanco*-Himalayan haplotype[b])	Himalayan wolf	Himalayas, Tibetan Plateau	no	[6][a]; [7][b]
C. lupus pallipes	Indian grey wolf	Southwestern Asia, Middle east	yes	[3,5]
Canis aureus lupaster/Canis lupus lupaster	the currently proposed African (golden) wolf/formerly golden jackal	Northern Africa	no	[8–10]
Canis aureus	golden jackal	Eurasia	yes	[3]

2.2. Collection of genetic material

A total of 104 samples from putative wolf scat and hair were collected along a total transect length of 605 km. The majority of transects consisted of opportunistic searches of the study area, and included also systematic prey distance sampling transects of two 1.5 km transects per 4 × 4 km grid cell. Ridge lines, streams and valley floors are topographic features known to be preferred travelling routes for wolves and were extensively searched [21].

Scats were swabbed and stored in Isohelix solution (Isohelix Ltd) and hair was stored with silica desiccant in a paper envelope. For genetic sampling, the faecal surface was scrubbed with a swab and then rinsed in the Isohelix solution, with this process being repeated two to three times. The swabbing was done on the outer shiny layer of the tapered end of the wolf scat. Scat age classification was adapted from Jackson & Hunter [22] and only recent to fresh wolf scats were sampled. The samples were kept out of sunlight in fresh temperatures until transfer to the genetic laboratory where they were stored in a freezer at −20°C. All further genetic laboratory protocols can be found in the electronic supplementary material.

2.3. Phylogenetic analysis

Sequences generated from Nepal were compared with canid reference sequences from around the globe obtained from the NCBI GenBank database (electronic supplementary material, tables S2 and S3). We constructed phylogenies using Bayesian and maximum-likelihood methods for D-loop and cytochrome *b* sequences. The dataset analysed consisted of 72 canid D-loop sequences from this study collected in Nepal and 148 canid D-loop reference sequences from NCBI GenBank. For cytochrome *b*, the dataset consisted of 24 sequences from this study collected in Nepal and 104 reference sequences from NCBI GenBank. Sequences were aligned using the ClustalW algorithm implemented in GENEIOUS v. 8.1.8 to a consensus length of 242 bp for D-loop and of 508 bp for cytochrome *b* with final corrections done by eye. Phylogenies were built with MRBAYES [23] with 11 002 tree building iterations (D-loop: chainlength 1 100 000, subsample frequency 200, burn-in-length 110 000, samples analysed 4951; cytochrome *b*: chainlength 1 100 000, subsample frequency 200, burn-in-length 110 000, samples analysed 4951), and maximum-likelihood phylogenies were built with PAUP* [24] (100 bootstrapping replications). The phylogenies were rooted with red fox (*Vulpes vulpes,* Linnaeus, 1758) sequences collected in the study area (NCBI GenBank accessions KY996531 and KY996535). Additional phylogenies built with Neighbour-joining and Tamura-Nei models of genetic distance showed the same arrangement and are found in the electronic supplementary material, figures S1 and S2. Haplotype networks were drawn with the software tool PopART (http://popart.otago.ac.nz) using Median-joining and TCS networks [25]. Evolutionary divergence between the Himalayan wolf, Holarctic grey wolf, African wolf, golden jackal, coyote and red fox was calculated with the software MEGA using the maximum composite likelihood distance between groups of nucleotide sequences [26,27]. For a subset of high-quality samples from the study area in Humla (Nepal), final intron sequences of the zinc-finger X-chromosomal (ZFX) and Y-chromosomal (ZFY) genes were generated following protocols described elsewhere [8,28–30]. For the primer sequences used for the zinc-finger protein gene analysis, see the electronic supplementary material, table S4. To test the method and verify our results, samples of African wolf [31] and Holarctic grey wolf were also sequenced at both these zinc-finger protein genes. Owing to the long PCR fragments involved (greater than 834 bp) and inevitable DNA degradation in non-invasive samples, it was only possible to obtain high-quality sequences of the sex genes for a small subset of the samples: five complete and one partial sequence for the Y-chromosome, and nine complete sequences for the X-chromosome.

3. Results

We found that the Himalayan wolf forms a distinct monophyletic clade to the Holarctic grey wolf, supported by both the D-loop mtDNA and the cytochrome *b* mtDNA phylogeny (figure 2*a*,*b*), similar to previous studies by Sharma *et al*. [6] and Aggarwal *et al*. [7]. In addition, the results from the zinc-finger protein gene sequences at the Y-chromosome (1176 bp) and the X-chromosomes (514 bp) independently support these findings from the mtDNA data.

We found 76 unique canid D-loop haplotypes in the analysed dataset. Of these, 62 were wolves (including Holarctic grey wolf *C. lupus* spp., African wolf *C. aureus lupaster* and Himalayan wolf *C. himalayensis*), while the other 14 canid haplotypes of dhole (*Cuon alpinus,* Pallas, 1811), African wild dog (*Lycaon pictus,* Temminck, 1820), coyote, golden jackal (*C. aureus*, Linnaeus, 1758), side-striped jackal (*Canis adustus,* Sundevall, 1847) and red fox were used to put the collected data in a wider canid phylogenetic context. The Himalayan wolf clade contained 11 unique D-loop haplotypes (figure 2*a*), of which three haplotypes were found in the study area in Humla (Nepal): 'Himalayan wolf haplotype 1' (13 samples, NCBI GenBank accession KY996529), 'Himalayan wolf haplotype 2' (56 samples, NCBI GenBank accession KY996530) and 'Himalayan wolf haplotype 3' (seven samples, NCBI GenBank accession KY940301). The complete haplotype dataset used in the analysis with accession numbers is found in the electronic supplementary material, tables S1–S3. The monophyletic Himalayan wolf

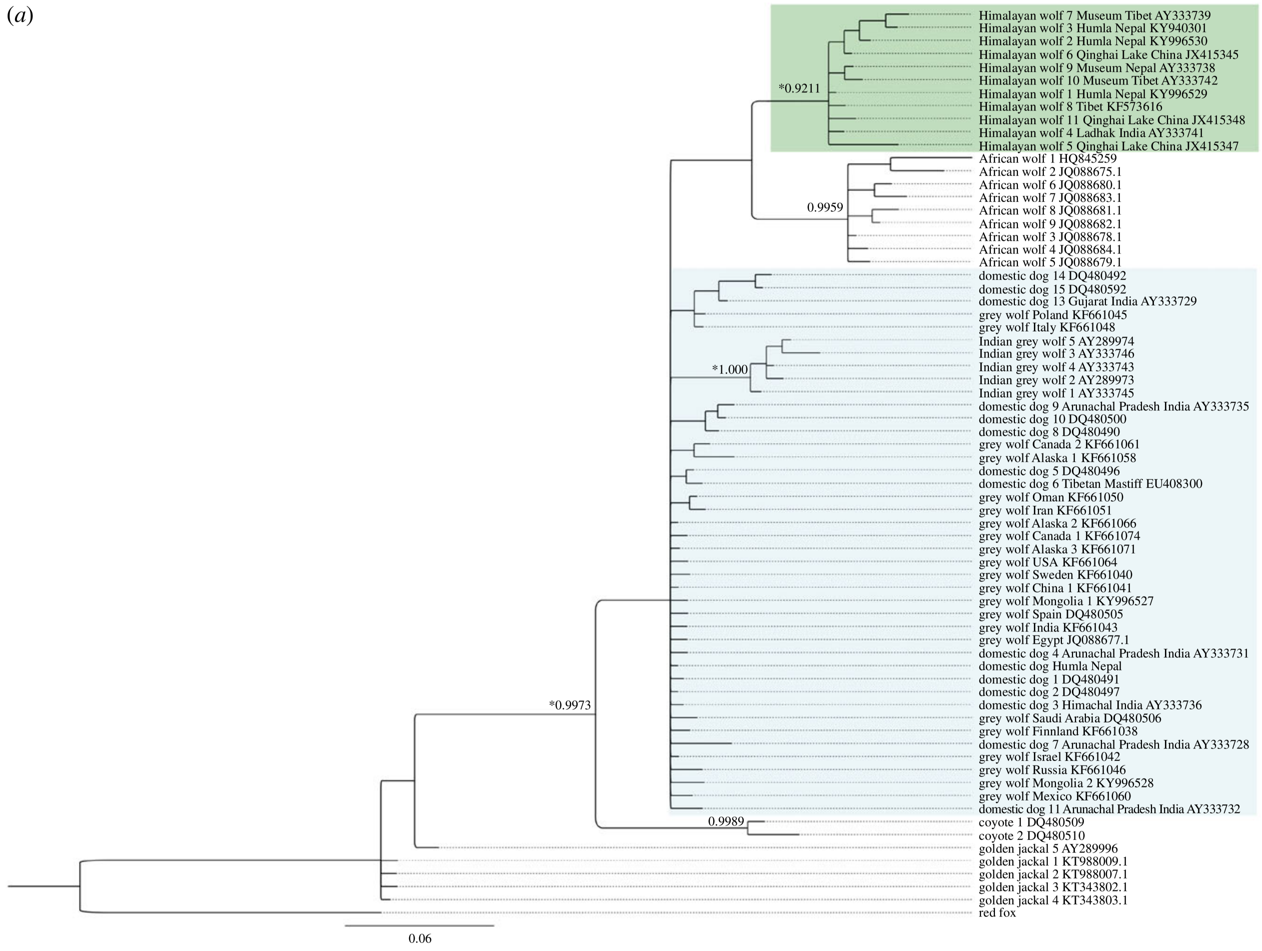

Figure 2. Bayesian phylogeny based on (*a*) 242 bp D-loop mtDNA sequences and (*b*) 508 bp cytochrome *b* mtDNA sequences with Bayesian posterior probability values at nodes. Asterisks at nodes indicate greater than or equal to 80% bootstrap support based on maximum-likelihood analyses and greater than or equal to 0.90 posterior probability from Bayesian inference. The Himalayan wolf (green) is a distinct monophyletic clade relative to the Holarctic grey wolf clade (blue) which also includes domestic dogs. Haplotypes without accession numbers are sequences generated in this study (electronic supplementary material, table S1).

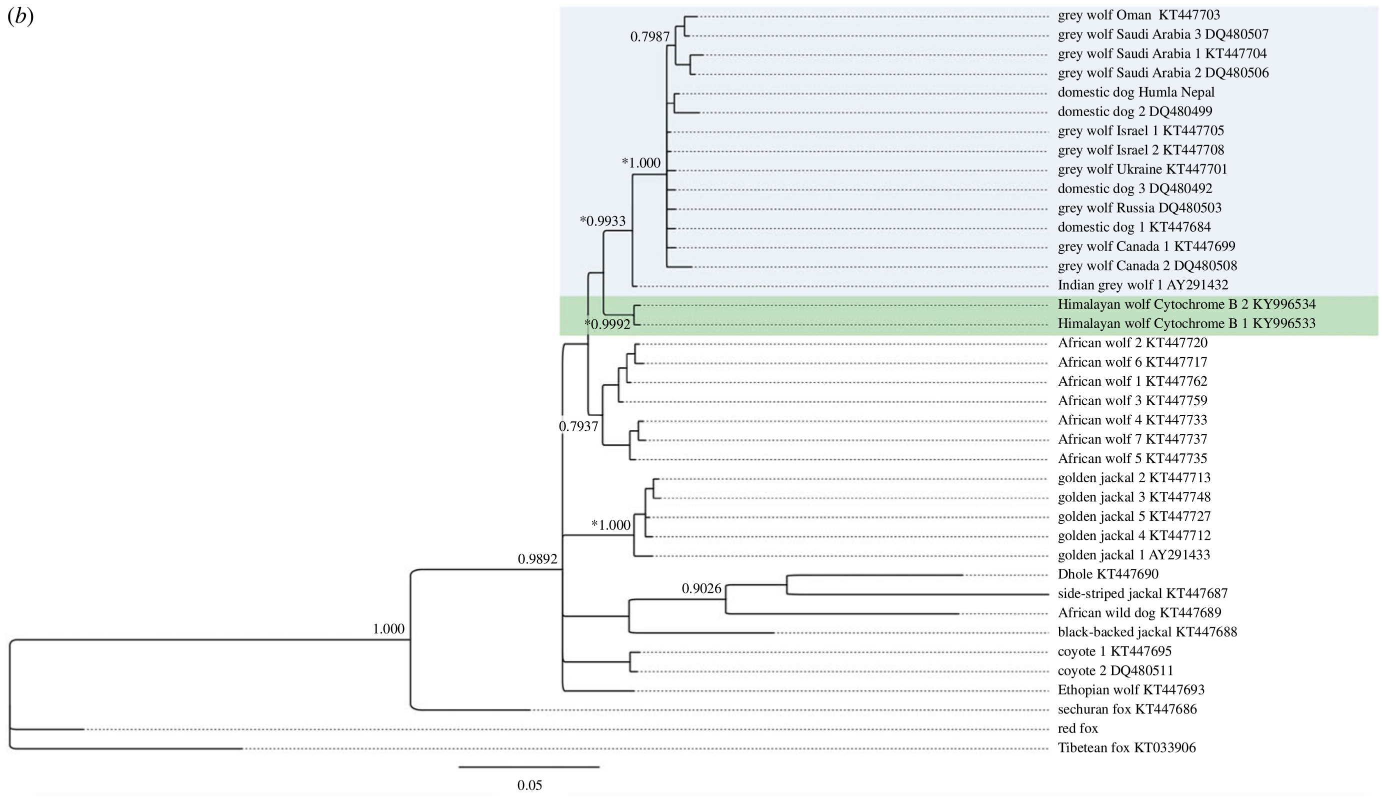

Figure 2. (*Continued.*)

clade is supported with posterior probability/maximum-likelihood bootstrap of greater than 0.92/85 values for D-loop and posterior probability/maximum-likelihood bootstrap greater than 0.99/93 values for cytochrome *b* (figure 2*a*,*b*). Bayesian and neighbourhood-joining phylogenies based on D-loop and cytochrome *b* sequences showed the same structure (figure 2; electronic supplementary material, figure S2). Furthermore, the Himalayan wolf clade was supported similarly in both Median-joining and TCS haplotype networks (figure 3*a*,*b*).

Two Nepalese Himalayan wolf cytochrome *b* haplotypes were found in the samples from the study area in Humla (Nepal) which differed from Holarctic grey wolf haplotypes with 14 transitions and two transversions in the nucleotide sequence. For D-loop, the nucleotide differences between Holarctic grey wolf and the haplotype 'Himalayan wolf D-loop 1' were 12 transitions, 14 transitions for haplotype 'Himalayan wolf D-loop 2' and 16 transitions for the haplotype 'Himalayan wolf D-loop 3'.

Another unique canid haplotype was found in three samples collected in Humla (Nepal), of which two samples were deliberately collected as domestic dog. From this putative domestic dog mitochondrial genome, we found one unique D-loop 'Domestic dog D-loop Nepal 1' (NCBI GenBank accession KY996526) and one unique cytochrome *b* haplotype 'Domestic dog Cytochrome B 1' (NCBI GenBank accession KY996532). This dog haplotype clusters with domestic dog *C. lupus familiaris* and Holarctic grey wolf *C. lupus* spp. haplotypes from around the globe in both D-loop and cytochrome *b* phylogenies (figure 2*a*,*b*).

The D-loop haplotype 'Himalayan wolf D-loop 1' found in Humla (Nepal) is identical to Himalayan wolf sequences deposited on NCBI GenBank by Sharma *et al*. [6] and Aggarwal *et al*. [7] (electronic supplementary material, tables S2 and S3; figure 2*a*). These sequences used by Aggarwal *et al*. [7] identical to 'Himalayan wolf D-loop 1' derive from captive animals in the Padmaja Naidu Zoo in India which descend from two to three wild captured wolves caught in the Trans-Himalayan region without a more specific capture location available [7,32]. The sequences used by Sharma *et al*. [6] identical to 'Himalayan wolf D-loop 1' originate from a captive individual caught in Spiti Valley (Himachal Pradesh, India), approximately 400 km northwest from the study area in Humla (Nepal).

Our analysis indicates that the Himalayan wolf lineage might be found as far north as Qinghai Lake in Qinghai Province in the People's Republic of China. This is indicated by our finding that unpublished D-loop sequences originating from Qinghai Lake on the Tibetan Plateau in the People's Republic of China and derived from NCBI GenBank were identical to Himalayan wolf D-loop haplotypes found in Nepal in this study (no further information could be obtained). These sequences from Qinghai Lake were designated as *C. lupus chanco* but matched with the haplotypes found in Nepal as follows: i.e. 'Himalayan wolf D-loop 1' identical with JX415352 and JX415350; 'Himalayan wolf D-loop 2' identical with JX415351; 'Himalayan wolf D-loop 3' identical with JX415343. In addition, other samples originating from Qinghai Lake and retrieved from NCBI GenBank present three additional unique haplotypes within the Himalayan wolf clade that were not found in Humla (Nepal) (i.e. JX415348, JX415345 and JX415347).

The understandable confusion around wolf scientific naming in this region is apparent from sequences on NCBI GenBank coming from wider locations on the Tibetan Plateau and attributed to different scientific names, i.e. either *C. l. chanco or C. l. laniger*, which all do cluster within the Himalayan wolf clade in this study (e.g. *C. l. chanco* NCBI GenBank accessions*:* AY333738, AY333739, AY333740, AY333741, AY333742, JX415343, JX415344, JX415345, JX415347, JX415348, JX415350, JX415351 and JX415352; *C. lupus laniger* NCBI GenBank accession*:* KF573616).

By contrast, Mongolian grey wolf (*C. l. chanco*) samples from individuals in the Zürich Zoo and originating from the Great Gobi B in Mongolia (R Zingg 2016, personal communication) clustered within the Holarctic grey wolf clade (figure 2*a*). It is currently uncertain based on the data we present here whether *C. l. chanco* should be considered as a distinct group within the Holarctic grey wolf as *C. l. chanco* samples do not seem to form a monophyletic clade.

Evolutionary divergence estimates between the D-loop sequences show a slightly greater distance between Holarctic grey wolf/Himalayan wolf (0.069) than between Holarctic grey wolf/African wolf (0.066), while the cytochrome *b* sequences show a greater distance between Holarctic grey wolf/African wolf (0.044) than between Holarctic grey wolf/Himalayan wolf (0.038) (table 2).

One unique haplotype for each the ZFY and the ZFX sequence was found in the Himalayan wolf samples collected in the study population in Humla (NCBI GenBank accessions: MF101862 and MF101863). For the Holarctic grey wolf and African wolf samples, we found the same ZFY/ZFX haplotypes as in Koepfli *et al.* [8]. Comparing the results of the ZFY final intron sequence among Himalayan, Holarctic grey and African wolf supports that the Himalayan wolf forms a distinct wolf lineage. The Himalayan wolf ZFY is different from the Holarctic grey wolf at position 1010, where both Himalayan and African wolf share the nucleic acid T, rather than the G found in Holarctic grey wolf. A

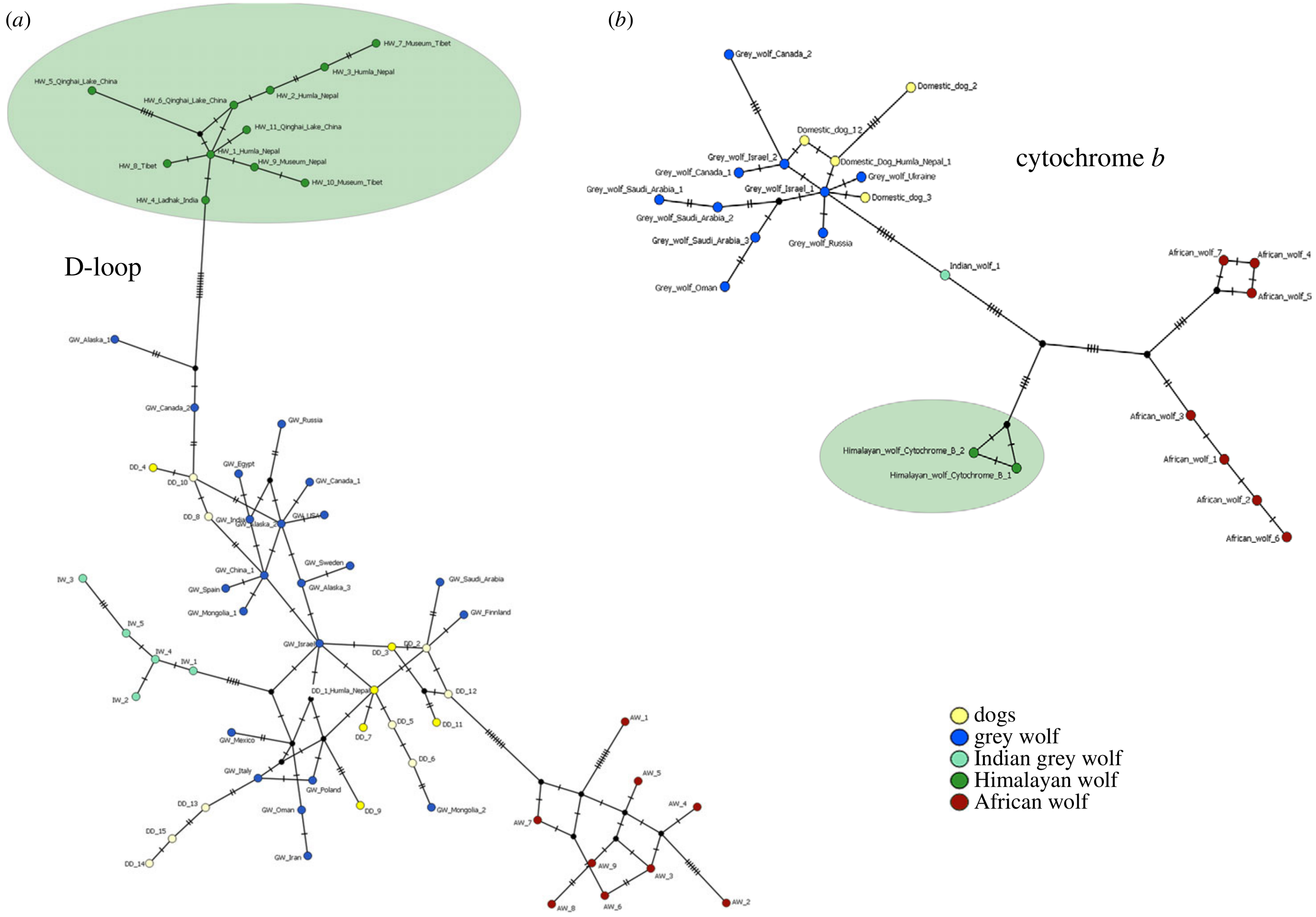

Figure 3. Median-joining networks based on (*a*) 242 bp D-loop haplotypes of *Canis* species and (*b*) 508 bp cytochrome *b* haplotypes of *Canis* species. Himalayan wolves (HW, green), African wolves (AW, red) form monophyletic clades. The grey wolf complex clusters with grey wolves from around the globe (GW, blue) including Indian grey wolves (IW, light blue) and domestic dogs (DD, yellow).

Table 2. Evolutionary distances (maximum composite likelihood analysis with MEGA) between Himalayan wolf, African wolf, Holarctic grey wolf, golden jackal, coyote and red fox for D-loop and cytochrome *b* mtDNA sequences.

	Holarctic grey wolf		Himalayan wolf		African wolf		golden jackal		coyote	
	D-loop	cyt *b*	D-loop	cyt *b*	D-loop	cyt *b*	D-loop	cyt *b*	D-loop	cyt *b*
Himalayan wolf	0.069	0.038								
African wolf	0.066	0.044	0.062	0.028						
golden jackal	0.118	0.076	0.127	0.052	0.136	0.051				
coyote	0.099	0.062	0.128	0.050	0.103	0.050	0.124	0.056		
red fox	0.328	0.179	0.366	0.176	0.332	0.175	0.300	0.180	0.341	0.170

30 bp indel shared by both Himalayan and Holarctic grey wolf is not found in the African wolf (table 3 and figure 4). For the ZFX final intron sequence, we found identical haplotypes for both the Himalayan wolf and the African wolf from Kenya. By contrast, the Holarctic grey wolf, including domestic dogs, shows a haplotype different at two positions from the Himalayan wolf and the African wolf (indel at position 328, substitution at 425; table 4).

4. Discussion

This study provides genetic evidence in support of the distinct Himalayan wolf lineage found in Central Asia, the formal taxonomic recognition of which is pending due to limited data from contemporary wild populations. Our results confirm findings of previous studies from the broader Himalayan region, largely based on museum specimens and zoo animals [6,7,13,33], which place the Himalayan wolf as a distinct monophyletic lineage relative to the Holarctic grey wolf. This study expands the existing data on the Himalayan wolf with sampling of a contemporary living wolf population in a previously unconfirmed location in northwestern Nepal. Further, this study updates the currently limited understanding of the distribution range of the Himalayan wolf by integrating available genetic and geographic data from previous studies.

We show the genetic distinctness between the Himalayan wolf and the Holarctic grey wolf based on 242 bp D-loop mtDNA and 508 bp cytochrome *b* mtDNA sequences. Further, the analyses of the X- and Y-linked zinc-finger protein gene (ZFX: 514 bp and ZFY: 1176 bp) sequences support the results emerging from the mtDNA (figure 4). Hence, multiple independent pieces of genomic evidence indicate that the Himalayan wolf forms a distinct monophyletic wolf lineage to the Holarctic grey wolf similar to the recently posited African wolf. At the mtDNA genes tested here, the level of divergence from the Holarctic grey wolf to the Himalayan wolf is similar to that of the African wolf (table 2). Within the Himalayan wolf population in our study area in Humla (Nepal), we found one haplotype for each of the X- and Y-linked zinc-finger protein gene (ZFX and ZFY) sequences. Fewer haplotypes are expected at this gene sequence, as the sex chromosomes are a slow evolving area, and a similar situation was found for the African wolf (tables 3 and 4) [8]. The Himalayan wolf emerges as a basal distinct monophyletic wolf lineage to the Holarctic grey wolf in phylogenies derived from cytochrome *b*, an arrangement which is additionally supported by the intermediate placement of the Himalayan wolf between the African wolf and Holarctic grey wolf at ZFY and the sharing of a ZFX haplotype with the African wolf. At D-loop, the Himalayan wolf forms a distinct monophyletic clade within the Holarctic grey wolf clade (figure 2*a*,*b* and tables 3 and 4), but it does not occupy a basal position with respect to the Holarctic grey wolf. Sequencing of longer fragments of D-loop (currently 242 bp) may resolve this conflict.

The Himalayan wolf lineage is more divergent from the Holarctic grey wolf than the IUCN recognized Indian grey wolf subspecies (*C. lupus pallipes*), which forms a monophyletic clade nested within the Holarctic grey wolf complex [6,10]. Thus, in this context, the current and previous studies suggest the need for adjusting the taxonomy of the Himalayan wolf in recognition of its genetic uniqueness but also imply more generally the need for revision of wolf subspecies and units of conservation concern.

Based on its clear phylogenetic distinction and older age of divergence relative to the Holarctic grey wolf and for consistency within the existing IUCN naming system, the Himalayan wolf merits at minimum classification at subspecies level of special conservation concern (i.e. *C. lupus himalayensis;* and possibly *C. himalayensis* as proposed by Aggarwal *et al*. [7]). Future assessments of more nuclear data,

Table 3. Informative positions found in the final intron sequences of the zinc-finger Y-chromosomal (ZFY, 1176 bp) results of Himalayan wolf, compared with African wolf, Holarctic grey wolf, golden jackal and coyote. Accession numbers for NCBI GenBank reference samples used in the analysis are provided.

			ZFY final intron									
			23	123	193–201	236–446	880–909	1010	1036–1037	1056	1094	
seq. source	species	*n*	A/G	1 bp insertion	9 bp insertion	210 bp SINEII insertion	30 bp deletion	T/G	2 bp insertion	G/A	T/C	haplotype
NCBI GenBank	coyote *C. latrans* (JN663892.1)	1	A	—	—	—	30 bp	T	—	G	C	E
	Holarctic grey wolf *C. lupus* Canada (KT448254.1)	1	A	—	—	—	30 bp	G	—	G	T	C
	golden jackal *C. aureus* Serbia (KT448259.1)	1	G	A	9 bp	210 bp	—	T	TA	A	T	A
	golden jackal *C. aureus* Israel (KT448266.1)	1	G	A	9 bp	210 bp	—	T	TA	A	T	
	African wolf *C. aureus lupaster* Kenya and Morocco (KT448270.1, KT448267.1)	2	A	—	—	—	—	T	—	G	T	B
this study	African wolf *C. aureus lupaster* Morocco	1	A	—	—	—	—	T	—	G	T	
	Himalayan wolf *C. l. himalayensis* Nepal (MF101862)	5 and partial	A	—	—	—	30 bp	T	—	G	T	D
	Holarctic grey wolf *C. lupus* Europe and Mongolia (captive Zurich Zoo)	2	A	—	—	—	30 bp	G	—	G	T	C

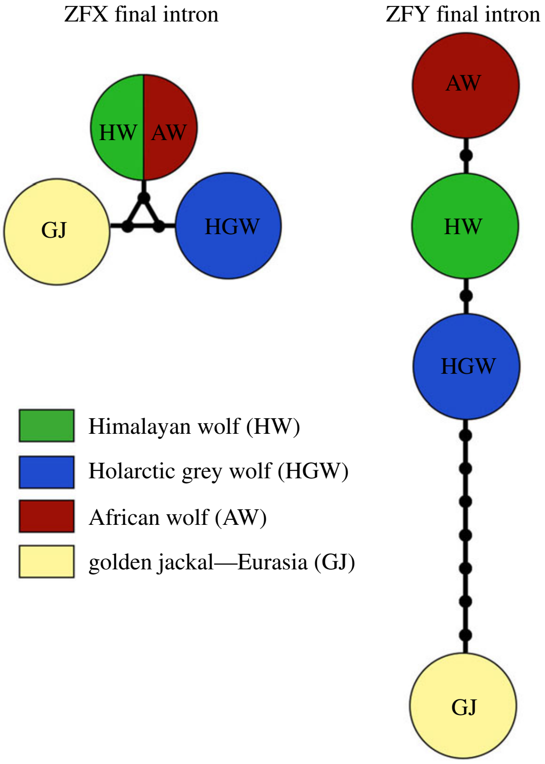

Figure 4. Haplotype network showing the ZFY and ZFX final intron sequences of Himalayan wolf (green), African wolf (red), Holarctic grey wolf (blue) and golden jackal (red). The black dots on the internode represent indels and substitutions between the haplotypes.

Table 4. Informative positions found in the final intron sequences of the ZFX (514 bp) results of Himalayan wolf, compared with African wolf, Holarctic grey wolf and golden jackal. NCBI GenBank accession numbers are provided.

			ZFX final intron			
			328	381	425	
seq. source	species	*n*	1 bp insertion	T/A	A/G	haplotype
NCBI GenBank	golden jackal *C. aureus* Israel (KT448243.1)	1	G	A	A	A
	African wolf *C. aureus lupaster* Kenya (KT448251.1)	1	G	T	G	B
	Holarctic grey wolf *C. lupus* Canada (KT448225.1)	1	—	T	A	C
this study	Holarctic grey wolf *C. lupus* Europe	1	—	T	A	C
	Holarctic grey wolf *C. lupus* Mongolia	4	—	T	A	
	Holarctic grey wolf *C. lupus* Arabian	1	—	T	A	
	dog *C. lupus* Nepal	2	—	T	A	
	Himalayan wolf *C. l. himalayensis* Nepal (MF101863)	9	G	T	G	B

and critically the factors causing reproductive isolation (if they indeed exist) might then further validate the taxon's status as a full species (i.e. *C. himalayensis*).

Our findings show that wolf individuals from the Tibetan Plateau and the Himalayan region, which had historically been assigned to *C. l. chanco* or *C. l. laniger*, fall within the Himalayan wolf lineage, while wolf individuals from Mongolia, also assigned as *C. l. chanco*, phylogenetically group within the Holarctic grey wolf complex. Thus, the subspecies name *C. l. chanco* (often attributed the common name 'Mongolian grey wolf') seems accurately used for grey wolves found in northern parts of Central Asia such as Mongolia. However, the justification for its own subspecies *C. l. chanco* is limited considering

Figure 5. Himalayan wolf adults in Humla, Nepal. Photograph (*a*) shows a pale-coloured wolf individual, and (*b*) a black-coloured wolf individual (© Geraldine Werhahn).

the data here presented, as the 'accurately' named *C. l. chanco* sequences group within the monophyletic Holarctic grey wolf clade, but without forming an own distinct monophyletic group within that.

The Mongolian grey wolf and the Himalayan wolf seem to be found in different parts of Central Asia. The currently available data indicate that the distribution of the Himalayan wolf lineage extends west of Kashmir valley (India) across the Himalayan Mountains, with samples from wild individuals available from Kashmir valley (India) [6], Spiti Valley and Ladakh (India) [6,7], Humla (Nepal) (this study) and the Annapurna Conservation Area in Mustang (Nepal) [13]. An advanced understanding of the Himalayan wolf distribution range derives from including all genetic data from the region in our analysis as available on NCBI GenBank. Our analysis suggests that the range of the Himalayan wolf lineage extends north from the Himalayas across the Tibetan Plateau as far as Qinghai Lakes in Qinghai Province in the People's Republic of China, while the Mongolian grey wolf (*C. l. chanco*—if it can be considered as such, see above) is found in Mongolia. This raises questions about the distribution boundaries between the Himalayan wolf and the grey wolf in Mongolia, and also what evolutionary processes might be maintaining them.

Historically, the naming of wolves in the Himalayan region has been based on scattered observations. Shrotryia *et al.* [1] include a historical overview of the scientific naming of wolves in this region briefly summarized in the following: Hodgson [34] provided a first description of what seemed a Himalayan wolf and attributed it to *C. laniger*. Blanford [35] then merged this taxon with *C. lupus*. They described the wolf variety found in Tibet and Ladakh as pale coloured with woolly underfur and also mentioned black individuals [1]—both morphologies were frequently observed in the study area in the Himalayas of Nepal (figure 5). Later, Pocock [36] described *C. laniger* as a *C. lupus* subspecies and combined it with the more widely distributed *C. lupus chanco.* The voucher for the genetic sequence HW9_Museum Nepal (figures 2*a* and 3*a*) is a museum specimen in the Natural History Museum in South Kensington (UK) from the collection by B. Hodgson from Nepal (NCBI GenBank accession AY333738; BM58.6.24.61) [6]. The voucher for the genetic sequence HW7_Museum Tibet (figures 2*a* and 3*a*) is a museum specimen in the Natural History Museum in South Kensington (UK) collected in Tibet by A.H. Savage-Landor and described as a black animal (NCBI GenBank accession AY333739; BM99.12.29.1) [6].

4.1. Applying species concepts

The contemporary recognized Holarctic grey wolf subspecies show no reciprocally monophyletic clades, with the exception of the Himalayan wolf and Indian grey wolf, likely due to glaciation and dispersal events in the past [11,37]. Leading up to its divergence, the Himalayan wolf seems to have formed an independently evolving metapopulation lineage based on the mitochondrial data, which is a primary criterion for defining species status under the unified species concept [38]. If we were to define taxa according to the genetic and phylogenetic species concept, this monophyletic lineage showing 3.8% divergence from Holarctic grey wolf (table 2) could be used to justify that the Himalayan wolf is a distinct species. Studies on other mammalian taxa [39] conclude that greater than 2–11% divergence in cytochrome *b* is indicative of conspecific populations and possibly valid species, and therefore merits additional studies concerning species status in these cases. However, at least 26 different species concepts exist [40]. Inherent to them all is that a species is a cohesive cluster of individuals representing a different lineage as a result of at least partially different evolutionary paths. Species concepts suitable also for conservation should take fitness into account, and as such, the species concepts considered above are not generally thought to be the most suitable for making conservation-relevant decisions about taxonomy. The ideal species concept for conservation purposes maximizes benefits for the species in terms of reproductive fitness, sustains evolutionary adaptation processes and facilitates conservation. The Himalayan wolf, as indicated in the current and previous studies, fulfils the criteria of the phylogenetic, the evolutionary and the unified species concepts [40]. To test the biological species concept and differential fitness species concept which are more appropriate for conservation purposes [40], more nuclear genomic data, including in best case also functional genes, from across the Himalayan wolf's range should be analysed. Such data could then also be used to assess geographic range boundaries with the Holarctic grey wolf, and evaluate the evolutionary processes that maintain these species boundaries. Other fitness relevant data in the form of biogeographic, phenotypic and karyotypic evidence might further add to the argument if these become available. Investigation into whether hybridization with the domestic dog is occurring may also be of conservation concern, although it may prove hard to disentangle from introgression from the Holarctic grey wolf as this may also occur.

Given the mounting genetic evidence surrounding the Himalayan wolf and African wolf lineage, it seems inevitable that a wider revision of canid taxonomy on the Eurasian continent and North Africa may be required in due course.

5. Conclusion

We present genetic evidence for the Himalayan wolf lineage collected from a contemporary wild wolf population in northwestern Nepal. Our study adds to the growing evidence around the evolutionarily distinct Himalayan wolf with substantial field collected genetic data combined with genetic and geographic data from previous studies, and is the first to include evidence from sex chromosomes. The genetic evidence (mtDNA cytochrome *b*, mtDNA D-loop and sex chromosomes) point to a distinct monophyletic position of the Himalayan wolf with respect to the Holarctic grey wolf. We infer from this and previous studies that the Himalayan wolf lineage deserves taxonomic recognition at subspecies level (i.e. *C. lupus himalayensis*). Given further research, especially involving nuclear DNA, elevation to *C. himalayensis* as proposed by Aggarwal *et al*. [7] may be justified. Adjusting the taxonomy of the Himalayan wolf to its phylogeny will not only adequately reflect its genetic distinctness, but is also essential to advance future research into the genetics, ecology and conservation of the Himalayan wolf.

Ethics. All required fieldwork permits were obtained from the Department of National Parks and Wildlife Conservation Nepal, and the research was conducted in coordination and accordance with the respective National Park Managements.

Data accessibility. All sequence data supporting this publication are deposited on NCBI GenBank under the accessions (KY996526–KY996535, KY94030, MF101862 and MF101863). All accession numbers are included in the text and figures of the main article, and tables of the electronic supplementary material. The electronic supplementary material accompanying this article contains details of the genetic analysis method, additional figures and a complete list of all data used in the phylogenetic analyses with accession numbers and references.

Authors' contributions. G.W. has collected the field data, participated in data analysis, carried out sequence alignments, participated in the design of the study, conceived the study and drafted the manuscript. H.S. has developed the genetic analysis protocols, participated in data analysis, carried out sequence alignments and participated in drafting the manuscript; J.K. has participated in developing the genetics protocol and participated in data analysis; J.J. and S.B. carried out the molecular laboratory work and participated in data analysis; N.K. has collected field data, participated in data analysis and participated in drafting the manuscript; C.S.-Z. and D.W.M. have contributed to conceiving the study, participated in the design of the study and helped draft the manuscript. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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Chapter 3.

THE UNIQUE GENETIC ADAPTATION OF THE HIMALAYAN WOLF TO HIGH-ALTITUDES AND CONSEQUENCES FOR CONSERVATION

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The unique genetic adaptation of the Himalayan wolf to high-altitudes and consequences for conservation

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ABSTRACT

The Himalayan wolf seems uniquely adapted to life at high-altitudes of the Himalayas and the Tibetan Plateau. Through a non-invasive survey we confirm the presence of the Himalayan wolf across the Nepalese Himalayas and its phylogenetic distinctness based on mitochondrial and nuclear DNA. We use the data generated from 287 scat and hair samples combined with a reference dataset including canid samples from around the globe. The Himalayan wolf forms a genetically distinct lineage based on 1) 242bp of D-loop and 508bp of cytochrome *b* (mtDNA), 2) the ZF gene of both sex chromosomes, 3) a microsatellite panel of 17 nuclear loci, and 4) four non-synonymous SNPs in four hypoxia pathway related (functional) nuclear genes. The SNP analysis indicates a genetic adaptation to cope with the hypoxic stresses in the high altitude habitats which we did not find in the Holarctic grey wolf. Based on analysis of divergence time from full mitochondrial genomes we estimate that the Himalayan wolf diverged from the Holarctic grey wolf complex 691,000–740,000 years before the present day. We provide first insights into the population status of the Himalayan wolf in Nepal with nuclear genotyping revealing counts of 12, 16, and 2 wolf individuals in the three study areas Humla (384 km^2), Dolpa (1,088 km^2), and Kanchenjunga Conservation Area (368 km^2) respectively. The methods presented here offer a complete toolkit for the non-invasive monitoring of this wolf lineage. Nepal holds a significant population of this unique wolf across its Himalayan landscapes and we recommend the country takes a leading role on its protection.

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1. Introduction

Conservation science recognizes genetic, species and ecosystem diversity as the top three forms of biodiversity that require conservation attention (McNeely et al., 1990). Taxonomic units provide important guidance in decision-making processes of conservationists and policy makers. But designating species boundaries can be challenging and is subject to different criteria depending on the species concept applied (Baker and Bradley, 2006; Frankham et al., 2012; Zachos, 2018; Zachos et al., 2013).

Canids, especially grey wolves (*Canis lupus* Linnaeus, 1758), can be particularly challenging with regards to species and subspecies designation due to hybridization among lineages (and also with domestic dogs (*Canis lupus familiaris*)), large dispersal ranges, and historic population radiations and declines due to glaciation events (Sillero-Zubiri et al., 2004). Consequentially wolf taxonomy is the subject of an ongoing debate (e.g. Rutledge et al., 2015; vonHoldt et al., 2016; Wilson et al., 2000).

The Himalayan wolf is a little understood wolf lineage found in the Himalayas and the Tibetan Plateau of Asia (Werhahn et al., 2017b). Different names have been used to describe this wolf lineage in the past (Table 1) but to date the formal taxonomic classification is pending. The Himalayan wolf forms a monophyletic wolf lineage basal to the Holarctic grey wolf complex (Sharma et al., 2004; Aggarwal et al., 2007; Shrotryia et al., 2012; Koepfli et al., 2015; Werhahn et al., 2017a,b). It appears to have diverged before the radiation of the contemporary Holarctic grey wolf which took place approximately 800,000–300,000 years before present (Sotnikova and Rook, 2010; Tedford et al., 2009; Vilà et al., 1999). Recent studies indicate that contemporary Holarctic grey wolves all originate from a Beringian wolf population expansion that took place at the end of the Last Glacial Maximum, a process driven by the large ecological changes during the late Pleistocene (Ersmark et al., 2016; Koblmüller, 2016; Loog et al., 2018).

The evaluation of the conservation status of this wolf, and the subsequent implementation of conservation actions, is hindered by scarce data on taxonomic status, distribution and ecology. Although the scientific evidence supporting its genetic uniqueness has been accumulating in recent years, reliable populations estimates are lacking, ranging from 350 individuals in Jammu and Kashmir and Himachal Pradesh (~70,000 km^2 of habitat) (Fox and Chundawat, 1995) to several thousands in Tibet and Qinghai as calculated in Fan et al. (2016) with the generalized phylogenetic coalescent sampler method from 6 samples from the region; based on geographic location these populations likely represent the Himalayan wolf lineage (Werhahn et al., 2017b).

The Himalayan wolf has been documented at 3,900–5,600 m asl across the continuous landscape of the Himalayas and genetic evidence confirmed its presence in the Himalayas and the Tibetan Plateau (Werhahn et al., 2017a,b). Life at these high elevations presents two major metabolic challenges: severe oxidative stress and elevated metabolic rates (Beall, 2007; Gou et al., 2014; Zhang et al., 2014). As a consequence genomic changes due to adaptation to the montane environment have been found in other taxa, such as the *Caprini* (Hassanin et al., 2009).

Further it is hypothesized that Tibetan mastiff (domestic) dogs may have acquired hypoxia adaptation through hybridization with wolves on the Tibetan Plateau (Gou et al., 2014; Zhang et al., 2014). Similarly it is proposed that Tibetan nomads have acquired their hypoxia adaptation from hybridization with Denisovan hominids (Huerta-Sanchez, 2014).

In this study we set out the genetic evidence for the Himalayan wolf using mitochondrial and nuclear data derived from a non-invasive (faecal sample) survey from three study sites spanning the entire Nepalese Himalayas (~750 km). In Werhahn et al. (2017a) we presented evidence that the level of genetic differentiation from the Holarctic grey wolf at mitochondrial D-loop and cytochrome *b* genes and sex chromosome markers (ZFY, ZFY genes) was comparable to that in other recognised subspecies of *Canis lupus* and the postulated African wolf (Gaubert et al., 2012; Koepfli et al., 2015; Rueness et al., 2015). However, evidence for justifying further taxonomic elevation to species level in addition requires nuclear genomic evidence, and preferably from functional genes from which differential fitness effects could be inferred (Frankham et al., 2012; Vasemägi and Primmer, 2005). Functional genes can provide an understanding of the evolutionary mechanisms that have triggered local adaptations and eventual genetic differentiation. There are over 26 species concepts in use, but those suitable

Table 1
Overview of different Latin names used in the past for describing the Himalayan wolf lineage. The formal taxonomic classification of the Himalayan wolf is pending; the valid Latin taxon will be assigned according to taxonomic priority ruling.

Latin Taxon	Origin and year	Region	Common names	Literature
Canis laniger	Hodgson, 1847	China (Tibet, Qinghai, Gansu)	Tibetan wolf	(Hodgson, 1847; Shrotryia et al., 2012; Smith, 1840)
Canis chanco	Gray, 1863	Indian and Nepalese Himalaya, China, Mongolia[a]	Himalayan wolf, Tibetan wolf, Mongolian wolf	(Chetri et al., 2016; Chetri et al., 2017; Gray, 1863; Sharma et al., 2004)
Canis filchneri	Matschie, 1907	China (Tibet, Qinghai, Gansu)	Tibetan wolf	(Filchner, 1903; Bocci et al., 2017)
Canis (lupus) himalayensis	Aggarwal, 2003	Indian and Nepalese Himalaya	Himalayan wolf, Tibetan wolf	(Aggarwal et al., 2007; Werhahn et al., 2017a, 2017b)

[a] Historically *Canis lupus chanco* had also been used to describe the Holarctic grey wolf clade that is found in the Mongolian region which does not belong to the Himalayan wolf lineage.

for conservation purposes should take fitness into account (Frankham et al., 2012). This requirement is met by the Biological Species Concept (Mayr, 1942) and the Differential Fitness Concept (Frankham et al., 2012; Hausdorf, 2011), which we will discuss below. We address this requirement by providing extensive scientific evidence on the Himalayan wolf with mitochondrial and nuclear genome data. We include nuclear functional genes related to the hypoxia pathway, which are likely involved in the adaptation to the selective pressures of life at high altitudes with reduced atmospheric oxygen.

Specifically, we: 1) present insights into Himalayan wolf phylogeny and population genetics, 2) explore the genetic adaptation to the hypoxic conditions at high altitudes, 3) assess population status in three Nepalese Himalayan study sites, and 4) present a toolkit for conservation genetics to investigate phylogenies and monitor elusive species in difficult environments based on non-invasive sampling.

2. Methods

2.1. Study areas and sample collection

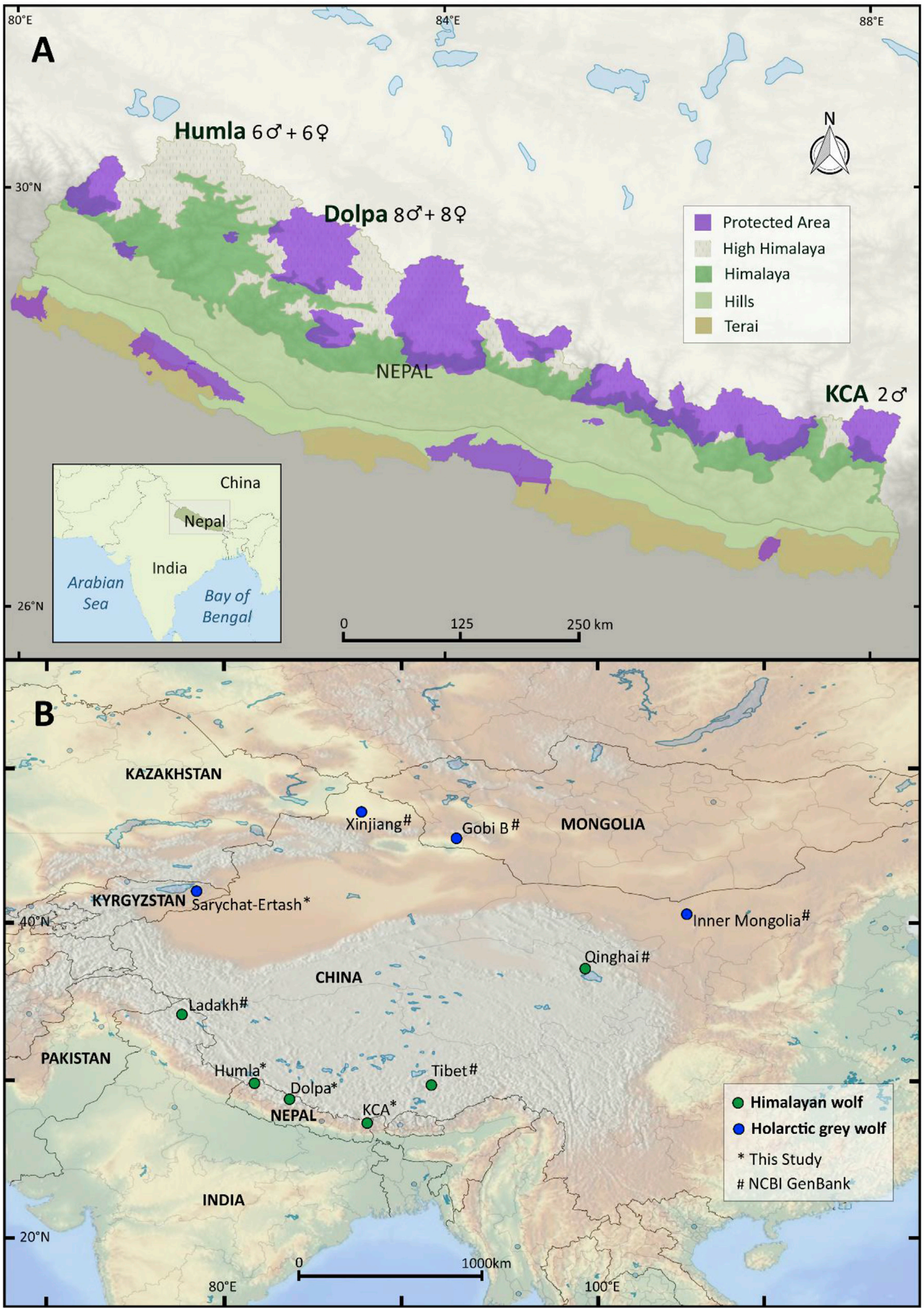
We conducted field work in three study areas across the Himalayas of Nepal. These include Humla and Dolpa districts in the north-western Nepalese Himalayas, and Kanchenjunga Conservation Area (KCA) in the north-eastern Nepalese Himalayas during the spring and summer periods of 2014–2016 (Fig. 1). The study area in Humla (29.97°-30.36°N, 81.50°-82.05°E) and Dolpa (29.00°-29.58°N, 82.89°-83.44°E) districts are both situated within the arid zones of the Nepalese Himalayas and comprise alpine grasslands and alpine steppe habitats with altitudes ranging from 3,600m to 5,600 m asl (Miehe et al., 2016). The study area in KCA (27.53°-27.88°N; 87.84°-88.13°E) is situated within the Inner Valleys of the eastern Himalayas (Miehe et al., 2016) (Fig. 2. A) and is composed of subalpine forests in the lower regions and alpine grassland habitats in the upper regions with altitudes ranging from 2,400m to 5,156 m asl. We searched for non-invasive carnivore samples in form of scats and hair over a total of 1,770 km. In 2015, we walked 605 km in Humla in an area of 384 km^2. In 2016, we walked 810 km in Dolpa over an area of 1,088 km^2 and 355 km in KCA over an area of 368 km^2. Our sample collection focussed on wolf, but we also sampled scats from red fox *(Vulpes vulpes* Linnaeus, 1758), Tibetan fox *(V. ferrilata* Hodgson, 1842), snow leopard (*Panthera uncia* Schreber, 1775), and Eurasian lynx (*Lynx lynx* Linnaeus, 1758). For genetic reference we opportunistically collected scat samples from local domestic dogs *(Canis lupus familiaris)* in the three study areas, which likely are from the dominant breed in the region, i.e. the Tibetan Mastiff. We have also included dog reference sequences from diverse breeds as obtained from NCBI GenBank.

2.2. Samples and analysis of genetic material

Overall, we collected a total of 287 samples (104 in Humla, 139 in Dolpa and 44 in KCA).

We also included in our analysis a set of samples from different populations/species: i) seven African wolf/golden jackal from North Africa (taxonomic classification pending, see (Gaubert et al., 2012; Koepfli et al., 2015; Rueness et al., 2015)) tissue samples from the Al Ain Zoo in the United Emirates and four from Morocco. African wolves are another taxonomically unresolved monophyletic *Canis* clade; ii) four wolf scat samples from Sachyat-Ertash in the Issyk Kul region of Kyrgyzstan to refine the distribution range and to explore a potential hybrid zone; iii) fifty scat and tissue samples from Iberian wolf *Canis lupus signatus* as reference (Large Carnivore Initiative for Europe, 2007); iv) six blood samples from European wolves, v) six tissue samples from Mongolian wolves; vi) six tissue samples from Ethiopian wolf (*C. simensis* Rüppell, 1840) as a comparison of another high altitude canid specialist living at 3,000–4,500 m asl (Marino, 2003).

Fig. 1. An adult Himalayan wolf in Humla district of Nepal (© Geraldine Werhahn).


A
80°E
84°E
88°E
30°N
26°N
Humla 6♂ + 6♀
Dolpa 8♂ + 8♀
KCA 2♂
NEPAL
N
Protected Area
High Himalaya
Himalaya
Hills
Terai
China
Nepal
India
Arabian Sea
Bay of Bengal
0
125
250 km
B
KAZAKHSTAN
Xinjiang#
Gobi B#
MONGOLIA
KYRGYZSTAN
Sarychat-Ertash*
Inner Mongolia#
40°N
Qinghai#
CHINA
Ladakh#
PAKISTAN
Humla*
Dolpa*
Tibet#
NEPAL
KCA*
INDIA
Himalayan wolf
Holarctic grey wolf
* This Study
# NCBI GenBank
20°N
0
1000km
80°E
100°E

An overview of the samples analysed within this study is presented in Supplementary Material Table S7. Refer to Werhahn et al. (2017a) for details on the field sampling procedure and lab methods for DNA extraction. Part of the genetic results from Humla (i.e. mitochondrial DNA and ZFX/ZFY) were reported in (Werhahn et al., 2017b) and are reported here only where relevant for completeness.

2.3. Phylogenetic analysis of mtDNA

Phylogenetic analysis of mtDNA was based on 242bp of D-loop and 508bp of cytochrome *b* sequences generated according to methods outlined in Werhahn et al. (2017b). For the cytochrome *b* sequences analysis we analysed a subset of those samples with unique D-loop sequences. Sequence data were quality controlled and edited using Geneious version 8.1.8. Besides the unique haplotypes generated from our field collected data we used a large number of additional reference samples of canids, mainly wolves, from NCBI GenBank for the phylogenetic analysis. These references also include samples from adjacent populations such as wolves from Xinjiang, Qinghai, Inner Mongolia and Tibetan Autonomous Region of China, but also wolves from Mongolia, India and eastern China (see Supplementary Material Tables S2 and S3). We constructed phylogenies with Bayesian (Mr Bayes (Huelsenbeck and Ronquist, 2001)) and maximum likelihood methods (PAUP* (Swofford, 2004)). Haplotype networks were drawn with PopART using Median-Joining Networks (Bandelt et al., 1999; Leigh and Bryant, 2015). Divergence estimates were calculated with Maximum Composite Likelihood in the software MEGA (Kumar et al., 2015; Tamura et al., 2004). The divergence time, i.e. age of nodes, was estimated with the software BEAST (Drummond et al., 2012) using 21 full mtDNA sequences of Holarctic grey wolf and Himalayan wolf, and one dhole (*Cuon alpinus* Pallas, 1811) and two coyote (*Canis latrans* Say, 1823) sequences as outgroups (GenBank accessions are found in Fig. 4 and originate from the following studies: Björnerfeldt et al., 2006; Koblmüller, 2016; Meng et al., 2009; Pang et al., 2009; H. Zhang and Chen, 2011; Zhang et al., 2014). The Himalayan wolf samples included in this divergence time analysis all shared either Himalayan wolf D-loop haplotype 2 or Himalayan wolf D-loop haplotype 4 and Himalayan wolf cytochrome B 1 as found in the Himalayan wolf samples from Nepal collected in this study. The KHY + G model was assumed for the nucleotide substitution and the ‘Bayesian SkyGrid’ and ‘Coalescent Exponential growth’ used as Tree prior.

2.4. nDNA microsatellite analysis

We used a specifically developed microsatellite multiplex protocol with 17 loci in four panels to analyse our genetic samples. In the development phase of the used microsatellite marker panel we checked for cross-amplification with all the potential prey species (Ghazali et al., 2016). For details on microsatellite primers, panels and PCR conditions see Supplementary Material Table S4.

For the microsatellite analysis a subset of the sample set used for the mtDNA analysis were used. See the Supplementary Material Table S10. To ensure we were working with higher quality samples, only the samples for which D-loop sequences could be obtained were processed for genotyping. To guard against errors introduced by allelic drop-out, we repeated each sample a minimum of three times. For scoring the microsatellite data we applied the following rules:

1) For homozygous genotypes we accepted three matching repeats.
2) For heterozygous genotypes we accepted two matching repeats.

In case of ambiguity we ran two more repeats per sample. One positive and at least two negative controls were run on all plates of samples to ensure proper calibration of the data. To check the data quality we estimated the mismatch between individual microsatellite reruns: we had 10.4% of missing loci data; of the scored loci we found mismatches between runs due to allelic dropout in 2.5% of the loci and in 2.1% due to other causes.

Microsatellite profiles were scored in Geneious (https://www.geneious.com/), scoring of the profiles was conducted at least twice to ensure consistency.

For the microsatellite analysis, basic quality control of the data was conducted with the Genalex plug-in for excel (Peakall and Smouse, 2006, 2012), the allelematch package of R (Galpern, Manseau, Hetting P., Smith and Wilson, 2012; R Core Team, 2018) was used for clustering of microsatellite genotypes, and the adegenet package of R was used for creating DAPC plots (Jombart, 2008; Jombart and Ahmed, 2011). The AMOVA was calculated in Genalex with 999 permutations. The Structure software package was used to estimate admixture using the admixture model and correlated allele frequencies settings for running simulations. We used a burn-in of 500,000 and MCMC replicates of 1,000,000 after burn-in (Pritchard et al., 2000). The optimal population number K was estimated by plotting K vs. Ln P(D) and verified with structure harvester (Earl and

Fig. 2. **A)** Humla, Dolpa and Kanchenjunga Conservation Area (KCA) study areas across the Nepalese Himalayas with the number of wolf individuals detected through genotyping. The potential Himalayan wolf range in Nepal spans throughout the entire ‘High Himalaya’ habitats and adjacent parts of the ‘Himalaya’ habitats. **B)** Himalaya wolf distribution, with mtDNA verified samples in green and Holarctic grey wolf in blue. Sample locations originate from this study and following studies: (Fan et al., 2016; Koblmüller, 2016; Meng et al., 2009; Pilot et al., 2010; Sharma et al., 2004; Thalmann et al., 2013; Werhahn et al., 2017b; H. Zhang and Chen, 2011; Zhang et al., 2014). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

vonHoldt, 2012). We sexed our samples with the genetic protocol of Sastre et al. (2009) which indicates male (presence of Y chromosome) with amplification at 113-114bp, and the presence of X chromosome with amplification at 186-203bp. The presence of X chromosome in absence of a Y chromosome was treated as a female. The allele calling for the sex analysis was scored according to two identical repeats for the male and three identical repeats for the female.

We used the package capwire in R (Pennell and Miller, 2012) to estimate Maximum Likelihood population sizes from the genotyping datasets obtained from the samples from Humla and Dolpa.

2.5. ZFX/ZFY analysis

We analysed sequences from the final intron of the zinc-finger X-chromosomal (ZFX) and Y-chromosomal (ZFY) genes to investigate phylogenetics. Refer to Werhahn et al. (2017b) for details on the ZFX/ZFY analysis where we applied the methods adapted from (Koepfli et al., 2015; Nakagome et al., 2008; Tsubouchi et al., 2012; Verma and Singh, 2003).

2.6. Functional genes: hypoxia sequencing

We designed primers to target short (6-73bp) regions of the nuclear genome that contain non-synonymous SNPs in four genes (ANGPT1, RYR2-1, RYR2-2, EPAS1) suspected to be involved in the hypoxia pathway and shown to segregate between highland and lowland canids within China (Zhang et al., 2014). We specifically designed primers to yield short (<200bp) PCR products suitable for degraded sample types such as scat samples. For details on primers used see the Supplementary Material Table S5.

3. Results

3.1. mtDNA phylogenetics of the Himalayan wolf

For D-loop we generated 72 canid sequences from Humla (published in Werhahn et al., 2017a), 89 canid sequences from Dolpa, and 13 from KCA. For cytochrome *b* we generated a subset of 24 canid sequences from Humla (published in Werhahn et al., 2017a), 16 from Dolpa, and 7 from KCA (Figs. 2 and 3, Table 2).

In Humla we found Himalayan wolf D-loop haplotypes 1–3, while in Dolpa we found Himalayan wolf D-loop haplotypes 3–5, and in KCA, we found only Himalayan wolf D-loop haplotype 5 (Fig. 3A.). In the dog samples we found four new D-loop dog haplotypes and four new cytochrome *b* dog haplotypes for Nepal. For NCBI GenBank accessions see Supplementary

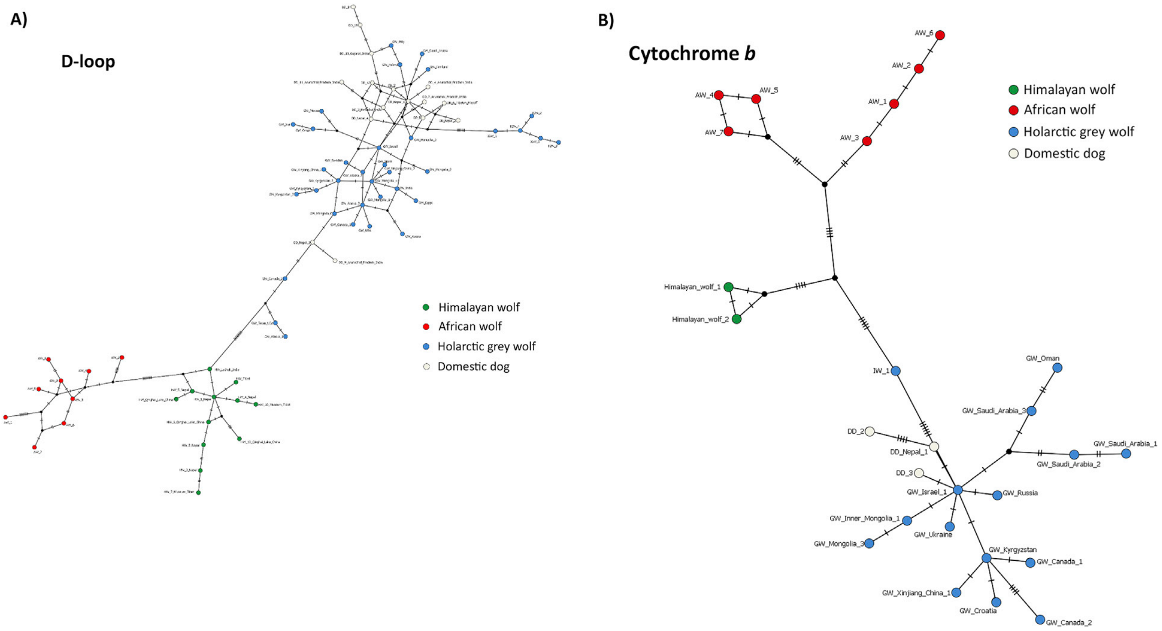


Fig. 3. Haplotype networks for A) D-loop, and B) cytochrome *b*. Himalayan wolf is shown in green, African wolf in red (AW), and Holarctic grey wolf in blue (GW for grey wolf, IW for Indian wolf *Canis lupus pallipes*), and DD for domestic dog shown in white. For the corresponding phylogenetic trees see Supplementary Material Figs. S1 and S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

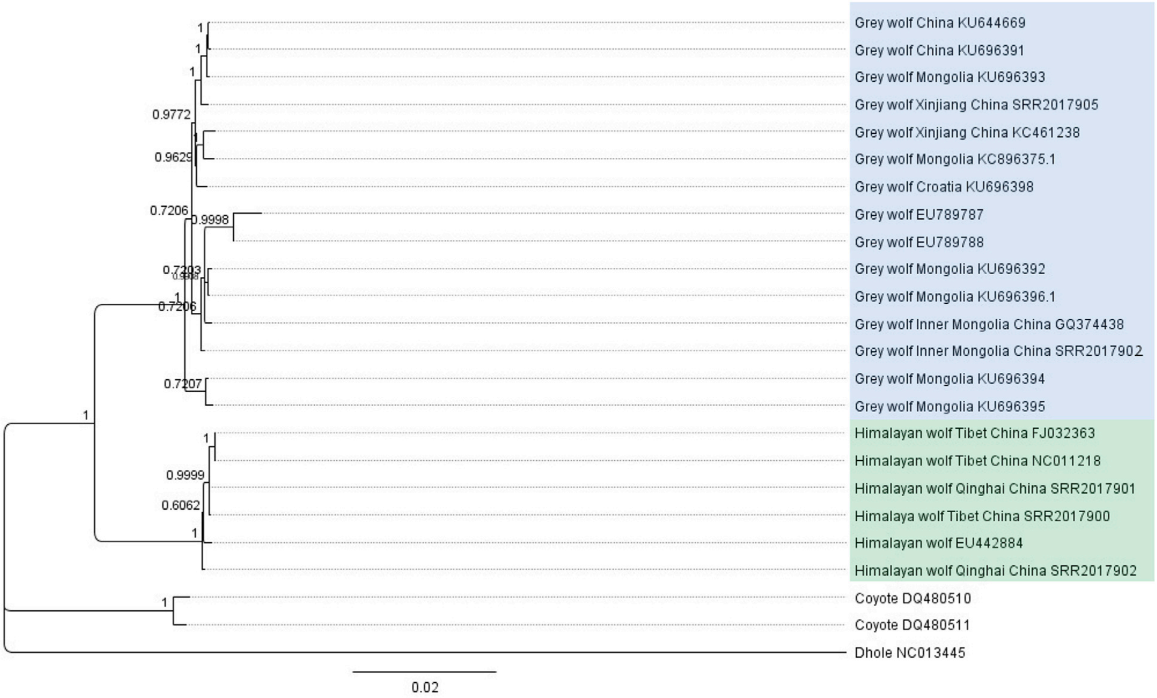


Fig. 4. Bayesian phylogeny built with full mitochondrial genome sequences showing posterior probabilities at nodes.

Material Table S1. For an overview of the results per amplified sample across the different analyses consult Supplementary Material Table S10. For divergence estimates based on mtDNA across the different considered wolf populations see Table 3.

3.2. Divergence estimate for the Himalayan wolf

Based on analysis of the full mitochondrial genome in the software BEAST we estimated the divergence time of the Himalayan wolf from the Holarctic grey wolf complex at 691,000–740,000 years before present (691,000 years before present using the Tree prior ‘Coalescent: Exponential growth’, 740,000 years before present using the Tree prior ‘Bayesian SkyGrid’) (Fig. 4). Divergence estimates based on this full mtDNA dataset calculated in MEGA are shown in Table 4.

3.3. Microsatellite results

All 17 loci were polymorphic across the considered wolf populations. Population-wise diversity statistics are shown in Table 5 and Table 6, and complete statistics per locus and population is found in Supplementary Material Table S6.

We found four populations ($K = 4$) in our microsatellite dataset as analysed with Structure and the R package adegenet. The Structure analysis at $K = 2$ splits first the Himalayan wolf from all other samples (including grey wolves from Europe, Mongolia and Kyrgyzstan; Iberian wolf, African wolf). This is followed by splitting the African wolf at $K = 3$, and then Iberian wolf at $K = 4$ (Fig. 5), and at $K = 5$ the Kyrgyz wolf samples. The DAPC plot differentiates the Iberian wolf at $K = 5$ but not at $K = 4$, while it splits the Kyrgyz wolf samples at $K = 4$. In contrast Structure differentiates the Iberian wolf at $K = 4$ while the Kyrgyz wolf samples only split at $K = 5$ (Tables 5 and 5, Fig. 5). Allele ranges and private alleles per population are shown in Fig. 6. The individual identification based on genotyping was combined with the sexing data to reveal individuals and sex per study area. Minimum total counts and Maximum Likelihood population estimates are shown in Table 8 and Fig. 2A.

3.4. Cost-effective microsatellite panel for Himalayan wolf monitoring research

For future monitoring research we propose a more cost-optimized panel of 10 loci identified in this study to serve two purposes: 1) differentiate the Himalayan wolf from the Holarctic grey wolf, and 2) to identify unique individuals within the sampled area. This selection of the 10 best performing loci is based on the probability of identity, allelic diversity and private alleles in the Himalayan wolf population. The 10 loci are listed in the following and are also highlighted in the Supplementary

Table 2
Haplotype overview of the samples collected in the three study areas, Humla, Dolpa, and KCA in Nepal.

Haplotype	Lineage	Humla[a]	Dolpa	KCA
No. of D-loop sequences generated		79	93	14
		No. of sequences in all samples including repeats (Haplotype frequency per site in % based on unique wolf individuals, wolf only and only for D-loop #)		
Himalayan wolf D-loop 1	Himalayan wolf[d]	13 (18.2%)	1	0
Himalayan wolf D-loop 2	Himalayan wolf[d]	56 (72.7%)	0	0
Himalayan wolf D-loop 3	Himalayan wolf[d]	7 (9.1%)	37 (50%)	2[b]
Himalayan wolf D-loop 4	Himalayan wolf[d]	0	49 (50%)	0
Himalayan wolf D-loop 5	Himalayan wolf[d]	0	1[b]	12 (100%)
Domestic dog D-loop Nepal 1	*C. lupus familiaris*	3[a]	1[c]	
Domestic dog D-loop Nepal 2	*C. lupus familiaris*	0	1[c]	
Domestic dog D-loop Nepal 3	*C. lupus familiaris*	0	1[c]	
Domestic dog D-loop Nepal 4	*C. lupus familiaris*	0	1[c]	
Domestic dog D-loop Nepal 5	*C. lupus familiaris*	0	1[c]	
No. of cytochrome-b sequences		16	16	14
Himalayan wolf cytochrome B 1	Himalayan wolf[d]	11	12	7
Himalayan wolf cytochrome B 2	Himalayan wolf[d]	3	0	0
Domestic dog cytochrome B Nepal 1	*C. lupus familiaris*	2	1	0
Domestic dog cytochrome B Nepal 2	*C. lupus familiaris*	0	1	0
Domestic dog cytochrome B Nepal 3	*C. lupus familiaris*	0	1	0
Domestic dog cytochrome B Nepal 4	*C. lupus familiaris*	0	1	0
ZFX/ZFY				
Himalayan wolf Nepal ZFY	Himalayan wolf[d]	5 and partial[a]	7	2
Himalayan wolf Nepal ZFX	Himalayan wolf[d]	9[a]	7	1

[a] Published in (Werhahn et al., 2017b).
[b] No individual ID for this sample obtained.
[c] Domestic dog samples were only collected for comparative purposes, and therefore frequencies are not given for dogs, while the cytochrome *b* was tested only on a subset and hence also no haplotype frequencies are given.
[d] Taxonomic classification pending.

Table 3
Divergence estimates based on unique haplotypes of 242 bp of D-loop and 508 bp of cytochrome *b* gene sequences calculated with MEGA. The Holarctic grey wolf contains samples from North America and Eurasia (Supplementary Material Tables S2 and S3).

	N D-loop/Cyt *b*	Himalayan wolf		Holarctic grey wolf		Iberian wolf		Indian wolf		African wolf		Golden jackal	
		D-loop	Cyt *b*	D-loop	Cyt *b*	D-loop	Cyt *b*	D-loop	Cyt *b*	D-loop	Cyt *b*	D-loop	Cyt *b*
Himalayan wolf	13/2												
Holarctic grey wolf	32/16	0.111	0.039										
Iberian wolf	3/1	0.129	0.034	0.038	0.005								
Indian wolf	5/1	0.128	0.023	0.088	0.018	0.098	0.016						
African wolf	9/7	0.108	0.028	0.082	0.044	0.095	0.040	0.114	0.028				
Golden jackal	5/5	0.150	0.053	0.167	0.079	0.183	0.073	0.179	0.061	0.185	0.051		
Ethiopian wolf	3/3	0.180	0.048	0.123	0.062	0.145	0.057	0.138	0.050	0.120	0.046	0.162	0.054

Material Table S4: FH2137, C20.253, FH2001, CPH09, CPH12, FH2010, FH2161, C09.173, CPH04, C13.758. We found a probability of identity of 2.2E-04 with this panel of 10 best performing loci in this study (Table 7).

3.5. *ZFX/ZFY final intron sequences*

The testing of additional Himalayan wolf samples for the ZFX and ZFY final intron sequences from the study areas in Humla, Dolpa and KCA confirm the findings of (Werhahn et al., 2017b). The Iberian wolf samples and three tested domestic dog samples from Nepal share the ZFY haplotype with the Holarctic grey wolf from Canada, Europe and Mongolia. This is in line with data from other genes where the domestic dogs clusters within the Holarctic grey wolf complex and the Iberian wolf is considered a grey wolf subspecies. Interestingly the three tested wolves from Kyrgyzstan share the ZFY haplotype with the Holarctic grey wolf, but the ZFX haplotype with the Himalayan wolf (Supplementary Material Tables S8 and S9).

3.6. *Hypoxia adaptation of the Himalayan wolf*

For the Himalayan wolf from Nepal (this study) we found distinct genetic variants on non-synonymous loci in all four tested genes that are suspected to be linked to the hypoxia pathway, as also found for wolves from Tibet tested in Zhang et al.

Table 4
Divergence estimates, indicative of genetic distance among lineages, based on the full mtDNA from Fig. 4 and calculated in MEGA.

	Coyote	Himalayan wolf	Grey wolf
Coyote			
Himalayan wolf	0.05		
Grey wolf	0.05	0.03	
Dhole	0.106	0.110	0.109

Table 5
Summary statistics per populations. Mean and standard error of genetic diversity indices for 17 autosomal microsatellite loci in different wolf populations. N = number of individuals; Na = number of alleles; Ne = number of effective alleles; He = expected heterozygosity, Ho = observed heterozygosity), F =fixation index; PA = number of private alleles.

Lineage (Population)		N	Na	Ne	Ho	He	F	PA
Himalayan wolf[a]	**Mean**	23.294	4.706	2.887	0.557	0.573	0.067	12
	SE	1.714	0.381	0.327	0.062	0.051	0.054	
African wolf[a]	**Mean**	6.588	2.176	1.745	0.445	0.350	−0.242	7
	SE	0.243	0.196	0.155	0.077	0.057	0.053	
Holarctic grey wolf	**Mean**	3.529	2.118	1.905	0.691	0.390	−0.790	3
Canis lupus spp. (Kyrgyzstan)	**SE**	0.212	0.225	0.165	0.112	0.064	0.048	
Holarctic grey wolf	**Mean**	2.000	2.824	2.616	0.794	0559	−0.433	4
Canis lupus spp. (Europe)	**SE**	0.000	0.214	0.228	0.075	0.047	0.081	
Holarctic grey wolf	**Mean**	2.000	2.471	2.153	0.765	0.493	−0.525	4
Canis lupus spp. (Mongolia)	**Se**	0.000	0.151	0.143	0.076	0.042	0.044	
Iberian wolf	**Mean**	15.000	4.588	2.949	0.584	0.611	0.025	9
Canis l. signatus	**SE**	0.000	0.394	0.241	0.044	0.043	0.046	
Domestic dog	**Mean**	2.647	3.059	2.535	0.608	0.526	−0.166	0
Canis l. familiaris	**SE**	0.170	0.277	0.240	0.081	0.058	0.092	

[a] Taxonomic classification pending.

Table 6
Pairwise AMOVA F_{st} estimates among Himalayan wolf (with populations from Humla, Dolpa and KCA), African wolf, Holarctic grey wolf (Mongolia, Europe and Kyrgyzstan), and Iberian wolf based on 17 microsatellite loci.

	Himalayan wolf			Holarctic grey wolf			
		Humla	Dolpa	African wolf	Mongolia	Europe	Kyrgyzstan
Himalayan wolf							
Humla							
Dolpa		0.092					
KCA		0.163	0.049				
African wolf	0.371						
Holarctic grey wolf							
Mongolia	0.257			0.426			
Europe	0.233			0.385	0.182		
Kyrgyzstan	0.263			0.477	0.383	0.317	
Iberian wolf	0.283			0.339	0.179	0.121	0.329

(2014), when compared to the other wolf lineages tested here (i.e. Holarctic grey wolf from Mongolia and Europe, Iberian wolf, African wolf, Ethiopian wolf). Wolves from Qinghai and Kyrgyzstan show a mixture of the Himalayan wolf and the Holarctic grey wolf haplotypes in the four tested SNP genes (Table 9).

4. Discussion

This study provides further evidence for the genetic distinctiveness of the Himalayan wolf from the Holarctic grey wolf, based on mitochondrial DNA (D-loop and cytochrome *b*) and nuclear DNA (ZFX and ZFY, non-synonymous SNPs within candidate hypoxia genes, and microsatellites). The Himalayan wolf differs from the Holarctic grey wolf in a set of functional genes suspected to be related to the hypoxia pathway (Zhang et al., 2014). These are genes which seemingly are involved in the adaptation to life at high altitudes where less atmospheric oxygen is available. We substantiate the evidence that this Himalayan wolf is found specifically in the high altitude habitats of the Tibetan Plateau and the Himalayas.

We found a divergence time estimate of 691,000–740,000 years before present for the Himalayan wolf. Matsumura et al. (2014b) estimated that the Himalayan wolf has diverged from other wolf-dog clades 549,800 [495,100–605,600] years before present based on full mitochondrial genomes. Sharma et al. (2004) estimated its time of divergence at more than 800,000 years before present based on 440 bp mitochondrial DNA of the cytochrome *b* gene sequences. Regardless of which

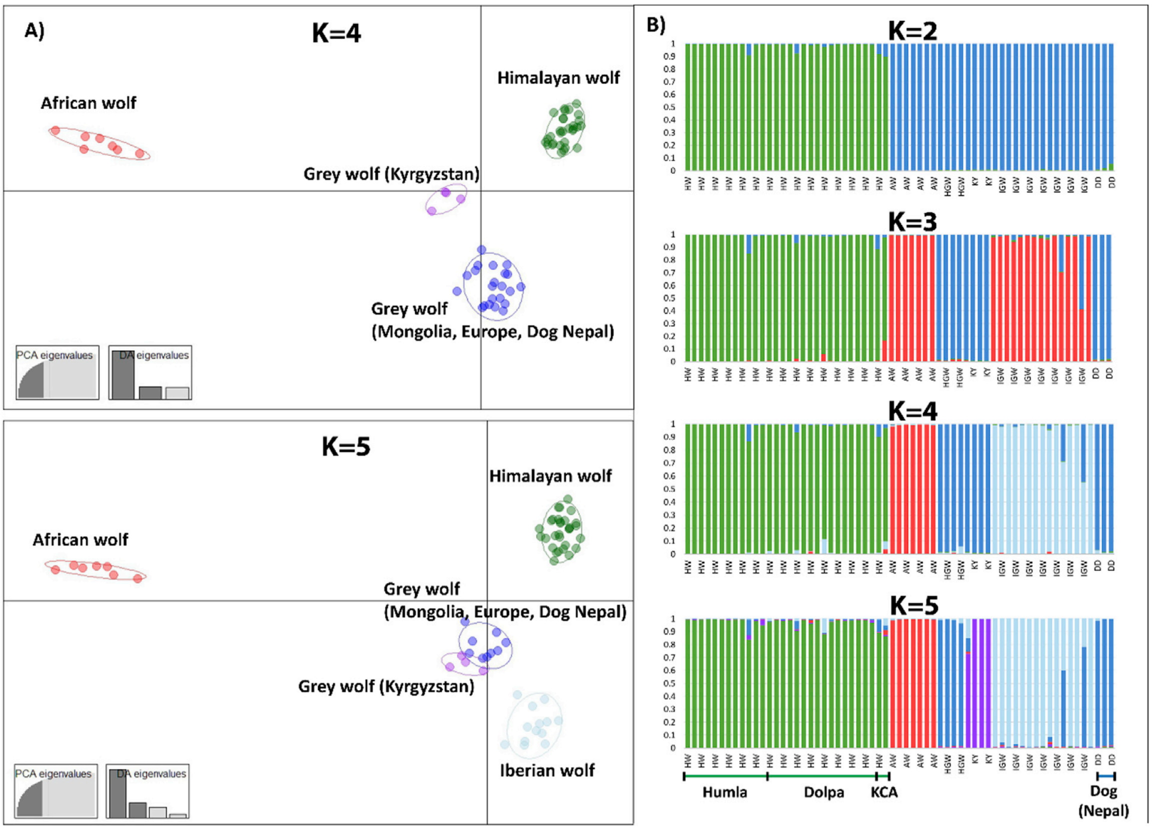


Fig. 5. **A)** DAPC with four and five populations (K = 4 and K = 5) showing the Himalayan wolf, the African wolf, grey wolf including wolves from Europe and Mongolia, the Iberian wolf, and Kyrgyz wolf samples. **B)** STRUCTURE plots with Himalayan wolf (HW from Humla, Dolpa, and KCA) splitting at K = 2; African wolf (AW) at K = 3, the Iberian wolf (IGW) at K = 4, and Kyrgyz wolf samples (KY) at K = 5. HGW comprises grey wolves from Europe and Mongolia (HGW), and also domestic dogs from Nepal (DD) assemble within this Holarctic grey wolf cluster.

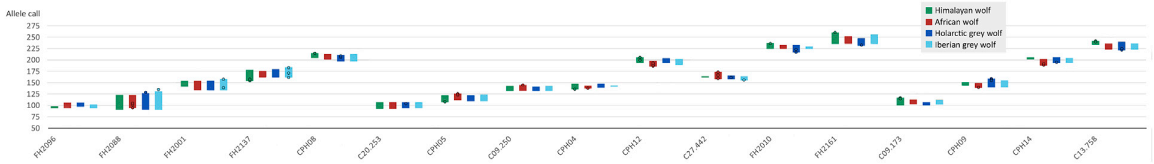


Fig. 6. Allele range and private alleles per population as indicated by the circles. The Holarctic grey wolf includes samples from Europe, Mongolia and Kyrgyzstan (For full details on allele frequencies see Supplementary Table S6.

Table 7
Probability of identity (PI) and Probability of identity of siblings (PIsib) across all 17 loci, the 10 and 8 best performing loci for monitoring Himalayan wolf, and the 8 worst performing loci for Himalayan wolf (HW).

		All 17 loci	10 recommended loci	8 best loci	8 worst loci
Himalayan wolf[a]	**PI**	4.4E-12	1.2E-09	2.6E-08	7.9E-04
	PIsib	1.6E-05	2.2E-04	8.9E-04	3.6E-02
African wolf[a]	**PI**	2.5E-12	5.6E-09	1.1E-07	1.0E-04
	PIsib	9.9E-06	3.4E-04	1.3E-03	1.5E-02
Holarctic grey wolf	**PI**	6.0E-16	3.5E-11	1.2E-09	3.0E-06
Canis lupus spp.	**PIsib**	6.6E-07	9.0E-05	4.2E-04	3.4E-03
Iberian wolf	**PI**	3.4E-13	5.9E-10	2.2E-08	9.6E-05
Canis lupus signatus	**PIsib**	5.0E-06	1.7E-04	7.7E-04	1.4E-02

[a] Taxonomic classification pending.

Table 8
Minimum number of individuals found in the three study areas based on 17 microsatellite loci. Maximum Likelihood population sizes per study area as calculated with capwire in R.

Study area and year	No. of individuals	Sex	Density[a] per 100 km^2 based on total count	Total ML-Population size (95% confidence interval)
Humla 2015	12	6 female, 6 male	3.125	60 (43–83) individuals
Dolpa 2016	16	8 female, 8 male	1.47	28 (15–51) individuals
KCA 2016	2	2 males	0.54	NA

[a] Densities to be considered as approximates. The number of individuals per 100 km^2 was obtained by faecal samples collected in the study areas searched. A precise density estimate is to be obtained by revealing the home range of individuals and packs.

divergence time estimate considered, all estimates support the notion that the Himalayan wolf separated as an independent lineage before the radiation of the Holarctic grey wolf complex.

The genetic distinctness of the Himalayan wolf from the Holarctic grey wolf is further supported by microsatellite data analysis at 17 loci. In line with our previous study we found that the Himalayan wolf, the African wolf and the Holarctic grey wolf each have a unique ZFY gene haplotype, while for the ZFX the Himalayan wolf and African wolf share a haplotype which is distinct from the Holarctic grey wolf (Werhahn et al., 2017b).

4.1. Himalayan wolf distribution

We find evidence across multiple markers that there is introgression in Nepal from another wolf and/or domestic dog lineage (Fig. 5/Table 9). On the eastern fringes of the Tibetan Plateau this is possibly higher as indicated by many animals from Qinghai being heterozygous for the SNPs within the supposed hypoxia pathway related genes. This may indicate the presence of hybridization at the boundaries of different wolf populations or hybridization with domestic dog (Table 9). Due to the close relatedness of domestic dog to Holarctic grey wolf, it is difficult to dissociate which group this introgression has come from, it may be grey wolf and domestic dog. But it seems likely, given their presence across all study areas, that domestic dogs play a role and this should be considered a conservation concern for the Himalayan wolf across its range (Hennelly et al., 2015). The microsatellite marker panels used were not specifically designed to distinguish between domestic dog and Holarctic grey wolf and to do this would likely require both higher density nuclear data and comprehensive reference data from wolf and dog populations which exceed the extent of this study. This should be a priority in the future.

We refine the understanding of the Himalayan wolf's wider distribution range by verifying its occurrence across locations in the high altitude habitats of the Tibetan Plateau and the Himalayas. This is based on genetic samples from different studies originating from Tibet and Qinghai province of China which all cluster with the Himalayan wolf lineage. In contrast, wolves found in the lower altitudes of Inner Mongolia and Xinjiang Province of China, and Mongolia cluster with the Holarctic grey wolf species complex (Fig. 2. B and Fig. 3).

4.2. High altitude ecological adaptation of the Himalayan wolf

The Himalayan wolf appears specifically adapted to life in the high altitude ecosystems of Asia (Zhang et al., 2014). We found differentiation in the Himalayan wolf compared to the Holarctic grey wolf in the hypoxia pathway suspected related genes EPAS1, ANGPT1 and RYR2, all of which are considered to be linked to hypoxia adaptation. All three genes showed non-synonymous fixed mutations in the Himalayan wolves (Zhang et al., 2014). The EPAS1 is a hypoxia pathway suspected related gene and directly regulates key genes such as erythropoietin (EPO) and the vascular endothelial growth factor (VEGF) (Patel and Simon, 2008), it is associated with decreased blood flow resistance, which may help to improve haemorheologic fitness and in humans it is associated with differences in haemoglobin concentration at high altitude (Gou et al., 2014; Huerta-Sanchez, 2014). ANGPT1 can increase tissue vascularization which promotes increased oxygen delivery (Prabhakar and Semenza, 2012). The gene RYR2 is linked to cardiac excitation-contraction regulation, i.e. heart function (Gillespie and Fill, 2013).

These three genes also exhibit differentiation in high altitude human populations, which suggest, perhaps unsurprisingly, that similar evolutionary drivers of natural selection have shaped wolves and humans of the Tibetan Plateau (Huerta-Sanchez, 2014; Li et al., 2014; Zhang et al., 2014). The same modifications of the EPAS1 gene were found in Tibetan mastiff dogs which led to the proposition that these dogs may have acquired hypoxia adaptation through hybridization with high-altitude wolves (Gou et al., 2014; Zhang et al., 2014) and this is confirmed in this study with the Tibetan mastiff dog samples collected in our Nepalese study areas.

The Himalayan wolf across Nepal showed the same differentiation on the hypoxia suspected related genes as wolf samples from Tibet (China) and partly found in the samples from Qinghai Tibet (China) analysed by (Zhang et al., 2014), all of which are attributed to the Himalayan wolf lineage based on mtDNA and nDNA. It is hypothesized that the harsh conditions in the high altitude environment have resulted in the evolution of the most distinct wolf population in the Old World as represented in the Himalayan wolf (Fan et al., 2016).

Table 9
Results on the hypoxia suspected related genes across the different tested locations and populations. The Himalayan wolf lineage shows diversification in these genes compared to other wolf lineages. T* is inferred from dog whole reference genome.

Gene (Locus†)		**ANGPT1**	**EPAS1-1**		**RYR2-1**		**RYR2-2**	
Samples								**Reference**
Location	**Lineage***	**8141664**	**48630137**	**48630159**	**2589113**	**2589135**	**2778722**	
Summary								
Nepal	**Himalayan wolf**	C/T C	G	A	C	T	G	This Study
Tibet (China)	**Himalayan wolf**	C	G	A	C	T*	G	Zhang et al., 2014
Qinghai (China)	**Himalayan wolf**	C/T	G/T	A/G	C/G	T*	C/G	Zhang et al., 2014
Kyrgyzstan	**Grey wolf**	T	G/T	A/G	G	T	C	This Study
Nepal	**Dogs**	T	G	A	G	T*	C	This Study
Xinjiang (China)	**Grey wolf**	T	T	G	G	T*	C	Zhang et al., 2014
Inner Mongolia (China)	**Grey wolf**	T	T	G	G	T*	C	Zhang et al., 2014
Mongolia	**Grey wolf**	T	T	G	G	T	C	This Study
Europe	**Grey wolf**	T	T	G	G	T	C	This Study
Iberia, Spain	**Grey wolf**	T	T	G	G	T	C	This Study
North Africa	**African wolf**	T	T	G	G	T	G	This Study
Ethiopia	**Ethiopian wolf**	T	T	G	G	C	C	This Study
Sample per location and lineage								
Nepal (67 Samples)								
3 Nepal samples	**Dogs (Nepal)**	T	G	A	G	T*	C	This Study
26 Nepal samples	**Himalayan wolf**	C	G	A	C	T*	G	This Study
30 Nepal samples	**Himalayan wolf**	C/T	G	A	C	T*	G	This Study
1 Nepal sample	**Himalayan wolf**	C/T	G	A	C	T*	C	This Study
1 Nepal sample	**Himalayan wolf**	C	G/T	A/G	C	T*	G	This Study
4 Nepal samples	**Himalayan wolf**	C	G	A	C	T*	C/G	This Study
1 Nepal sample	**Himalayan wolf**	C/T	G	A	C/G	T*	C/G	This Study
1 Nepal sample	**Himalayan wolf**	C	G	A	G	T*	fail	This Study
Tibet	**Himalayan wolf**	C	G	A	C/G	T*	C/G	Zhang et al., 2014
	Himalayan wolf	C	G	A	C	T*	G	Zhang et al., 2014
	Himalayan wolf	C/T	G	A	C	T*	G	Zhang et al., 2014
	Himalayan wolf	C	G	A	C	T*	G	Zhang et al., 2014
Qinghai	**Himalayan wolf**	C/T	G/T	A/G	C/G	T*	C/G	Zhang et al., 2014
	Himalayan wolf	C/T	G	A	C	T*	G	Zhang et al., 2014
	Himalayan wolf	C	G	A	C/G	T*	C/G	Zhang et al., 2014
	Himalayan wolf	C/T	G/T	A/G	C/G	T*	C/G	Zhang et al., 2014
	Himalayan wolf	C	G/T	A/G	C	T*	G	Zhang et al., 2014
	Himalayan wolf	C/T	G/T	A/G	C/G	T*	C/G	Zhang et al., 2014
	Himalayan wolf	C/T	G/T	A/G	C/G	T*	C/G	Zhang et al., 2014
	Himalayan wolf	C/T	G/T	A/G	C/G	T*	C/G	Zhang et al., 2014
	Himalayan wolf	T	G	A	G	T*	C	Zhang et al., 2014
	Himalayan wolf	C	G	A	C/G	T*	C/G	Zhang et al., 2014
Kyrgyztan	**Grey wolf**	T	G/T	A/G	G	T	C	This Study
	Grey wolf	T	G/T	A/G	G	T	C	This Study
	Grey wolf	T	G/T	A/G	G	T	fail	This Study
	Grey wolf	T	T	G	fail	fail	fail	This Study
Xinjiang	**Grey wolf**	C/T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014

	Grey wolf	C/T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	C/T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	C/T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	G/T	A/G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	G/T	A/G	G	T*	C	Zhang et al., 2014
Inner Mongolia	**Grey wolf**	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
	Grey wolf	T	T	G	G	T*	C	Zhang et al., 2014
Mongolia	**Grey wolf**	T	T	G	G	T	C	This Study
Europe	**Grey wolf**	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
Northern Spain	**Grey wolf**	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	fail	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
	Grey wolf	T	T	G	G	T	C	This Study
North Africa	**African wolf**	T	T	G	fail	fail	G	This Study
North Africa	**African wolf**	T	T	G	fail	fail	G	This Study
Ethiopia	**Ethiopian wolf**	T	T	G	G	C	C	This Study
	Ethiopian wolf	T	T	G	G	C	fail	This Study
	Ethiopian wolf	T	T	G	G	C	C	This Study
	Ethiopian wolf	T	T	G	fail	fail	C	This Study
	Ethiopian wolf	T	T	G	G	C	C	This Study
	Ethiopian wolf	T	T	G	G	C	fail	This Study

† Aligned to the dog complete genome (boxer genome, CanFam3.1); * Lineage based on mtDNA.

Wolves in Inner Mongolia (China, Zhang et al., 2014), Xinjiang (China, Zhang et al., 2014) and Mongolia, where the landscape is at considerably lower altitudes (~1,000–2,000 m asl), do not show this differentiation on the hypoxia suspected related genes and in line with this they cluster within the Holarctic grey wolf complex in our mtDNA and nDNA analysis.

The tested wolf samples from Kyrgyzstan group within the Holarctic grey wolf complex based on mtDNA and the ZFY analysis, while in the tested hypoxia-pathway suspected related genes, the ZFX and the microsatellite data, the Kyrgyz wolf samples show an intermediate type between the Himalayan wolf and the Holarctic grey wolf. However these results for the Kyrgyz wolf samples need to be considered as preliminary given the low sample size ($n = 4$) from this region. These samples originate from Sachyat-Ertash in the Issyk Kul region of Kyrgyzstan with habitats above 3,000 m asl. Given the proximity and connection of these Kyrgyz mountains with the Tibetan Plateau and the Himalayas through the Tian Shan, Pamir, Karakoram, Hindu Kush Mountain ranges, it is possible that at least part of the grey wolf population in Kyrgyzstan has acquired partly differentiated hypoxia related genes through gene flow from the Himalayan wolf. This could be indicative of a hybrid zone between the two lineages but requires further data from larger regions within this presumed hybrid zone.

The tested Ethiopian wolf samples do not share the same SNPs that confer hypoxia adaptation with the Himalayan wolf. If the Ethiopian wolf shows genetic adaptation to high altitudes (3,000–4,500 m), then this must be reflected in mutations at different SNP loci (i.e they are not shared by decent with the Himalayan wolf). The unique haplotype for the Ethiopian wolf found in this study on the RYR2-1 SNP gene may be indicative of this (see Table 9).

4.3. *Evolutionary causes for Himalayan wolf divergence*

From the evidence we present here, it emerges that the Himalayan wolf has evolved as a unique lineage with specific adaptation to life at high altitudes, not found in the Holarctic grey wolf. This is also reflected in its distribution range which seems to be defined by the high altitude ecosystems of the Himalayas and the Tibetan Plateau.

We propose that the environmental cline as found with increasing altitude in combination with the natural intraspecific competition occurring in wolves may have played a significant role in the speciation of the Himalayan wolf into a unique wolf lineage.

Sympatric speciation can be triggered by various environmental mechanisms (Bolnick and Fitzpatrick, 2007; Dieckmann and Doebeli, 1999; Doebeli and Dieckmann, 2000; Kawecki, 1997), and can occur based on evolutionary branching along environmental clines of moderate steepness given the presence of intraspecific competition (Doebeli and Dieckmann, 2003; Leonard, 2014) which is well documented among wolf packs (Mech and Boitani, 2003). Distance on a continental scale can cause a pattern of isolation likely related to climate and habitat (Geffen et al., 2004). Consequently, an environmental cline such as extreme altitudes in the case of the Himalayan wolf can provide a potent mechanism for the evolutionary differentiation from the ancestors of the Holarctic grey wolf complex.

Kawecki (1997) argues that evolution of ‘habitat races’ can be driven by genetic variation due to loci that affect fitness in one habitat and are neutral or nearly so in others, such as the genes responsible for hypoxia adaptation in the Himalayan wolf.

If our hypothesis is correct that the high altitude environment has been an important ultimate cause for the divergence of the Himalayan wolf lineage, it is also likely to be one of the most important proximate mechanisms for its persistence besides the Holarctic grey wolf.

4.4. *Results in the context of species concepts*

Evidences in this and other phylogenetic studies confirm that the Himalayan wolf is a highly divergent wolf lineage of the Asian high altitudes (Aggarwal et al., 2007; Fan et al., 2016; Gaubert et al., 2012; Koepfli et al., 2015; Leonard et al., 2007; Matsumura et al., 2014a; Pilot et al., 2010; Rueness et al., 2015; Sharma et al., 2004; Werhahn et al., 2017b; Zhang and Chen, 2011; Zhang et al., 2014). The Himalayan wolf is currently not assigned a taxon. Evidence justifying it as a subspecies is elaborated here and elsewhere (see Aggarwal et al., 2007; Sharma et al., 2004; Werhahn et al., 2017b). Importantly, this study informs the question of whether the Himalayan wolf should be classified as a species by providing data on genetic differentiation found on multiple sites of the nuclear genome including functional genes suspected to be related to the hypoxia pathway.

The Biological Species Concept and the Differential Fitness Species Concept are considered appropriate species concepts for conservation purposes (Frankham et al., 2012, 2017). For the respective definitions see (Mayr, 1942) and (Hausdorf, 2011). In the framework of these species concepts our results point towards the Himalayan wolf's eligibility as a species. We base this suggestion on the isolation between the Himalayan wolf and the Holarctic grey wolf species complex as evidenced by the genetic diversification across different regions of the mitochondrial and the nuclear genome. However, we do assume and find indications that hybridization between the Himalayan wolf and Holarctic grey wolf may occur where conditions favour, e.g. in the adjacent mountain ranges around the extreme heights of the Tibetan Plateau and the high Himalayan regions, and that a hybrid zone(s) may exist. Further surveys across the range are required to elucidate this. We hypothesize that the high altitude adaptation was a crucial driver in the genetic diversification and presents an important proximate mechanism in the persistence of the Himalayan wolf and the Holarctic grey wolf despite hybridization in the mountainous boundary areas around its high altitude distribution range. This is further corroborated by the Himalayan wolf presenting a monophyletic lineage with a 3.9% divergence on the mtDNA cytochrome *b* gene from the Holarctic grey wolf which could be further used to justify the Himalayan wolf as a distinct species (Table 3) (Bradley and Baker, 2001; Werhahn et al., 2017b).

Besides the here elaborated genetic differences described for the Himalayan wolf, Hennelly et al. (2017) found that the vocalization of the Himalayan wolf differs significantly from the Holarctic grey wolf. Future studies should also explore differences in the morphology, ecology and behaviour of the Himalayan wolf in comparison to the Holarctic grey wolf.

5. Conclusion

The evidence presented here shows that the Himalayan wolf is genetically distinct and basal to the Holarctic grey wolf based on multiple neutral and functional genes. The Himalayan wolf shows a genetic adaptation to the hypoxic conditions in the Asian high altitude ecosystems that is unique amongst wolves. Therefore, our results solidify the need to classify the Himalayan wolf as a taxon of special conservation concern.

Our study further delivers an applied genetic methods toolkit based on non-invasive samples that can a) clarify phylogenies, b) inform conservation genetics, and c) be used for population monitoring of the Himalayan wolf. For future cost-optimized research around the Himalayan wolf we recommend a panel of 10 microsatellite loci that can differentiate the Himalayan wolf from the Holarctic grey wolf (including domestic dogs), and identify unique wolf individuals.

Conservation action for the Himalayan wolf is required and of global conservation interest. Nepal, with its northern arch dominated by the Himalayan mountain range, holds a considerable Himalayan wolf population. These dramatic inaccessible high Himalayan landscapes may present important habitat refuges for the Himalayan wolf. Hence we recommend that Nepal commences to play a leading role in Himalayan wolf conservation and thereby acts as a role model to the other range countries.

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Data accessibility statement

The GenBank accession numbers for all new haplotypes generated in this study are found in the Supplementary Material Table S1. Overviews of all reference data used are found in Supplementary Material Tables S2 and S3 and details on results of the samples used in this study across the considered markers are shown in Supplementary Table S10.

Author contributions

GW conceived of the study, performed field research, data analysis and wrote the paper. HS conceived of the study, developed novel analytical tools, supported data analysis and writing the paper. MG performed genetic analysis and supported data analysis. DK supported data analysis and writing the paper. AMS supported data analysis and writing the paper. JJ performed genetic analysis and supported data analysis. NK performed field research and supported writing the paper. JVLB supported data analysis and writing the study. TR supported data analysis and writing the paper. SK supported data analysis and writing the paper. CSZ supported conceiving of the study and supported writing the paper. DWM supported conceiving of the study and supported writing the paper.

Declaration of interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

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Chapter 4.

HIMALAYAN WOLF DISTRIBUTION AND ADMIXTURE BASED ON MULTIPLE GENETIC MARKERS

Himalayan wolf distribution and admixture based on multiple genetic markers

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Abstract

We explore the phylogeography of the Himalayan wolf (also called the Tibetan wolf) using multiple genetic markers applied on a landscape scale dataset and relate our findings to the biogeographic history of the region.
We present a large scale, non-invasive study of Himalayan wolves from across their estimated range. We analysed 280 non-invasive wolf scat samples from the Tibetan Plateau region in western China, Kyrgyzstan and Tajikistan at the mtDNA loci, and a subset for 17 microsatellite loci, four non-synonymous SNPs in three hypoxia-pathway related functional nuclear genes, and ZF genes on both sex chromosomes.

Our results corroborate previous studies showing that the Himalayan wolf forms a distinct wolf with a genetic adaptation to high-altitudes and a prevalence above 4,000m elevation. We found a correlation between the hypoxia adaptation and the divergent Himalayan wolf mtDNA haplotype. This wolf lineage is found across the Tibetan Plateau of Qinghai and the Tibetan Autonomous Region (TAR) and the Nepalese Himalayas. We identified a region of admixture between the Himalayan and grey wolves at the boundary of their distributions where the Tibetan Plateau elevation gradually drops.

The Himalayan wolf forms a reciprocally monophyletic wolf lineage, with a unique adaptation to high-altitude based on multiple genetic markers. We hypothesize that the uplifting of the region in geographic history has triggered the diversification of the Himalayan wolf and that the lineage maintains its high-altitude niche, in part, through the genetic hypoxia adaptation.

We conclude that the Himalayan wolf merits taxonomic recognition and designation as an ESU.

Keywords admixture, China, Himalayan wolf, Nepal, phylogeography, population structure, Tibetan Plateau

Introduction

The Himalayan wolf (also called Tibetan wolf) was demonstrated to form an evolutionarily distinct wolf that has diversified as an independent lineage from the common ancestor of the Himalayan and Holarctic grey wolves (Aggarwal et al., 2007; Sharma et al., 2004; Werhahn et al., 2018, 2017a). The Himalayan wolf lineage presents as a monophyletic clade, distinct and basal to the Holarctic grey wolf and its subspecies, based on mitochondrial genetic analysis (Aggarwal et al., 2007; Koepfli et al., 2015; Sharma et al., 2004; Werhahn et al., 2018). In contrast, results of nuclear genome analysis (Fan et al. 2016) suggests that these wolves form a recent clade within the Holarctic grey wolf complex.

The Himalayan wolf lineage exhibits ZF protein gene haplotypes on both sex chromosomes of the nuclear genome that are distinctive from those found in the Holarctic grey wolf complex. Specifically, the ZF protein gene haplotype on the X chromosome is shared with the African wolf, recently posited as *Canis lupaster* (Alvares et al., 2019; Viranta et al., 2017; Koepfli et al., 2015), while the ZF protein gene haplotype on the Y chromosome found in the Himalayan wolf is unique among all the other tested wolf lineages (Werhahn et al., 2017a, 2018). The Himalayan wolf lineage exhibits mutations on four non-synonymous SNPs in three hypoxia pathway related (functional) nuclear genes (vonHoldt et al., 2017; Werhahn et al., 2018; Zhang et al., 2014). These are genes suspected to be responsible for high-altitude adaptation, i.e. the evolution of physiological mechanisms to cope with lifelong hypoxic conditions in high-altitude habitats (Storz et al., 2010). Different mammals are specifically adapted to life at high-altitudes and similar genetic hypoxia adaptations have been found in the Tibetan people that share these high-altitude ecosystems (Beall, 2007; Huerta-Sanchez, 2014; Peng et al., 2011; Yi et al., 2010), domestic yaks *(Bos grunniens)* (Qiu et al., 2012), and in Tibetan mastiff dogs (*Canis lupus familiaris)*, that may have acquired them via hybridization with Himalayan wolves (Li et al., 2014).

The Himalayan wolf is estimated to have diverged as an independent lineage from the wolf-clade between 0.55-0.8 Ma (based on full mitochondrial genome analysis Matsumura et al. (2014) estimated it at 0.549Ma ago and Werhahn et al. (2018) at 0.69-0.74Ma ago; Sharma et al. (2004) estimated it at more than 0.8Ma ago based on 440bp of the cytochrome *b* gene). These estimates would place the divergence of the Himalayan wolf as prior to the radiation of the modern grey wolf estimated at 0.5-0.3 Ma (Sotnikova and Rook, 2010; Tedford et al., 2009). To the contrary of these estimates, Fan et al. (2016), using nuclear genomic data, estimated the divergence times for wolves as being much more recent, projecting the split of Eurasian and North American wolves at 12,500 years before present, followed by divergence of Eastern (including Himalayan wolves) and Western Eurasian wolves at 11,700 years ago.

Sharma et al. (2004) and Aggarwal et al. (2007) were among the first to genetically describe the Himalayan wolf lineage. At the time, the data for these wolves came primarily from the Himalayas, and later evidence corroborated that the lineage is also found across the Tibetan Plateau (Werhahn et al., 2018). The scientific and common names used for these wolves have differed across studies and on databases such as GenBank, including *C. l. chanco*, *C. l. laniger, C. l. himalayensis*, *C. l. filchneri*, Himalayan wolf, and Tibetan wolf (Aggarwal et al., 2007; Bocci et al., 2017; Chetri et al., 2016; Gray, 1863; Hodgson, 1847; Matschie, 1908; Sharma et al., 2004; Shrotryia et al., 2012; Werhahn et al., 2017a, 2017b, 2018).

Alvares et al. (2019) recently recommended *Canis lupus chanco* and Himalayan wolf for its scientific and common name, respectively, and for simplicity in this study it is referred to as Himalayan wolf.

The Himalayan wolf is found in the alpine grassland and tundra habitats of High Asia in the Himalayas and Tibetan Plateau (Werhahn et al., 2018, 2017b). Further diagnostic traits of the Himalayan wolf include a feeding ecology specialized to the high-altitude prey community (Werhahn et al., 2019), and a vocalization that is differentiated from those of Holarctic grey wolves (Hennelly et al., 2017).

Members of the genus *Canis* hybridize readily under favourable circumstances and disperse over large distances, two characteristics that facilitate gene flow and are responsible for the relatively low population diversification in Holarctic grey wolves (Dufresnes et al., 2019; Fan et al., 2016; Pilot et al., 2010; Vilà et al., 1999). Gopalakrishnan et al. (2018) highlighted the role of interspecific hybridization in the evolution of the canid family, especially the genus *Canis*. It is therefore interesting to understand why the Himalayan wolf diversified in evolutionarily history and how it has remained distinctive in the northern hemisphere in spite of its adjacent range to the widely distributed Holarctic grey wolf (Boitani et al., 2018).

Here we advance the understanding of the Himalayan wolf by providing landscape scale genetic and genomic data to explore 1) its distribution and admixture with grey wolves, and 2) possible mechanisms for its diversification and maintenance in a biogeographic context. Our study provides a large-scale analysis to explore the Himalayan wolf's phylogeny and distribution. These results contribute to the database for considering a formal taxonomic recognition of this wolf and inform conservation planning.

Methods

Study areas

We collected the data for this study across multiple areas of the Asian high mountain landscape, including the Tibetan Plateau of China: Sanjiangyuan National Nature Reserve (Zhaqing and Namsai Township, Zadoi County, Yushu Prefecture, Qinghai province) and Qomolangma National Nature Reserve in the TAR, Qilianshan mountains (Gansu and Qinghai Provinces) and Sichuan; different mountainous areas of Central Asia: Santai National Forest in Bortala Mongol Autonomous Prefecture (Xinjiang Autonomous Region, China), Zorkul Reserve of Tajikistan, and Jeti-Oguz region of Kyrgyzstan (Figure 1 and Table 1). For the comprehensive presentation of genetic and genomic data, we included the previously published data from three study areas in the Himalayas of Nepal (see Werhahn et al. (2018)). The habitats on the Tibetan Plateau comprise alpine tundra and grassland habitats. The habitats in Tajikistan and Kyrgyzstan comprise alpine steppe and wetlands, and the area of Bortala, Xinjiang, comprises open grassland, sparse shrubs and coniferous forest.

The primary carnivore species in the region include Holarctic grey wolf, Himalayan wolf, snow leopard (*Panthera uncia*), red fox (*Vulpes vulpes*), Tibetan fox (*V. ferrilata*), Pallas's cat (*Otocolobus manul*), Eurasian lynx (*Lynx lynx*), brown bear (*Ursus arctos*), and domestic dogs (*C. l. familiaris*).

Table 1. Study areas with sample size, year collected, habitat type, average elevation of the collected samples, and literature if previously published. Of the total of 470 collected samples, 280 samples were from wolves and were successfully sequenced.

Study region	Samples	Year collected	Habitat type	Elevation [m]	Literature
Sanjiangyuan (Qinghai, China)	165	2018, 2014	Alpine grassland and steppe	4404	
Tibet (TAR, China)	93	2015-2017	Alpine grassland and steppe	4650	
Qilianshan Mountains (China)	43	2014-2017	Grassland, shrubland, and coniferous mixed forest	3725	
Sichuan (China)	73	2014-2017	Forest (oak-conifer) and grasslands	3929	
Bortala (China)	7	2016	Grassland, shrubs, coniferous forest	525	
Jeti-Oguz (Kyrgyzstan)	6 (incl. 4 from (Werhahn et al., 2018)	2017	Alpine grassland and steppe	3107	(Werhahn et al., 2018)
Zorkul (Tajikistan)	30	2017	Alpine grassland and steppe	4294	
Himalayas (Nepal)	53	2015-2016	Alpine grassland and steppe	4813	(Werhahn et al., 2018)

Collection of genetic material

We collected genetic samples in the form of putative wolf scat samples along opportunistic transects focusing on ridges, streams and valley floors in all study areas, as these topographic features are important traveling routes for carnivores. We noted GPS position and associated habitat features for each sample. For detailed information of all samples refer to Table S4 in the supplementary material.

Genetic analysis

The following loci were examined for mtDNA: 242bp D-loop and 508bp cytochrome *b* as they separate the divergent Himalayan wolf from the Holarctic grey wolf (see Figure 3 in Werhahn et al. (2018)). A total of 280 wolf samples were successfully sequenced at the mtDNA loci following methods and PCR conditions in Werhahn et al. (2017a). A subset of 110 samples were successfully genotyped at 17 microsatellite loci previously screened as Himalayan wolf, using the same methods as in Werhahn et al. (2018). Of these, 18 samples had to be discarded due to missing data and 6 were later discarded as duplicate genotypes (i.e. scat from the same individual) resulting in 86 microsatellite genotypes from across the Tibetan Plateau of China, Tajikistan and Kyrgyzstan. This dataset was then combined with the 37 samples of Himalayan wolf from Nepal, European and Mongolian grey wolf, and domestic dogs published in Werhahn et al. (2018) to provide a comprehensive microsatellite dataset for the Himalayan wolf across the sampled range.

For the Zinc-finger (ZF) analysis we have developed new shorter primers to improve success rate (supplementary material Table S9) adopting the laboratory procedures described in Werhahn et al. (2017a). For the hypoxia pathway-related SNPs we looked specifically at the ANGPT1, EPAS1, RYR2-1 and RYR2-2 genes (Werhahn et al., 2018; Zhang et al., 2014), using the methods reported in Werhahn et al. (2018). We used Geneious version 8.1.8 for editing, quality control, building Bayesian phylogenies from mtDNA sequences, and scoring the 17 microsatellites. For the Bayesian phylogeny building we included wolf reference samples of partial and full mitochondrial genomes as obtained from GenBank (see supplementary material Table S2 and S3). We conducted a genetic distance analysis comparison including coyote (*C. latrans,* GenBank Accessions: DQ480510, KF661096), Grey wolf from Europe and North America (GenBank Accessions: KF661089, KF661091, KU696409, KF661095, KU696411, KF661087, KF661076, KF661056, KF661090), Grey wolf from Mongolia, Inner Mongolia and Xinjiang (China) (GenBank Accessions*:* SRR2017905, KU696393, KU696392, KU696396, GQ374438, SRR20179, KU696395, KU693394), Himalayan wolf (GenBank Accessions: FJ032363, NC011218, EU442884, KF573616), African wolf (GenBank Accessions: KT378605, NC027956), Iberian wolf (*C. l. signatus*, GenBank Accessions: KU644670) and Indian wolf (*C. l. pallipes,* GenBank Accessions: KF661043, KF644666) based on full mtDNA in the software MEGA (Kumar et al., 2018).

For the analysis of the microsatellite data we used the Genalex 6.503 plug-in for Microsoft Excel for basic quality control, diversity statistics, Hardy-Weinberg testing, and calculating genetic distances between locations (Peakall & Smouse, 2006, 2012). The R packages allelematch and adegenet (Galpern et al., 2012; Jombart, 2008; Jombart and Ahmed, 2011; R Core Team, 2018) were used for clustering the microsatellite genotypes. The software package STRUCTURE (Pritchard et al., 2000) was used to estimate admixture among the different wolf populations. We used a burn-in of 500,000 and MCMC replicates of 1,000,000 after burn-in with three replicates for each tested K to check for stability. The optimal population number K was estimated by plotting K vs. Ln P(D) and the Evanno method run with 15 replicates for K=2-9 (Evanno et al., 2005). The software HP-Rare (Kalinowski, 2005) was used to calculate allelic richness for the 17 microsatellite loci. We conducted a Fisher Exact Test for Count data to check correlation between mtDNA lineage and the four hypoxia pathway related SNPs.

Results

Mitochondrial DNA and nuclear microsatellites

The Himalayan wolf from TAR and Qinghai showed diverged mtDNA haplotypes on the 242bp D-loop, 508bp cytochrome *b*, and full mitochondrial genome in line with the findings of Werhahn et al. (2018) around Himalayan wolves in Nepal. The lineage was supported in the Bayesian phylogenies based on D-loop (posterior probability 1), cytochrome *b* (posterior probability 0.8443, supplementary material Figure S1), and the phylogeny based on full mtDNA (posterior probability 0.989, Figure 2). Himalayan wolf mtDNA haplotypes were prevalent across the Himalayas and the Tibetan Plateau (Figure 1A). When combining the mtDNA and microsatellite data we found a consistent pattern of admixed individuals (Figure 1B) at the distribution edges with admixed animals all showing mtDNA of Himalayan wolf and microsatellites allelic patterns showing admixture between Holarctic and Himalayan wolves (Figure 3). Structure analysis of microsatellite data from individuals sampled across the Himalayas, the Tibetan Plateau, and the surrounding mountain ranges in Tajikistan and Kyrgyzstan suggested an optimal K = 5 (Figure 3). Genetic distances (Nei unbiased) between locations are shown in Table 2 and allelic richness in supplementary material Table S7. See supplementary material Table S8 for full details on allele frequency per population.

Hypoxia Adaptation

The Himalayan wolf lineage predominated above 4,000m elevation, whereas grey wolves were present at lower elevations (Figure 4). We successfully amplified four SNP loci of hypoxia pathway-related genes for 59 samples from across TAR, Sichuan and Qinghai in China, and Tajikistan and Kyrgyzstan (Figure 1C, supplementary material Table S4). When considering the hypoxia pathway results combined with the mtDNA and microsatellite data of the same individuals, we found that the hypoxia adaptation present in the Himalayan wolf of the Nepalese Himalayas and TAR appeared to be linked with a diagnostic mtDNA Himalayan wolf haplotype. Fisher's Exact Test for Count data showed significant correlation of the divergent alleles at each of the four tested hypoxia related SNPs and the divergent Himalayan wolf mtDNA haplotypes (ANGPT1: p-value = 2.072^{e-1}; EPAS: p-value = 0.0018; RYR2-1: p-value < $2.2e^{-16}$; RYR2-2: p-value = $3.264e^{-07}$).

Our results further show that admixed individuals, characterised by mtDNA of Himalayan wolf and intermediate nuclear DNA, share the hypoxia adaptation, especially on the EPAS gene (Figure 1). Some admixed individuals showed grey wolf variants at particular loci, but usually only one gene had a characteristically grey wolf allele while the rest presented the characteristic Himalayan wolf hypoxia adaptation (Figure 1C, supplementary material Table S5). We also visualized each hypoxia gene allele versus elevation of sampled individuals (Figure 5) to gain separate insights into potential adaptive selection for each gene locus.

Zinc Finger Results

We successfully amplified 14 samples for ZFY and 49 for ZFX from Qinghai, TAR, Sichuan (China) and Tajikistan. The results from wolves in TAR and Qinghai corroborated the previously described unique Himalayan wolf haplotypes for both the ZFX and ZFY

(Werhahn et al., 2018). There were a few observed exceptions to this pattern that may indicate introgression (supplementary material Table S6). These exceptions to the general pattern in the ZFY were the following: one Himalayan wolf from Qinghai showed the haplotype typical of grey wolf; one admixed individual from Qinghai showed the Himalayan wolf haplotype; another admixed individual from Qinghai showed the grey wolf haplotype. The exceptions on the ZFX were the following: one Himalayan wolf from Qinghai and two admixed individuals from each Qinghai and Sichuan showed the grey wolf haplotype. Two admixed individuals from Qinghai and Sichuan, two admixed individuals from Qilianshan, and two grey wolves from Qilianshan and Qinghai showed the Himalayan wolf haplotype.

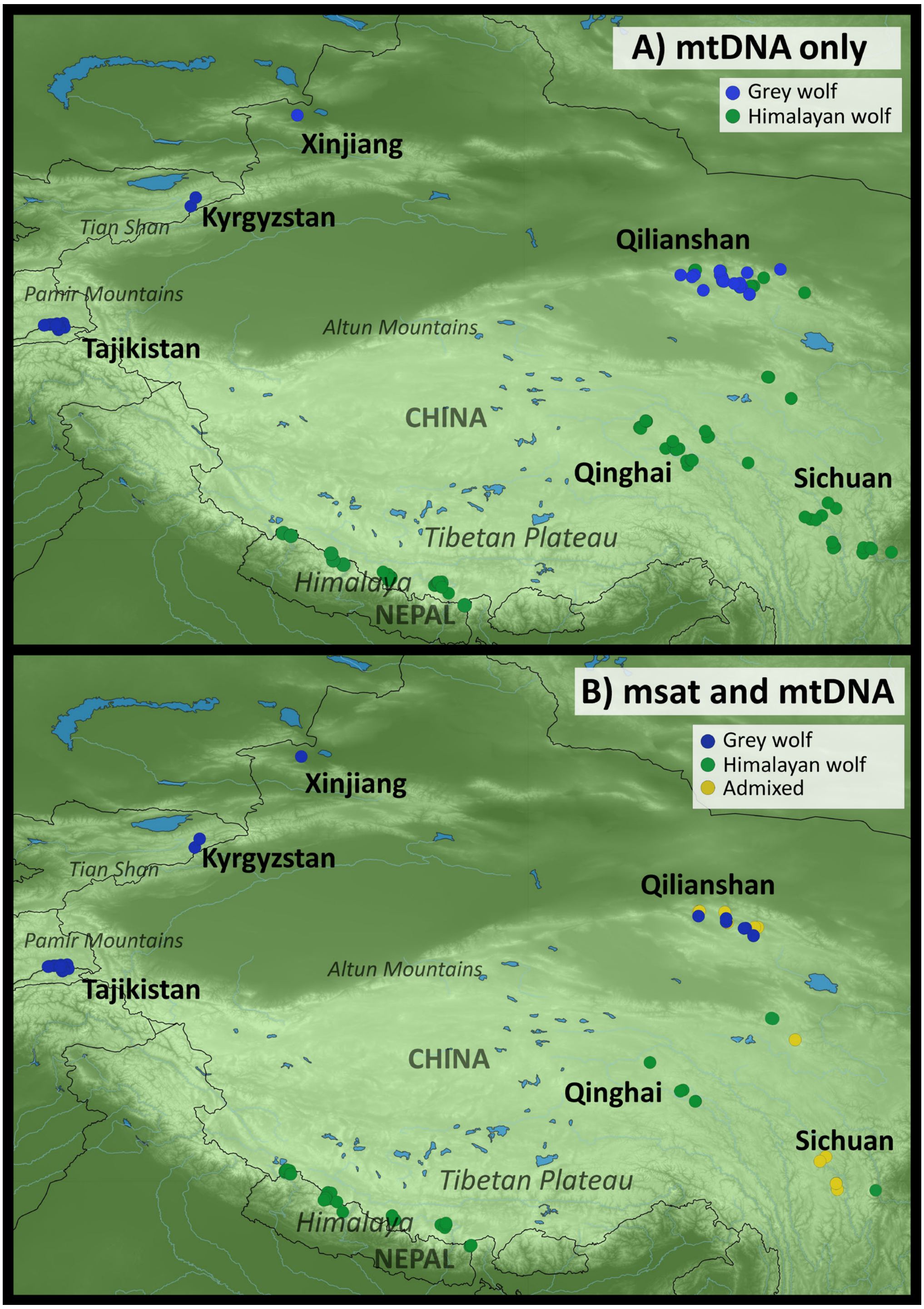

Figure 1. Map showing the region of the Himalayas and the Tibetan Plateau with study areas and specific sample locations. **A)** shows the mtDNA of samples, and **B)** shows the microsatellite and mtDNA results combined per sample. The two maps illustrate that admixed individuals are found in the north-eastern edge regions of the Tibetan Plateau, i.e. in Qilianshan mountains and Sichuan, where the habitat gradually changes to lower lying habitats where grey wolves predominate.

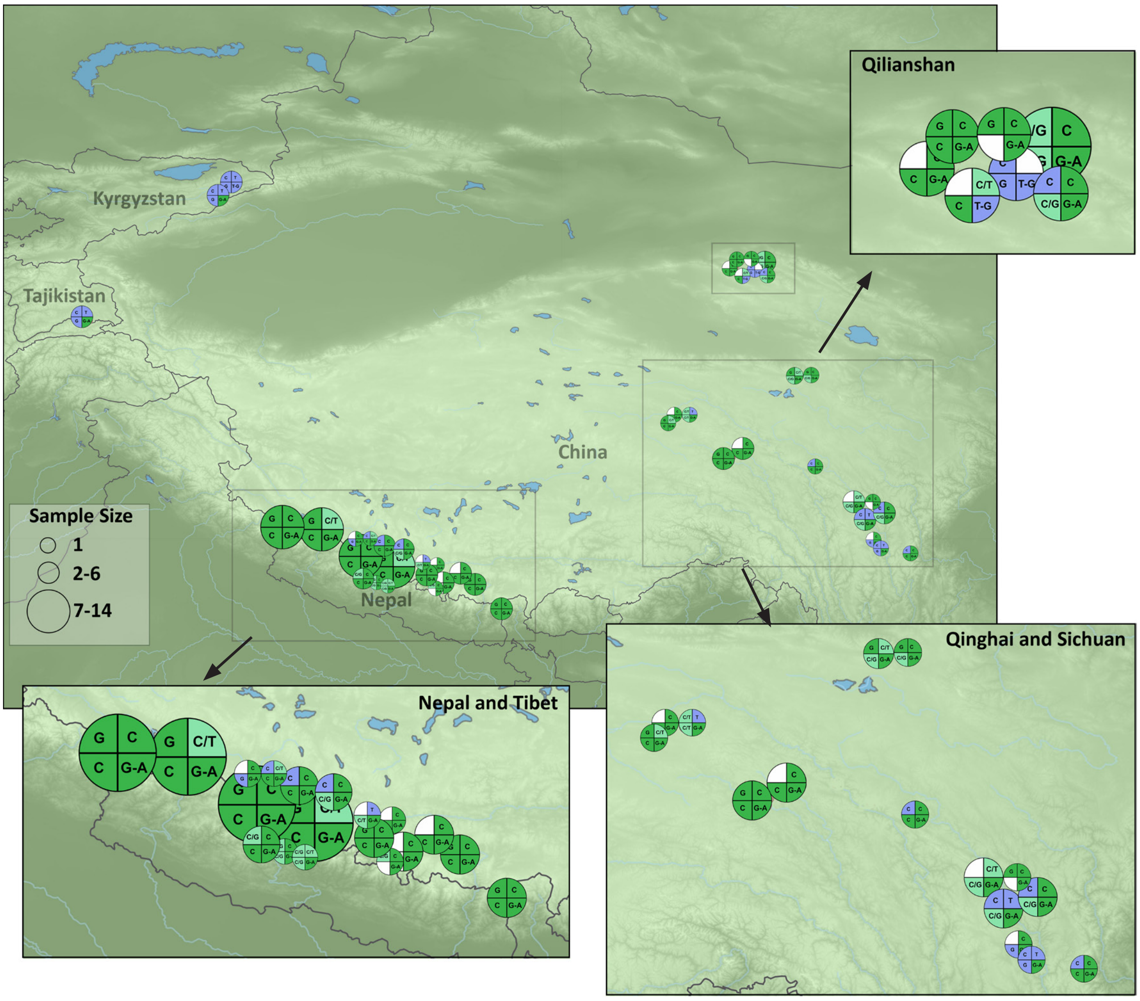

Figure 1. C) Hypoxia-pathway related SNPs per indiviual sample and geographic origin. Letters in the circles indicate SNP genotypes of three hypoxia-related genes, and size of the circle shows the number of samples with the genotypes. Clockwise starting from top right of each circle: ANGPT1, EPAS1, RYR2-1, RYR2-2. Dark green indicates allelles characteristic of the Himalayan wolf type, pale green heterzygous alleles characteristic of the Himalayan wolf type, blue the grey wolf type, and white indicates missing data (for full details see supplementary materials S5).

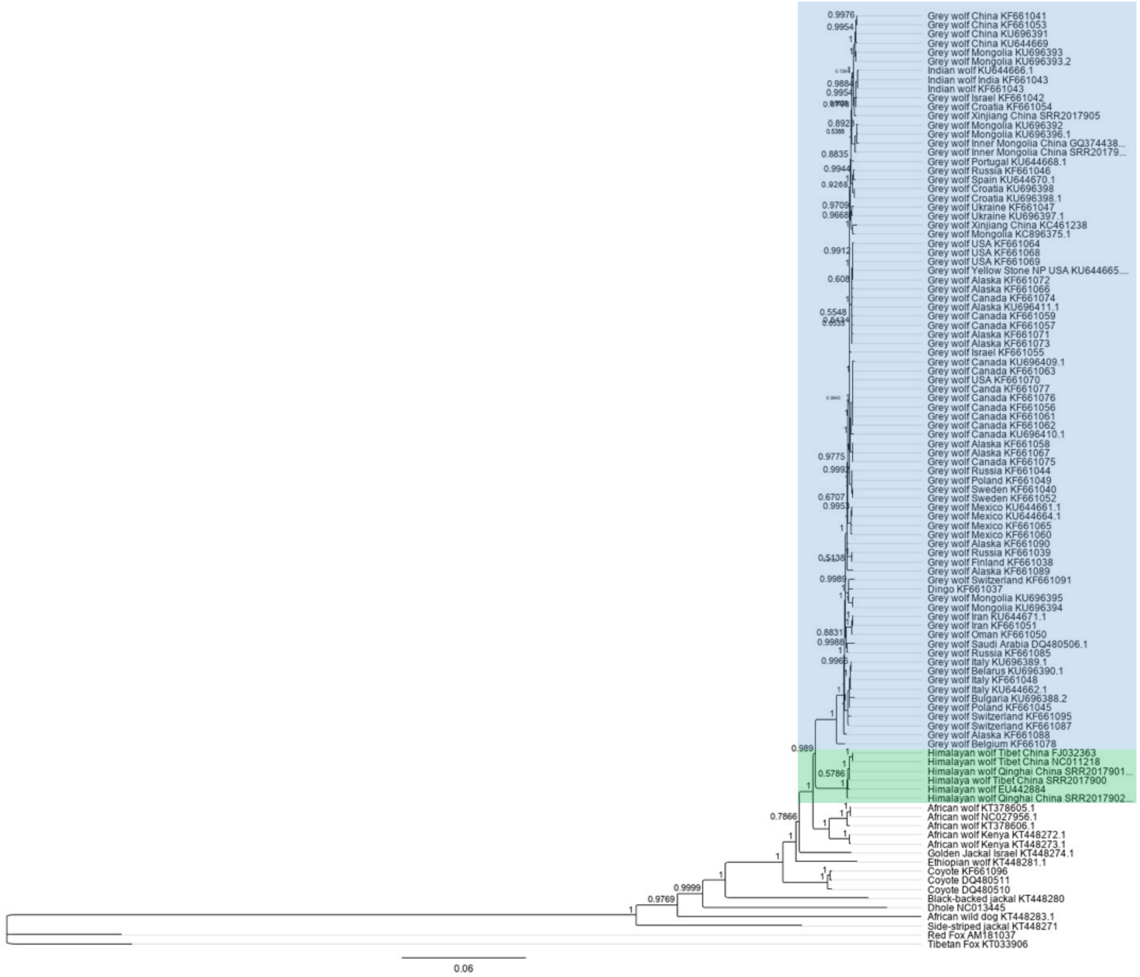

Figure 2. Canid phylogeny based on full mitochondrial genomes with GenBank accession numbers (also see Figure 4 in Werhahn et al. (2018)).

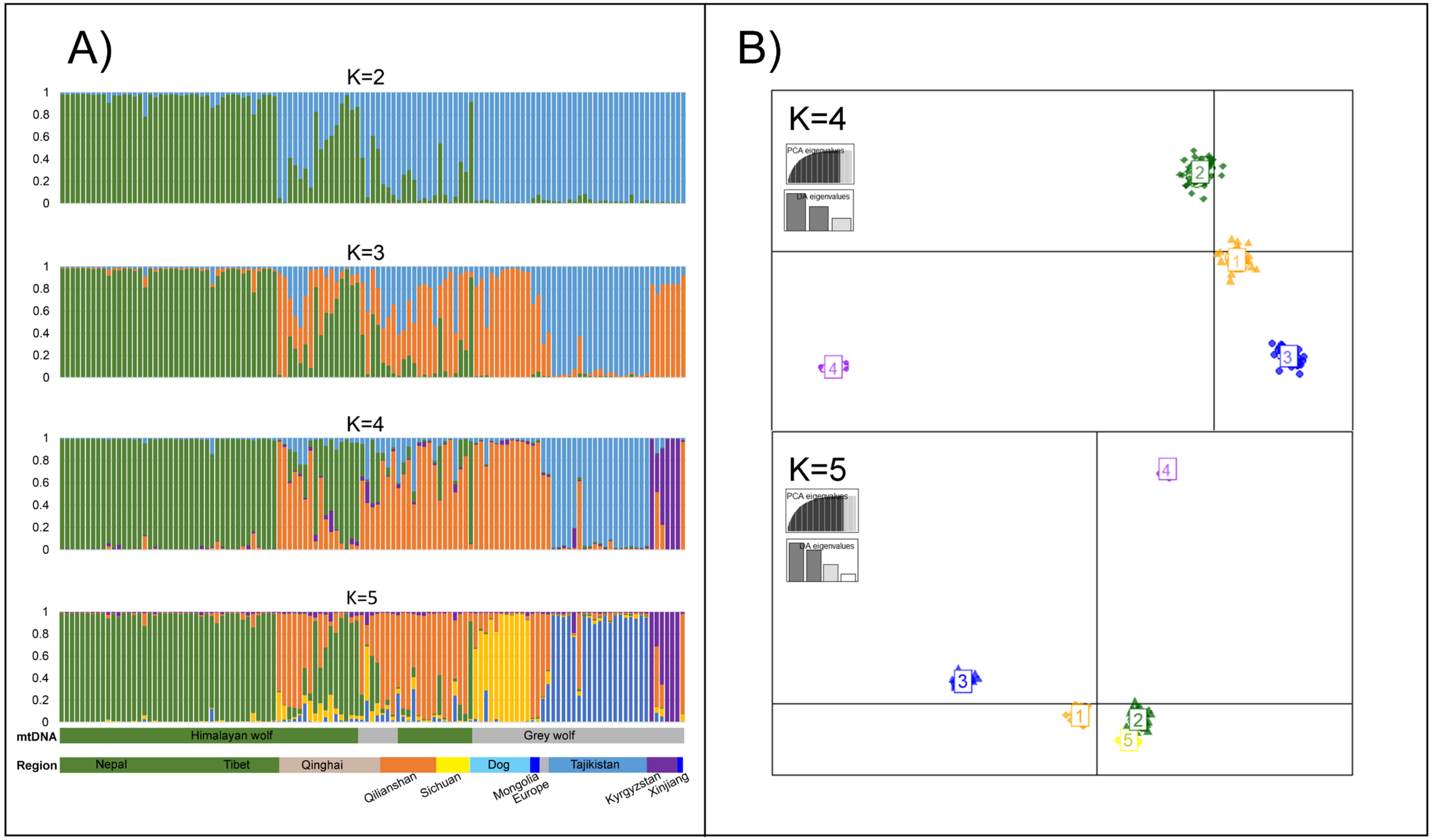

Figure 3. A) Structure plot of microsatellite results for K=2-5, with K=5 most supported. At the bottom the mtDNA lineage and geographic origin of samples is provided. B) The corresponding DAPC plot with the following geographic origin of wolves at K=4: 1) Qinghai, Sichuan, Qilianshan, Xinjiang, Mongolia and Europe and including the Tibetan mastiff dogs; 2) Nepal and Tibet; 3) Tajikistan; 4) Kyrgyzstan. At K=5 the clusters represent wolves from: 1) Qinghai, Sichuan, Qilianshan, 2) Nepal and Tibet; 3) Tajikistan; 4) Kyrgyzstan; 5) Europe, Mongolia, Xinjiang, Qinghai, Sichuan and including Tibetan mastiff dogs.

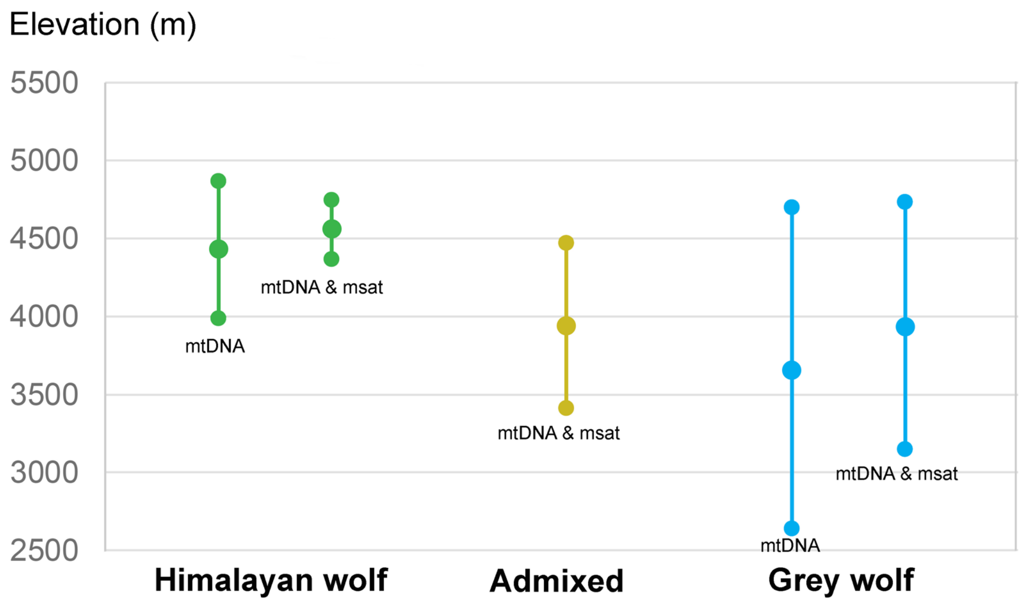

Figure 4. Mean altitude with standard deviation of genetically verified Himalayan wolves, admixed individuals and grey wolves sampled in China, Tajikistan and Kyrgyzstan. Admixed individuals are characterised by the mtDNA of Himalayan wolf and intermediate microsatellites.

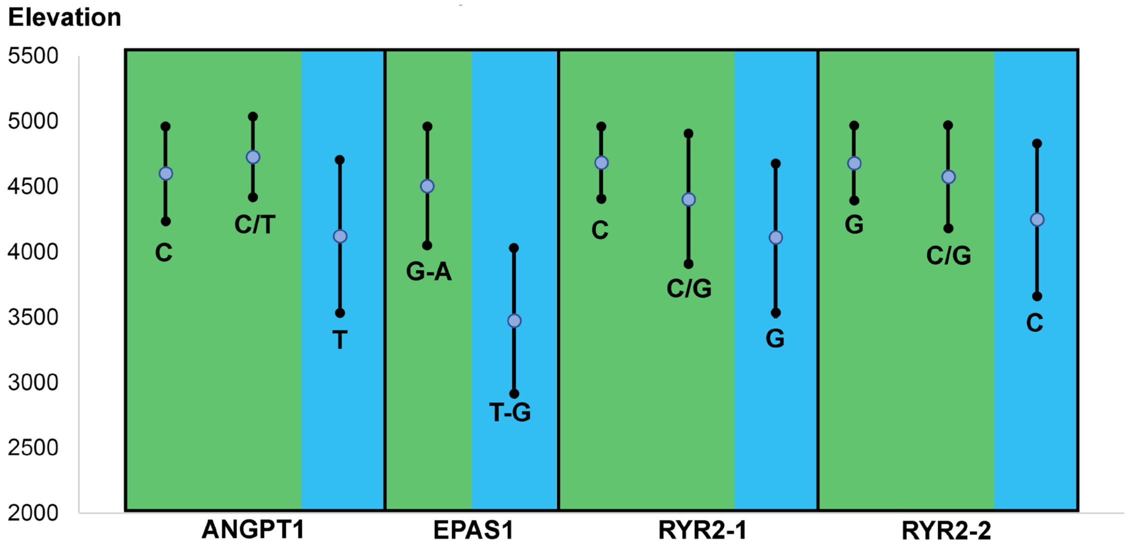

Figure 5. Mean and standard variance of elevation for wolf samples carrying different alleles of hypoxia-pathway related genes. For EPAS1 the allele combination for the two tested regions were fixed, i.e. individuals with the hypoxia adaptation consistently showing allele G and A for the two regions, whereas for grey wolf lineages without the adaptation it was T and G. In constrast at other genes heterzygotes were present with the slash (e.g A/T) indicating the presence of both alleles. More information is shown in supplementary material Table S5.

Table 2. Genetic distances (Nei unbiased) among wolf populations from the sampled geographic regions at 17 microsatellites with sample size N in parentheses.

Nepal & Tibet (N=43)	**Dog (Tib. Mastiff, N=11)**	**Mongolia & Xinjiang (N=3)**	**Europe (N=2)**	**Kyrgyzstan (N=6)**	**Qilianshan (N=11)**	**Qinghai (N=21)**	**Sichuan (N=7)**	
0.105	0.000							Dog (Tib. Mastiff)
0.215	0.307	0.000						Mongolia & Xinjiang
0.365	0.424	0.193	0.000					Europe
0.252	0.250	0.516	0.430	0.000				Kyrgyzstan
0.296	0.231	0.634	0.806	0.448	0.000			Qilianshan
0.135	0.199	0.256	0.333	0.277	0.213	0.000		Qinghai
0.498	0.567	0.738	0.537	0.431	0.756	0.349	0.000	Sichuan
0.415	0.588	0.664	0.731	0.506	0.638	0.261	0.233	Tajikistan

Table 3. Net genetic distance in % of different canid lineages based on the full mitochondrial genome calculated with MEGA.

	Holarctic grey wolf (N=9)
Coyote (N=2)	4.15
African wolf (N=3)	2.55
Himalayan wolf (N=4)	2.45
Grey wolf Mongolia (N=8)	0.04
Indian wolf (N=2)	0.22
Grey wolf Xinjiang (China) (N=2)	0.06
Iberian wolf (N=1)	0.20

Discussion

Our landscape scale analysis illustrates the divergence of the Himalayan wolf based on multiple genetic markers, the distribution of the Himalayan wolf lineage across the high altitudes of the Himalayas and the Tibetan Plateau, and a genetic adaptation to cope with the hypoxic conditions in these habitats. Our results, combined with previous studies (Sharma et al., 2004; Werhahn et al., 2018), indicate that the core distribution of the Himalayan wolf lies in the high Himalayas of Nepal and India (its presence in Bhutan remains to be verified), and spreads across the Tibetan Plateau of TAR and large parts of Qinghai with a prevalence in habitats above 4,000m. The adaptation to high altitudes in Himalayan wolves is shown across four SNPs in the three functional genes tested, which was consistent within the wolf populations of the Himalayas and TAR.

Our dataset indicates that the Himalayan wolf lineage is found across the continuous high-altitude habitats of the Himalayas and the Tibetan Plateau. In the surrounding lower elevation regions of Kyrgyzstan, Mongolia and Eastern China the Holarctic grey wolf is found instead. A detailed distribution analysis is recommended for future research to 1) identify areas with the highest habitat suitability for the Himalayan wolf, and 2) designate the core habitat and corridors for connectivity that are important for Himalayan wolf conservation.

Microsatellite data revealed that the Himalayan wolves in the Nepalese Himalayas and TAR show very limited admixture whereas populations toward the edges of the Tibetan Plateau (i.e. northern Qinghai, Qilianshan, and Sichuan) show signs of admixture with grey wolves. Admixture analysis of nuclear microsatellite data at K=2 suggested there is little gene flow between Himalayan and Holarctic grey wolves. At K=3-5 the wolves from Tajikistan and Kyrgyzstan and domestic dogs split successively from the grey wolf complex.

Populations towards the northern and eastern edges of the distribution (Qilianshan, northern Qinghai, and Sichuan) indicated admixture between the Himalayan wolf and the grey wolf at lower elevation habitats. The distribution to the south is likely to be more discrete due to the steep habitat cline from the Himalayas to the lowlands of the Indian subcontinent. We found consistent genetic diversification across multiple markers in the Himalayan wolf from TAR, Qinghai, and the Himalayas from the adjacent grey wolf populations. This finding is in line with other wolf phylogeographic studies (Ersmark et al., 2016; Fan et al., 2016; Pilot et al., 2010; Rueness et al., 2011; Zhang et al., 2014). Worthy of noting, however, is a slight imprecision in Ersmark et al. (2016) where figure 1 at the bottom shows the Himalayan wolf clade, but the respective samples grouped in Clu76 were not from Mongolia but from the Qinghai Tibetan Plateau and the samples from China were from TAR (see Meng et al., 2009). Thalmann et al. (2013), in their global analysis of wolves and dogs, excluded two aberrant Himalayan wolf samples from their analyses ‘since their phylogenetic positioning suggests only a distant relationship to all extant grey wolves’. A clearer resolution of the distinctiveness of the Himalayan wolf may have also been obfuscated because many large scale phylogeographic studies of Holarctic wolves do not include samples from the Asian high-altitudes (e.g. Gopalakrishnan et al., 2018; Loog et al., 2018; Pilot et al., 2019) and as a result have overlooked the diversified Himalayan wolf lineage.

Fan et al. (2016) included Himalayan wolf samples in their study of global wolf genomic diversity. This study suggests a recent ancestry of all extant wolf taxonomic groups and places the Himalayan wolf into a recent clade within the Holarctic grey wolf complex (see ‘Highland wolves’ in Figure 3 and 6 in Fan et al., (2016)). The placement of the Himalayan wolves in this nuclear genomic phylogeny differs from that of mitochondrial phylogenies (Aggarwal et al., 2007; Koepfli et al., 2015; Sharma et al., 2004; Werhahn et al., 2018, 2017a). There are at least two possible explanations for this inconsistency. First, the mitochondrial and nuclear genomes follow different evolutionary pathways, maternal vs. bi-parental, respectively (Gopalakrishnan et al., 2018; Groot et al., 2016), and hence the two datasets reflect different demographic phylogenetic histories of the Himalayan wolf relative to grey wolves (e.g. see Cahill et al. (2013).

Nevertheless, the nuclear genomic markers analysed in this study (SNPs on the functional genes involved in hypoxia adaptation, the ZF protein gene on the Y and X sex chromosome) are characteristically different for the Himalayan wolf lineage. Similarly, microsatellite data, insightful to understand population structure across the region, also differentiates the Himalayan wolf.

Another explanation may be that the sampled wolf individuals included in the study by Fan et al. (2016) may be of admixed origin. This possibility only becomes evident through the additional insight provided in the present study, where the data indicate 1) the existence of a hybrid zone at the distribution edges, and 2) that admixed individuals have mtDNA and hypoxia pathway related SNPs characteristic of the Himalayan wolf but nuclear DNA profiles intermediate with grey wolves. The genomic data on the 'Highland wolves' in Fan et al. 2016 is based on two samples from Tibet and two from Qinghai (same samples as used in Zhang et al., 2014). The samples originate from captive wolf individuals (Luobulingka Zoo in Tibet and Xining Zoo in Qinghai) but were wild born (supplementary material, Zhang et al., 2014). Given the results of the present study, the specific geographic location of where these animals were wild born is relevant for the interpretation of the data. This is especially the case for the individuals from Qinghai as northern regions of Qinghai, including Xining (situated at 2,270m elevation), lie within the region of admixture. Similarly, for the animals in Luobulingka Zoo in Lhasa Tibet (situated at 3,650m elevation), this is close to the eastern edge of Himalayan wolf distribution (Figure 1). Hence, the results of this present analysis indicate that the geographic location of animals used for genomic work around Himalayan wolves is highly relevant.

Here, we revealed the existence of an admixture zone and provided important insight into the genetic profiles of these admixed individuals to inform how future genomic studies should sample. Specifically, future genomic work should include full genome data from multiple contemporary wolf individuals of verified geographic origin from within the Himalayan wolf core distribution and from the admixture zone at the distribution edge.

High-altitude adaptation

The low oxygen availability at high altitudes present continuous environmental stress in the form of severe lifelong high-altitude hypoxia (Dosek et al., 2007), a selective pressure that has shaped the genomes of species found in these ecosystems and led to functional and physiological adaptations to cope with the conditions (Polle and Rennenberg, 1992). In mammals, including humans, exposure to high altitudes results in decreased partial pressure of oxygen and an increased formation of reactive oxygen and nitrogen species (RONS) which cause oxidative damage to lipids, proteins and DNA (Maiti et al., 2006). Adaptive responses have fine-tuned the physiological mechanisms that mitigate the destructive effects of free radicals (Maiti et al., 2006). However, the genetic mechanisms underpinning long-term survival of humans in these environmental extremes remains poorly understood (Moore, 2001; Peng et al., 2011; Simonson et al., 2010). The situation is similar for the Himalayan wolf, as only recently the genetic and physiological mechanisms involved in high-altitude adaption have been revealed (vonHoldt et al., 2017; Werhahn et al., 2018; Zhang et al., 2014). This wolf is found primarily above 4,000m, an elevation at which less than 12.7% effective oxygen is available, in contrast to the 21.9% of effective oxygen available at sea level (West et al., 2007). In line with previous work (vonHoldt et al., 2017; Werhahn et al., 2018; Zhang et al., 2014), this study showed differentiated alleles in the Himalayan wolves from across the Tibetan Plateau on four SNPs of three functional hypoxia pathway related genes, EPAS1 and ANGPT1, which increase oxygen delivery, and RYR, which strengthens heart function (Zhang et al., 2014). These functional mutations were correlated with the Himalayan wolf mtDNA haplotypes and indicate an adaptive advantage in hypoxic environments for the Himalayan wolf versus wolves without the mutations.

Tibetan Mastiff dogs, the characteristic dog breed of these high-altitude environments, has acquired high-altitude adaptation through specific adaptive introgression from the wolves to the dogs (Li et al., 2014; vonHoldt et al., 2017). In our samples, wolves and dogs from the Himalayas and the Tibetan Plateau, as well as admixed individuals, all had the same hypoxia adapted allele at the EPAS1 gene (supplementary material Table S5). The admixed individuals at the distribution edges shared the mtDNA of Himalayan wolves and tended to have hypoxia adaptation through fixation of, usually, the EPAS1 gene, corroborating the findings of Gou et al. (2014) and vonHoldt et al. (2017). The grey wolves in Kyrgyzstan and Tajikistan showed little indication for admixture with the Himalayan wolf, but they showed introgression on hypoxia pathway-related genes, especially the EPAS1, which is likely to be adaptive as these wolves live in higher elevation habitats with some degree of connectivity with the Himalayas and the Tibetan Plateau.

Speciation and biogeography

The Pleistocene epoch (2.588- 0.0117 Ma) was characterized by cycles of glaciation, which dramatically reshaped global biodiversity patterns, eliminating terrestrial biota from many mid- to high-latitude areas of the planet (Wallis et al., 2016). The region overlapping with the range of Himalayan wolves has undergone significant orogenic activity through the collision of continental plates, resulting in the formation of the Himalayas and the uplift of the Tibetan Plateau. This led to dramatic habitat changes and the creation of new ecological niches, which facilitated speciation through mechanisms such as divergent selection and adaptation (Liu et al 2013). Coinciding with these dramatic biogeographic changes in the region is the estimated divergence time (based on the mitochondrial genome) for the Himalayan wolf at 0.55-0.8 Ma (Matsumura et al., 2014; Sharma et al., 2004; Werhahn et al., 2018).

Specifically one of the last widespread, rapid uplift episodes of the Qinghai–Tibetan Plateau, the Kunlun-Huanghe Tectonic Movement, took place between 1.1 and 0.6 Ma while glaciations developed only around the high mountains on the Tibetan Plateau, but not the whole plateau surface (Li et al., 2014; Li and Fang, 1999; Zhou et al., 2006). Such a combination of geological and climatic events may have posed major evolutionary pressure for the Himalayan wolf to adapt to environmental challenges and diverge as an independent lineage splitting from the ancestors of the wolf-dog clade (Matsumura et al., 2014; Sharma et al., 2004; Werhahn et al., 2018). Similarly, other taxa in the region, such as Himalayan brown bears *Ursus arctos isabellinus*, show diversification estimated at a similar time period (Lan et al., 2017).

Speciation by environmental cline

Kawecki (1997) states that the evolution of lineages can be driven by genetic variation due to genetic loci that affect fitness in one habitat and are neutral or nearly so in others, such as the genes suspected to be responsible for hypoxia adaptation in the Himalayan wolf. Further, speciation can be facilitated through strong habitat clines (Doebeli and Dieckmann, 2003), such as the dramatic altitudinal difference that separates the Tibetan Plateau and Himalayas from surrounding, lower-lying regions. The related change in climate, habitat type, prey composition, and geographic distance contribute to isolation, genetic drift and subsequent divergence as the lineages evolve independently (Geffen et al., 2004; Leonard, 2014; Pilot, 2006).

We hypothesize that the high-altitude environment was an important selective pressure leading to the differentiation of the Himalayan wolf and its unique genetic adaptations to cope with the hypoxic conditions.

This adaptation and specialization to life in the high altitudes may give it a fitness benefit over grey wolf populations, and hence may be an evolutionarily mechanism reinforcing the divergence and maintenance of the Himalayan wolf’s monophyletic state. This maintenance as a distinct lineage is interesting when considering two prominent wolf characteristics: long dispersal distances (often over hundreds of kilometres and up to 1000km documented (Ciucci et al., 2009)) and ready hybridization with other wolf-like canids when conspecifics are lacking (Kusak et al., 2018; Pacheco et al., 2017). Both these characteristics can lead to polyphyletic lineages and homogenization of populations through gene flow. It is hypothesized that the Himalayan wolf’s specific genetic adaptation to life in the extreme high altitudes gives it an adaptive advantage and fitness benefit over the grey wolf.

Admixture at the distribution boundaries

Hybridization is ubiquitous in nature (Stanton et al., 2019). It occurs between wild animals and their domestic relatives and among related wild species (Adams et al., 2003; Pacheco et al., 2017; Randi, 2008) with the latter having an important role in the evolution of the canid family (Gopalakrishnan et al., 2018; Pilot et al., 2019).

Himalayan wolves show a consistent genotype of hypoxia adaptation across Nepal, TAR and Sichuan, while grey wolves found in Qilianshan did not show the hypoxia adaptation. Our results indicate that the divergent Himalayan wolf mtDNA haplotype is linked with the genetic hypoxia adaptation. We found admixed individuals at the edges of the Tibetan Plateau, i.e. in Qilianshan mountains (Gansu and northern Qinghai province) and Sichuan province. Admixed individuals showed mtDNA haplotypes and a genotype of hypoxia adaptation characteristic of Himalayan wolf yet had microsatellite profiles of intermediate states. These admixed individuals may have a fitness advantage by maintaining the mtDNA and hypoxia genes from the Himalayan wolf. To date, it is not understood if this advantage posed by hypoxia adaptation in the high altitudes is neutral or negative in lower elevation habitats.

Historically, wolves were present in most of continental China (Wang et al., 2016). The admixture in Sichuan may originate from grey wolves found in the lower areas of eastern and south-eastern China. The habitats in northern Qilianshan are characterized by lower elevations with open grasslands mixed with barren and desert areas, and there we found only grey wolves and admixed individuals (Figure 1). In these habitats the hypoxia adaptation may be less advantageous and the detected introgression may originate from grey wolves from Inner Mongolia and Mongolia to the North, or from dispersal through the Altun Mountains connecting Qilianshan and the mountains of Central Asia (i.e. the Tian Shan and Pamir mountains in Tajikistan and Kyrgyzstan). The pattern of admixed individuals and grey wolves found in Qilianshan (lower elevation habitats), versus only admixed individuals, and no grey wolves, in Sichuan (high elevation habitats) points towards the importance of elevation in predicting the presence of Himalayan wolves.

The width of an admixture zone is thought to be a function of the distance travelled from birth place to place of first reproduction and the degree of natural selection against admixed individuals (Barton and Hewitt, 1989, 1985; Wayne et al., 2004). Based on our results, the admixture zone is between 660 and 900km wide but likely varies strongly with topography and elevation.

Baker and Bradley (2006) propose that two phylogenetic groups represent different species when hybridization is restricted to a limited geographic area, an admixture belt, and outside the admixture belt the two respective phylogenetic groups are defined by well-supported monophyletic clades based on mitochondrial and nuclear genetic variation.

The admixed individuals, facilitating gene flow and adaptive introgression in the contact zones between taxa, may be important to conserve evolutionarily processes which are increasingly recognized (Stanton et al., 2019), including in the delineation of protected areas (IUCN, 2016). Similar cases of two related taxa separated by altitude with an admixture belt in-between are also found in plants (Choler et al., 2004) and birds (Cheviron and Brumfield, 2009; DuBay and Witt, 2014). Jeong et al. (2014) showed that adaptive introgression can be a mechanism to adapt to new or changing environments. However, results here allow only for preliminary insights into the admixture zone. It is further complicated by regionally high densities of domestic dogs in the study areas, and hence hybridization is expected to take place among the Himalayan wolf, domestic dogs and the grey wolf.

Taxonomy and conservation

This study corroborates the accumulating evidence that the Himalayan wolf merits taxonomic recognition (Aggarwal et al., 2007; Alvares et al., 2019; Sharma et al., 2004; Werhahn et al., 2018, 2017a). This is based on these wolves having a monophyletic ancestry in a geographically discrete region and exhibiting a diagnostic evolutionary characteristic (i.e. the high-altitude adaptation) which is not found in other populations of the same species (Medicine et al., 2019).

The phylogeny of ‘Highland wolves’ from Tibet and Qinghai, based on genomic data (Fan et al. 2016), also indicate that the lineage is monophyletic and diversified. These are presumably synonymous with Himalayan wolves (but see above). However, in contrast to mitochondrial phylogenies, Fan et al. (2016) placed them as a more recent branch within the Holarctic grey wolf. Future genomic research may reveal the cause for these contrasting phylogenies.

The recent species level recommendation by Alvares et al. (2019) for the African wolf (*Canis lupaster*; Viranta et al., 2017) supports a need for greater taxonomic consistency within the canid family. The Himalayan wolf appears similarly deeply diverged as the African wolf, and more deeply diverged in mtDNA as compared to currently recognized grey wolf subspecies, such as the Indian and Iberian wolf (Table 3).

The current evidence supporting taxonomic recognition for the Himalayan wolf includes the following mitochondrial and genomic evidence: 1) mitochondrial genome data (Werhahn et al., 2018), 2) nuclear genome data: four SNPs on three hypoxia pathway related genes, ZF on both sex chromosomes, and microsatellite data from 17 loci (Werhahn et al., 2018), 3) a discrete distribution range with admixture zones at the edges (present study), 5) differentiated vocalization (Hennelly et al., 2017), and 6) qualitatively described morphological differences (Hodgson, 1847, and also see Janssens et al., (2016)), although a systematic morphometric study is recommended.

Based on this array of evidence, taxonomic recognition may be supported according to the Unified Species Concept (Queiroz, 2007), the Differential Fitness Species Concept (DFSC) (Hausdorf, 2011), and the Biological Species Concept (BSC; Mayr, 1942). The DFSC defines ‘species as groups of individuals that are reciprocally characterized by features that would have negative fitness effects in other groups and that cannot be regularly exchanged between groups upon contact.’ So this may include a lineage which has adaptive advantage over other lineages and where hybridization would have negative effects on the survival of one lineage; this may be the case for the Himalayan wolf but future studies should address the fitness consequence of hypoxia adaptation at low altitudes. To further inform the taxonomy of these wolves a systematic morphological study is recommended, as well as studies including full genome data of multiple contemporary wolf individuals from verified geographic locations to complement the existing mitochondrial and nuclear genome evidence.

Gopalakrishnan et al. (2018) conclude, for mammals in general and for canids in particular, that rather than being isolated entities that evolve along treelike phylogenies, species are interlinked, and evolve through interactions in network-like topologies that are connected through gene flow. Hence like other traits used in taxonomy (e.g. biogeography, morphology, ecology and behaviour), genomic evidence is expected to be of gradual rather than discrete nature when used to describe different taxa.

Stanton et al. (2019) argues that conserving adaptive potential is a priority for conservation. Further delay of urgently needed conservation interventions due to taxonomic inertia will have negative effects, as the conservation of the Himalayan wolf and its habitat may remain hindered until the taxonomy is resolved. In parallel and independent of the taxonomic classification, the Himalayan wolf population fulfils the criteria to be designated as an 'evolutionary significant unit' (ESU) based on its discrete and separated distribution and the diversification on a wide array of genetic markers (Conner and Hartl, 2004; Moritz, 1994). Delineating the Himalayan wolf as an ESU will facilitate conservation on the ground in the time being of deciding on an appropriate taxonomy.

While genetic evidence underlining the Himalayan wolf's distinctiveness progresses, information regarding this wolf's population size, status, ecology, behaviour and distribution range remain scarce. Appropriate scientific recognition, such as a new taxonomic classification, and subsequent assignment on the IUCN Red List, could catalyse this much needed research. This recognition could also serve to focus regional and international conservation communities on the plight of the Himalayan wolf, its habitat, and the imperilled ecosystem in which it lives.

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Chapter 5.

HIMALAYAN WOLF DIET AND THE IMPORTANCE OF WILD PREY

Himalayan wolf diet and the importance of wild prey

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Abstract

Carnivore predation on livestock and game species leads to human-carnivore conflict. Thus, understanding the foraging ecology of threatened carnivores is important for conservation planning. We explore the summer diet of the Himalayan wolf, and of sympatric carnivores, based on the analysis of 257 field collected and genetically confirmed scat samples collected across three study areas in the Himalayas of Nepal (Humla, Dolpa, and Kanchenjunga Conservation Area) and two study areas on the Tibetan Plateau of China (Zhaqing and Namsai Township). We compared the prey species consumed to the relative availability of wild and domestic prey species. Himalayan wolves tend to use more wild than domestic prey, smaller (e.g., Tibetan gazelle, *Procapra picticaudata*) over larger sized wild ungulates (e.g. White-lipped deer, *Cervus albirostris*), and plains-dwelling (Tibetan gazelle) over cliff-dwelling ungulates (naur, *Pseudois nayaur*). Tibetan gazelle was consistently over-proportionally used by the Himalayan wolf and smaller mammals such as Himalayan marmot (*Marmota himalayana*), woolly hare (*Lepus oiostolus*) and pikas (*Ochotona* spp.) are important supplementary food resources. Himalayan wolves used less livestock relative to the availability, and livestock which showed a seasonal high abundance, that exceeded many-fold the abundance of wild prey species during the summer study period. Given this seasonally high livestock abundance, depredation by Himalayan wolves is inevitable and a major conservation concern. Habitat encroachment and depletion of wild prey populations are important drivers of this conflict. But we found that livestock was avoided when wild prey was available, a finding that can direct conservation. We conclude that the protection of Himalayan wolves, and other sympatric carnivores can be enhanced by a) securing healthy wild prey populations (ungulates and small mammals) through setting aside wildlife habitat refuges, and b) more sustainable livestock herding including reduced livestock loads, and improved herding practices and protection.

Key words: *Canis lupus chanco,* conservation, depredation conflict, foraging ecology, Himalayan wolf, wolf diet

Introduction

Wolves (*Canis lupus*), like other large carnivores, come into conflict with humans. At the root of human-wolf conflict is the wolf's predatory habit, and therefore competition with humans over livestock and game (Naughton-Treves et al., 2003; Newsome et al., 2016). This requires conservation management strategies that foster coexistence with an increasing human population (Treves and Karanth, 2003). Any such strategies must be multifactorial, delivering healthy wildlife populations and habitats, and sustainably managed livestock loads, pastureland use, and livestock protection, and requires a good understanding of carnivore foraging ecology. After centuries of wholesale wolf eradication grey wolf populations are recovering in North America and Europe, partly motivated by the perceived benefits of their ecological services (Newsome et al., 2016; Ripple et al., 2014, 2013; Ripple and Beschta, 2012).

Himalayan wolves are a genetically distinct wolf lineage unique to the Asian high altitudes of the Himalayas and the Tibetan Plateau (Werhahn et al., 2018); habitats which are recognized as a biodiversity hotspot (Lamoreux et al., 2006; Olson and Dinerstein, 1998; Pimm et al., 2014; Watson et al., 2016). The Himalayan wolf is increasingly being recognised as a taxon in need of protection (Werhahn et al., 2017a) but little remains known of its ecology or populations status. Evidence of its phylogenetic uniqueness is mounting (Aggarwal et al., 2007; Sharma et al., 2004; Shrotryia et al., 2012; Werhahn et al., 2018; Werhahn et al., 2017b). The Himalayan wolf's taxonomic classification is pending but recently recommended as *Canis lupus chanco* until full genomes verify the existing evidences which all indicate species eligibility (Alvares et al., 2019; Werhahn et al., 2017a; Werhahn et al., 2018; Werhahn et al., 2019). Besides these wolves, snow leopards (*Panthera uncia*) and red foxes (*Vulpes vulpes*) are important carnivores in these high-altitude ecosystems (Jnawali et al., 2011). Snow leopards and wolves are reported to be the main depredation conflict causing carnivores (Chetri et al., 2017; Kusi et al., 2019; Suryawanshi et al., 2014) but only snow leopards receive scientific and conservation attention (Devkota et al., 2013; Lyngdoh and et al., 2014; McCarthy and Mallon, 2016; Oli, 1993). And the red fox is a smaller mesopredator less studied in the region (Hoffmann and Sillero-Zubiri, 2016).

We focus our investigation on the Himalayan wolf's dietary habits across the Himalayan range of Nepal and in the Tibetan Plateau in Sanjiangyuan National Nature Reserve in Qinghai, China. We then compare the diet of wolf and snow leopard to shed light on the depredation conflict which is similarly reported for the two species but receives differing attitudes by local people (Kusi et al., 2019). We further include the red fox, a little studied mesopredator in this region (Hoffmann and Sillero-Zubiri, 2016), to understand dietary niche partitioning among these three important Himalayan carnivores. We hypothesize that the wolves and snow leopards share diets high in ungulate content with a high dietary niche overlap where the relative amounts of livestock and wild prey consumed reflect their respective abundance in the landscape. Whereas the red fox is hypothesized to forage mainly on smaller wild mammal species with little livestock consumed.

We report on these carnivores' estimated summer diet and relate what they consumed to the relative abundance estimates of the respective prey species in the habitats. We thereby deliver insights into important prey species for the Himalayan wolf, reveal prey use, including characterisation of livestock depredation in the high-altitude habitats, and thus draw conservation inferences.

Methods

Study areas

We collected data in three study areas in the Himalayas of Nepal and two study areas in the Sanjiangyuan National Nature Reserve of the Tibetan Plateau of Qinghai, China (Figure 1 and Table 1). Humla and Dolpa are situated within the arid zones of the Nepalese Himalayas and comprise alpine grasslands and alpine steppe habitats, while Kanchenjunga Conservation Area (KCA) is situated within the Inner Valleys of the eastern Himalayas (Miehe et al., 2016). Zhaqing and Namsai Township (Zadoi County, Yushu Prefecture, Qinghai) are situated on the Tibetan Plateau. Carnivore species in these high-altitude habitats include the Himalayan wolf, snow leopard, red fox, Tibetan fox (*V. ferrilata*), Pallas's cat (*Otocolobus manul*), Eurasian lynx (*Lynx lynx*), brown bear (*Ursus arctos*), and domestic dog (*Canis familiaris*). Wild prey species observed in the study areas include kiang (*Equus kiang*), naur, Tibetan gazelle, Tibetan argali (*Ovis* *ammon hodgsoni*), white-lipped deer *(Cervus albirostris)*; and the smaller Himalayan marmot, woolly hare, several species of pika (*Ochotona* spp.), and rodents. Livestock species kept in the study areas were yak (*Bos grunniens*), jhoppa (yak-cow hybrid, *Bos grunniens-Bos taurus*), horse (*Equus ferus caballus*), goat (*Capra hircus*), and occasionally sheep (*Ovis aries*) (Figure 2).

Livestock is usually brought up to the summer pasture lands from May to September. The herding regime varies according to species: Yaks and their hybrids are usually kept in small (5-10 animals) to larger (10-100 animals) herds with a herder loosely present in the vicinity. Smaller stock like goats and sheep are usually kept in larger herds of 25-100 (but up to 300) animals and are generally much closer herded and guarded compared to yaks. Horses, mostly used as a means of transport, are left unguarded in the pasture lands for multiple months at a time in small groups of 2-6 animals.

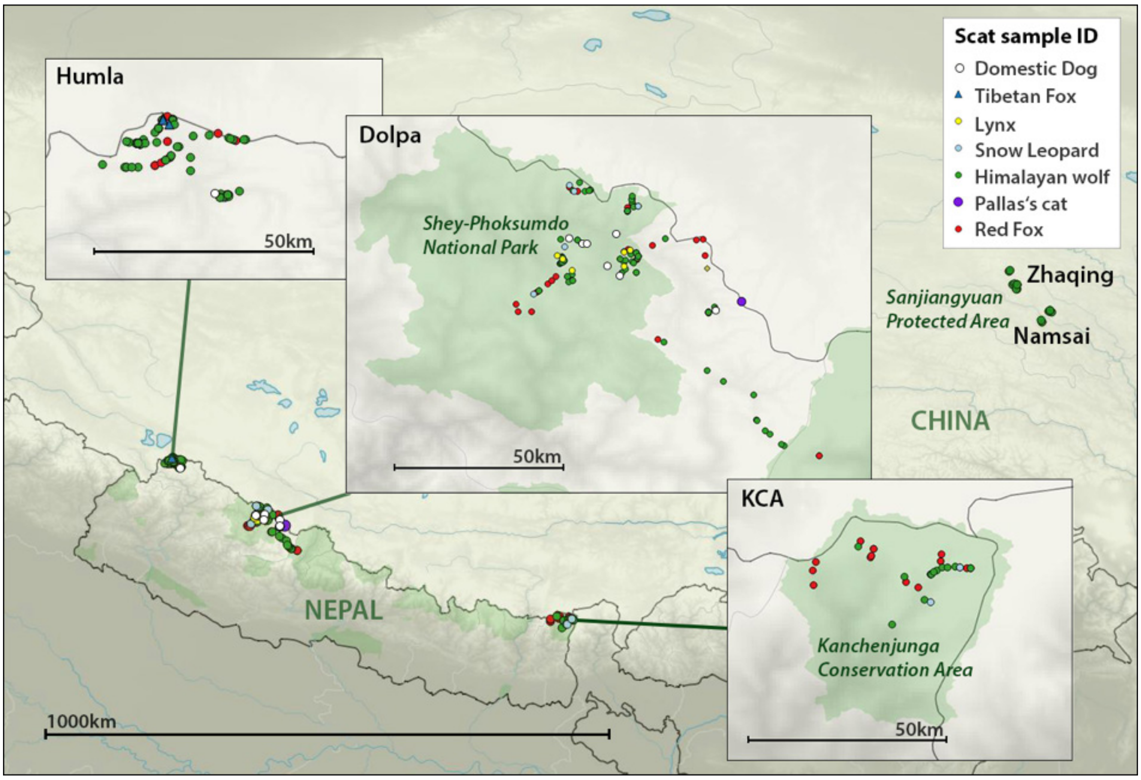

Figure 1. Study areas in Nepal: Humla (currently situated outside the Nepalese national protected area system), Dolpa (within Shey-Phoksundo National Park), and KCA (a community managed Conservation Area). Study areas in in Sanjiangyuan National Nature Reserve, Tibetan Plateau of Qinghai Province, China were Zhaqing and Namsai Township.

Table 1. Study areas, indicating details of distance transects and field observed wild prey species. Transects were spatially replicated with two transects per grid.

Study Area	**Year**	**Month**	**Elevation (m)**	**Study area size (km2)**	**Number of transects**	**Transect length (km)**	**Wild prey species observed**
Humla (Nepal)	2015	Jul/Aug	4,560-5,120	384	38	57	kiang, Tibetan gazelle, Tibetan argali, Himalayan marmot, naur, woolly hare
Dolpa (Nepal)	2016	May/Jun/Jul	3,850-5,540	1088	153	230	Tibetan argali, Himalayan marmot, naur, woolly hare
KCA (Nepal)	2016	Sept	3,980-5,150	368	50	75	Himalayan marmot, naur, woolly hare
Namsai (China)	2017	Aug	4,360-4,770	175	12	18	Himalayan marmot, naur, woolly hare
Zhaqing (China)	2017	Aug	4,380-4,800	452	31	47	white-lipped deer, Himalayan marmot, naur, Tibetan gazelle, woolly hare

Sample collection and preparation

We opportunistically collected samples across the Nepalese Himalayas and the Tibetan Plateau that were then genetically tested for species. This led to a total of 257 included scat samples during the summer season, 240 samples from the Nepalese Himalayas and 17 from the Tibetan Plateau (Table 2). In Nepal we collected scats also from other carnivores such red fox, Tibetan fox, snow leopard, Eurasian lynx, domestic dog, and Pallas's cat (Figure 2). Wolf samples were identified in the field according to scat appearance (size and form) and odour (wolf scats have a characteristic odour that aids identification). These were later verified by mtDNA analysis, as field misclassification can be high (Chetri et al., 2017; Jumabay-Uulu et al., 2014; Weiskopf et al., 2016). Genetic samples were swabbed from the surface of the tapering scat end and stored in an isohelix solution (for details refer to Werhahn et al. (2017a)). Diet samples were stored in paper envelopes and thoroughly sun-dried. They were then washed to dissolve the soluble remains by wrapping each sample individually in a stocking and rinsing it with water. This left only the insoluble scat contents such as hair, bones, soil, stones, vegetation and other solid fragments, which were thoroughly dried in preparation for microscopic analysis.

Table 2. Overview of genetically verified samples and species per study area.

Species/Study area	**Humla**	**Dolpa**	**KCA**	**Namsai**	**Zhaqing**	**Total**
Himalayan wolf	69	77	13	9	8	176
Snow leopard*	0	5	3			8
Red fox	13	28	14			55
Eurasian lynx#*	0	6	0			6
Pallas's cat#*	0	1	0			1
Tibetan fox†*	3	0	0			3
Domestic dog	1	7	0			8
TOTAL	86	124	30	9	8	257

*Sample size less than N=10. # Reported in (Werhahn et al., 2018). † Genetic findings reported in Werhahn et al. (2016).

Microscopic dietary analysis

We built a hair reference collection for all potential domestic and wild prey species found across the study areas. We used a modified point-frame method (Ciucci et al., 2004) to randomly select 40 diet items (hair, bone, stones, etc.) per sample. We scattered each scat sample on a gridded tray and selected the item at each grid intersection with tweezers for identification. These hair items were fixed to a slide with nail polish and inspected under a microscope (Bresser Science TRM-301,40x-1000x) at 40x magnification. The hairs were assigned to consumed species based on cuticular cell arrangements, medullary patterns, relative lengths and overall appearance with our reference collection (Appendix C) and literature (Bahuguna et al., 2010; Ciucci et al., 2004; Klare et al., 2011; Oli, 1993; Teerink, 1991). Bone items, tissue fragments and larger plant material was stored in annotated plastic bags. For the microscopic analysis of the Nepalese samples we worked in a pair with two microscopes which allowed cross-verification in case of doubt. A systematic crosscheck whereby both observers scored the same samples (n=9) resulted in a confidence of >90% overlap in assigning the hair in scats to the same species.

Vegetation included any kind of plant material and was commonly entire grass/sedge blades. Rodents, pikas (*Ochotona* spp.), and insectivores were pooled in a ‘small mammal’ category, whereas yak, cows and jhoppa were pooled in a ‘yak/ cow’ category.

Statistical analysis of diet data

To determine the adequacy of overall sample size and sample size per study area we calculated the Brillouin’s index (Brillouin, 2013) for each sample, ran a bootstrap resampling for 1000 samples, and then determined sample size at which an asymptote was reached for diet diversity in the plot of H_b versus increasing sample size according to the methods applied in Imbert et al. (2016). For the statistical analysis of the dietary data we used the following categories: naur, kiang, Tibetan gazelle, Tibetan argali, woolly hare, white-lipped deer, Himalayan marmot, small mammal, goat, yak/cow, horse, vegetation, stone, soil, and plastic. We calculated the Frequency of Occurrence (FoO) per food item and biomass of food consumed according to the recommendations of Klare et al. (2011). Both approaches have their advantages and limitations: FoO may over-represent small mammals as it does not reflect the relative proportion of items ingested, which can be better approximated through biomass models, while biomass models have limitations based on the study animals used for developing the models (Klare et al., 2011). For the biomass model calculation we used the relative frequency as equivalent of relative volume and used the later in the biomass calculation model developed for grey wolf based on feeding trials (Floyd et al., 1978; Weaver, 1993). To estimate food ingested per prey species we used the biomass calculation by Weaver (1993) Bio_{Wea}: $Y = 0.439 + 0.008X$, where Y = the biomass of prey consumed to produce a scat, and X = the average body weight of each prey species. This model is a refinement of the model developed by Floyd et al. (1978). For snow leopards we used the model by Ackerman et al. (1984). For red foxes we used the model coefficients used by Rühe et al. (2008). We calculated the dietary niche breadth with the Levins Index: $\boxed{B = 1/(\sum \mathrm{p}i^2)}$, where pi is the relative frequency of ingested food item by predator p (Colwell and Futuyma, 1971; Levins, 1968). For the dietary niche overlap between the Himalayan wolf, red fox and snow leopard we used Pianka Index (Pianka Index ranges from 0 to 1, from no overlap to complete overlap) (Loveridge and Macdonald, 2003; Pianka, 1975, 1973). We calculated the Jacobs Index for assessing prey use based on the relative biomass eaten relative to the biomass availability of the same ungulate in the study area (Bocci et al., 2017; Lyngdoh and et al., 2014). We then performed a bootstrap resampling with 1000 replicates to estimate mean and confidence interval for the Jacobs index, to check if 0= no selection falls within the confidence limits.

Abundance of wild and domestic prey

Prey abundance estimation of wild and domestic ungulate prey in the landscapes was conducted with distance sampling (Buckland, 2004). The distance samples were collected by visual detection while walking along two randomly placed 1.5km long transects per grid with the two transects representing spatial replication. Transects were conducted in the morning and late afternoon to evening hours when generally herbivores are most active. Grid cell size in Nepal (Humla, Dolpa, and KCA) was 4x4km, while in China (Zhaqing and Namsai Township) it was 5x5km. Wherever possible we placed the distance sampling transects randomly in the grids through pre -assigned random starting points. Field logistics and landscape features (e.g. cliffs) did however at times constrain transect layout and forced us to situate them along more feasible features such as valley floors. These transect layout constraints occurred at random across the study areas and remain a possible source of bias in our estimates of ungulate densities. The distance sampling data were analysed with the package ‘unmarked’ in R (Chandler et al., 2011). For all assessed ungulate species we used the same model with a half-normal detection function. For each transect we also noted human pressure as represented by number of humans sighted and mean elevation.

Relative abundance of marmots across study areas was estimated by counting the number of burrows within a radius of 10m (~314.2m^2 of area) at the start, the midpoint and the end of the distance sampling transects, and the number of direct sightings along the entire transect. The counted burrows and direct sightings were summed into a relative marmot index per distance sampling transect which in turn was averaged per study area.

Results

Collected scats were correctly field assigned to the genetically verified carnivore species in 76% of scats from Dolpa, in 82% of scats from Humla, and in 64% of scats from KCA. The samples incorrectly assigned in the field were between wolf, snow leopard, red fox and Tibetan fox. Our overall sample size of 176 wolf samples was large enough to draw dietary diversity inferences from our data given the minimum sample size across all study areas as indicated by the Brillouin Index being 96 samples. The sample size was also sufficient for the Nepalese study sites (i.e. a minimum of 68 required for Dolpa, 68 for Humla, and 9 for KCA) but sample size for the two sites in Qinghai (minimum of 49 needed for Namsai, and 81 for Zhaqing) was not sufficient for robust statistical analysis and hence the results for Namsai and Zhaqing are to be considered as preliminary trends only. An overview of the genetic identification of species producing each scat is given in Table 2, while dietary results are reported in Table 3 and 4. Plant parts from the genus *Myricaria* ssp. were found in three wolf scats resulting in a FoO of 1.88 for the wolf scats.

Himalayan wolf individuals sampled in Qinghai China had the widest dietary niche breadth (Levins Index = 5.65; N=17), followed by wolves in Nepal (Levins Index 5.12;

N=159), snow leopards (Levins Index=3.25; N=8, but note the sample size <10) and the meso-predatory red fox with a more narrow dietary niche (Levins Index = 1.86; N=55). The red fox scats contained mainly marmots, woolly hare and small mammals.

Pianka Index for dietary niche overlap between carnivores in Nepal was 0.19 for the Himalayan wolf and the red fox, and 0.81 for the Himalayan wolf and the snow leopard.

The Jacobs Index indicates a tendency for the Himalayan wolf to proportionally use more wild prey compared to livestock, and to over-proportionally use the smaller plain dwelling Tibetan gazelle, a trend supported in the study areas and the mean over all study areas (Figure 3 and supplementary material Table S1). Livestock was very abundant in all study areas during the summer and exceeded the biomass of wild prey several fold (supplementary material Table S1). However, the average Jacobs index and 95% confidence interval for 1000 bootstrap resamples was

-0.17 ±0.01, hence indicating a slight negative bias in our Jacobs Index data. This may be a result of the large livestock numbers in the landscape, which were generally avoided, and thereby leading to larger negative Jacobs indices, when compared to the values found for wild prey.

Figure 2. Carnivores and and their prey considered in this study: A) Himalayan wolf, B) snow leopard, C) Eurasian lynx, D) red fox, E) Tibetan fox, F) domestic dog (Tibetan mastiff). Herbivore species found in the study areas: G) Naur, H) Tibetan argali, I) Tibetan gazelle, J) Kiang, K) Himalayan marmot, L) Plateau pika (*Ochotona curzoinae*), M) Woolly hare, N) White-lipped deer, O) domestic yak, P) domestic horse, Q) domestic goat (Photographs by GW).

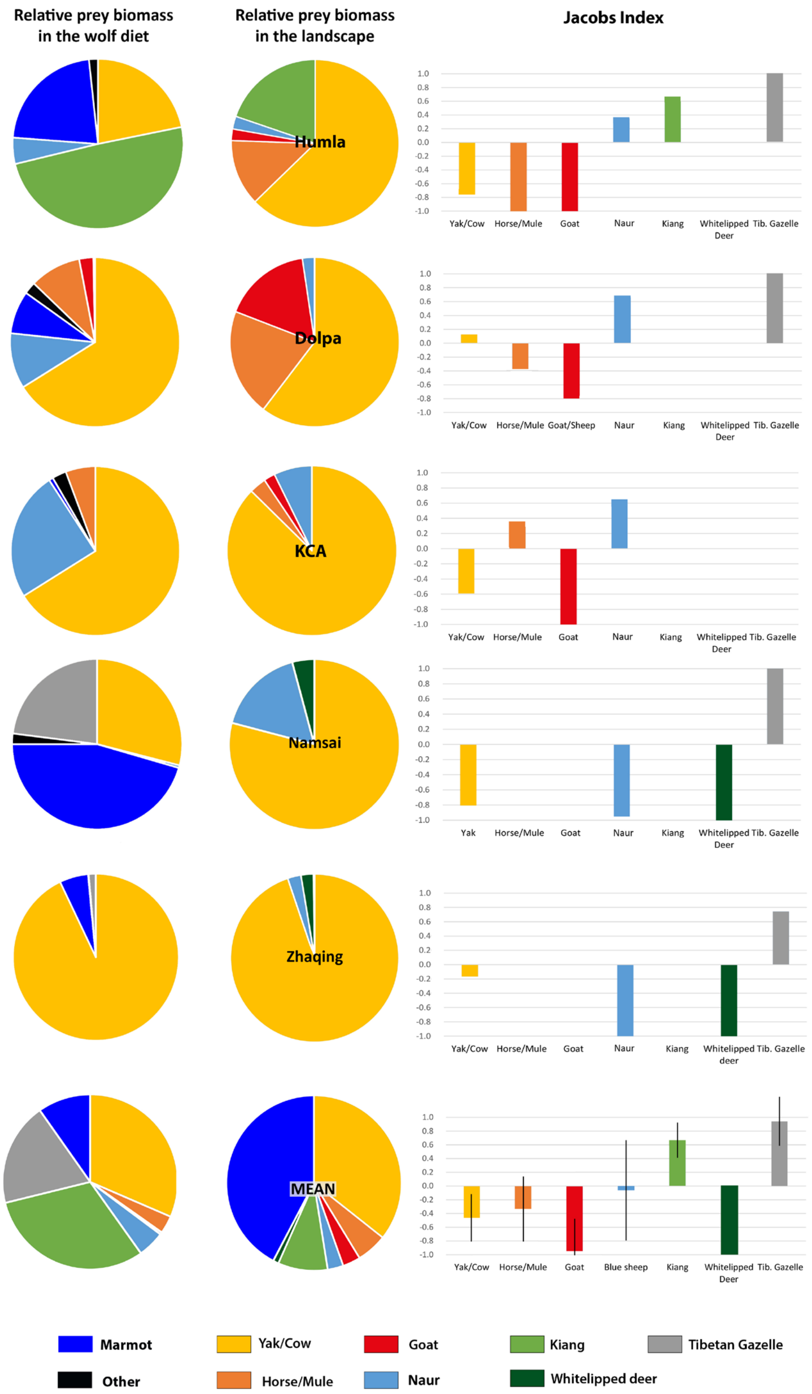

Figure 3. Prey in the wolf diet (relative biomass in %), prey abundance in the landscape (relative biomass in %), and the Jacobs Index for prey selection during the summer (-1 indicates prey avoidance and +1 indicates prey selection) per study area and mean with SD. For more details see supplementary material Table S1. Please note that the wolf scat sample sizes used for the analysis for Namsai (N=9) and Zhaqing (N=8) were less than 10 and therefore are only to be considered preliminary trends.

Table 3. Carnivore summer diet across the three study areas in Nepal for Himalayan wolf (HW), snow leopard (SL), and red fox (RF). Relative proportions eaten (%) (RPE), relative biomass eaten (%) RBE, and relative number of prey individuals eaten (%) (RNPE). Please note that sample sizes lower than N=10 are flagged with * and need to be considered as preliminary trends.

	Dolpa HW (N=77)			**Dolpa SL (N=5) ***			**Dolpa RF (N=28)**			**Humla HW (N=69)**			**Humla RF (N=14)**			**KCA HW (N=13)**		
	RPE	**RBE**	**RNPE**	**RPE**	**RBE**	**RNPE**	**RPE**	**RBE**	**RNPE**	**RPE**	**RBE**	**RNPE**	**RPE**	**RBE**	**RNPE**	**RPE**	**RBE**	**RNPE**
Yak/Cow	34.7	66.2	2.3	2.5	11.1	0.2	0.7	33.3	0.5	8.0	17.3	1.0				32.5	63.5	1.0
Horse	3.8	10.1	0.2	0	0	0	0	0	0	0	0	0	0	0	0	2.5	6.7	0.1
Goat	4.1	2.2	0.7	0	0	0	2.7	14.5	1.9	0	0	0	0	0	0	0	0	0
Kiang										18.9	52.9	1.7	0	0	0			
Naur	15.4	11.0	1.7	16.7	28.0	2.1	2.8	29.9	1.9	6.5	5.2	1.0	6.0	57.6	4.3	35.0	25.7	1.7
Tibetan gazelle	0.7	0.3	0.2							1.6	0.8	0.6						
Marmot	21.2	8.5	10.8	62.1	59.1	36.8	7.0	18.8	10.1	50.2	22.8	36.5	16.7	40.3	25.0	2.3	0.9	0.5
Small mammals	3.3	1.2	82.9	2.1	1.8	60.9	77.3	2.9	84.7	1.6	0.7	58.2	62.3	2.1	70.7	8.5	3.1	96.7
Hare	1.1	0.4	1.2	0	0	0	0.6	0.7	0.9	0.7	0.3	1.2	0	0	0	0	0	0

Table 3 continued. KCA in Nepal and the study areas in China: Namsai and Zhaqing for the Himalayan wolf (HW) scat samples.

	KCA SL (N=3) *			KCA RF (N=14)			Namsai – HW (N=8)*			Zhaqing – HW (N=9)*		
	RPE	**RBE**	**RNPE**	**RPE**	**RBE**	**RNPERN**	**RPE**	**RBE**	**RNPE**	**RPE**	**RBE**	**RNPE**
Yak/ Cow	0	0	0	0.4	6.0	0.3	16.1	29	0.6	15.6	92.9	22.7
Horse	40.0	96.9	1.3				0	0	0	0	0	0
Goat	0	0	0	0.5	1.1	0.4	0	0	0	0	0	0
Kiang												
Naur	0.8	0.6	0.04	23.4	91.8	17.1	0.3	0.5	0.04	0	0	0
Tibetan gazelle	0	0	0				12.8	23	7.6	4.1	1.4	5.9
Marmot	1.3	0.5	0	0.2	0.2	0.3	25.3	45.5	32.3	33.8	5.6	49.1
Small mammals	7.5	2.5	98.7	71.3	1.0	82.0	0.8	1.5	58.6	15.3	0.1	22.3
Hare	0	0	0	0	0	0	0.3	0.5	0.9	0	0	0

Table 4. Frequency of Occurrence per food item in % as found in the summer diet. Himalayan wolf results are shown pooled and separate for the Nepalese study areas, while the results are pooled for the other species and the study areas in China. Please note that sample sizes lower than N=10 are flagged with * and need to be considered as preliminary trends.

Row Labels	Yak/ Cow	Horse	Goat	Kiang	Naur	Tibetan gazelle	Marmot	Small mammal	Hare	Vegetation	Bird	Insect	Soil	Stone	Plastic	Unknown
Himalayan wolf (Dolpa)	57	3.90	11.7	0	24.7	1.3	36.4	9.1	1.3	65.0	2.6	0.00	10.4	27.3	0.00	24.7
Himalayan wolf (Humla)	14.3	0.00	0	20.0	10.0	5.7	64.3	7.1	1.4	68.6	0.00	4.3	14.2	21.4	1.4	11.4
Himalayan wolf (KCA)	46.2	15.4	0.00	0.00	76.9	0.00	23.1	30.8	0.00	69.2	0.00	0.00	15.4	23.1	0.00	7.7
Himalayan wolf (Nepal)	37.5	3.13	5.6	9.38	22.5	3.1	47.5	10.0	1.3	66.9	1.3	1.9	12.5	24.4	0.62	17.5
Snow leopard*	12.5	25			25		50	25		62.5	12.5		12.5	37.5		25
Tibetan fox*								100.00	33.33	66.67				33.33		33.33
Red fox*	5.17		5.17		18.97		20.69	93.10	5.17	50.00	18.97	24.14	8.62	8.62	3.45	17.24
Pallas's cat*,#								present	present	present				present		
Eurasian lynx*,#			33.33				16.67	66.67						33.33		
Domsetic dog*	50.00		12.50		12.50	12.50	25.00					12.50	12.50	25.00	12.50	12.50
Himalayan wolf (China)	76.47					5.88				70.59		35.29	47.06	17.65	23.53	52.94

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Discussion

Our study confirms our hypothesis of a high dietary niche overlap during summer between Himalayan wolves and snow leopards, whereas red foxes tend to feed on smaller wild prey species. Contradictory to our hypothesis of wolves feeding on livestock and wild prey relative to their abundance in the landscape, we find that Himalayan wolves then to relatively use more wild ungulates when compared to livestock.

Specifically, across our study areas we found a tendency of the Himalayan wolf to 1) use more wild prey than livestock, even though the seasonal relative biomass of livestock exceeded that of wild prey several fold, and to 2) over-proportionally use the small plains-dwelling Tibetan gazelle (13.2-15kg, Jacobs Index = 0.94), followed by the larger plains-dwelling kiang (250-400kg, Jacobs Index = 0.64), whereas the use for the cliff-dwelling naur (35-75kg, Jacobs Index = -0.06) was positive in the Nepalese study sites but negative in the study areas on the Tibetan Plateau which however only indicate a preliminary trend due to the low sample size in Namsai and Zhaqing. This trend may possibly driven by the higher availability of Tibetan gazelle in the latter study areas (Figure 3). The Tibetan gazelle is a comparably small wild ungulate found across the Tibetan plateau (IUCN SSC Antelope Specialist Group, 2016) with its distribution range approximately corresponding to that for the Himalayan wolf (Werhahn et al., 2018). The Holarctic grey wolf shows a comparable tendency towards smaller sized ungulate prey species (e.g. roe deer *Capreolus capreolus* (10-25kg) selected in Europe (Hosseini-Zavarei et al., 2013; Marucco et al., 2008; Reig and Jedrzejewska, 1988; Rigg and Gorman, 2004), and white-tailed deer *Odocoileus virginianus* (40-125 kg) in North America (Fuller, 1989)). Marmots are an important summer food source of the Himalayan wolf (Table 6) and similarly other smaller mammals such as woolly hare, pikas, and rodents may be important year-round sources.

Where Tibetan gazelle are absent or rare, other wild prey are used, e.g. kiang and naur in Humla, and naur in Dolpa. The cliff dwelling Tibetan argali was present at low numbers and only occasionally sighted in Humla and Dolpa (Kusi et al., 2018; Werhahn et al., 2015), but never found in the wolf scats. Similarly, white-lipped deer, despite being locally abundant in the two Tibetan study areas, was not represented in the Himalayan wolves' diet. Indeed, grey wolf pack size is likely correlated with local prey size (Fuller, 1989; Jędrzejewski et al., 2002). The observed Himalayan wolf packs (mean five animals - Werhahn et al., 2017b) are smaller than grey wolf packs (6-12 animals, Sillero-Zubiri et al., 2004), possibly reflecting relatively small prey size.

Goats are an appropriately sized prey for wolves, but they were intensively tended by herders. Goats were avoided across the study areas with the exception of Dolpa. Their representation in the wolf diet in Dolpa might be related to the limited wild ungulate community there, with a low density of naur and the biomass of wild prey dwarfed by that of livestock (41.7 times the biomass of livestock vs. wild ungulates). Our results show that yaks and yak-cow hybrids are frequently consumed by the wolves in all study areas, and indeed were ubiquitously present at high densities. Yaks are less rigorously tended than goats. A herdsman generally accompanies yaks, but the herd disperses over a large area while calves are seasonally abundant. Liu and Jiang (2003) studied wolf diet in Qinghai Tibet and found that yak, hare and small rodents are the important prey species of these (presumed Himalayan) wolves in the summer, and yak, sheep and hare in the winter. The authors found few seasonal differences in the wolves' diet and concluded that livestock remains in the wolves' scats can largely be attributed to scavenging on the grounds that few livestock went missing during the study period. Doubtless carcasses were available to the wolves in the harsh high-altitude conditions of our study sites, but the proportion of scavenged food to fresh kills cannot be discerned by scat analysis. The yak remains in red fox scats surely originate from scavenged carcasses. Insofar as our study reports on Himalayan wolf diet only during the summer months, when livestock predation is most intense (Kusi et al., 2019), future studies of seasonal variations in the wolf's diet will be informative.

The dietary niche overlap that we report between snow leopard and Himalayan wolf (Pianka Index = 0.81) was relatively high and comparable to observations of these sympatric carnivores in Kyrgyzstan (Pianka Index = 0.91) (Jumabay-Uulu et al., 2014), or in the Pamir mountains (Pianka Index = 0.87) (Wang et al., 2014). The red fox on the other hand had a comparably narrow dietary niche mainly foraging on smaller mammals and less overlap with the wolves. Our results for snow leopard diet are however only indicative due to small sample size but complement the findings of Chetri et al. (2017), Jumabay-Uulu et al. (2014), Shrestha et al. (2018), and Weiskopf et al. (2016).

Vegetation is a frequent component of carnivore diet as it provides fibres to help digestion, cleans the digestive tracts of parasites and hair, and can provide supplemental nutrients (Mech and Boitani, 2003; Rigg and Gorman, 2004; Wang et al., 2014). We frequently found grass/sedge vegetation in the wolf scats and found the small twigs of false tamarisk *Myricaria* sp. plants in three wolf scats. False tamarisk has also been detected repeatedly in snow leopard scats in other studies (Bocci et al., 2017; Chetri et al., 2017; Devkota et al., 2013; Wang et al., 2014). It may be relevant that local human communities use this plant to treat stomach-ache, uterine bleeding and food poisoning (Kala, 2006; Lama et al., 2001).

However it has to be emphasized that some of the scats used for this diet analysis may belong to wolves of the same pack and therefore they are not entirely independent from each other, thus reducing the effective wolf diet sampling size further and likely reflecting the summer diet of only a handful of Himalayan wolf packs. And this present study represents the summer diet of these packs only. It further must be noted that the biomass conversion factors used have been developed for North American wolves and thus are an approximation.

Use of wild prey and livestock

Our data indicate that the Himalayan wolves use wild prey species more when compared to livestock during the summer period despite the seasonally overwhelming abundance of yaks and goats in the study areas (see also Chetri et al. (2019)). Similarly, studies from different regions around the globe find indications that grey wolves use more wild than domestic prey (Meriggi and Lovari, 1996; Rigg and Gorman, 2004; Wang et al., 2014). A review of wolf dietary studies over 15 years in southern Europe shows that wild ungulates are preferred by grey wolves over livestock and that the presence of multiple wild prey species can reduce livestock depredation (Meriggi and Lovari, 1996). That review also emphasised the importance of effective livestock protection and grazing management in reducing predation on livestock (Meriggi and Lovari, 1996). However it has to be noted that domestic prey is not equally available to the wolves compared to wild prey species, given that livestock is often guarded to varying extents by a shepherd and sometimes guarding dogs. So the direct comparison of wild prey and livestock numbers in the landscape can be imprecise. In the Nepalese Himalayan context, especially goats are herded more closely and are likely the least available to wolves, whereas yaks are herded loosely with the herds spreading over entire valleys, and horses are not herded but left to graze for multiple month at a time without a herder present in the pasturelands. Hence in future wolf diet studies this difference in availability of livestock versus wild prey for the wolves should receive more detailed attention. Other studies have concluded that where wild prey is available only a minority of wolf packs kill livestock (Jedrzejewski et al., 2003; Muhly et al., 2003; Treves et al., 2003, 2001). The results from southern Europe suggest that grey wolves switch their diets away from domestic species if more wild ungulates are available (Newsome et al., 2016). In line with optimal foraging theory, all ungulates are sufficiently profitable to wolves, making encounter rate a critical determinant of prey selectivity (Huggard, 1993). Depleted prey populations are an important driver for wolves to turn to livestock. Consequently maintaining and restoring wild ungulate populations should be a key priority for Himalayan wolf as for grey wolf conservation (Ripple et al., 2014).

Recommendations for conservation: Importance of intact wild prey populations

Wild ungulate populations worldwide face many threats including habitat destruction and encroachment, unsustainable human hunting activity, competition with livestock, and disease transmission by livestock (Karimov et al., 2018; Newsome et al., 2016; Ripple et al., 2015). In our study areas in the Himalayas and the Tibetan Plateau we observed habitat encroachment, competition with large numbers of livestock, and illegal poaching as immediate concerns for the wild herbivore populations. Wild prey has to compete with the encroaching livestock and as a consequence may move to other areas (Karimov et al., 2018). The observed densities of naur in our Nepalese study areas (0.8-3.1 sheep/km^2) were lower than the 6.6–10.2 sheep/km^2 in the Annapurna Conservation Area documented by Oli (1994). The naur is a widely distributed wild ungulate across our study region (Harris, 2014) and presents an important wild ungulate prey for the Himalayan wolf and the sympatric snow leopard (Chetri et al., 2017). The kiang in Nepal is restricted to few trans-Himalayan habitats in Humla and Mustang (Jnawali et al., 2011). And similarly, the Tibetan gazelle in Nepal is restricted to a few individuals in the trans-Himalayan habitats of Humla and Mustang (Jnawali et al., 2011; Werhahn et al., 2015) though this species appears more common on the Tibetan Plateau habitats of China (IUCN SSC Antelope Specialist Group, 2016). While Tibetan gazelles to date are not reported from Dolpa this species was found in a wolf scat from Dolpa, collected from alpine steppes very close to the Tibetan plateau habitats in the neighbouring Tibetan Autonomous Region (TAR) of China. Based on the far roaming nature of wolves, it is possible that the wolf killed a Tibetan gazelle on the Chinese side while depositing the scat on the Nepalese side (a possibility that underlines the importance of trans-boundary conservation).

Conservation priorities should be improving wild ungulate populations in these high altitude ecosystems by a) protection of favourable wild herbivore habitat refuges (Lyngdoh and et al., 2014), b) sustainable management of pastureland use, livestock numbers and improving livestock protection, and c) combating illegal poaching of ungulates and carnivores.

Further the vulnerability of livestock in the high-altitude habitats of the Himalayas and the Tibetan Plateau may be reduced by more rigorous presence of herders. And the night protection of corralled herds may be improved by a combined use of fladry (Musiani et al., 2003), and electric, audio and visual deterrents (Newsome et al., 2016). The safeguarding of smaller prey species (hare, marmot, pika) is another priority for the conservation of Himalayan wolves and indeed snow leopard, foxes and Eurasian lynx, based on the importance of these small mammals in diet of the studied carnivores.

Conclusion

Successful protection for large carnivores requires protecting entire ecosystems (Estes et al., 2011). Understanding their feeding behaviour is an important element of planning the conservation of Himalayan wolves, because of their dependence and impact on both wild and domestic prey, and the associated conflict with people. Our study suggests that predation on livestock increases where wild prey is scarce during summer. It emerges that the restoration and maintenance of healthy wild prey populations, in combination with effective livestock protection, are fundamental for the conservation of Himalayan carnivores.

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Chapter 6.

CANIDS CHALLENGE TAXONOMY: A REVIEW OF ASIAN WOLVES

Canids challenge taxonomy: a review of Asian wolves

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Abstract

Wolf-like *Canis* taxa challenge taxonomy because their species boundaries and their distribution ranges can be continuous. Species delineation is currently not based on consistent criteria, but a consistent taxonomy is critical, given its importance for assigning legal protection, conservation priorities, and financial resources.

We carried out a qualitative review of the wolf lineages described for Asia from historical to contemporary time and considered relevant morphological, ecological and genetic evidence, with the aim to clarify contemporary wolf lineages within the context of the larger phylogenetic group of the Canidae. We present full mitochondrial phylogenies and genetic distances of the discussed lineages. Working towards a taxonomy that is consistent within the canid family, we applied a traffic light system to evaluate the evidence. We found support for the presence and taxon eligibility of Holarctic grey, Himalayan, Indian, and Arabian wolves in Asia, and recommend a taxonomic revision of the canid family based on consistent criteria and guidelines.

Keywords: Arabian wolf, *Canis lupus arabs*, *Canis lupus chanco*, *Canis lupus laniger*, *Canis lupus pallipes*, Himalayan wolf, Indian wolf, Mongolian wolf, phylogeny

Introduction

Canids (Order Carnivora, Family Canidae), like many other mammalian groups, are characterised by gene flow between taxa in the evolutionary past and present (Gopalakrishnan et al., 2018). Taxonomic delineations in the group are the subject of ongoing change and debate, especially in the wolf-like *Canis* lineages. New phylogenetic studies rapidly and continuously update and challenge our understanding of species and subspecies due to quickly advancing genetic methods. Hence the total number of 37 species recognized within the family Canidae is a point of some contention (Macdonald and Sillero-Zubiri, 2004; Sillero-Zubiri et al., 2004; Wang et al., 2004). Wolves hybridize when circumstances favour (Adams et al., 2003; Dufresnes et al., 2019; Gottelli et al., 1994; Hennelly et al., 2015; Kusak et al., 2018; Pacheco et al., 2017), such as lack of conspecific mates, and they disperse over large distances (Geffen et al., 2004; Mech et al., 1995) both of which facilitates gene flow. A re-evaluation of contemporary wolf lineages with consistent criteria is thus advisable (Zrzavý and Ricankova, 2004), especially given the new evidence around wolf lineages in Asia, Africa and North America (e.g. Rutledge et al., 2015; Viranta et al., 2017; vonHoldt et al., 2016; Werhahn et al., 2019). Historically species designation and the evolutionary placement relied on morphological measurements. A type specimen is named as a reference to describe a particular species and is kept in a recognized scientific museum (Thiel and Wydeven, 2011). Today genetic analysis is augmenting our understanding of species delineation, the relationships among lineages, and phylogenies among species groups.

The debate of what a species is has moved beyond reproductively isolated lineages, and conservation is gradually recognizing the importance of preserving adaptive potential (e.g. Stanton, 2019) and genetic diversity (Biological Convention of Diversity, 2018; IUCN, 2016). Genetic variation in nature is gradual and differs in extent but not quality between species and populations (Hey and Pinho, 2012). Where experts draw the line between species compared to population is thus open to different schools of thought.

So taxonomy, assigning discrete species in a continuous world (Galtier, 2019), needs to carefully consider multiple criteria, such as the phylogeny and morphology, but it also needs to ensure the conservation and integrity of evolutionary lineages. But taxonomy is heterogeneous at present with regards to the criteria applied for species delineation, and this is particularly sensitive in endangered taxa, where species delineation has immediate consequences on management decisions, conservation, legislation and financing (Galtier, 2019).

Recently a revised taxonomy based on consistent criteria and guidelines has become available for the Felidae (Kitchener et al., 2017), while the reclassification of antelopes by Groves and Grubb (2011) has sparked the debate on appropriateness and consistency of the taxonomy for conservation (IUCN/SSC ASG, 2017).

Here we provide an overview of canid evolutionary history and of recent research on contemporary wolf-like *Canis* lineages found in Asia to inform a consistent canid taxonomy. But with new studies continuously emerging, any review can only momentarily claim completeness.

Evolution of the Family Canidae and the Genus *Canis*

The Canidae are part of the order Carnivora, a large group of predatory mammals. The Canidae comprise three subfamilies, Hesperocyoninae, Borophaginae and Caninae, of which only the latter is extant (Sillero-Zubiri et al., 2004). The genus *Canis* falls within the tribe Canini within the subfamily Caninae; *Canis* species are divided into jackal-like *Canis*, wolf-like *Canis* and coyote-like *Canis*. In Asia we find multiple wolf-like *Canis* lineages with the Asian golden jackal *Canis aureus* belonging to the jackal-like *Canis*.

The Caninae evolved in the early Oligocene around 34-32 Ma ago, and first members of the tribe Canini appeared in the medial Miocene approximately 11 Ma ago (Sillero-Zubiri et al., 2004). The *Canis-Lycaon* clade within the Canini appeared in North America near the Miocene-Pliocene boundary between 6 and 5 Ma ago. At about the same time, the first records of canids began to appear in Europe. In this period from the Miocene-Pliocene boundary to the late Pliocene, multiple *Canis* (“dog” in Latin) species evolved and went extinct in both continents (Sillero-Zubiri et al., 2004). By the early Pliocene, approximately one million years ago, the coyote *Canis latrans* had evolved in North America (Wang and Tedford, 2008).

The grey wolf *C. lupus* appeared in the middle Pleistocene, approximately 0.8-0.3 Ma ago in the Arctic North (Sotnikova and Rook, 2010; Tedford et al., 2009; Vilà et al., 1999). The evolution of the *Canis* then becomes more complex due to a series of radiation events, range expansions and contractions subjected to the fluctuating climate and glaciation that affected the entire circumpolar fauna. *Canis* lineages in Eurasia underwent an extensive radiation and range expansion during the late Pliocene, resulting in multiple, closely related species in Europe, Africa and Asia (Sillero-Zubiri et al., 2004). In North America the Pliocene produced the coyote as an endemic form. While the contemporary Holarctic grey wolves (*C. lupus* spp.), the Ethiopian wolf (*C. simensis*), jackals (*C.aureus*, *Lupulella mesomelas*, *L. adusta*), dhole (*Cuon alpinus*), and African wild dog (*Lycaon pictus*) evolved in the Eurasian and African continents (Sillero-Zubiri et al., 2004).

During the evolutionary history spanning the ancestors of the wolf-dog clade in the early to middle Pleistocene (Tedford et al., 2009) to the contemporary Holarctic grey wolf, different lineages such as the Himalayan wolf (Werhahn et al., 2018), the African wolf (*C. lupaster*, Rueness et al., 2011), and the Indian grey wolf *C. l. pallipes* (Sharma et al., 2004) diverged as monophyletic sister clades.

Extant *Canis* species evolved under different ecological circumstances than those which exist at present. The grey wolf today is considered the top predator in much of the Holarctic, but for hundreds of thousands of years prior, the wolf coexisted as mesopredator with multiple predators as large as, or larger than, itself (Sillero-Zubiri et al., 2004).

Wolf-like canids are characterized by slender bodies with long legs, adapted for chasing prey. They have elongated muzzles with the canid typical dental formula: I 3/3, C1/1, P4/4, M2/3 =42 (except dholes *Cuon alpinus* which have 40 teeth) (Castelló, 2018). They all have 2n=78 chromosomes (Wayne, 1993; Wayne et al., 1987).

Methods

We reviewed the literature on wolf-like *Canis* lineages in Asia to provide an overview of latest research and explore taxon eligibility within the context of the larger Canid phylogenetic group. A total of 92 papers resulted from systematically searching the available English literature on Google Scholar with the search terms of historical and contemporary *Canis* species' scientific names (Table 1), and a search for *Canis lupus* + country name (following Newsome et al. (2016)). All relevant studies from 1990 onwards where included, but a few older studies and historical accounts relevant for taxonomy and morphology were also included. There were eight older studies in Russian and Mandarin that were not considered. A traffic light system was applied per taxa, based on the number of studies in support of a given taxon. Studies were allocated to three categories: morphology, genetics, and ecology/behaviour (Table 1). Those relevant to taxonomy were examined in detail for the quality of the research, such as sample size and methodology.

We conducted a genetic distance analysis based on full mtDNA and the cytochrome *b* gene only (because it is often used in phylogenetic studies) in the software MEGA (Kumar et al., 2018) for the considered lineages. And we built a Bayesian phylogeny based on full mtDNA (Huelsenbeck and Ronquist, 2001) in Geneious Version 2019.1.1. to complement the findings gleaned from the literature and further investigate taxon eligibility.

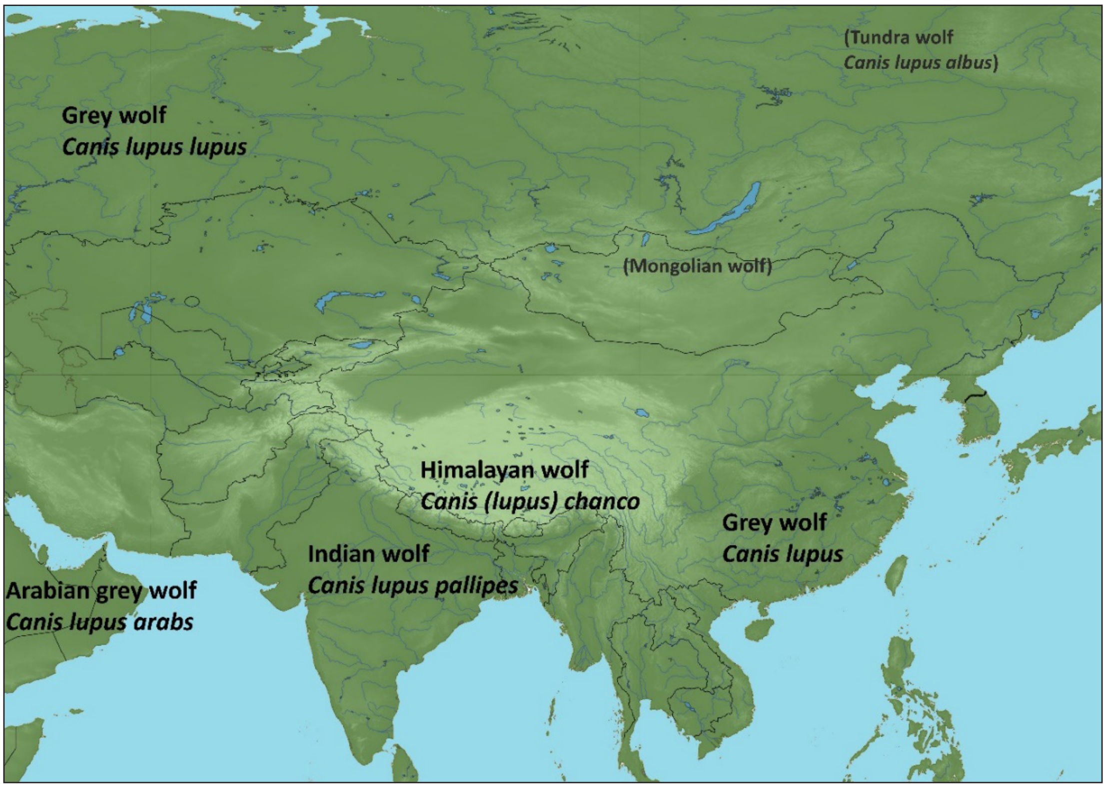

Figure 1. Wolf lineages in Asia. Lineages listed in the literature but with little contemporary support are shown in parentheses (see Table 1).

Wolf lineages of Asia

Holarctic grey wolf (*Canis lupus* spp.)

It was once the most widely distributed terrestrial mammal, found across the entire northern hemisphere, but today its range has been reduced by a third (Mech and Boitani, 2003). Due to its still wide distribution, large and stable populations the Holarctic grey wolf is listed as Least Concern by the IUCN Red List (Boitani et al., 2018). In the past there were as many as 38 subspecies reported for *C. lupus* (Wozencraft, 2005), of which only 10 subspecies are recognized by the IUCN today, five in North America and five in Eurasia (Table 1) (Boitani et al., 2018).

The Holarctic grey wolf *C. l. lupus* subspecies occupies large parts of Europe, with *C. l. signatus* (Cabrera, 1907) found in the Iberian peninsula and *C. l. italicus (*Altobello, 1921) in Italy, France and Switzerland (Boitani et al., 2018). *C. l. lupus* dominates Asia according to Boitani et al. (2018) from Mongolia across China and the Himalayan Mountains, but the Himalayan wolf *C. l. chanco* is mentioned as proposed for the Himalayan range. Also recognized are the Indian wolf *C. l. pallipes* for the Indian subcontinent and the Arabian wolf *C. l. arabs* for the Arabian peninsula. Pilot et al. (2010) found that except for Indian and Himalayan wolf lineages, contemporary worldwide grey wolves show little evolutionary significant diversification in terms of monophyletic clades with allopatric distributions. Wolves are highly mobile predators with dispersal distances reaching over 1,000 km (Geffen et al., 2004; Mech et al., 1995). Consequently, during interglacial periods, wolf populations could rapidly expand into favourable habitats leading to population admixture that obscured past phylogeographic structure caused by Ice Age isolation (Vilà et al., 1999).

Today, the Holarctic grey wolf comprises two main haplogroups: haplogroup 1 dominant in North America, and haplogroup 2 which dominated Eurasia and North America in the Pleistocene but was later completely outnumbered by haplogroup 1 in North America and partially outnumbered in Eurasia (Pilot et al., 2010). This shift was likely influenced by the changing ecological conditions occurring after the Last Glacial Maximum (LGM) which peaked around 26,500 years ago (Leonard et al., 2007; Pilot et al., 2010). Today only haplogroup 1 occurs in contemporary North American wolves, but all ancient wolf samples from Western Europe that dated from between 44,000 and 1,200 years before present belonged to haplogroup 2. Hence Pilot et al. (2010) suspected the long-term predominance of haplogroup 2 in the European region. Leonard et al. (2007) showed that mtDNA haplotypes of Pleistocene wolves from eastern Beringia belonged to a distinct haplogroup not occurring in contemporary North American wolves but corresponding to haplogroup 2 in Pilot et al. (2010), and some of the ancient European and Beringian wolves shared a common haplotype (Leonard et al., 2007).

Studies indicate a dramatic population decline of grey wolves beginning at least ~30,000 years ago and a rather recent common ancestry of extant grey wolves, suggesting that wolves existing before that time were phylogenetically distinct (Fan et al., 2016; Freedman et al., 2014; Leonard et al., 2007; Thalmann et al., 2013). Recent work further suggest that contemporary Holarctic grey wolves all originated from a Beringian wolf population expansion that took place at the end of the Last Glacial Maximum (between 26,500-19,000 years ago), with the expansion driven by the considerable ecological changes of the time (Ersmark et al., 2016; Koblmüller et al., 2009; Loog et al., 2018). Within the Holarctic grey wolf complex, the highest diversity is found in wolves from Europe, China and Russia (Ersmark et al., 2016).

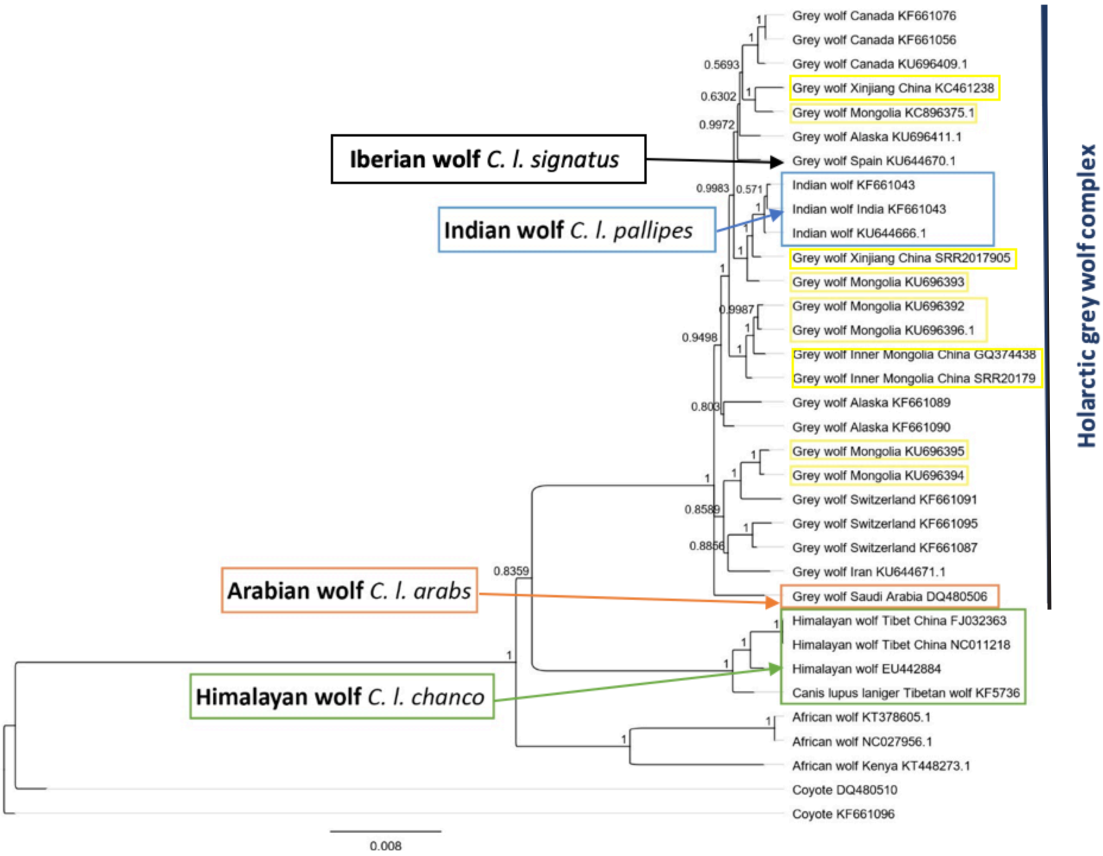

Figure 2. Bayesian phylogeny based on the full mitochondrial genome with GenBank accession numbers. The Indian wolf (blue), the Arabian wolf (orange, but note N=1 only allows pre-eliminary inference), and Himalayan wolf (green) are monophyletic, whereas the wolf samples from the Mongolia and Inner Mongolia region (yellow) are polyphyletic. This phylogeny indicates that, 1) the Indian and Arabian wolf form a clade within the Holarctic grey wolf, 2) wolves in Mongolia do not form a monophyletic clade and thus subspecies recognition is not supported, and 3) the Himalayan wolf forms a monophyletic clade basal to the Holarctic grey wolf and thus taxon recognition is supported.

Table 1. Overview of historical and current wolf lineages proposed for Asia. Evidence for taxon support are listed in colour coded classes:

🟩 dark-green indicates studied and supported by more than ≥4 studies,
▧ light-green indicates studied and supported but less <4 studies,
🟨 yellow indicates not studied,
▩ grey indicates studied but not supported.

Colour coding is not applied for extinct lineages and synonyms. Contemporary lineages supported by evidence as phylogenetically distinct are in bold. Lineages marked with * are recognized by Boitani et al. (2018) in the current IUCN Red List assessment. Morphological evidence refers to studies based on skull measurements, genetic evidence includes mitochondrial and nuclear genetic studies, and ecological evidence includes studies that reveal distinct behaviour and/or ecological adaptations.

Latin Name	Distribution Region	Common Name	Morphological evidence	Genetic evidence	Ecological evidence	Additional references
C. l. lupus* Linnaeus, 1758	Northern Hemisphere circumpolar	Eurasian grey wolf, Holarctic grey wolf, grey wolf, gray wolf	🟩 Morphometric skull measurements (Nowak, 2003; Paquet and Carbyn, 2003; Therrien, 2005; Wozencraft, 2005)	🟩 -mtDNA and nDNA (Boitani et al., 2018; Ersmark et al., 2016; Gopalakrishnan et al., 2018; Mech and Boitani, 2003; Pilot et al., 2010)	🟩 Ecology and behaviour of Holarctic grey wolf widely studied (e.g. Mech and Boitani, 2003)	Boitani et al. (2018); Mech and Boitani (2003); Sillero-Zubiri et al. (2004)
C. l. albus Kerr, 1792	Eurasia‘s tundra and forest-tundra zones in northern Russia	Tundra wolf	▧ Morphometric skull measurements (Nowak, 1995)	🟨	🟨	Nowak (2003); Sillero-Zubiri et al. (2004)
C. l. communis Dwigubski, 1804	Central Russia	Russian wolf	▧ Morphometric skull measurements	🟨	🟨	Nowak (2003); Sillero-Zubiri et al. (2004)

Latin Name	Distribution Region	Common Name	Morphological evidence	Genetic evidence	Ecological evidence	Additional references
C. l. cubanensis Ognev, 1923	east central Asia, Caspian Sea, Caucasus	Caucasus wolf, Caspian Sea wolf	Morphometric skull measurements (Nowak, 1995)	-mtDNA and nDNA (~65 samples) (Pilot et al., 2014)		Nowak (2003); Sillero-Zubiri et al. (2004)
C. l. hattai (extinct) Kishida 1931	Hokkaido, Japan	Ezo wolf	Morphometric skull measurements (Ishiguro et al., 2010; Nowak, 2003)	-mtDNA (2 samples) (Ishiguro et al., 2010; Koblmüller, 2016; Matsumura et al., 2014)		Sillero-Zubiri et al. (2004)
C. l. hodophilax (extinct) Temminck 1839	Honshu, Japan	Japanese wolf	Morphometric skull measurements (Nowak, 2003)	-mtDNA (8 samples) (Ishiguro et al., 2009; Koblmüller, 2016; Matsumura et al., 2014)		Sillero-Zubiri et al. (2004)
***C. l. pallipes* *** Sykes,1831	Indian subcontinent	Indian wolf	Morphometric skull measurements (Blanford, 1888; Mivart, 1890; Nowak, 1995)	-mtDNA evidence (~50 samples) -0.1% genetic distance on the cyt *b* gene from the Holarctic grey wolf (Table 2) (Aggarwal et al., 2007; Sharma et al., 2004)	Differentiated vocalisation (Hennelly et al., 2017)	Boitani et al, (2018); Nowak (2003); Sillero-Zubiri et al. (2004)

Latin Name	Distribution Region	Common Name	Morphological evidence	Genetic evidence	Ecological evidence	Additional references
C. l. chanco Gray, 1863 synonym: laniger Hodgson, 1847 filchneri Matschie,1907 himalayensis (Aggarwal, 2003)	Accurate contemporary geographic use: Himalayas (India and Nepal); Tibetan Plateau of Qinghai and the Tibetan Autonomous Region (China) Past geographic use not accurate given contemporary use: *C. l. chanco* was used for the grey wolf populations in Mongolia and northern China which do not belong to the Himalayan wolf lineage. E.g. see Wang et al. (2016)	Himalayan wolf Synonyms: Tibetan wolf, Plateau wolf	Morphometric measurements of the mandibular coronoid process (Janssens et al., 2016)	-mtDNA and nDNA evidence (>100 samples) -3.84% genetic distance on the cyt *b* gene from the Holarctic grey wolf (Table 2) (Aggarwal et al., 2007; Chetri et al., 2016; Ersmark et al., 2016; Fan et al., 2016; Li et al., 2011; Matsumura et al., 2014; Sharma et al., 2004; vonHoldt et al., 2017; Werhahn et. al, 2018; Werhahn et al., 2019, 2017a; Zhang et al., 2014)	Differentiated vocalisation (Hennelly et al., 2017); discrete distribution in Asian high-altitude ecosystems above 4000m a.s.l. (Werhahn et al., 2019)	Alvares et al. (2019); Boitani et al. (2018); CITES (2017)
C. l. campestris Dwigubski,1804 synonym: chanco Gray, 1863 (but recently recommended for the Himalayan wolf	Lowlands north of the Tibetan Plateau, i.e. Inner Mongolia and Mongolia	Mongolian wolf		-mtDNA evidence (1 sample) Zhang et al, (2013b) provides full mtDNA of one animal (used in Figure 2), but no phylogenetic research		

Latin Name	**Distribution Region**	**Common Name**	**Morphological evidence**	**Genetic evidence**	**Ecological evidence**	**Additional references**
C. l. desertorum Bogdanow, 1882	Xinjiang	Chinese Shinjang wolf, Steppe wolf		-mtDNA evidence (1 sample) Zhang et al., (2013a) provides full mtDNA of one animal (used in Figure 2) but no phylogenetic research		Wang et al. (2016)
C. l. arabs* Pocock, 1934	Arabian Penisula	Arabian wolf	Morphometric skull measurements (Nowak, 2003)	-mtDNA evidence (>100 samples) - 0.43% genetic distance on the cyt *b* gene from the Holarctic grey wolf (Table 2) (Bray et al., 2014)		Boitani et al. (2018)

Table 2. Net genetic distance in % based on the whole mitochondrial genome and only the cytochrome *b* (cyt *b*) gene as calculated with MEGA. The coyote, African wolf, and Iberian wolf are included as comparison of a recognized species, a recommended species and grey wolf subspecies respectively. The grey highlighted column shows the genetic distance between the Holarctic grey wolf and considered lineages.

	Holarctic grey wolf		**African wolf**		**Himalayan wolf**		**Grey wolf Iran**		**Grey wolf Mongolia**		**Indian wolf**		**Grey wolf Xinjiang (China)**		**Iberian wolf**	
	mtDNA	cyt b	mtDNA	cyt b	mtDNA	cyt b	mtDNA	cyt b	mtDNA	cyt b	mtDNA	cyt b	mtDNA	cyt b	mtDNA	cyt b
Holarctic grey wolf (N=9)																
Coyote (N=2)	4.15	6.60														
African wolf (N=3)	2.55	3.11														
Himalayan wolf (N=4)	2.45	3.84	2.59	2.48												
Grey wolf Iran (N=1)	0.30	0.45	2.80	3.60	2.77	4.31										
Grey wolf Mongolia (N=8)	0.04	0.09	2.59	3.19	2.56	4.10	0.37	0.52								
Indian wolf (N=3)	0.22	0.10	2.79	3.23	2.84	4.12	0.56	0.53	0.14	0.14						
Grey wolf Xinjiang (China) (N=2)	0.06	-0.02	2.61	2.95	2.69	3.94	0.43	0.44	0.02	0.05	0.05	0.00				
Iberian wolf (N=1)	0.20	0.20	2.76	3.22	2.71	3.93	0.56	0.71	0.18	0.32	0.33	0.35	0.20	0.18		
Grey wolf Arabia (N=1)	0.35	0.43	2.80	3.60	2.77	4.31	0.66	0.88	0.41	0.52	0.58	0.53	0.45	0.44	0.61	0.71

Focusing on Asia, Wang et al. (2016) described five wolf lineages for China, but not all were supported by the evidence: *C. l. chanco, C. filchneri, and C. l. desertorum* (in Table 1, but listed according to their contemporary use) and two more *C. lupus* lineages: *C. l*. Nei-Mongol form in Inner Mongolia (western and mid part) and *C. l*. South-China form in Anhui, Jiangsu, Zhejiang, Jiangxi, Fujian, Guangdong, Hunan, Guizhou, Yunnan, Hubei and Sichuan.

We found evidence for the presence of two distinct wolf lineages in China, the grey wolf and the Himalayan wolf, with the latter found in the high altitudes of western China (Fan et al., 2016; Matsumura et al., 2014; Werhahn et al., 2018; Zhang et al., 2014). Full mitochondrial DNA analysis (Figure 2) and the genetic distance analysis (Table 2) suggested that the wolf lineages in Xinjiang (Zhang et al., 2013a), Inner Mongolia and Mongolia do not form a monophyletic clade within the Holarctic grey wolf, and hence taxonomic distinction is not supported. Below, we take a closer look at historical and contemporary wolf lineages considered for Asia.

Eurasian wolf *C. l. lupus*

The Eurasian wolf is found in large parts of Eurasia, including northern Europe, Russia, Mongolia, Kyrgyzstan, Kazakhstan, and the lowlands of northwestern China (Boitani et al., 2018). This nominal subspecies was also recognized by Nowak (1995) based on skull morphology. However, in-depth genetic and ecological studies on wolves in Central Asia and north-eastern Asia are recommended to further verify the distribution of this taxon.

Tundra wolf *C. l. albus*

The Tundra wolf of northern Russia was listed by Nowak (2003 and 1995) but is not included in the recent *C. lupus* Red List assessment (Boitani et al., 2018). Mech (1974) stated that the type locality was Jenisea of the east of former USSR. The subspecies was recognized by Nowak (1995) based on skull morphology, but we found no further evidence supporting the taxon.

Russian wolf *C. l. communis*

This subspecies is listed in Sillero-Zubiri et al. (2004) but is not included in the recent *C. lupus* Red List assessment (Boitani et al., 2018). The subspecies was recognized by Nowak (1995) based on skull morphology, but we find no further supporting evidence for the taxon.

Caucasus wolf *C. l. cubanensis*

The Caucasus wolf is listed by Sillero-Zubiri et al. (2004) but is not included in the recent *C. lupus* Red List assessment (Boitani et al., 2018). Wolves in the Caucasus region are found in the geographic boundaries between Europe and Asia. We found one recent study (Pilot et al., 2014) that investigated the genetic distinctness of Caucasus wolves and concluded that these wolves were genetically connected with Eurasian wolf populations and shared the same demographic trends. The Caucasus region wolves shared mtDNA haplotypes with both Eastern European and West Asian wolves, suggesting past or ongoing gene flow. The study is based on 65 invasive and non-invasive samples analysed for 660 bp of mtDNA control region and 14 microsatellite loci, as well as four individuals analysed for 167,989 autosomal genome-wide SNPs (Pilot et al., 2014). The subspecies was recognized by Nowak (1995) based on skull morphology, but we found no other supporting evidence for the taxon.

Japanese wolf *(C. l. hodophilax,* extinct) and Ezo wolf *(C. l. hattai,* extinct)

Two lineages of grey wolves were historically found in Japan, i.e. the Japanese or Honshu wolf and the Ezo wolf, but rapidly went extinct around 100-120 years ago. Of these two, the Japanese wolf is considerably older based on mitochondrial DNA and is believed to have colonized the Japanese archipelago in the Late Pleistocene (ca. 25,000–125,000 years ago), while the Ezo wolf arrived in Japan later <14,000 years ago (Ishiguro et al., 2010, 2009; Matsumura et al., 2014). Ishiguro et al. (2009) analysed eight samples of the Japanese wolf for ~590 bp of the mtDNA control region, and two Ezo wolf samples were analysed for ~600 bp of the mtDNA control region (Ishiguro et al., 2010). Nowak (2003) recognized the morphological distinctness of the two lineages.

Indian wolf *C. l. pallipes* (synonym: *C. indica)*

The Indian wolf *C. l. pallipes* is recognized by Boitani et al. (2018) as a grey wolf subspecies, characteristic to the arid and semi-arid lowlands of the Indian subcontinent. It shows divergent mtDNA haplotypes that form a monophyletic clade within the Holarctic grey wolf complex (Figure 2) (Aggarwal et al., 2007; Fan et al., 2016; Pilot et al., 2010; Sharma et al., 2004; Werhahn et al., 2017a), but we did not find any published nuclear genetic data. Aggarwal et al. (2007) analysed five samples for D-loop mtDNA (1140 bp) and 16S rRNA gene (560 bp), and two samples for cytochrome *b* (1300 bp), Ersmark et al. (2016) used the samples by Aggarwal et al. (2007), and Sharma et al. (2004) analysed 45 samples for 440 bp mtDNA control region.

Estimated divergence times range between 0.27-0.4 Ma ago based on molecular clock analysis of mitochondrial DNA (Aggarwal et al., 2007; Sharma et al., 2004).

The Indian wolf is similar in appearance to the European wolf, but smaller, more slightly built, and has shorter fur of greyish-red to reddish-white colour with little to no underfur (Castelló, 2018). The Indian wolf generally lives in smaller packs rarely exceeding 6-8 individuals. It typically feeds on antelopes, rodents, and hares (Blanford, 1898; Jhala, 1993; Jhala and Giles, 1991).

Our phylogenetic analysis (Table 2, Figure 2) supports the Indian wolf as subspecies with genetic distance values comparable to the Iberian and Arabian wolf. Sharma et al. (2004) had proposed *C. indica* as its scientific name (e.g. NCBI GenBank Taxonomy as *Canis indica*) but species level recognition is not supported by the evidences.

Formerly this lineage was reported from southwest Asia to the Indian subcontinent (Nowak, 1995; Sillero-Zubiri et al., 2004), but recent research suggests that wolves in Waziristan of Pakistan and westwards (e.g. Iran, Oman, Israel, and Turkey) genetically group with the Eurasian grey wolf clade (Bray et al., 2014; Ersmark et al., 2016; Hamid et al., 2019; Sharma et al., 2004). In the past wolves in Iran were considered the same lineage as the Indian wolf but this is not supported by any study. The full mitochondrial phylogeny (Figure 2) shows that the Iranian wolf sample does not cluster with the Indian wolf. Khosravi et al. (2012) showed minor morphological variations of the skull of Iranian wolves but they do not provide morphological support for a different wolf subspecies in the region; their genetic lineage was not verified. Our genetic distance analysis (Table 2) and phylogeny (Figure 2) included only one wolf sample from Iran but suggested that these wolf populations would merit further in-depth genetic studies as they seem diversified and neither cluster with Arabian nor Indian wolves.

Arabian wolf *C. l. arabs*

The Arabian wolf *C. l. arabs* (Pocock, 1934) is a desert-adapted grey wolf subspecies found in the Arabian peninsula and recognized as a subspecies (Boitani et al., 2018; Hefner and Geffen, 1999). It is genetically distinct from the Indian wolf and more closely associated to the European wolf (Bray et al., 2014)*.* Bray et al. (2014) analysed 15 blood samples of captive animals and 88 tissue samples of road kills for mitochondrial DNA, specifically ~400-800 bp of the cytochrome *b* gene region and a ~ 300 bp fragment of the control region. The type locality for *C. l. arabs* is in Ain in S.E. Arabia (Mech, 1974).

Formerly, but not conforming with the recent genetic evidence on distribution range, *C. l. pallipes* was used to describe wolves in Arabia and Iran (e.g. Khosravi et al., 2013; Wronski and Macasero, 2008).

The genetic distance analysis (Table 2) and phylogeny (Figure 2, but including only one Arabian wolf sample) supported subspecies classification with genetic distance values in the same decimal range as the other recognized grey wolf subspecies. Arabian wolves are usually greyish beige in colour but melanistic individuals are frequent (Islam et al., 2019). Nowak (2003) recognized the morphological distinctness of the Arabian wolf.

Mongolian wolf

In the past the wolves of Mongolia have been treated as a different subspecies of grey wolf (Wilson and Reeder, 2005), but this subspecies has been dropped in other recent literature, such as Boitani et al. (2018), Sillero-Zubiri et al. (2004), and Wozencraft (2005).

The naming of the Mongolian wolf, historically often called *C. l. chanco,* has been ambiguous, as *C. l. chanco* has also been used to describe a completely different wolf lineage, namely that of the Tibetan plateau and the Himalayas, the so called Himalayan or Tibetan wolf. Recently Alvares et al. (2019) recommended that *C. l. chanco* should be used exclusively for the Himalayan wolf of the Asian high-altitudes, which forms a distinct clade outside the Holarctic grey wolf complex. In contrast, the Mongolian wolf, i.e. the wolf populations in Mongolia and in the geographically close Inner Mongolia and Xinjiang provinces of China, genetically group within the Holarctic grey wolf complex, but are polyphyletic and only show a shallow diversification (Figure 2, Table 2). Hence subspecies recognition for the wolf populations in the wider Mongolian region is not indicated. This is in line with the recent dropping of the subspecies and considering these populations as part of *C. l. lupus.* If a subspecies designation were to be considered, the previous synonyms of *C. l. chanco* as applied to the Mongolian wolf must be considered: *C. l. desertorum (*Bogdanow, 1882) and *C. l. campestris (Dwigubski, 1804).* Some authors list *desertorum* as synonym for *campestris* (e.g. Mech (1974), whereas in Zhang et al. (2013a) *desertorum* is used for wolves in Xinjiang province of China, and in Zhang et al., (2013b) *campestris* for wolves in Mongolia. But the type locality of *campestris* appears to be between the Black and Caspian Seas, Kirghizia, to Yenesei (Mech, 1974), therefore sometimes also referred to as Caspian wolf. To tackle remaining uncertainties, it would first need to be genetically and/or morphologically proven that wolves in Xinjiang and/or Mongolia are eligible for subspecies status, contrary to evidences and if so, whether they belong to one subspecies. And then the type specimen would require verification with regards to geographic origin and genetic lineage.

But taxon recognition is not supported as the wolves in Mongolia and Inner Mongolia present little diversified polyphyletic clades within the Holarctic grey wolf complex (Fan et al., 2016; Werhahn et al., 2017a; Zhang et al., 2014). And neither our genetic distance analysis (Table 2) nor our phylogeny (Figure 2) support taxonomic recognition for wolves in Mongolia and Xinjiang, given their comparably small genetic distance from the Holarctic grey wolf.

Himalayan wolf *(C. l. chanco; synonym: C. laniger, C. filchneri, C. himalayensis)*

The Himalayan wolf presents a phylogenetically distinct wolf clade characteristic to the Asian high-altitudes (Aggarwal et al., 2007; Sharma et al., 2004; Shrotryia et al., 2012; Werhahn et al., 2019; Werhahn et al., 2018, 2017a). It is found in habitats above

4,000m elevation in the Himalayas and the Tibetan Plateau (Aggarwal et al., 2007; Chetri et al., 2016; Sharma et al., 2004; Werhahn et al., 2019). The clade is supported by multiple large scale studies on wolf phylogeny and phylogeography, e.g. Ersmark et al. (2016), Fan et al. (2016), Gaubert et al. (2012), Pilot et al. (2010), and Rueness et al. (2011), and forms a monophyletic clade basal to the Holarctic grey wolf complex (Fan et al., 2016; Leonard et al., 2007; Matsumura et al., 2014; Pilot et al., 2010; Werhahn et al., 2019, 2018, 2017a). Sharma et al. (2004) included 23 samples of the Himalayan wolf lineage analysed for 440 bp of the mtDNA control region. Aggarwal et al. (2007) included 16 Himalayan wolf samples analysed at the mtDNA D-loop, cytochrome *b* and 16S rRNA. Of these, one sample originated from a wild animal, while eight samples were from zoo animals likely duplicating at least in part the samples used in Sharma et al. (2004); the remaining seven samples originated from collection specimens. Zhang et al. (2014) analysed 14 samples of assumed Himalayan wolves at 26 microsatellite makers and 25 SNPs (including three hypoxia-related genes), and full genomes for four assumed Himalayan wolf individuals. Fan et al. (2016), used the same samples as Zhang et al. (2014), and in contrast to other studies placed the Himalayan wolf lineage as the most recent clade within the Holarctic grey wolf complex in the maximum likelihood phylogeny based on whole genome SNP data. vonHoldt et al. (2017) also used the samples from Zhang et al. (2014) with the addition of one new sample and analysed them for admixture at the hypoxia related EPAS gene. A careful verification of the origin and lineage of these repeatedly used samples is recommended, given that they originate from zoo animals with unconfirmed capture origin; furthermore, these facilities lay at the edges or outside of Himalayan wolf range as described in Werhahn et al. (2019).

Werhahn et al. (2017a, 2018 and 2019) analysed 82 Himalayan wolf non-invasive samples for 17 microsatellite loci and for four non-synonymous SNPs in three hypoxia-pathway related functional nuclear genes, a subset for ZF genes on both sex chromosomes, and >280 samples at the mtDNA loci. In their study on Japanese wolf lineages, Matsumura et al. (2014) included available mitochondrial DNA samples belonging to the Himalayan wolf lineage from the study by Meng et al. (2009) and Pang et al. (2009), and found that the wolves from Tibet “differ from other wolf and dog samples and form a remarkably different clade.” These findings were also supported by the studies by Li et al. (2014 and 2011) on Tibetan Mastiffs including wolf samples.

The divergence time for the Himalayan wolf from the ancestors of the wolf-dog clade is estimated at between 0.69-0.80 Ma ago based on molecular clock analysis of the mitochondrial DNA (Matsumura et al., 2014; Sharma et al., 2004; Werhahn et al., 2018). This suggests that the Himalayan wolf is considerably older than current and extinct extinct grey wolves belonging to the haplogroups 1 and 2 in Pilot et al. (2010). They do not give divergence time estimates for haplogroups 1 and 2, but all ancient wolf samples from central and western Europe (all belonging to haplogroup 2) date no older than 44‘000 years before present. The Himalayan wolf is considered to have existed as a distinct lineage before the radiation of the contemporary Holarctic grey wolf (Rueness et al., 2011) and its distinction is also reflected in differences in howl acoustics (Hennelly et al., 2017). Genetic distance analysis (Table 2) implied a similar genetic distance between the Holarctic grey and Himalayan wolf as between the Holarctic grey and African wolf, with the latter recently recommended as species *Canis lupaster* by Alvares et al. (2019). Further the genetic distance of the Himalayan to the Holarctic grey wolf is much larger than for the recognized grey wolf subspecies (Table 2). Given the need of taxonomic consistency within the canid family, these findings imply that the Himalayan wolf needs to be recognized at the same taxonomic level as the African wolf, i.e. at species level as *Canis chanco*. Nevertheless, Álvares et al. (2019) recommend subspecies level *C. l. chanco* for the Himalayan wolf until further full genome work is carried out to verify the existing evidence.

Detailed and systematic morphological studies for the Himalayan wolf are required. Differences in the mandibular coronoid process were described in Janssens et al. (2016), and Hodgson (1847) provided a historical description of the overall appearance and differences between this wolf of Tibet and the wolves of Europe.

While the scientific name for the Himalayan wolf was recently recommended as *C. l. chanco* by Alvares et al. (2019), different scientific names have been used over the past decades. Previously, Wilson and Reeder (2005) mentioned these wolves as *C. l. filchneri* (Matschie, 1906) (Filchner, 1903). Pocock (1941) referred to it as woolly wolf *C. l. chanco* (Gray, 1963) and *C. l. laniger* (Hodgson, 1847) as a synonym (Gray, 1863; Hodgson, 1847). NCBI GenBank currently lists *C. l. chanco* as the Mongolian wolf

(NCBI GenBank Taxonomy, *Canis lupus chanco*) and separately *C. l. laniger* as the Tibetan wolf (NCBI GenBank Taxonomy, *Canis lupus laniger*). Furthermore, recent studies (including the authors‘ earlier work) have referred to it as *C. himalayensis* (Aggarwal et al., 2007; Werhahn et al., 2017a, 2017b), however this is a *nomen nudum* and taxonomically is not valid. The type locality of *C. chanco* is the Chinese Tartary which comprised present day China and Mongolia. Thus, the genetic lineage of the holotype needs verification. The type locality for *Lupus laniger* by Hodgson (1847) is noted as Tibet, but as Mech (1974) points out could refer also to little Tibet in Kashmir. The type locality of *Lupus filchneri* by Filchner (1903) is Siningfu, Kansu, China. Given the latest study by Werhahn et al. (2019), Kansu, i.e. referring to present day Gansu, may lie in the admixture zone between Himalayan and grey wolf. Wang et al. (2016) in their review on wolves in China used *C. l. chanco* according to past (now outdated) usage, i.e. using *C. l. chanco* for the wolf lineage in Mongolia and northern China. The authors described this subspecies in the following Chinese provinces, Heilongjiang, Jilin, Liaoning, Inner Mongolia (eastern part), Hebei, Beijing, Shandong, Henan and Shanxi, but these populations more likely belong to *C. l. lupus*.

A relevant implication for research practice given this past name ambiguity is that when considering existing studies on Himalayan wolves and data from databases (e.g. NCBI GenBank), the geographic origin of the sample used needs to be carefully considered whereas the provided scientific name is less reliable to infer the true genetic lineage.

Discussion

Genetic, species and ecosystem diversity are the top three forms of biodiversity recognized for conservation (IUCN, 2016; Jenkins, 1988) while the conservation of evolutionary and ecosystem processes are increasingly recognized as essential for biodiversity conservation (Stanton et al., 2019). Diversified populations, irrespective of taxonomy, are important for biodiversity conservation as they represent evolutionary potential within a species (Haig et al., 2006) allowing them to adapt and meet future challenges such as disease, climatic change, and shifts in resource availability.

Admixture and hybridization in *Canis*

Introgression has been important in the evolution of the canid family (Gopalakrishnan et al., 2018) and gene flow among lineages may be important for evolutionary processes. Wolf species delineation is complex not only due to a long history of admixture between different wolf lineages, also including domestic dogs *C. familiaris*, but also past range contractions and expansions due to glaciation (Pilot et al., 2010). The extent of interbreeding varies and illustrates the adaptability and flexibility of wolves.

Wolf-dog hybridization is challenging to detect given the genetic similarity between domestic dogs and grey wolves (dogs group within the Holarctic grey wolf complex), and requires the use of large numbers of genetic markers. Dufresnes et al. (2019) found very little dog introgression in the recolonizing wolf populations in the Alps based on 11 microsatellite markers and the Y sex chromosome (also see Vilà and Wayne (1999)). In contrast, Fan et al. (2016) found considerable admixture between dogs and wolves based on 34 full genome sequences at 9–28× coverage, with up to 25 % of Eurasian wolf genomes showing signs of dog ancestry. Galaverni et al. (2017) found no sharp subdivisions between non-admixed grey wolves and hybrids in Italian populations, which the authors interpret as recurrent hybridization and deep introgression which might have started mostly at the beginning of the population re-expansion of grey wolves. But high levels of recent dog introgression were detected in an Italian wolf population from a central Italian agricultural landscape in Salvatori et al. (2019) based on 16-49 multi-locus genotypes. Pacheco et al. (2017) document a scenario of multiple and widespread Iberian wolf-dog hybridization events at the population level during one breeding season in northern Spain. But they also find a clear maintenance of wolf genetic identity, evidenced by the sharp genetic identification of pure individuals, which suggests the resilience of wolf populations to a small amount of hybridization. The authors consider that real-time population level assessment of hybridization provides a new perspective on the wolf conservation debate (Pacheco et al., 2017), and this will also be useful to identify the variability of wolf-dog hybridization across different landscapes. Wolf-dog hybridization is expected to increase with numbers of feral dogs, whereas intact wolf populations with enough breeding partners may mitigate it.

Despite many uncertainties around wolf-dog hybridization, wolves and dogs have certainly influenced their recent phylogenetic history through admixture in both directions. In some events this might lead to potentially enhancing adaptive effects, such as in the Tibetan Mastiff dog of the high-altitude Tibetan Plateau areas where the breed is thought to have gained the genetic hypoxia adaptation by interbreeding with the high altitude wolves of the region (Li et al., 2014). Nevertheless, hybridization between wolves and feral dogs poses a conservation challenge that requires research and consensus on the best management practice (Donfrancesco et al., 2019).

Gene flow is also documented among contemporary wild canids, e.g. among the red wolf *C. rufus* and coyote (Adams et al. 2003, 2007), among North American grey wolf subspecies (Sinding et al. 2018, vonHoldt et al. 2016), and indications for gene flow are found between the Himalayan and grey wolf (Werhahn et al. 2019). Wolves are highly mobile animals and dispersal ranges can be considerable (Ciucci et al., 2009; Mech et al., 1995). This high mobility further influences the degree of interspecific hybridization and gene flow. The width of a hybrid zone is proposed as a function of the distance travelled from birth to place of first reproduction and the degree of natural selection functioning against hybrids (Wayne et al., 2004).

Baker and Bradley (2006) propose that two phylogenetic groups represent different species when hybridization is restricted to a limited geographic area, a stable hybrid belt, and outside the hybrid belt the two phylogenetic groups are defined by unique, conclusively supported monophyletic clades based on mitochondrial and nuclear genetic variation. This view is also supported by the fact that stable hybrid zones between species are documented across many taxa (Barton and Hewitt, 1989, 1985). Similarly, Hausdorf (2011) concludes that one of the most important insight with regards to species concepts is that reproductive barriers are semipermeable to gene flow and that species differentiation takes place despite ongoing gene flow. Hence differentiation between populations maintained despite gene flow strengthens the case for considering the populations as different species.

Species concepts

A ‚species' is a useful concept in a continuous nature (Galtier, 2019). The definability of species varies across taxonomic groups. Whereas historically species were considered entities that are completely isolated, today we know that reproductive barriers can be semipermeable to gene flow, that species can differentiate despite ongoing interbreeding, and even that a single species can originate polyphyletically by parallel evolution (Hausdorf, 2011). Genetic methods are increasingly gaining importance for informing taxonomy but, as is also inherent in morphological methods, individuals do not necessarily cluster in well-defined entities and intermediate individuals are frequently observed (Galtier, 2019). A standardized taxonomic approach considering genetic, morphological, and ecological methods is required. Most importantly, the species concepts applied should be consistent within neighbouring taxonomic groups at minimum, and needs to be conducive for conservation by maximizing benefits for the species in terms of reproductive fitness, sustain evolutionary adaptation processes, facilitate conservation, and maximize persistence in the face of changing environments (Frankham et al., 2017).

There are at least 27 - 30 applied species concepts, leading to inconsistent species delineation and disparate groupings, i.e. a heterogenous taxonomy overall, but with considerable financial, legal, biological and conservation implications (Frankham et al., 2017, 2012; Galtier, 2019; Zachos, 2018). A broad species definition may lead to management actions supporting the crossing of distantly related groups and consequently outbreeding depression, whereas excessive splitting may preclude genetic rescue of small inbred populations with low genetic diversity. From the management perspective it means combined versus separate management (Frankham et al., 2017). While for the respective animals species delineation has major consequences, the practice is currently inconsistent (Galtier, 2019; Tobias et al., 2010) and drags historical baggage.

In the following a brief outline of the more recent and commonly used species concepts (SC): The Biological SC (Mayr, 1942) is based on populations that are reproductively isolated, but has to be reconsidered given the wide occurrences of gene flow and stable hybrid belts between taxa (Barton and Hewitt, 1985). The Genetic SC focuses on genetic isolation rather than reproductive isolation (Baker and Bradley, 2006). The phylogenetic species concept defines species as the smallest definable cluster of individuals with shared ancestry (Cracraft, 1983). The Unified SC by Queiroz (2005) argues that all modern species concepts have a common element, namely a species is a “separately evolving metapopulation lineages”. And the Differential Fitness SC by Hausdorf (2011) proposes that “species can be defined as groups of individuals that are reciprocally characterized by features that would have negative fitness effects in other groups and that cannot be regularly exchanged between groups upon contact.”

Gene flow is a useful indication of reproductive isolation (Galtier, 2019), but lineage differentiation and speciation, can take place despite gene flow (Baker and Bradley, 2006) and in some cases gene flow may even be important (Gopalakrishnan et al., 2018). Often within species it is considered beneficial but between species as a threat (Galtier, 2019). It is considered positive when it is used to achieve genetic rescue, but negative when it comes to hybridization among species that we want to preserve (e.g. dog and wolf hybridization), and this is very much influenced by the existing taxonomy (Frankham et al. 2012). So, what is a species and where do we draw the line?

Hey and Pinho (2012) state: ”Species as evolutionary lineages are expected to show greater evolutionary independence from one another than populations within species”. The authors investigate gene flow and divergence time as measures for species differentiation and conclude that both these measures show overlapping distributions for pairs of species and for pairs of populations within species but that both measures combined may be used to develop a repeatable tool for species diagnostics (Hey and Pinho, 2012).

Specialists agree that species delineation is arbitrary (Galtier, 2019; Hey, 2006). But the concept of a species is important and it is especially important to get it right, as legislation, conservation and the non-specialist science community rely on these taxonomic divisions and need species, as stated by Galtier (2019), as a ”simplified representation of natural variation”‘. Mace (2004) proposes to reduce the taxonomic inconsistencies by a) standardizing the rules for delineation and b) choosing an approach to delineate units for conservation recovery planning that recognizes the dynamic nature of natural systems. Mace (2004) further states that this needs a new kind of collaboration among conservation biologists, taxonomists and legislators.

Species delineation influences many applied issues, particularly wildlife conservation, as exemplified by lists of endangered species upon which legislators rely (Hey, 2006; Macdonald, 2019). Wolves belong to a taxonomic group that can exhibit continuous species boundaries. The resulting difficulties for species delineation and consequences for conservation are illustrated by the situation around wolves in North America which is subject to long standing debate (e.g. see Chambers et al., 2012; Cronin et al., 2015; Rutledge et al., 2015; vonHoldt et al., 2016; Weckworth et al., 2010; Wilson et al., 2000). Haig et al. (2006) states that listing subspecies under the Endangered Species Act is an increasing source of conflict in science and policy, due to the complex processes involved in speciation which needs to be presented in simple terms for applicable legislation. Taxonomy is unfortunately sometimes being politicized as seen in the Eastern wolf-Grey wolf listing/delisting debate (Rutledge et al., 2015). But conservation needs to be empowered to protect evolutionary unique populations irrespective of taxonomic decisions, and hence it is critical that taxonomy and politics are disentangled. vonHoldt et al. (2016) argues “for a more balanced approach that focuses on the ecological context of admixture and allows for evolutionary processes to potentially restore historical patterns of genetic variation.”

But despite all the difficulties described, taxonomic groupings are key to conservation efforts and there seems no way around them because they allow listing of species, subspecies, and evolutionary significant units (ESU) in the listings of the global conservation authorities, such as the IUCN Red List of Threatened Species, appendices in the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES), and TRAFFIC (wildlife trade monitoring network) (Haig et al., 2006). These lists in turn allow us to track species recovery and loss.

Taxonomic conclusions for Asian *Canis* lineages

Our review and genetic analysis support the taxonomic eligibility of a) the Himalayan wolf at species level, b) the Indian wolf and Arabian wolf at subspecies level, and c) the presence of the Holarctic grey wolf (*C. l. lupus*) in large parts of Asia. But studies on wolves in central Asia are few and in-depth wolf studies for Central Asia, Pakistan, Mongolia and eastern Russia are advised. Further, the wolf populations in Iran merit detailed studies, especially with regards to their genetic lineage.

When considering taxon level consistency within canids we find insights in the genetic distance analysis (Table 2). The coyote shows 6.6 % genetic distance on the cytochrome *b* gene from the Holarctic grey wolf. The Himalayan wolf and African wolf show comparable distances from the Holarctic grey wolf with 3.8 % and 3.1 % respectively. In contrast the genetic distance from the Holarctic grey wolf to the recognized subspecies is much smaller with the Indian wolf at 0.1 % and the Arabian wolf at 0.43 % genetic distance. To note is also the Iranian wolf sample at 0.45 % genetic distance.

Bradley and Baker (2001) found for mammals (particularly rodents and bats) that >5 % distance on the cytochrome *b* is typically observed between morphologically recognized mammal species. Our results imply that for canids, the species level is drawn at similar but lower genetic distance which is reasonable given that the group is especially characterised by gene flow across lineages (Gopalakrishnan et al. 2018).

What is next

A consistent revision of *Canis* taxonomy is recommended due to potential taxonomic heterogeneity within the group and various new insights around canid phylogeny in recent studies. The revision should be based on consistent criteria that are applied across the entire canid family and ideally are comparable to those used in revised taxonomies of other mammal groups.

In order to achieve this a systematic landscape scale sampling of morphometric and genetic characters of wolf-like canids in Asia is recommended as available data on these populations is comparably less than from wolves of other parts of the world. Specifically, we recommend sampling multiple male and female wolf individuals per lineage with verified geographic origin locations and with the sampling spatially distributed across the estimated range. These sampled individuals should be examined for their skull morphology and their genetic and genomic profiles. The morphometric study should be done in systematic manner in accordance with the methods used by Viranta et al. 2017. In addition, a full genome analysis is recommended with individuals from across each of the supported lineages‘ range with multiple sampled individuals from the core of the distribution and the distribution edges to understand the lineages, their distribution and admixture at the boundaries. Such a full genome analysis should also include data from European and North American wolves, and coyotes and golden jackals as references.

Admixture zones between different taxa of wild animals should be acknowledged in taxonomy and conservation for their potential importance in allowing the continuation of evolutionary processes through gene flow and thereby allowing future adaptation to changing conditions. Conservation policy must find ways to incorporate and safeguard such admixed populations, and taxonomy must find ways to incorporate admixed populations in the delineation process.

In addition, in depth studies on the ecology and behaviour of the different wolf lineages of Asia are recommended.

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Chapter 7.

GENERAL DISCUSSION

General Discussion

The existence of the Himalayan wolf as a distinct wolf lineage is intriguing from an evolutionary perspective.

This thesis explores the phylogenetic distinction and taxonomy of the Himalayan wolf and reveals an unexpected adaptation to life in the Asian high-altitudes that is unique among canids. A case is made for taxonomic recognition and conservation as a matter of priority. Additionally, novel data on the Himalayan wolf's dietary habits and prey use is presented and discussed which, combined with insights into the most pressing threats, informs the development of a much-needed conservation strategy for the species.

Synthesis of results

The Himalayan wolf forms a monophyletic lineage basal to the Holarctic grey wolf. The genetic results show a deep divergence of this ancient canid based on a wide array of genetic and genomic markers, and illustrates its eligibly for taxon recognition (Werhahn et al., 2017a, 2018; Werhahn et al., 2019a). The Himalayan wolf's unique genetic adaptation to cope with the harsh hypoxic conditions in the high-altitudes gives it an adaptive fitness benefit over the grey wolf (Werhahn et al., 2019a). This genetic adaptation to life in the extreme high-altitudes is hypothesized to be the main mechanism responsible for the maintenance of the Himalayan wolf lineage. The molecular clock analysis suggests that this wolf diversified as an evolutionarily independent lineage from the ancestors of the wolf dog clade around 0.69 – 0.74 Ma ago, that is, at the beginning of the radiation period of the modern Holarctic grey wolf estimated at 0.70–0.30 Ma ago (Sotnikova and Rook, 2010; Tedford et al., 2009; Vilà et al., 1999; Wang and Tedford, 2008; Werhahn et al., 2018).

Insights into the distribution of the Himalayan wolf are presented and reveal that this lineage is not only found in the Himalayas as initially thought, and from where it derives its name, but that it is also found across the high-altitude habitats of the Tibetan Plateau, a finding that is also ecologically supported by the continuity of habitat and the prey community. Specifically, the Himalayan wolf is found across the Himalayas and the Tibetan Plateau in habitats above 4,000m elevation (Werhahn et al., 2019a). The distribution range extends from the western Himalayas in northern India to the Eastern Himalayas in Nepal and likely to Bhutan, and to the Tibetan Plateau especially in the Tibetan Autonomous Region (TAR) and Qinghai Tibet in China. But its presence in Bhutan has yet to be genetically confirmed. Large areas of the north-western Tibetan Plateau also still require sampling to verify the wolf lineage present there, and to further investigate the admixture zone identified at the edges of the Tibetan Plateau to the north-east and east, i.e. northern Qinghai, Gansu and Sichuan, where grey wolf and Himalayan wolf distributions meet (Werhahn et al., 2019a). There is a discrete geographic division between the two, with the Himalayan wolf lineage prevalent above 4,000m and the grey wolves below, with admixed individuals found between 3,400-4,400m.

The Himalayan wolf presents a deeply diversified lineage based on genetic (D-loop and cytochrome *b* mtDNA) and genome-wide data (ZF on both sex chromosomes, four hypoxia pathway related functional SNPs, and a microsatellite panel of 17 loci). Whereas the admixed individuals all presented with mtDNA and hypoxia adaptation characteristic of the Himalayan wolf but with intermediate microsatellite profiles. The admixture zone between the Himalayan and grey wolf at the edges of their distribution may hold evolutionarily significant populations that allow for continuing adaptive evolutionary processes. Such stable admixture zones are documented in a wide range of species (Barton and Hewitt, 1985). Such populations need to be acknowledged for their potential evolutionary and ecological value and need to be incorporated in conservation planning, legislation and taxonomy. Hybridization and genetic introgression among canid lineages is a natural ongoing process important to the canid family (Gopalakrishnan et al., 2018) as it allows evolutionary processes and adaptation to changing conditions. And such adaptive potential is increasingly recognized for its importance in nature (Stanton et al., 2019).

Hybridization should be examined in detail in future studies with regards to the admixture between Himalayan and grey wolves, which occurs at the distribution boundaries based on the results of this thesis research. However, hybridization between wolves and dogs is also likely to occur in these habitats. And while admixture among wild related taxa can be considered natural, hybridization between wolves and domestic dogs is a conservation concern that merits detailed studies. The microsatellite data in this research indicated limited introgression of dog genes in the Himalayan wolf populations, and similar occurrence at low level is documented elsewhere (Dufresnes et al., 2019; Fan et al., 2016, Pacheco et al., 2017). The amount of hybridization between Himalayan wolves and dogs is expected to vary with densities of feral dogs and the health of the wolf population.

The results provide answers to the research questions asked in the introduction. The first question addressed the cause for the reproductive isolation and the estimated divergence time for the Himalayan wolf. The results of Chapter 3 and 4 indicate that the high-altitude adaptation may be an important cause for the genetic diversification of the Himalayan wolf from the Holarctic grey wolf. But such an adaptive benefit does not lead to complete reproductive isolation and accordingly Chapter 4 indicates the existence of an admixture belt at the distribution boundaries to the north and northeast of the estimated Himalayan wolf distribution range. The divergence time for the Himalayan wolf is estimated between 0.691-0.74Ma before present based on the molecular clock analysis in Chapter 3.

The second question asked whether the phylogenetic distinctness justifies taxonomic recognition of the Himalayan wolf. The results indicate that the Himalayan wolf merits taxonomic recognition at subspecies, and possibly species, level. Subspecies level recognition can be justified based on the genetic diversification of the Himalayan wolf population which is found in a geographic discrete high-altitude region and showing a specific adaptation to this habitat. The genetic distance comparison of different *Canis* lineages in Chapter 6 (Table 2) implies that the diversification found in the Himalayan wolf mitochondrial genome is comparable to the diversification of currently recognized canid species rather than to that of subspecies. However, whether the lineage deserves species level recognition shall be informed by future full genome work including data of multiple wolf individuals from the core and edges of the estimated Himalayan wolf distribution (Chapter 4).

The third question asked what mechanisms may be maintaining the genetic distinctness of the Himalayan wolf. The research findings of this thesis indicate that the genetic adaptation to hypoxia may be maintaining the Himalayan wolf lineage because this adaptation is hypothesized to give the wolves a fitness benefit in the high-altitude habitats. This hypothesis is supported by the consistent pattern of these wolves occurring above 4,000m elevation while at lower elevations the grey wolf lineage dominates. While this thesis research did not investigate behavioural or reproductive aspects that may keep the lineages apart, such mechanisms seem less likely to be the main mechanism given the flexibility and reproductive compatibility of *Canis* species (i.e. Mech, 2014). Based on the described distribution in Chapter 4, a large-scale effective geographic barrier in form of rivers or mountains could not be identified. The altitudinal cline in conjunction with the drop in available oxygen may be responsible for the Himalayan wolf distribution, but the Taklamakan desert to the northwest may function as geographic barrier in that region.

The fourth question concerned the distribution range of the Himalayan wolf. The large scale data analysed in Chapter 4 indicates that this wolf lineages is found in the high-altitudes of the Himalayas and the Tibetan Plateau with a prevalence in habitats above 4,000m elevation. The lineage was not found in the surrounding lower elevation habitats and also not in the mountain ranges of Central Asia, although some introgression of the hypoxia pathway related gene alleles was found in some of the grey wolf samples tested from there.

The fifth question addressed the main prey species and dietary habits of the Himalayan wolf (Chapter 5). The results showed an over-proportional use of wild prey over domestic animals compared to their respective abundance in the landscape, and a strong reliance on marmot, Tibetan gazelle, and blue sheep as prey species. The Tibetan gazelle was consistently used over-proportionally across the study areas. Livestock was often consumed by wolves, it was seasonally very abundant in all study areas, and displaced wild prey from wolf habitats. The Himalayan wolf showed a dietary ecology specialized to the high-altitude prey community of Asia. The results on the dietary habits (Werhahn et al., 2019b) in combination with the social survey study investigating human-carnivore conflict (Kusi et al., 2019, Appendix A) can inform conservation action from regional to local level in Nepal and other range countries.

The sixth question asked how the insights from this Himalayan wolf research relate to contemporary global wolf taxonomy and what lessons may be learnt from it. The results from the analysis of the mitochondrial genome of the Himalayan wolf in context of the

Holarctic grey wolf indicated that the Himalayan wolf has diversified as an independent lineage before the radiation of modern Holarctic grey wolves and has a more deeply diversified mitochondrial genome than currently recognized grey wolf subspecies. The findings underline the importance of taxonomic consistency within the canid family. Taxonomic consistency in mammals merits attention from the scientific community, as currently neither a consistent system nor criteria are in place to decide on mammalian taxonomies (Garnett and Christidis, 2017).

Further, the Himalayan wolf lineage is an example of the challenges that a discrete taxonomy faces in an often gradual nature. *Canis* lineages are characterized by a complex evolutionary history (Gopalakrishnan et al., 2018; Pilot et al., 2019) and this makes the interpretation of genetic and genomic data challenging. The choice of molecular markers is important, and studies are advised to use methods that allow comparability of the results across different studies and taxa (Groot et al., 2016). The genetic methods used in this thesis were developed with this in mind. For example the inclusion of the ZF protein gene marker in Chapters 2 to 4 was to allow comparability of the results to those around the African wolf in Koepfli et al. (2015). Similarly, full mitochondrial genomes as well as individual markers such as the cytochrome *b* and D-loop genes were analyzed to ensure wide comparability.

The findings around the Himalayan wolf and the pending taxonomic decision underline the call for empowering conservation to protect biodiversity in a timely manner that is disentangled from taxonomic decisions (Haig et al., 2006; Mace, 2004; vonHoldt et al., 2016). And finally, the findings emphasize the need to find ways to incorporate admixed populations among wild related species in taxonomy and conservation and acknowledge their evolutionary and other potential values.

Wolf taxonomic discourse around the globe

Taxonomic discourse is taking place around different wolf lineages in North Africa and North America. Importantly, what taxonomy is trying to classify is a snapshot in time of an evolutionary process of either speciation or dissolution (Medicine et al., 2019). There is no right answer to taxonomic decisions (Zachos, 2018), but there is the scientific and ethical mandate for taxonomy to make the best decisions based on the available data and make those decisions based on consistent criteria applied across the family and taxa involved.

The African wolf of North Africa has recently been posited as an own wolf taxon (Koepfli et al., 2015; Rueness et al., 2015, 2011; Viranta et al., 2017); recommended as *C. lupaster* by Alvares et al. (2019)) with genetic, genomic and morphological evidences supporting the taxon. There is a morphological and ecological resemblance of the African wolf with the Eurasian golden jackal (*Canis aureus*) which has created contention among some experts but may be the result of convergent evolution (Alvares et al., 2019).

In North America debates around taxonomic status are taking place especially around the Red wolf *C. rufus* (Medicine et al., 2019; Phillips, 2018), the Mexican wolf *C. l. baileyi* (Boitani et al., 2018; Medicine et al., 2019), and Eastern wolves *C. l. lycaon* (Boitani et al., 2018; Chambers et al., 2012), but also around a potential additional subspecies of grey wolf in the Northwest pacific (e.g. *C. l. ligoni*; Cronin et al., 2015a, 2015b; Weckworth et al., 2010, 2005). The Plains wolf *C. l. nubilus*, Northern timber wolf *C. l. occidentalis* and Arctic wolf *C.l. arctos* are more widely accepted subspecies that occur in North America (Boitani et al., 2018).

Different lines of thoughts dominate the North American wolf debate. Either the Eastern and Red wolf are of hybrid origin from grey wolf and coyote (Roy et al., 1996; vonHoldt et al., 2016, 2011), or the Eastern wolf is different but not a different species (Wayne and Vilà 2003b; Koblmüller et al. 2009; Nowak 2009), or the Eastern wolf and the Red wolf form endemic North American wolf lineages that have been present before the arrival of the Old World grey wolves (Chambers et al., 2012; Kyle et al., 2006; Rutledge et al., 2015, 2012, 2010b, 2010a; Wilson et al., 2012, 2000). Wolves in eastern North America show differentiated morphology in that they display more gracile characteristics, such as differences in body mass and skull measurements and more slender snouts (Nowak, 2009; Thiel and Wydeven, 2011).

But different studies around these wolves reach different conclusions (e.g. Chambers et al., 2012; Hohenlohe et al., 2017; Medicine et al., 2019; Rutledge et al., 2015; vonHoldt et al., 2016, 2011; Wilson et al., 2000), depending on the genetic or genomic marker used, the statistical method used, the interpretation of the results, and the inclination of the researchers with regards to splitting or lumping. The situation around wolf phylogeny and taxonomy in North America is complex, also due to an evolutionary history of repeated radiation and shrinking of canid populations with the glaciation events, gene flow among lineages in historical and contemporary times, hybridization, and human caused population exterminations and habitat modifications (Medicine et al., 2019).

The Mexican wolf is currently considered the most distinct of the recognized subspecies of grey wolf in North America based on genetic and genomic analyses (Gopalakrishnan et al., 2018; Medicine et al., 2019; vonHoldt et al., 2016, 2011). It is also morphologically distinct based on skull measurements and exhibits a smaller body size (Nelson and Goldman, 1929; Nowak, 1995). It inhabits more arid ecosystems compared to the other grey wolf subspecies in North America, but its behaviour is similar to them (Medicine et al., 2019).

The Red wolf is a smaller-sized wolf of east-central North America. The debate around Red wolves originates in the inconclusiveness of the data with regards to the degree to which the lineage is different and recognizable as an own species or if it is of hybrid origin of coyote and grey wolf (Chambers et al., 2012; Sinding et al., 2018; vonHoldt et al., 2016, 2011; Wilson et al., 2000). While the currently managed reintroduced Red wolf is genetically more similar to coyotes, its genome contains large amounts of recent admixture and also some degree of genetic ancestry that neither traces back to contemporary coyotes nor grey wolves (Medicine et al., 2019; Rutledge et al., 2015; Sinding et al., 2018). Red wolves are behaviorally more similar to grey wolves. When mates are available they exhibit assortative mating, but there seems no reproductive isolation with neither grey wolves nor coyotes (Medicine et al., 2019). There are recognized morphological differences in this wolf from the grey wolf and coyote (Nowak, 2002). The Red wolf is currently considered an own wolf species by the ICUN (Phillips, 2018), but this is a point of some contention (Hohenlohe et al., 2017; vonHoldt et al., 2016). Evaluating the appropriate status of the Red wolf and Mexican grey wolf are further complicated because both populations went extinct in the wild, with limited samples available from before the extinctions and the currently managed populations tracing back to reintroductions based on a few founding individuals (Medicine et al., 2019).

The Eastern wolf (*C. l. lycaon*) is another differentiated wolf lineage of North America which is currently recognized as subspecies of grey wolf by Boitani et al. (2018). Some authors suggest that it is eligible for species recognition but acknowledge the close phylogenetic relatedness with Red wolves and suggest that both derive from a common ancestor with coyotes (Chambers et al., 2012; Grewal et al., 2004).

North American wolf phylogeny and taxonomy illustrates the fascinating complexity of evolutionary history and ongoing introgression among canid lineages which is further complicated through anthropological changes to populations and habitats in more recent times (Hailer and Leonard, 2008). The current taxonomic classifications of Red wolf, Mexican wolf and Eastern wolf are based on multiple lines of evidence including molecular, ecology and behaviour, and morphological data. Nevertheless, for these recognized taxa none of the individual lines of evidence were conclusive or entirely agreed upon (Medicine et al.,2019). A comprehensive comparative study of wolf lineages around the globe would be useful to illustrate how each of the recognized and considered lineages is diversified based on the traits relevant for taxonomy (i.e. molecular, ecological and behavioural, morphological, and biogeographical). For example, the currently available divergence time estimates for the Red wolf dated between 55,000-185,000 years before present (Medicine et al., 2019; vonHoldt et al., 2016) would indicate that this lineage has diverged considerably more recently compared to the Himalayan wolf with current estimates ranging between 549,800 and 800,000 years before present (Matsumura et al., 2014; Sharma et al., 2004; Werhahn et al., 2018).

While such a comparative global wolf study may lie in the future, current taxonomic decisions shall acknowledge a range of traits in combination with taking reference to other recognized wolf taxa.

Limitations and weaknesses of this research

The molecular data provided in this thesis research is limited in that it does not contain full genome data on the Himalayan wolves. Full genome data is increasingly being considered important for taxonomic classification. The dataset is also limited in that not the entire estimated distribution range of the Himalayan wolf is being sampled. Samples especially from the western and northwestern Tibetan Plateau and samples from northern Pakistan will be insightful to include in future studies. The genetic methods applied were of limited power to provide an in-depth understanding of wolf-dog hybridization.

Molecular clock analysis provides useful estimates of divergence times. Nevertheless, the method has its limitations due to assumptions inherent to the method. That is the method is based on the molecular clock hypothesis which assumes that mutation rates in any specific genetic regions are constant over evolutionary time. This provides the estimation of relative time distances between taxa which then needs calibration against independent evidence about dates in related taxa to then estimate divergence time of the target taxa (Benton and Donoghue, 2007; Kumar, 2005). The estimate will further depend on the genetic region used, which was the full mitochondrial genome in this thesis research (Chapter 3).

The methods used to understand dietary habits (Chapter 5) do not allow discrimination between predated and scavenged food items. I would have very much liked to overcome this limitation, but it is difficult in the field to observe many carnivore feeding and predation events. The dietary samples were limited in number and possibly sampled individuals of the same pack in some occasions. Consequently, not all samples may have been independent. If one assumes that all pack members feed on the same prey item at a given time, then one dietary scat sample per pack and feeding event should be used for analysis. The dietary study is further limited in reflecting only the summer diet. All these limitations imply that in-depth foraging studies are needed in the future. For the prey availability estimates in the landscape it would have been advantageous to conduct the double observer method (Suryawanshi et al., 2012) in addition to the distance sampling to then compare the resulting prey estimates of both methods.

While the goal from the beginning was to clarify the name confusion around the Himalayan wolf, the scientific articles resulting from this research may have added to it, unfortunately. This may be so because the scientific name proposed changed from Chapter 2 (*C. l. himalayensis*) to Chapter 4 (*C. l. chanco*). However the scientific name recommended was always carefully chosen according to best knowledge available at the given time.

Biogeographic history and speciation

The ultimate causes for the diversification of the Himalayan wolf from the ancestors of the Holarctic grey wolf complex are explored in the context of evolutionary history, biogeography and speciation. Speciation occurs along different trajectories, with allopatric, peripatric, parapatric and sympatric being among the main recognized modes for the evolution of species, but evolutionary biology is constantly unravelling exciting new insights about the ways that species evolve (Coyne and Orr, 2004). Different forms of selection pressures result in populations following different evolutionary pathways, such as ecological, reproductive or reinforcement selection that go back to the description of the role of natural selection for speciation by Charles Darwin (1859). But also genetic drift, i.e. the random frequency change of gene variants under random mating, and population bottlenecks, i.e. an environmental event drastically reducing population size, may be involved in speciation processes (Masel, 2011; Robinson, 2003).

Speciation in mammals produces a population of individuals that mainly reproduce with individuals from the same population (assortative mating) and that is usually characterised by some adaptation or specialisation to the niche they inhabit (Coyne and Orr, 2004). Speciation occurs over evolutionary time and in mammals often involves adaptive divergence. Different populations can be at different stages in the species formation process and hybridization and gene flow may be ongoing (Medicine et al., 2019; Orr and Smith, 1998).

In parapatric and sympatric speciation, lineage diversification takes place despite individuals of the two lineages coming into contact and the exchange of genes in the contact zones (Coyne and Orr, 2004; Dieckmann and Doebeli, 1999). In these modes of speciation, the reduced fitness of the heterozygotic individuals will over time select for behaviours or mechanisms that prevent reproduction. Such mechanisms are divided into pre- and post-zygotic reproductive isolation, i.e. those that occur before fertilization and those that occur after. Prezygotic isolation involves geographic, temporal, physiological or gametic isolation mechanism. Post-zygotic isolation involves non-viability of the zygote and hybrid infertility (Futuyma, 1998), but also reduced hybrid viability or fertility may drive lineage diversification.

Hybrids can have reduced viability or fertility and therefore gene flow is restricted between the two hybridizing lineages which can lead to the development of pre-mating isolation as mating with members of the own lineage is reproductively more successful (Medicine et al., 2019).

The data indicating admixture at the distribution boundaries of the Himalayan wolf and the Holarctic grey wolf implies hybridization between individuals of the two lineages there. No indication for hybrid infertility or reduced viability is described to date between the two lineages but has also not been studied. The present data indicates that admixture is restricted to the boundary regions which implies that the admixed individuals may have a fitness disadvantage keeping the two lineages distinct, as otherwise panmixia and no diversified lineage would be expected across the Himalayan wolf distribution range.

Reproductive isolation, in the strict sense, is thus not fulfilled in the case of the Himalayan wolf but may not be a conclusive criterion for species delineation in canids, as different species in the family produce viable offspring, such as wolves and coyotes in North America (Bohling et al., 2016; Mech et al., 2014). But nevertheless assortative mating, as found in red wolves (Bohling and Waits, 2015; Hinton et al., 2018), may drive lineage diversification and speciation. Assortative mating in Himalayan wolves may be investigated in experiments with captive individuals or with carefully designed field studies in the admixture region.

The molecular clock analysis based on the full mitochondrial genome suggests that the Himalayan wolf has split as an independent lineage approximately 0.69 – 0.74 Ma ago from the ancestors of the Holarctic grey wolf complex, a finding that is comparable with Himalayan wolf divergence time estimates in previous studies (Matsumura et al., 2014; Sharma et al., 2004). But why did the Himalayan wolf diversify? The evolution of biodiversity and the process of speciation are strongly linked to geological and climatic processes such as continental drift and the uplift of mountain chains, so called orogenic activity.

The time period when the Himalayan wolf diversified was also a time of major geologic change in the region with the uplifting of the Himalayas and the Tibetan Plateau caused by the collision of the Indian and Asian continental plates (Li et al., 2014; Li and Fang, 1999). The uplift started ~8 ± 1 Ma ago, followed by stepwise accelerated rising episodes at ~3.6 Ma, 2.6 Ma, 1.8–1.7 Ma, 1.2–0.6 Ma and 0.15 Ma ago. One of the last intense, rapid and widespread uplift episodes of the Qinghai–Tibetan Plateau, the Kunlun-Huanghe Tectonic Movement, is dated between 1.1 and 0.6 Ma, at the same time the Himalayan wolf diversified as a distinct wolf lineage. The uplifting of the Tibetan Plateau resulted in dramatic ecological habitat changes, such as a persistent stepwise accelerated enhancement of the East Asian winter monsoon, and drying and cooling of the Asian interior (Li et al., 2014). These ecological changes were accompanied by decreasing oxygen levels as the habitat was increasingly uplifted.

These processes created new ecological niches and thus facilitated speciation by divergent selection and adaptation (Liu et al., 2013). Biodiversity is unevenly distributed on earth, and hotspots of biodiversity are often in areas that have undergone orogenic activity in recent geological time. Higher biodiversity than expected is associated with mountains in general and has been reported for the Himalayas (Favre et al., 2015). As a result, the Himalayas and fringe mountain ranges of the Tibetan Plateau (i.e. Tian Shan, Pamirs, Hengduanshan) are listed among the main biodiversity hotspots for the Northern Hemisphere (Brooks et al., 2006; Favre et al., 2015; Myers et al., 2012, 2000).

Speciation along an environmental cline and high-altitude adaptation

A dramatic habitat cline together with intraspecific competition facilitates speciation (Doebeli and Dieckmann, 2003). The hypothesis is that the environmental cline presented by the transition to extreme high-altitudes, in combination with geographic distance and intraspecific competition, well documented in wolves (Mech and Boitani, 2003), caused the evolutionary differentiation of the Himalayan wolf from the ancestors of the Holarctic grey wolf complex.

If the hypothesis is correct that the high-altitude environment was an important ultimate cause for the differentiation of the Himalayan wolf, then it must also present an important proximate mechanism for its maintenance. The maintenance of the genetic integrity of the Himalayan wolf is surprising and difficult to explain apart from an adaptive fitness benefit, given that the Holarctic grey wolf dominates the large canid niche in most parts of the Northern Hemisphere. It is also surprising considering the prominent wolf characteristics: long dispersal distances (Ciucci et al., 2009; Mech et al., 1995) and ready hybridization with wolf-like taxa when conspecifics are lacking (Donfrancesco et al., 2019; Hennelly et al., 2015; Pacheco et al., 2017; Randi, 2008). Both these characteristics lead to genetic homogenization through gene flow.

But some mechanisms appear to prevent extensive gene flow between the Himalayan and grey wolf. What are the other possible explanations for why the Himalayan wolf exists, besides an adaptive fitness benefit acquired through hypoxia adaptation? Other mechanisms preserving the Himalayan wolf may be of behavioural, ecological, or geographic nature. The first two, behavioural or ecological mechanisms, are unlikely to be so drastic as to result in lineage separation given that wolves are flexible in their behaviour and ecology and adjust to different conditions with flexibility within individuals and populations (Macdonald et al., 2019, Appendix A). More effective may be a geographic mechanism, e.g. a habitat barrier or ecological cline. Any such habitat barrier would need to be drastic to prevent gene flow in wolves, as rivers and mountains would not stop a dispersing wolf (personal observation, Ciucci et al., 2009). But a wide desert may provide a potent mechanism for preventing dispersal, as well as an environmental cline that is best mastered by genetic adaptation. The Himalayan wolf distribution range is delimited by the Taklamakan desert (>400 km wide) to the northwest, and steep habitat clines to the southwest and south, while to the north and northeast the elevational decline of the Tibetan Plateau is gradual. The hypoxia adaptation likely gives the Himalayan wolf a long-term adaptive advantage and fitness benefit in the high-altitudes over the grey wolf found in the surrounding lower lying habitats of Mongolia, eastern China, central Asia and India. And this fitness benefit may preserve the Himalayan wolf lineage in the high-altitudes, despite gene flow in the stable admixture belt at the boundaries. However, it is possible that the hypoxia adaptation involves a cost at lower altitudes, but this remains unstudied. In other words, the dispersing grey wolves that have over the past thousands of years ventured into the high-altitudes dominated by Himalayan wolves, are disadvantaged in these habitats and as a result the incoming grey wolf genes did not gain much ground in the Himalayan wolf populations.

High-altitude adaptation, opposed to short term physiological acclimatisation, is an evolved physiological response to the low available oxygen at high-altitudes and is best studied to date in our own species (Beall, 2007; Beall et al., 2002; Huerta-Sanchez, 2014; Wu and Kayser, 2006; Xu et al., 2011). There are also indications for high-altitude adaptation in other species such as yaks (Qiu et al., 2012; Wang et al., 2006), goats of the genus *Capra* and the Himalayan thar *Hemitragus jemlahicus* (Hassanin et al., 2009), deer mice of the genus *Peromyscus* (Cheviron et al., 2012; Storz et al., 2010a), and birds (Faraci, 1991). But it is studied in only a minority of the many species living at high-altitudes (Storz et al., 2010b). And a lot remains to be understood about the different physiological routes the adaption can take and the underlying genetic components, as well as the fitness consequences of the hypoxia adaptation at lower altitudes (with translocation experiments being possible study approaches to the later).

Taxonomic implications

The Himalayan wolf forms a monophyletic wolf clade basal to the Holarctic wolf complex that is unique to the Asian high-altitudes. In addition to genetic and genomic evidence for this unique wolf lineage presented in this thesis (Werhahn et al., 2017a, 2018; Werhahn et al., 2019a) and elsewhere (Aggarwal et al., 2007; Koepfli et al., 2015; Matsumura et al., 2014; Rueness et al., 2011; Sharma et al., 2004), the Himalayan wolf also exhibits a howl variation that differentiates it from all other wolf lineages (Hennelly et al., 2017).

Given its phylogenetic and ecological distinction, the Himalayan wolf seems eligible for taxonomic recognition under multiple species concepts (SC), e.g. the Biological SC (Mayr, 1942), the Genetic and Phylogenetic SC (Cracraft, 1983), the Unified SC (de Queiroz, 2005), and the Differential Fitness SC (for details see Werhahn et al., 2019a/ Chapter 4 and Chapter 6). The Himalayan wolf population may fulfil the criteria of the Biological SC only in the wider sense as the lineage is diversified despite introgression at the distribution edges. The genetic diversification indicates genetic isolation between the lineages which is the main criteria of the Genetic SC. The Phylogenetic SC considers a lineage as eligible for taxonomic recognition if the population is characterized by monophyletic shared ancestry (Cracraft, 1983) which is implied by the data for the Himalayan wolf population. The population fulfils the criteria of the Unified SC stating that a species is a “separately evolving metapopulation lineage” (de Queiroz, 2005) through the genetic data indicating the diversification of this population from the grey wolf, the genetic adaptation of the population to high-altitudes and the resulting characteristic distribution in the high-altitudes but not in the surrounding lower lying regions. Finally, the Differential Fitness SC may be fulfilled by the hypoxia adaptation that is hypothesized to give the Himalayan wolves a fitness benefit in the high-altitudes over the grey wolves.

A best practice taxonomy may be informed by molecular, morphological, ecological and behavioural, and biogeographic evidences. This thesis provides data towards the molecular and biogeographic evidence base around the Himalayan wolf. It reveals these wolves' specific adaptation and characteristic occurrence in the high-altitude ecosystems of Asia. The research provides data on the full mtDNA and different genomic markers applied to a landscape scale dataset. The biogeographic evidence in this thesis illustrates that these wolves seem restricted to the Asian high-altitudes to which they have a genetic adaptation whereas the surrounding lower lying habitats are inhabited by a sister taxon, the grey wolf. The diversification of the Himalayan wolf lineage coincides with the time when the high-altitudes of the Himalayas and Tibetan Plateau came into existence and all lines of evidence support the notion that the population has followed a separate evolutionary pathway. In addition the study by Hennelly et al. (2017) finds differentiated vocalisation of the Himalayan wolf from other wolves which implies ecological and behavioural differences and possibly an underlying physiological difference in the vocal apparatus (which needs investigation). Systematic morphological studies especially with regards to skull measurements are required for the Himalayan wolf.

The Himalayan wolf is diversified across all tested mitochondrial and nuclear markers and is eligible for taxonomic recognition at minimum as subspecies. The eligibility for species is to be informed by additional full genome research of multiple contemporary wolf individuals from across the distribution range and admixture zones identified in Chapter 4 to supplement the findings by Fan et al. (2016). Taxonomic consistency is necessary within groups, i.e. lineages with similar levels of divergence require recognition at the same taxonomic level. This principle is highly relevant in the light of the recent recommendation of the African wolf at species level as *Canis lupaster* (Gaubert et al., 2012; Koepfli et al., 2015; Alvares et al., 2019; Rueness et al., 2011), and its comparable differentiation from the Holarctic grey wolf when compared to the Himalayan wolf across the genetic and genomic markers (Werhahn et al., 2019a; Werhahn et al., 2018, 2017a). Furthermore, the Himalayan wolf is more steeply diversified than other currently acknowledged grey wolf subspecies (Chapter 6). The current recommendation by Alvares et al. (2019) for subspecies recognition of the Himalayan wolf as *Canis lupus chanco* can be considered the *status quo* until reliable full genome analyses can verify existing conclusions. This is reasonable given the different evolutionary pathways of mitochondrial and nuclear DNA (Gopalakrishnan et al., 2018; Groot et al., 2016). However it may also be considered an unwarranted delay given the data from multiple nuclear and mitochondrial DNA markers both presumed neutral and functional (Aggarwal et al., 2007; Sharma et al., 2004; Werhahn et al., 2017a, 2018; Werhahn et al., 2019a/Chapter 2 and 3), differentiated vocalisation (Hennelly et al., 2017), and specific high-altitude prey use (Werhahn et al., 2019b/Chapter 4), all of which support the Himalayan wolf's distinction. Morphological differences from the grey wolf are notable as described by the early explorers (Gray, 1863; Hodgson, 1847; and also see Janssens et al., 2016), but a systematic morphometric study is needed.

The taxonomic recognition of the Himalayan wolf as well as its conservation are a matter of urgency, given these diverse but consistent lines of evidence. Extensive conservation threats are documented for these wolf populations (Kusi et al., 2019; Mishra, 1997; Werhahn et al., 2017b), unsurprising given the lack of both taxonomic recognition and conservation awareness. The population status and trends for the Himalayan wolf are not understood, but there were indications for declines in Nepal where wolf persecution is high due to livestock depredation and illegal wildlife trade while attitudes remain negative (Kusi et al., 2019; Werhahn et al., 2017b). The appropriate taxonomic classification is pivotal for the conservation of these wolves and will pave the way for future research including morphological, behavioural, and ecological studies.

Conservation implications from global to regional level

Canids are flexible and adaptive and as a family have fared relatively well in the challenging conditions of the Anthropocene (Macdonald et al., 2019). Nevertheless, canids face many threats, from disease transmission increasing with feral dog populations (Gompper, 2013), habitat reduction and land-use change, and persecution due to livestock retaliation (Boitani et al., 2018), and negative perceptions (e.g. Kusi et al., 2019). In some cases, such as the Himalayan wolf and wolf lineages of North America, the taxonomic uncertainty and resulting inertia, including the absence of legalisation, can prevent effective conservation (Gippoliti et al., 2018; Macdonald, 2019; Zhou et al., 2016). And there is the challenge to incorporate admixed populations between adjacent wild species in conservation planning (Allendorf et al., 2001; vonHoldt et al., 2016).

But there are also increasing opportunities to protect canids. For example the growing evidence that top predators like wolves help maintain ecosystem balance, health and biodiversity can act as incentive for their protection (Fortin et al., 2005; Licht et al., 2010; Ripple and Beschta, 2012). Such research around trophic cascade effects by predators is of rising interest (Pace et al., 1999; Ripple et al., 2014) and ever more of relevance in a world where the climate and biodiversity crisis are finally making it onto the political and economic agendas and the increasing need to find solutions. At the same time biodiversity conservation is increasingly being recognized as an important part of these solutions (Chami et al., 2019; Seddon et al., 2019). Wolves, through trophic cascade effects, support intact habitats (Licht et al., 2010; Ripple and Beschta, 2012), and intact forests and grasslands are recognized for their carbon storage capacity which supports climate change mitigation (Luyssaert et al., 2008; Martin et al., 2001; Smith, 2014). Further, intact carnivore populations may help buffer adverse effects of climate change on ecosystems. An example are the wolves of Yellowstone ensuring carrion availability to scavengers irrespective of changing snow fall patterns and the related changes in food supply from winter mortalities (Wilmers and Getz, 2005; Wilmers and Post, 2006).

On a regional level, a major challenge to the protection of the wildlife of High Asia is posed by the Belt and Road initiative which not only will disturb and fragment habitats but will also facilitate poaching by increasing motorized accessibility (Farhadinia et al., 2019). Wolves in High Asia currently receive little research attention and funding, little conservation action is in place, and consequently they are often perceived negatively by local communities (Kusi et al., 2019; Suryawanshi et al., 2014). In Central Asia wolf studies are few and usually in connection to snow leopard research (Karimov et al., 2018; Wang et al., 2014). In China ecological aspects of wolves currently receive little attention (in research written in the English language) while phylogenetic studies are being conducted, often using existing samples from collections (Fan et al., 2016; Wang et al., 2019, 2016; Zhang et al., 2014). Phylogenetic and ecological studies with field collected samples of contemporary free ranging wolves are recommended. In India and Nepal the wolves of the Himalayas are receiving a bit more research attention, especially with regards to livestock depredation and conservation consequences (Chetri et al., 2017, 2016; Subba et al., 2016; Suryawanshi et al., 2014; Werhahn et al., 2019a). The first genetic studies on the Himalayan wolf originate in India (Aggarwal et al., 2007; Sharma et al., 2004) and the topic has been further pursued in this thesis with a focus on Nepal (Werhahn et al., 2018; Werhahn et al., 2017a, 2017b) that later also took a regional perspective (Werhahn et al., 2019b). Another top predator in the region, the snow leopard, is an inspiring and positive example of conservation. The conservation awareness and research interest are larger for this charismatic felid and may be attributed to the extensive work over the past decades by multiple non-governmental organizations dedicated to snow leopard conservation, and the resulting funding and legislation improvements. However, the single species conservation approach has also created a situation on the ground, where one predator is highly valued by the local communities because the global conservation community is showing interest, while the other predators remain heavily persecuted. It is therefore advisable to expand the scope of such conservation work (e.g. financial schemes, predator proofing of infrastructure, wildlife population monitoring) to include the entire carnivore community and educate local communities about the value of biodiversity and the interconnectedness of the species in their ecosystem. Fortunately, research and conservation are increasingly also focusing on multi-species approaches (Clark and Harvey, 2002; Lindenmayer et al., 2007). But also this thesis is guilty of a rather single-species research approach.

So any conservation action in the Asian high-altitudes, as elsewhere, should take a holistic approach, including and considering the entire wildlife community, under ‘respectful engagement’ (sensu Macdonald and Tattersall (2001)) and the inclusion of local communities. An important aim of this work is to inform and facilitate an ethically just and long-term functional coexistence of humans and wildlife in the Asian high-altitudes. Traditional communities in the region represent uniquely preserved cultural treasures that, like the wildlife community, have long been protected by the region’s remoteness and harsh climatic conditions, but now increasingly face the pressures of modern civilization. The Himalayan habitats of Nepal present the opportunity to ‚do it right from an early stage‘, meaning this is the opportune time to implement functioning and sustainable measures to protect wildlife and the ecosystem at large, while equally preserving the traditional lifestyle of local communities. This presents many opportunities: for the Nepalese government to act as a role model for nature conservation in their unique Himalayan ecosystem, for local people to ensure the continuation of their traditional lifestyle while not being isolated from the modern world, and for local people and the Nepalese government alike to reap the financial benefits of sustainable nature tourism, associated trades (e.g. manufacture of handicrafts), and collection of medicinal herbs. The natural value of the region presents a major economic asset, that if handled with the appropriate respect and care, can benefit all involved stakeholders.

Human-wildlife conflict in these high-altitudes is compounded by the temporal and spatial overlap of livestock herding in the summer pasturelands and wolf denning in the late spring and early summer. The seasonally high abundance of livestock in the Himalayan wolf’s habitats results in the displacement of wild prey species which further increases depredation conflict. As a consequence, Himalayan wolves get killed in retaliation for livestock depredation as well as for illegal wildlife trade (Werhahn et al., 2017b).

The upper Humla region holds an exceptional wildlife community (Acharya et al., 2015; Kusi et al., 2018a; Kusi and Werhahn, 2018; Kusi and Werhahn, 2016; Werhahn et al., 2016, 2015), and it is thus considered for protection by the Nepalese government. A community conservation approach would offer the most promising approach in the Himalayan context (Chaudhary et al., 2015; Kusi et al., 2019), and has been successful elsewhere (Fernández-Giménez et al., 2015; Jackson and Wangchuk, 2004).

Shey Phoksundo National Park (SPNP) in Dolpa district of Nepal, comprising a beautiful Himalayan and Transhimalayan landscape, is a government-managed protected area established in 1984 in response to wildlife biologist George Schaller and his team describing the exceptional beauty of the area during their 1976 expedition. Schaller returned to Dolpa 40 years later, a few months after the expedition to the area for this thesis in 2016. Both teams found that the park was not meeting the international criteria for a national park, is lacking conservation efforts on all levels while anthropogenic pressure were high and neither managed nor sustainable (Kusi et al., 2019; Palden, 2017). Park management must take action to improve conservation efforts with close inclusion of the local communities and provide them with financial support; currently the designated conservation funds tend to get lost in a bureaucratic maze. It was further noted that in SPNP, conservation efforts were narrowly focused on snow leopard and blue sheep, whereas poaching of wolves and most other species were common practice and had led to a depleted natural world (Palden, 2017).

In comparison to SPNP, Kanchenjunga Conservation Area (KCA), a community managed conservation area (Chaudhary et al., 2015), presented a much more successful scenario of a protected area with effective conservation action based on community management. Wolves recently recolonized KCA, and local people expressed the need for financial support for livestock losses to wolf predation, along the lines of that in place for snow leopard depredation (Kusi et al., 2019). These findings further underline the need for holistic conservation and specifically the inclusion of all conflict-causing carnivores in financial schemes across the Himalayan range. Close involvement of local communities has proven successful in KCA (Kusi et al., 2019) and elsewhere in the Himalayas (Bista, 2018; Mishra et al., 2017) and must be a critical component of any future conservation programmes.

In Nepal there is a legal basis for depredation compensation for snow leopards and wolves (GoN, 2015, 1973), but all conflict-causing carnivores, i.e. lynx (*Lynx lynx*) and brown bear (*Ursus arctos isabellinus)*, must be included (Kusi et al., 2019, 2018b). Snow leopard conservation and insurance schemes are implemented locally, but local communities were generally not aware of their eligibility for depredation compensation by the Nepalese government (GoN, 2015; Kusi et al., 2019; Werhahn et al., 2017b). Conservation action in Nepal has multiple loose ends to continue improving conservation effectiveness. Existing insurance schemes must be expanded spatially, they must include the entire large carnivore guild, and the compensation needs to be equal to the value of a young animal of the respective livestock type (Kusi et al., 2019). Then, the process to obtain financial compensation after a loss needs to be practical and time-effective given the rugged and broken landscapes and far distant locations of officials that need to visit the scene to authorise compensation. This process, and thereby the local people‘s motivation to engage with conservation actions, may be greatly improved if depredation verification can be done based on photographic evidence provided by the herders (with standardized indications such as carcass appearance, bite wound extent and locations, and surrounding scats). With this documentation, the herder should be given access to depredation compensation. Facilitating and streamlining this process for the Himalayan context will increase the local communities' willingness to participate in conservation schemes, and also gives herders the opportunity to make use of the lost animals‘ carcass after photographic documentation while it remains fresh, rather than risk the animals' meat going to waste (e.g. decay or consumed by scavengers) in the time spent waiting for an official to arrive for verification. Herders can be further supported by providing training for improving livestock protection, such as building stronger night corrals, training herding dogs, encouraging rotational pastureland use and sustainable livestock numbers, and training on alternative sources of income, such as ecotourism, sustainable cultivation of medicinal herbs, and woolen handicrafts (Kusi et al., 2019; Mishra et al., 2017, 2003)

Future research and conservation requirements

Based on these findings, future research priorities should consider the following topics: A) large-scale full genome study that uses samples from multiple contemporary wolf individuals from across the distribution range including samples from the core distribution and from the admixture zone at the boundaries; B) large-scale studies to better understand the distribution range and admixture between Himalayan and grey wolves at the boundaries; C) Himalayan wolf population status and trends across its range including an overall estimation of population size; D) a systematic morphometric study on skulls to allow comparison with Holarctic grey wolf subspecies and to inform taxonomy; E) detailed studies into hybridization between Himalayan wolf, grey wolf and domestic dogs and conservation consequences; F) studies into spatial and behavioural ecology to understand habitat and resource requirements and inform conservation and management strategies; and G) detailed studies into human-wildlife conflict and illegal wildlife trade, and respective mitigation approaches.

Formal next steps required are the taxonomic classification, assigning the Himalayan wolf an IUCN Red List status, and updating its CITES listing, where currently the grey wolf is listed in Appendix II with the exception of the populations in Bhutan, India, Nepal and Pakistan listed in Appendix I (CITES, 2017). The designation as an ESU is recommended and justified based on the criteria of these wolves forming a population with specific adaptation and of distinct evolutionary history (Moritz, 1994).

Conclusion

This thesis informs the evolutionary pathways and recognition of the Himalayan wolf as a distinct taxon and advances the awareness for the conservation of this wolf and its habitats. The research illustrates through multiple lines of evidence the phylogenetic distinction of the Himalayan wolf as an evolutionary older wolf lineage that is eligible for taxonomic recognition. It reveals an adaptation to the low oxygen levels in the Asian high-altitudes unique to this wolf and it is hypothesized that this adaptive benefit maintains the ancient Himalayan wolf and has driven its speciation. Based on these findings, carnivore taxonomists and the global conservation community should now seek to implement the required steps: 1) update the taxonomic classification of the Himalayan wolf, and 2) carry out an IUCN Red List assessment.

Himalayan wolves use wild prey over-proportionally compared to livestock, but seasonally high livestock densities facilitate human-wildlife conflict. Future efforts to conserve the Himalayan wolf and its ecosystem must ensure that intact wild prey populations are maintained or enhanced through management of sustainable livestock husbandry and combating poaching and illegal wildlife trade. With these measures in place, the Himalayan wolf can become a powerful and charismatic conservation ambassador for protecting some of the last intact large wilderness areas on our planet as found in the Asian high-altitudes.

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Appendix A.

ADDITIONAL PUBLICATIONS AND OUTPUTS

Additional publications and outputs

The field work for this doctorate research brought me and my team to very remote regions in the Himalayas of Nepal and the Tibetan Plateau of China. As dedicated naturalists we recorded all possible observations of mammals, birds and plants. Some of these findings have resulted in scientific publications, one book, and a short video documentary. The details of these are found below.

From the wild yak (*Bos mutus*) rediscovery to the Nepalese five-rupee bank note

My photograph taken during the exciting moments of rediscovering the wild yak for Nepal is now shown on the latest Nepalese five-rupee bank note released in 2017 (Figure A-1).

Figure A-1. The new Nepalese five-rupee bank note (left) showing the wild yak (right) rediscovered by our research team in Humla during 2014 and 2015.

Short Film

Werhahn G. 2017. The Himalayan wolf in Nepal.

A 9-minute documentary. https://youtu.be/TilOuJaV1wM

Book

Kusi N. and **Werhahn G.** 2016. ***Humla - Journey into the hidden Shangri-La***. Kathmandu: Himalayan Map House. Book.

Additional Publications

Werhahn G., Kusi N., Sillero-Zubiri C., and Macdonald D.W. 2017 **Conservation implications for the Himalayan wolf *Canis (lupus) himalayensis* based on observations of packs and home sites in Nepal**. Oryx.

Kusi N., Siller-Zubiri C., Macdonald D.W., Johnson P.J., and **Werhahn G**. 2019. **Perspectives of traditional Himalayan communities on fostering coexistence with Himalayan wolf and snow leopard.** *Accepted for publication in Conservation Science and Practice.*

Werhahn G., Kusi N., Man Sherchan A., Karmacharya D., Manandhar P., Manandhar S., Bhatta T. R., Joshi J., Bhattarai S., Sharma A. N, Kaden J., Ghazali M., and Senn H. 2018**. Eurasian lynx and Pallas's cat in Dolpa district of Nepal: Insights into genetics, distribution and diet from non-invasive sampling.** Cat News N°67.

Macdonald D.W., Campbell L.A.D., Kamler J.F., Marino J., **Werhahn G.,** and Sillero-Zubiri C. 2019. **Monogamy: cause, consequence or corollary of success in wild canids?** Frontiers in Ecology and Evolution.

Conservation implications for the Himalayan wolf *Canis (lupus) himalayensis* based on observations of packs and home sites in Nepal

Geraldine Werhahn, Naresh Kusi
Claudio Sillero-Zubiri and David W. Macdonald

Abstract We provide insights into pack composition and den site parameters of the Himalayan wolf *Canis (lupus) himalayensis* based on observations of free-ranging wolves in three study areas in Nepal. We combine this with a social survey of the local Buddhist communities regarding human–carnivore conflict, to draw inferences for conservation practice in the Nepalese Himalayas. We recorded eight wolf packs (with an average composition of two adults and three pups), and found five home sites in high-altitude shrubland patches within alpine grasslands at 4,270–4,940 m altitude. There was a spatial–temporal overlap of wolf home sites and livestock herding during spring and summer, which facilitated human–wolf conflict. The litters of three out of five wolf packs found in Dolpa during 2016 were killed by local people in the same year. In Nepal compensation is offered for depredation by snow leopards *Panthera uncia*, with associated lowering of negative attitudes, but not for depredation by wolves. We recommend the implementation of financial and educational conservation schemes for all conflict-causing carnivores across the Himalayan regions of Nepal.

Keywords *Canis (lupus) himalayensis*, conflict, conservation, denning, Himalayan wolf, Nepal, social survey, wolf pack

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Introduction

The Himalayan wolf is a distinct lineage occurring in the Himalayas and on the Tibetan Plateau. Genetic evidence supporting its classification as a separate taxon, *Canis (lupus) himalayensis*, is accumulating (Sharma et al., 2004; Aggarwal et al., 2007; Werhahn et al., 2017). The distribution range, population status and ecology of this wolf remain poorly known (Fox & Chundawat, 1995; Jnawali et al., 2011). Potential wolf habitat exists across large parts of the Nepalese Himalayas (> 70%) but the size of the wolf population in the country remains unknown; there are genetically verified records from Mustang, Dolpa and Humla (Subba, 2012; Chetri et al., 2016; Subba et al., 2017; Werhahn et al., 2017), and sign surveys have indicated wolf presence in Manaslu, Api Nampa and Kanchenjunga Conservation Areas (Subba et al., 2017; GW & NK, pers. obs., 2016).

The wolf is protected as a priority species in Nepal under the National Parks and Wildlife Conservation Act 2029 (1973) and is categorized nationally as Critically Endangered (GoN, 1973; Jnawali et al., 2011). Nepal's wildlife damage relief guidelines include the wolf in the national compensation scheme in its first amendment (GoN, 2015), but this legal basis awaits implementation.

Little is known about the denning ecology of the Himalayan wolf in the remote and rugged high-altitude landscapes of the Himalayas and the Tibetan Plateau (Werhahn et al., 2017) but similarities to the Holarctic grey wolf *Canis lupus* spp. are expected (Mech & Boitani, 2003).

Methods

This study was conducted in Humla and Dolpa districts in north-western Nepal, and Kanchenjunga Conservation Area in north-eastern Nepal, during the spring and summer seasons of 2014–2016 (Fig. 1). The Humla site lies outside the protected area system, whereas large parts of the Dolpa site are situated within Shey Phoksundo National Park, and Kanchenjunga Conservation Area is a community-managed conservation area. All three study areas are used seasonally by herders in the late spring and summer to graze yak *Bos grunniens*, cattle *Bos taurus* and goats *Capra aegagrus hircus* (Bauer, 2004). The vegetation in these areas is dominated by alpine grasslands and steppes interspersed with patches of shrubland (Miehe et al., 2016), at 3,600–5,600 m altitude. In Humla we searched 384 km^2 for wolves (walking 420 km in 2014 and 605 km in 2015), in Dolpa 1,088 km^2 (walking 262 km in 2015 and 810 km in 2016), and in Kanchenjunga Conservation Area 368 km^2 (walking 355 km in 2016).

We collected data on wolf packs through direct observations, examination of home sites, and reports of sightings by local people, which were later verified by examining distances between recorded home sites. Home sites,

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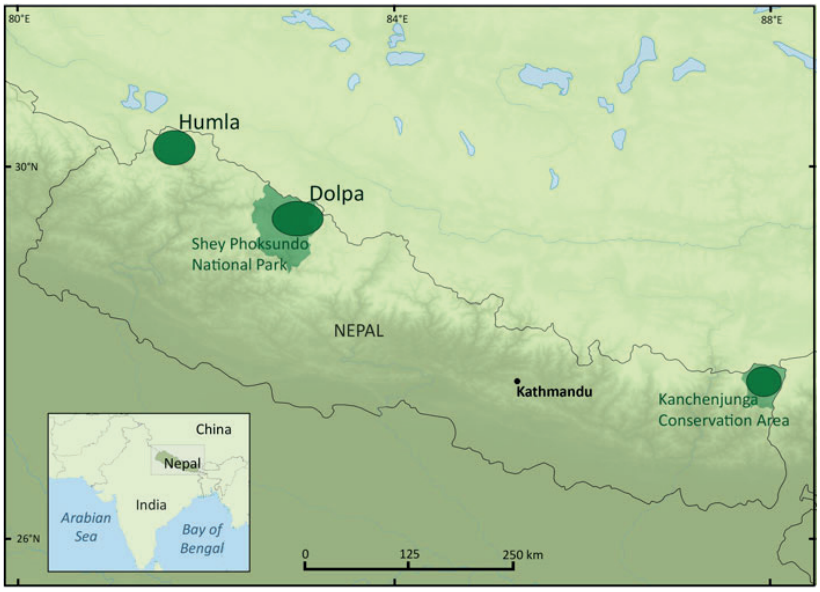

FIG. 1 Locations of the three study sites in the Himalayas in Nepal: Humla (outside the protected area network), Dolpa (partly within Shey Phoksundo National Park), and the community-managed Kanchenjunga Conservation Area.

comprising den and rendezvous sites, were characterized by abundant recent scats of pups and adults, along with bones and other food remains. Den sites additionally comprised a den cave (Mech & Boitani, 2003).

To investigate human–carnivore conflict we conducted semi-structured social surveys during the field work expeditions of 2014–2016, with 72 respondents from the local Buddhist communities (32 (44%) in Humla, 27 (38%) in Dolpa, and 13 (18%) in Kanchenjunga Conservation Area). Respondents comprised herders (67%), monks/lamas (10%), teachers (4%), business people (11%), construction workers (5%) and hoteliers (3%). Respondents were 20–78 years of age and comprised 25% females and 75% males. Survey questions explored perceptions of trends in the wolf population and depredation incidents, and spatial and temporal depredation hotspots, and respondents were asked to rank predators based on the magnitude of depredation problems they cause.

Results

The eight packs encountered in Nepal consisted, on average, of five individuals: an adult pair accompanied by a mean of 2.9 ± SD 0.6 pups of the current year (Table 1, Plate 1). According to our observations, Himalayan wolves in Nepal give birth between mid April and mid May (n = 4). The mean distance between the home sites of four adjacent wolf packs in Dolpa was 19.6 ± SD 5.5 km (n = 4). All home sites were situated in patches of rolling alpine shrubland dominated by *Caragana*, *Lonicera*, *Astragalus*, *Salix* and *Juniperus* spp. within alpine grasslands and in proximity to water (Table 2).

In Dolpa and Kanchenjunga Conservation Area a perceived increase in the wolf population was reflected in a perceived increase in depredation by wolves. In Humla, however, a perceived decrease in the wolf population correlated with a perceived decrease in depredation (Figs 2 & 3); local people attributed this decrease in depredation largely to the lower numbers of livestock kept now compared to the past. In Humla and Dolpa snow leopards were ranked as the most problematic carnivore, followed by wolves, and vice versa in Kanchenjunga Conservation Area. In Humla, depredation by wolves was reported to be at its highest during the summer. Respondents in Kanchenjunga Conservation Area reported the highest depredation rates in winter, followed by spring/summer, whereas no seasonal pattern emerged in Dolpa (Fig. 3).

Discussion

The social life of Himalayan wolves is characterized by small packs (i.e. family groups; Plate 1), similar to packs of Holarctic grey wolves, golden jackals *Canis aureus* and coyotes *Canis latrans* (Sillero-Zubiri et al., 2004). The Himalayan wolf packs observed were comparatively smaller than Holarctic grey wolf packs, which commonly comprise 6–12 individuals (Sillero-Zubiri et al., 2004). Factors potentially influencing pack size may include prey abundance and size, wolf mortality, and prompt dispersal of yearlings (Fuller, 1989; Jędrzejewski et al., 2002).

The home sites used by these Himalayan wolf packs were probably chosen for their quality in respect to hiding cover, structural diversity and water accessibility, similar to recorded characteristics of grey wolf den sites (Trapp et al., 2008).

Table 1 Pack composition of eight Himalayan wolf *Canis (lupus) himalayensis* packs in three areas in Nepal: Dolpa (within and outside Shey Phoksundo National Park), Humla and Kanchenjunga Conservation Area (Fig. 1), with type of evidence, and year recorded.

Location	Pack composition	Type of evidence	Year
Dolpa district			
Shey Phoksundo National Park			
Komas	2 adults, 3 pups*	Reported by local people & personal observation	2016
Bhijer	2 adults, 3 pups	Personal observation	2016
Nisalgaon	2 adults, 2 pups*	Reported by local people & personal observation	2016
Polte	2 adults, 3 pups	Personal observation	2016
Charka Tulsi	Unknown no. of adults, 3 pups*	Reported by local people	2016
Humla district			
Gyau	Unknown no. of adults, 3 pups	Personal observation	2015
Ngin	2 adults, 4 pups	Personal observation	2014
Taplejung district			
Lhonak (Kanchenjunga Conservation Area)	2 adults, 3 pups	Reported by local people	2016

*Pups of the year killed through smoking the den

Plate 1 A Himalayan wolf *Canis (lupus) himalayensis* pack photographed near Bhijer village in Dolpa district of Nepal (Fig. 1) in 2016. (a) A female and her three pups in the morning, and (b) the female suckling her pups. (Photograph by Geraldine Werhahn)

The high-altitude shrublands used by the packs for denning are also used by local people to graze livestock in late spring and summer, a time when the pups are a few weeks old and are restricted to home sites. Pupping packs are less mobile and need ample food. This spatial–temporal overlap of wolf denning with herding, which often involves large numbers of livestock being brought in seasonally, gives rise to human–wolf conflict (Mishra, 1997; GW & NK, pers. obs., 2014–2016). Livestock competes with, and may displace, wild prey species such as blue sheep *Pseudois nayaur*, kiang *Equus kiang* and argali *Ovis ammon*. Consequently human–wolf conflict is intensified during late spring and summer in the pasturelands.

The social surveys indicated that no compensation for wolf depredation was provided in any of the three study sites, whereas compensation for snow leopard depredation

TABLE 2 Characteristics of four den sites and one rendezvous site of Himalayan wolves in Dolpa and Humla districts, Nepal (Fig. 1), with type of vegetation, dominant topography, distance to water, elevation, aspect, year of recording, and district.

Location	Home site type	Vegetation	Dominant topography	Distance to water (m)	Altitude (m)	Aspect	Year
Dolpa							
Komas	Den	Shrubland	Hillslope	1,250	4,270	North	2016
Bhijer	Den	Shrubland	Hillslope	50	4,790	South	2016
Nisalgaon	Den	Shrubland	Hillslope	40	4,650	South-west	2016
Polte	Rendezvous	Shrubland/ grassland	Valley floor	20	4,915	Flat	2016
Humla							
Gyau	Den	Shrubland	Hillslope	280	4,940	South	2015

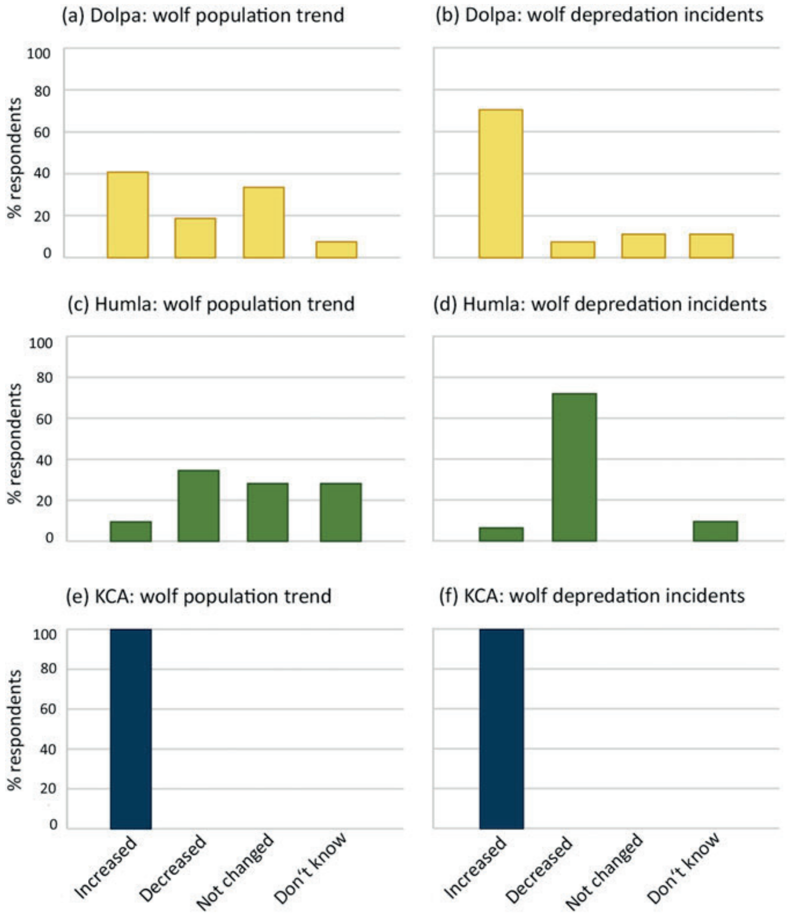

FIG. 2 Wolf *Canis (lupus) himalayensis* population and depredation trends as reported by local respondents during structured social surveys in Dolpa (n = 27), Humla (n = 32), and Kanchenjunga Conservation Area (KCA; n = 13).

is provided in parts of Shey Phoksundo National Park and in Kanchenjunga Conservation Area (Subba et al., 2017). Killing wolves is often considered to be an act of service towards the entire community, as it is assumed to prevent future livestock depredation.

The entire litters of three out of five wolf packs found in Dolpa in 2016 were killed that same year by local people. The pups were smoked to death by lighting a fire inside the den and blocking the entrance with stones (GW & NK, pers. obs.; local people, pers. comm., 2016). Schaller (1982) similarly reported persecution of wolves in Dolpa, with local people gathering each spring to look for wolf dens and kill the pups. However, killing wolves is often not an effective means of decreasing livestock depredation and can be counter-productive, because of a perturbation effect (Tuyttens & Macdonald, 2000) arising from the disrupted pack structure and territorial

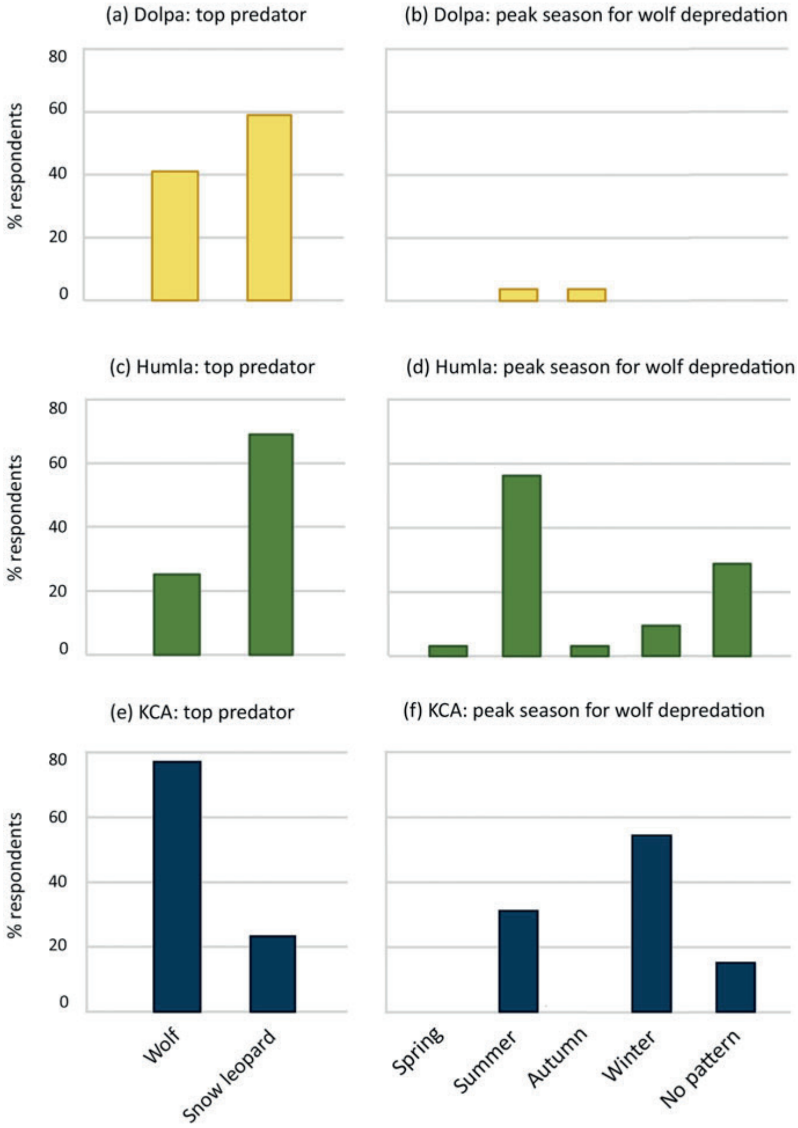

FIG. 3 The most problematic predator and the season with the most incidents of wolf depredation, as reported by local respondents during structured social surveys in Dolpa (n = 27), Humla (n = 32) and Kanchenjunga Conservation Area (n = 13), Nepal (Fig. 1).

configuration (Wielgus & Peebles, 2014; Chapron & Treves, 2016; Treves et al., 2016).

To date, carnivore conservation in the Himalayas has focused on snow leopards, with insurance schemes and awareness programmes implemented locally (Mishra et al., 2003; Subba et al., 2017). In Kanchenjunga Conservation Area, where conservation programmes exist for the snow leopard and blue sheep, respondents expressed a positive attitude towards the snow leopard. These communities stated that they would support wolf conservation only if they received similar financial compensation for depredation by wolves. It is therefore crucial for wolf conservation to expand implementation of compensation to cover depredation by wolves, and the legal basis for this is already in place in Nepal's wildlife damage relief guidelines (GoN, 2015). We further recommend that governmental and non-governmental organizations working on carnivore conservation in the Himalayas of Nepal include all conflict-causing carnivores in their conservation programmes. This may involve facilitating self-financed depredation insurance schemes, protecting wild prey populations and managing livestock numbers, improving livestock protection, and increasing conservation awareness (Mishra, 1997). Payments to encourage coexistence can be a useful tool to increase local tolerance towards carnivores (Dickman et al., 2011). The implementation of conservation payments for species that are difficult to monitor and occur in remote areas (e.g. the snow leopard, Himalayan wolf and Eurasian lynx *Lynx lynx* in the Himalayas) can be tied to tangible performance criteria, such as maintaining the habitat and populations of wild prey species (e.g. blue sheep), with payments being contingent upon communities not killing any predators (Mishra et al., 2003; Nelson, 2009).

A first step towards the conservation of the Himalayan wolf in Nepal is to include it in existing conservation programmes, followed by expanding these across the entire region. These programmes can be informed by our insights into Himalayan wolf denning ecology, temporal–spatial wolf depredation patterns, and the associated perceptions of local communities.

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Author contributions

GW collected the field data, conducted the data analysis, conceived and designed the study, and drafted the article. NK participated in data collection and analysis, and in drafting the article. CSZ and DWM helped to conceive and design the study, and draft the article.

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Biographical sketches

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Perspectives of traditional Himalayan communities on fostering coexistence with Himalayan wolf and snow leopard

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Abstract

The Himalayan wolf *Canis* sp. and snow leopard *Panthera uncia* are found in the Nepalese Himalayas where conservation efforts target the latter but not the former. We conducted semi-structured questionnaire surveys of 71 residents in upper Humla, upper Dolpa and Kanchenjunga Conservation Area (KCA) during 2014-2016 to understand people's knowledge, perceptions, attitudes and interactions with these two carnivores. We fitted a cumulative link mixed model to predict Likert scale ordinal responses from a series of Generalized Linear Mixed Models. Overall, attitudes were more positive towards snow leopards than wolves. Livestock depredation was the main predictor of the general negative attitude towards wolves (Estimate=-1.30873; p=0.029866) but there was no evidence for an effect for snow leopards (Estimate=-0.3640; p=0.631446). Agro-pastoralists had more negative attitudes than respondents with other occupations towards both carnivores and men had more positive attitudes than women. Among our study areas, respondents in the community-owned KCA had the most positive attitudes. Our findings illustrate the need to reduce human-carnivore conflict through a combined approach of education, mitigation and economic cost-sharing with respectful engagement of local communities. Specifically, to encourage more villagers to participate in livestock insurance schemes, they should be improved by including all large carnivores and adjusting compensation to the market value of a young replacement of the depredated livestock type. Carnivore conservation interventions should target the whole predator guild to achieve long-term success and to protect the Himalayan ecosystem at large.

Keywords: Compensation, conservation education, depredation, Himalaya, human-carnivore coexistence, Nepal

Introduction

The Himalayan wolf (currently recommended as *Canis lupus chanco* by Álvares et al., 2019; also see Werhahn et al., 2017b, 2018) and snow leopard are top carnivores coexisting in the Himalayas and the Qinghai-Tibetan Plateau (QTP) of Asia. As top carnivores, they regulate ecosystem health and processes through trophic interactions with mesopredators, herbivores and the vegetation, facilitating biodiversity and ecosystem resilience (Ripple et al., 2014), and may contribute to nutrient cycling by supporting scavenger diversity (Wilmers et al., 2003). Their beauty and charisma can provide economic benefits to local communities through tourism (Dickman et al., 2011; Maheshwari & Sathyakumar, 2019; Vannelli et al., 2019) and as ambassadors for conservation marketing (Macdonald et al., 2017). Hence, their conservation is of wider interest (Nowell & Jackson, 1996; Treves & Karanth, 2003), but human-carnivore conflicts often hinder conservation efforts.

Human perceptions of carnivores as threats to their livelihoods, combined with the negative impacts humans have on their habitats, and subsequently their survival, are important drivers of human-carnivore conflict (Kellert et al., 1996; Dar et al., 2009; Aryal et al., 2014). As around the globe, pastoralists in central Asia are persecuting carnivores in response to livestock depredation (Mishra & Fitzherbert, 2004; Lescureux & Linnell, 2013). But pastoral communities in south Asia and QTP China, by virtue of adherence to the basic tenets of Buddhism that include love, respect and compassion for all life forms (Karmapa & Dorje, 2011), are generally more tolerant of carnivores despite considerable loss of livestock to depredation (Suryawanshi et al., 2014). This is explained by the fact that the sacred mountains around Buddhist monasteries constitute safe havens for wildlife including snow leopards and wolves (Li et al., 2014). This positive influence of Tibetan Buddhism provides great potential to, hand in hand with science, strengthen conservation of wildlife and humans alike.

Physical (body size, morphology etc.) and behavioural traits (movement time and range, food habits, etc.) of carnivores, exposure to risks connected to the animals, together with social and cultural beliefs influence human perceptions and determine attitudes (Kellert et al., 1996). The mountain pastoralists in Asia usually have negative attitudes towards both snow leopards and wolves (Oli et al., 1994; Mishra, 1997; Bagchi

& Mishra, 2006; Namgail et al., 2007; Suryawanshi et al., 2014; Alexander et al., 2015; Chen et al., 2016; Din et al., 2017) because these carnivores frequently depredate on livestock, that form the backbone of household economy of pastoralists in the region (Rosen et al., 2012). In recent times, pastoralists in the region have shifted from subsistence to commercial agriculture and animal husbandry (Mishra, 1997; Bauer, 2004), increasing the economic value of livestock. Since the socio-economic consequences of livestock depredation by carnivores in economically marginalized pastoral communities are usually severe (Wang & Macdonald 2006; Aryal et al., 2014), livestock depredation is an important factor affecting hostility towards these carnivores. Also, livestock guarding practices in the region are aggravating the situation further, as yaks and horses are currently little tended and range freely in the pasturelands, facilitating the losses of livestock to carnivores (Mishra, 1997). Conservation interventions such as improved corrals and livestock vaccination (Nawaz & Mishra, 2016), financial incentives against carnivore-caused livestock mortalities (Mishra et al., 2016), and carnivore-based ecotourism (Vannelli et al., 2019) have proven effective in mitigating depredation losses in the region.

Conservation of large carnivores like wolves and snow leopards requires a better understanding of their conflict with humans (Namgail et al., 2007) because community perceptions and attitudes affect conservation effectiveness (Ferreira & Freire, 2009). In addition, a proper understanding of the human dimension and related social norms characterising wildlife conflicts is essential to inform management and ensure local support (Madden, 2004; Gelcich et al., 2006).

Many studies on human-carnivore conflicts in highland Asia have provided important conservation implications by understanding people’s attitudes towards carnivores; either by characterizing the attitudes (Oli et al., 1994; Bagchi & Mishra, 2006;Wang et al., 2006; Ferreira & Freire, 2009; Liu et al., 2011) or by identifying the drivers for the attitudes (Mishra, 1997; Suryawanshi et al., 2014; Li et al., 2015). But these studies usually present surveyors’ opinions as possible solutions rather than including those of local communities. In this study, we add to the efforts of identifying the drivers of attitudes and consider the opinions of the local communities while presenting the possible solutions; complementing that with recommendations from other studies and our own. Insights from the study have proven crucial in planning the conservation actions we look forward to implement by working closely with the local communities.

Throughout the Asian highlands, pastoralists' attitudes towards snow leopards and wolves differ: they show a comparatively better tolerance towards snow leopards than towards wolves (Mishra, 1997; Suryawanshi et al., 2013, 2014; Li et al., 2015; Din et al., 2017; Jamtsho & Katel, 2019; Maheshwari & Sathyakumar, 2019), even when snow leopards are responsible for higher economic loss than the wolves. Various factors like religion, income, education, species-specific characteristics and cultural factors can explain the differences (Mishra, 1997; Liu et al., 2011). Tolerance towards wolves is further worsened in the Pamirs of central Asia because wolves are considered as the main problem carnivore in the region (Mishra & Fitzherbert, 2004; Khan et al., 2014; Din et al., 2017).

Studies on attitudes towards carnivores in the Nepalese Himalayas, to date, have been limited to snow leopards (Oli et al., 1994; Gurung & Thapa, 2004; Schutgens et al., 2018; Hanson et al., 2018). This can be related to the fact that carnivore conservation in the Nepalese Himalayas has focused primarily on the snow leopard. Activities like carnivore population monitoring and raising conservation awareness, incentives for coexistence such as livestock insurance schemes, compensation schemes, predator-proof corrals, important policy and management documents like DNPWC (2017) and MOFSC (2017) target snow leopard only (notably excluding wolves). The recently amended wildlife damage relief guidelines (GON, 2015) grant compensation for livestock depredation by wolves also, but this provision is still awaiting implementation, and most Himalayan communities of Nepal are unaware of their entitlement to such compensations. This is possibly the first study from Nepal, revealing the differences in attitudes of local people towards wolves and snow leopards and seeking to understand the locally preferred solutions to mitigating human carnivore conflict to ensure that the solutions are sustainable and that they rightly address the local needs.

We conducted this study to provide insights into human-carnivore coexistence by i) understanding the attitudes of local communities towards the Himalayan wolf and snow leopard in the Nepalese Himalayas, ii) identifying key drivers for these attitudes, and iii) identifying locally-preferred conservation solutions to ensure local commitment to carnivore conservation. Based on the results, we discuss how conservation action can benefit from a more inclusive approach.

Methods

Study area

The study comprised three areas in the Nepalese Himalayas: upper Humla (30.19°-30.42°N, 81.48°-81.42° E) and upper Dolpa (28.97°-29.77°N, 82.49°-83.14°E) are located in the trans-Himalayan belt of north-western Nepal sharing an international border with the Tibetan Autonomous Region (TAR) of China, and Kanchenjunga Conservation Area (KCA, 27.48°-27.94°N, 87.65°-88.2°E) in north-eastern Nepal, bordering TAR and India, represents an alpine ecosystem. Upper Humla currently lies outside the protected area system, large parts of upper Dolpa fall into the government-managed Shey-Phoksundo National Park (SPNP) and KCA is owned and managed by the community.

Landscapes of the study areas vary from high altitude Himalayan valley floors, across steep mountain cliffs rising to the rolling grasslands of the Tibetan Plateau. Vegetation above 3,600 masl is dominated by dry alpine steppe rich in sedges and graminoids such as *Stipa* spp, *Carex* spp and *Kobresia* spp. Grasses and shrubs such as *Caragana brevifolia* and *Lonicera spinosa* dominate drier sites and rugged slopes (Miehe et al., 2016). Himalayan wolves and snow leopards coexist with other predators like Eurasian lynx *Lynx lynx*, Pallas's cat *Otocolobus manul,* Tibetan fox *Vulpes ferrilata*, red fox *Vulpes vulpes*, and brown bear *Ursus arctos* and prey on herbivores like Tibetan gazelle *Procapra picticaudata* and blue sheep *Pseudois nayaur* and small mammals like Himalayan marmot *Marmota himalayana* and woolly hare *Lepus oiostologous*. The study areas range in elevation between 3600-5600 masl. Precipitation is mainly in the form of snow.

Human communities in the study areas are mostly agro-pastoralists belonging to the Tibetan ethnic group whose dominant religion is Tibetan Buddhism (Bauer, 2004). Their livelihood is based on agriculture, livestock husbandry and collection of non-timber forest products. Livestock herders graze yaks *Bos grunniens*, cattle *Bos Taurus,* yak-cattle hybrids (dzos/jhoppas, *Bos spp*.), horses *Equus ferus coballus,* goats *Capra aegagrus hircus* and sheep *Ovies aries* in the alpine pastures above their villages during the late spring and summer seasons. Livestock is usually shifted among different pastures before bringing them down to the villages in the valley floors during winter.

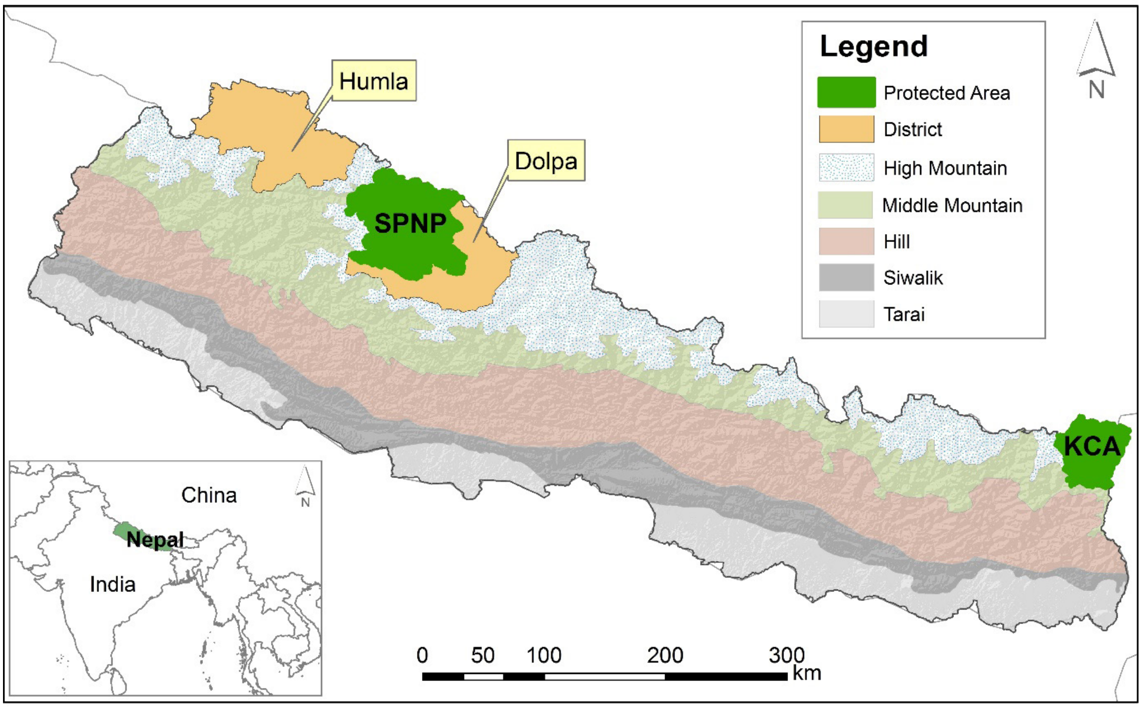

Figure 1. Study area location and physiographic division of Nepal. KCA and Dolpa are protected areas while Humla is not protected.

Sampling design

We conducted semi-structured questionnaire surveys (Newing et al., 2011) during the spring and summer seasons of 2014-2016 to collect data on local people's knowledge, perceptions, attitudes and interactions with Himalayan wolf and snow leopard. We used closed-format questions to minimise uncertainty (White et al., 2005) and to facilitate statistical analyses (Newing et al., 2011).

We applied convenience sampling (Etikan et al., 2016) to conduct the surveys due to the low density of humans in the remote study areas and general absence of people from their homes during the survey periods (because they were busy sowing crops in the agricultural lands, herding livestock or had temporarily migrated for business and labour work in nearby towns). We conducted surveys in all villages (identified through discussions with forest officers and park authorities) within each study area (i.e. five villages in upper Humla, 13 in upper Dolpa and four in KCA) to ensure our sampling was representative. Our survey dataset contained villagers of different age classes, occupations and gender in each study area (Table 1).

Data collection

Questionnaire surveys

We piloted the questionnaire in three villages of upper Humla in 2014 to test for its length, language, clarity and potential sources of bias (White et al., 2005). We then adjusted some questions and finalized the questionnaire to run the surveys in 2015 (upper Humla) and 2016 (upper Dolpa and KCA). We conducted the interviews, each lasting 30-40 minutes, in the local languages using reliable local translators.

We prepared a closed checklist of wildlife (carnivores, herbivores and small mammals) potentially found in the study areas following Jnawali et al., (2011). We used printed colour-photographs of different mammal species to ask each respondent about the species' presence/absence and to ascertain individual's knowledge about the natural environment in the study area. The use of photographs proved important, because the names given locally for some wildlife varied between study areas and between the villages within an area.

We divided the questionnaire into the following sections: (1) Wildlife understanding: Knowledge about wildlife found in the area and the population status (global and local) of Himalayan wolf and snow leopard; (2) Respondents background: age, gender, occupation, village, family size, religion, time in the area, origin, education level and travelling (outside the district) experience; (3) Snow leopard and Himalayan wolf: Experiences with the carnivores, their population trends, main prey and prey preference (wild versus domestic); (4) Depredation and economics: Livestock kept and mortality due to predation, current market value per head of livestock, problem predator ranking, reliance on livestock for income, social reaction norms to the appearance of a snow leopard or Himalayan wolf (near the villages and pastures); (6) Improving conflict: Existing practices to prevent livestock depredation, use of guard dogs, opinion on best methods to reduce depredation, livestock insurance and compensation schemes, knowledge/preference of other methods to improve the situation, assistance to commit to Himalayan wolf and snow leopard conservation; (7) Conservation and attitudes: Attitudes towards wildlife in general, snow leopard and Himalayan wolf; (8) Religious and medicinal use: Religious significance and use of wildlife body parts in the local culture and traditional medicines and local stories involving Himalayan wolf or snow leopard. Please refer to the supplementary material for the questionnaire form.

For the attitude section, we assigned a three-point Likert scale (-1 for disagree, 0 for maybe (neutral) and +1 for agree) to the responses. We calculated total attitude score by summing the attitude scores for the different questions. This generated a nine point ordinal response ranging from -5 (most negative attitude recorded) to +3 (most positive attitude recorded).

For the statistical analysis we pooled the occupation types into two categories: agro-pastoralist (including herder, labourer and farmer) and non-agro-pastoralist (including teacher, school manager, business and monk).

Statistical analysis

Our survey dataset comprised a total of 71 questionnaires (upper Humla: 30, upper Dolpa: 28 and KCA: 13). We used numerical codes for answers where possible or used the narrative responses as qualitative data. We used attitude scores as response variables and treated 'village' identity as a random factor to account for spatial clustering of respondents. We used Variance Inflation Factor (VIF) to explore collinearity among predictors using the rms package of R (Harrell Jr, 2019). Where evidence for high collinearity was present (VIF> 8) we excluded variables that were substantially collinear (Dormann et al., 2013). 'Schooling', for example, was excluded as a potential predictor because it was significantly associated with both 'gender' ($x^2 = 19.892$, p=0.0001) and 'occupation' ($x^2 = 58.75$, p=8.072e-11). Similarly, there was a strong evidence that 'travelling outside the district' was confounded with 'occupation' (Fisher's Exact test, P = 0.003); respondents whose principal occupation was 'business' were more likely to report travel. We therefore excluded 'travelling outside the district' as a predictor. Also we excluded 'originality' as a predictor because it was confounded with 'study area' (Fisher's Exact test, P= 0.0001). The 'livestock insurance scheme' was present only in KCA, and was therefore completely confounded with study area. We treated it as a characteristic of 'study area' and excluded it as a potential predictor. The number of respondents who claimed to have been threatened or attacked by a Himalayan wolf (n=1, 0.8%) or a snow leopard (n=3, 2.3 %) was negligible; hence, we excluded 'experiences with snow leopard or Himalayan wolf' as potential predictor. After excluding these and the collinear potential predictors, the final considered models included respondent characteristics (gender, occupation, whether they reported having experienced livestock attacks by snow leopard or Himalayan wolf, the study area and presence of compensation scheme) as predictor variables (Breslow & Clayton, 1993). See table 2 for description of the predictors.

We performed all analyses in R Version 3.4.3 (R Development Core Team, 2017). We fitted a cumulative link mixed random model with the clmm function in the 'ordinal' package of R (Christensen, 2018) to construct models predicting Likert scale ordinal responses from a series of Generalized Linear Mixed Models (GLMMs) because mixed models provide a more flexible approach for analysing non-normal data and accounts for random factors (Bolker et al., 2009). We used the R package 'effects' (Fox et al., 2019) to visualise the effect sizes for predictors of attitude.

We used an Information Theoretic approach for model selection because it enabled us to examine several competing models using both explanatory value and parsimony (Grueber et al., 2011). We used the Akaike's Information Criterion adjusted for small sample size (AICc) for ranking the models (Burnham & Anderson, 2002).

We calculated model weights by using the R package MuMIn (Barton, 2018) to evaluate relative model weights in the set of candidate models (Bolker et al., 2009). We also examined the parameter estimates of variables in the global models to assess the marginal significance of their effect on total attitude scores (the marginal effects indicating their effect on the response adjusting for all other effects in the model). As no single model was dominant we accounted for model uncertainty by using model averaging, based on model weights (Burnham & Anderson, 2002); potential problems with averaging arising from collinearity having been minimised by pruning collinear potential predictors (Cade, 2015). We averaged parameter estimates in all models up to a cumulative weight of 0.95. We explored model fit and diagnostics based on surrogate residuals using the R package 'sure' (Greenwell et al., 2017).

Results

Respondent characteristics

Most respondents (32.39%, n=23) were in the age class of 31-40 years old. Male respondents (76.06%, n=54) predominated over females, and agro-pastoralist (70.42%, n=50) was the dominant occupation. The average family size was 5.9 individuals (range 1- 12, median 6). Buddhism was the main religion (98.6%, n=70). Most respondents (97.18%, n=69) were originally from the respective study area with 95.77% (n=68) living there since childhood. The 'illiterate' class dominated schooling level (46.48%, n=33). Most respondents (97.18%, n=69) had travelled outside the district at least once (Table 1).

Table 1. Respondent characteristics in upper Humla, upper Dolpa and KCA. Main characteristics are given in italics followed by their categories where present.

	upper Humla	**upper Dolpa**	**KCA**
No. of respondents	**30 [#]**	**28**	**13**
Gender			
Male	22	22	10
Female	8	6	3
Age-class			
21-30	5	1	2
31-40	11	7	5
41-50	7	6	3
51-60	3	7	2
61-70	0	6	1
71-80	4	1	0
Occupation			
Agro-pastoralist	21	20	9
Non-agro-pastoralist	9	8	4
Average family size	7.8	5.2	4.8
Religion			
Buddhism	30	27	13
Bon-po	0	1	0
Time in the area			
Since childhood	30	26	12
>20 years	0	2	0
10-20 years	0	0	0
5-10 years	0	0	1
<5 years	0	0	0
Originally from the area?			
Yes	30	27	12
No	0	1	1
Schooling level			
Illiterate	14	14	5
Adult literacy class	0	0	0
Monastery education	1	9	0
Primary school	11	2	5
High school	4	3	2
University	0	0	1
Travelling outside the district			
Never	4	3	0
Once or twice	2	6	4
A few times	16	11	2
Often	8	8	7

[#] We included only 30 (of 32) surveys from upper Humla in the analysis. One respondent was interviewed twice and one could not provide any relevant information.

Table 2. Description of predictors.

Predictor	**Description**
Study area	Upper Humla: lies outside the Protected Area (PA) system Upper Dolpa: Most parts fall into the government-managed Shey-Phoksundo National Park Kanchenjunga Conservation Area: A PA managed and owned by the community
Occupation	Agro-pastoralists (includes farmers, herders and labourers) Non-agro-pastoralists (includes business, teacher, school manager and monk)
Gender	Male or Female
Livestock attacked by a wolf or a snow leopard	Reported (Yes) or Not reported (No)
Compensation scheme	Presence (Yes) or Absence (No)

Knowledge and perceptions

Both Himalayan wolf (92.96%, n=66) and snow leopard (94.37%, n=67) were reported to be present in all three study areas. Wolves were considered at risk of local extirpation by respondents in KCA (69.2%, n=9) and snow leopards by respondents in upper Humla (56. 7%, n=17). Average annual monetary loss per household reported due to livestock depredation was the highest in KCA (5,776 USD for 2015-2016), followed by Dolpa (3,112 USD for 2015-2016) and Humla (723 USD for 2014-2015) (Table 3).

Both wolves (97.18%, n=69) and snow leopards (88.73%, n=63) were ranked ‘high’ to ‘very high’ as problematic carnivores across all study areas. However, most respondents ranked snow leopards as the main problem carnivore in upper Humla and upper Dolpa while most respondents in KCA ranked wolf as the main problem carnivore (Table 4). Wolves were reported to be killed in Humla by 35.5% of respondents (‘sometimes’ (n=9), ‘rarely’ (n=2)) and by 21.4% respondents in Dolpa (‘sometimes’ n=4, ‘rarely’ n=2). The most prevalent motivation reported for this was to prevent future livestock loss (27.6%, n=21), in response to past attacks on livestock (5.3%, n=4) and to protect human safety (1.3%, n=1). The methods reported to be used to kill wolves included snare traps (13.1%, n=10), pit traps (9.2%, n=7), carcass poisoning (5.3%, n=4), smoking dens (3.9%, n=3), and cornering and stoning individuals (1.3%, n=1). Snow leopards were reported to be killed in Humla by 53.3% of respondents ('sometimes' (n=12), rarely (n=4)), with the motivating causes reported being to prevent future livestock loss (21.0%, n=16) and in response to past attacks on livestock (2.6%, n=2). Reports of methods used to kill snow leopards included pit traps (13.1%, n=10), carcass poisoning (3.9%, n=3), surrounding and stoning the animal (3.9%, n=3), snare traps (2.6%, n=2) and firearms (1.3%, n=1).

Factors affecting attitudes

Model-averaged parameter estimates provided evidence for a link between both 'study area' and 'occupation' on attitudes towards both carnivores. Livestock depredation was associated with more negative attitudes towards wolves (Table 5, Figure 2A, B). These were consistently included in the higher ranked models predicting attitudes, see Appendix 1.

People in KCA had more positive attitude towards the carnivores (Figure 2C, D): approximately 80% of respondents reported the highest attitude class concerning snow leopards compared to fewer than 20% in the other two study areas. A similar trend was observed for the Himalayan wolf: while hardly any respondent reported the highest attitude score in the other two study areas, close to 20% did in KCA. Also, men had more positive attitude towards the carnivores (Figure 2E, F). Agro-pastoralists had more negative attitudes towards both carnivores than respondents pursuing other occupations (Figure 2G, H). While for Himalayan wolves, responses were evenly distributed among the response classes, they were more skewed towards the positive attitude classes for snow leopards.

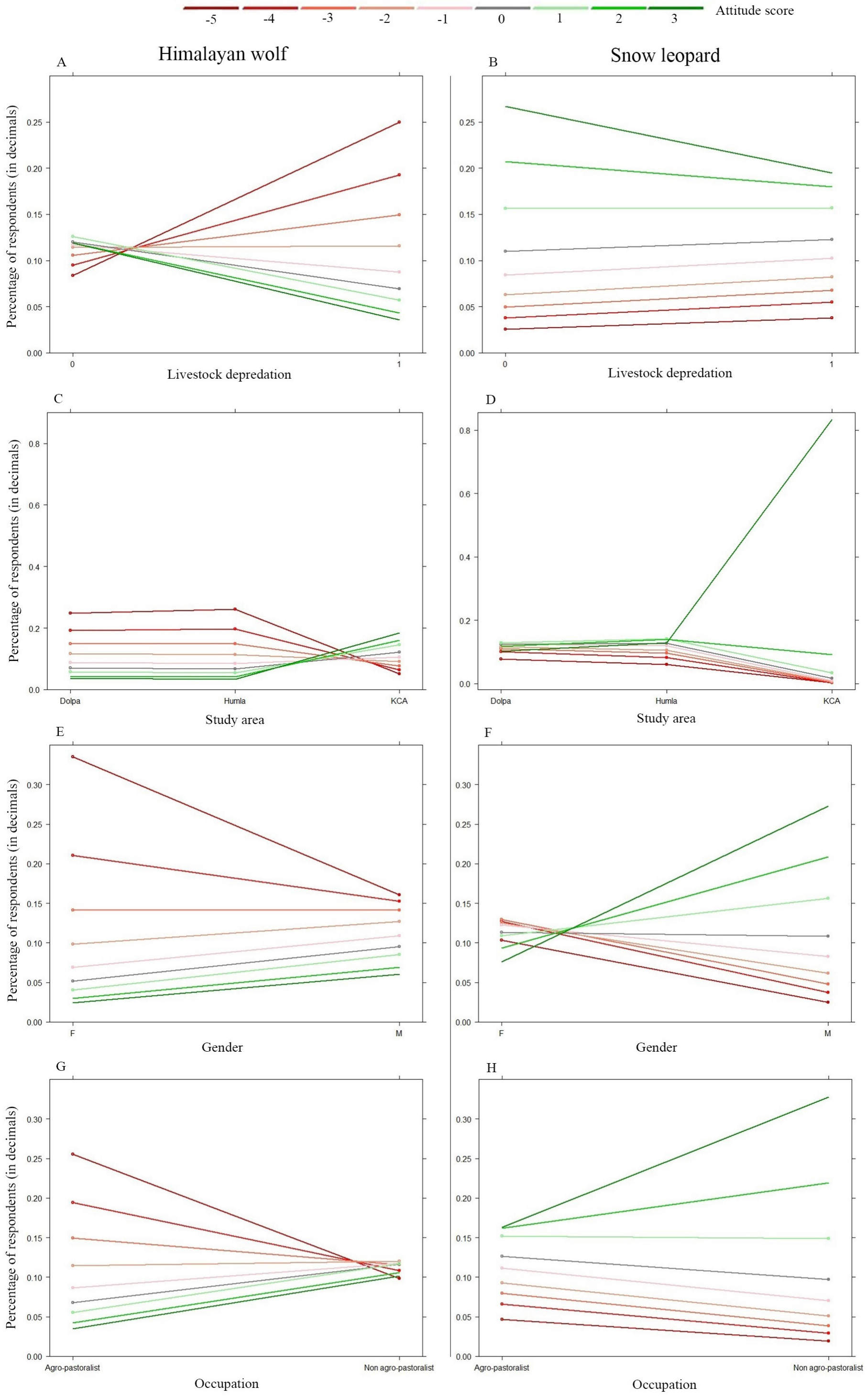

Figure 2. Effect plots illustrating the influence of predictors on attitudes towards wolves and snow leopards. A) and B) illustrate the effects of livestock depredation. Livestock depredation resulted into a clear negative attitude towards wolves but not for snow leopards. 0= livestock depredation not reported and 1= livestock depredation reported. C) and D) illustrate the effects of ‘study area’. People in KCA have a more positive

attitude towards the carnivores than in Dolpa and Humla. E) and F) illustrate the effects of 'gender'. Men have more positive attitude towards the carnivores than the women. M=Men and F= Female. G) and H) illustrate the effects of 'occupation'. Agro-pastoralists have more negative attitudes towards both the carnivores. 'yhw' represents ordinal attitude scores for Himalayan wolf while 'ysl' represents ordinal attitude scores for snow leopard. +3 represent the most positive attitude recorded while -5 represent the most negative attitude recorded.

Improving the conflict

Self-guarding of livestock was common in upper Humla (83.3%, n=25) and upper Dolpa (53.6%, n=15), while guard dogs were commonly used only in KCA (76.9%, n=10). Livestock night corrals were also reported as important in all study areas. Respondents considered guarding by a herder and use of corrals as the two most important measures to prevent livestock depredation (see Figure 3 for other measures used to prevent livestock depredation in the respective study areas). Additional methods mentioned by the respondents to improve the situation were: enclosing livestock corrals with nets, improving livestock corrals by building higher walls, installing flashing lights as visual deterrent, a siren that notifies of an approaching carnivore, and using recorded human voices as acoustic deterrent. Respondents highlighted two items as being essential prerequisites if they were to commit to carnivore conservation: construction materials to build stronger corrals (18.4%, n=14) and compensation equivalent to total loss (17.1%, n=13).

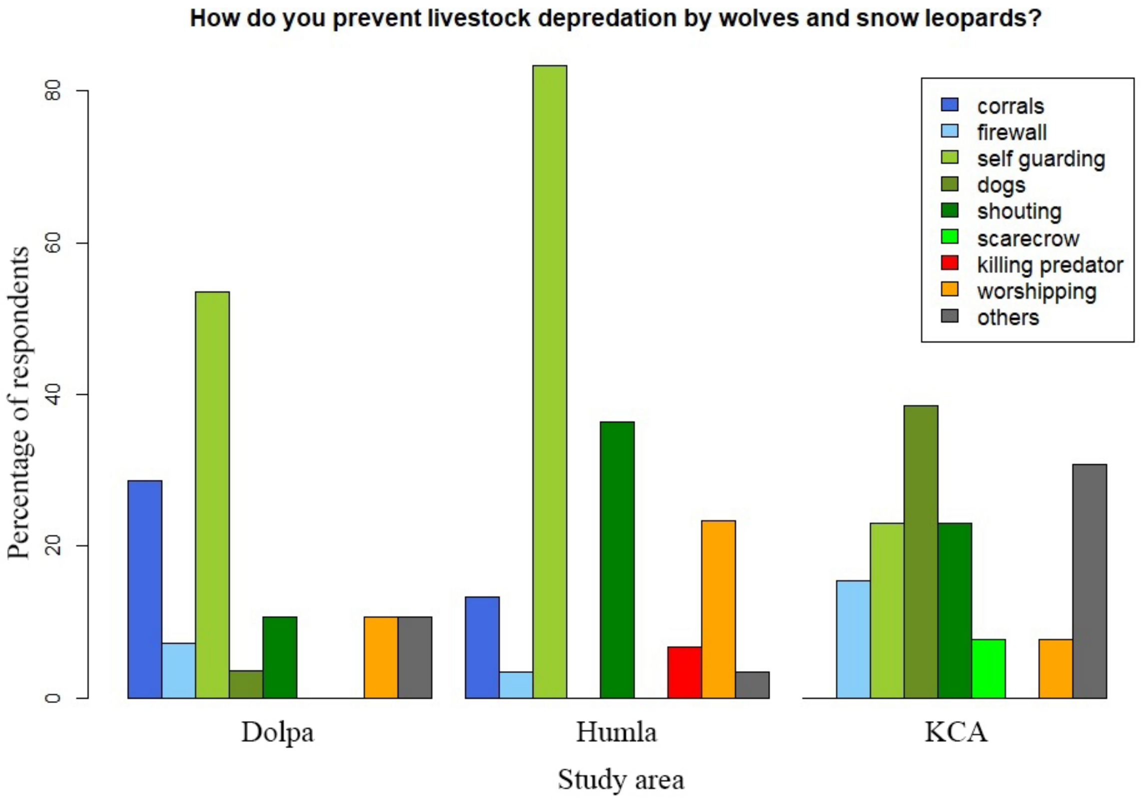

Figure 3. Measures applied for preventing livestock depredation in Humla, Dolpa and KCA. The reported methods include livestock corrals for night, use of guarding dogs, firewalls near corrals, self-guarding by herders, use of scarecrows, shouting and others (keeping all livestock together, avoiding depredation hotspots, using light system for night).

Table 3. Livestock economics showing average economic loss to Himalayan wolf and snow leopard in upper Humla, upper Dolpa, upper Mustang and KCA during 2014-2016.

		Average # of livestock lost (and its monetary value in USD) per household towards:		
		Himalayan wolf	**Snow leopard**	
Study area	Livestock type			Average price (USD) per head
Upper Humla	Yak	0.1 (75)	0.3 (225)	750
	Horse	0.2 (175.6)	0	878
	Goat & sheep	0	0.5 (247.5)	495
	Jhoppa	0	0	720
	Cattle	0	0	360
	Total Loss (USD)	250.6	472.5	
Upper Dolpa	Yak	0.7 (624.4)	0.5 (446)	892
	Horse	0.4 (679.6)	0.3 (509.7)	1,699
	Goat & sheep	1.6 (249.6)	3.1 (483.6)	156
	Jhoppa	0.1 (67.5)	0	675
	Cattle	0.1 (36.8)	0.04 (14.7)	368
	Total Loss (USD)	1657.9	1454.0	
KCA	Yak	2.6 (2399.8)	3.4 (3138.2)	923
	Horse	0	0	
	Goat & sheep	0.4 (33.2)	0.6 (49.8)	83
	Jhoppa	0.2 (154.6)	0	773
	Cattle	0	0	390
	Total Loss (USD)	2,588	3,188	

Table 4. Problem carnivore ranking for wolves and snow leopards according to the study areas. Figures in parentheses represent the percentage of respondents complimented by their numbers who reported a specific carnivore as the main problem carnivore.

Study area	Main problem carnivore
Upper Humla	Snow leopard (73.33%, n=22)
Upper Dolpa	Snow leopard (60.71%, n=17)
KCA	Wolf (76.92%, n=10)

Table 5. GLMM of ordinal attitude response towards a) Himalayan wolf and b) snow leopard, in upper Humla, upper Dolpa and KCA.

a) Himalayan wolf				
Model-averaged coefficients: (conditional average)				
Predictor	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
Livestock attacked by a Himalayan wolf	-1.30873	0.60258	2.172	0.029866*
Occupation (Non Agro-pastoralist)	1.18833	0.56456	2.105	0.035303*
Gender (M)	1.08325	0.57486	1.884	0.059516.
Study area (Humla)	0.07514	0.88891	0.085	0.932639
Study area (KCA)	1.82177	0.97961	1.860	0.062930.
Compensation scheme	0.57465	1.86629	0.308	0.758151
b) Snow leopard				
Model-averaged coefficients: (conditional average)				
Predictor	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
Occupation (Non Agro-pastoralist)	0.9536	0.5198	1.835	0.066564.
Gender (M)	1.5891	0.5712	2.782	0.005403**
Study area (Humla)	0.3789	0.6255	0.606	0.544685
Study area (KCA)	3.7957	0.8870	4.279	1.87e-05***
Livestock attacked by a snow leopard	-0.3640	0.7588	0.480	0.631446
Compensation scheme	-0.3222	1.4810	0.218	0.827753

· = $p \leq 0.1$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

Discussion

Villagers in the Himalayas of Nepal receive little support, either infrastructural or governmental, to help them face the material and financial consequences of coexisting with wolves and snow leopards. The combined circumstances of their dependence on livestock for livelihood, absence of support or adequate mitigation measures, and livestock losses all contribute to foster negative attitudes towards carnivores. The enthusiasm for conserving large carnivores amongst more affluent cross-sections of international society that rarely experience them in person (Macdonald et al., 2015) is generally not shared by those with first-hand experiences of livestock losses to carnivores. In the Himalayas of Nepal this understandable hostility has, in the case of snow leopards, been reduced by respectful attention to cultural and religious mores, but there has been no such attention to wolves. Comparison of local attitudes between our study areas revealed the greatest tolerance of carnivores in KCA, the only area that has adopted a community-owned conservation approach; this suggests to us that this approach should be trialled elsewhere.

Crucially, we found that while respondents in all study areas had more positive attitudes towards snow leopards than towards wolves, the impact on their attitudes of losing livestock was different between the two carnivores: it little affected attitudes to snow leopards, but radically worsened that towards wolves. This higher tolerance of snow leopards prevailed despite higher depredation by snow leopards than by wolves (Table 3). We see a combination of reasons responsible for this, including decades of conservation work to raise awareness for the snow leopard and financial support for reducing depredation conflict, and a high cultural and religious status of snow leopards; also reported by Suryawanshi et al., (2014) and Bhatia et al., (2017) in the Indian Himalayas and Alexander et al., (2015) in China. We interpret this as evidence that the iconic status of the snow leopard in local cultures may foster tolerance, and this hypothesis draws attention to the considerable relevance of religious influences on conservation (Gosler et al., 2013) remembering the respect in which snow leopards are held by Buddhism, the predominant religion in the region. For example, folklore in upper Dolpa holds that prominent Buddhist monks disguise themselves as snow leopards when visiting the Tibetan Plateau in search of rare medicinal herbs (Ale & Karky, 2002). Furthermore, considering their crepuscular nature, snow leopards are rarely seen, so another non-exclusive hypothesis for tolerance towards them might reflect the adage 'out of sight, out of mind'. And wolves are widely disliked (Dressel et al., 2015). Folkloric portrayals of wolves often characterise them as evil (Macdonald, 1987; Dickman, 2010), and the European psyche is surely touched by the childhood influence of *Little Red Riding Hood*. The prevalent attitude amongst monks in our survey, that snow leopards (but not wolves) should be protected also reflects the dislike for wolves. Kellert et al., (1996) speculate that animosity towards wolves may generally be enhanced by behaviours such as howling, pack living, greater diurnal visibility, and easily detectable denning sites (which, it occurs to us, may be associated with incriminating prey remains). The lower conservation status, for example on the IUCN Red List, of grey wolf *Canis lupus* as Least Concern (Mech & Boitani, 2010), compared to the snow leopard as Vulnerable (McCarthy et al., 2017), may affect capacity, or perceived need, to raise funds for their conservation (Courchamp et al., 2006; Suryawanshi et al., 2014). We foresee the wolf's standing in the Himalayas deteriorating, and persecution intensifying as a consequence of socio-economic changes in Asia, associated with increased global demand for cashmere, that is replacing large bodied livestock with smaller bodied cashmere-producing goats that are more vulnerable to wolf attacks (Namgail et al., 2007).

Agro-pastoralists, considering their dependence on livestock, were clearly, and unsurprisingly, more negative towards both wolves and snow leopards. They reported monetary loss due to livestock depredation equivalent to 15.8% of Nepalese per capita income in upper Humla, 125.2% in KCA and 67.5% in upper Dolpa. Per capita income of Nepal between 2014-2016 varied from 2,266-2,298 USD (World bank, 2018). While the losses in upper Humla are comparable to earlier studies in similar landscapes of Nepal (Oli et al., 1994) and Bhutan (Wang & Macdonald, 2006), they are much higher for upper Dolpa and KCA. The possibilities cannot be ruled out that some respondents could have misidentified a different carnivore species responsible for each predation event as either snow leopard or wolf and that some might have attributed deaths due to disease to carnivore depredation (Li et al., 2015) leading to overestimation of depredation losses. There are also chances that livestock that died due to reasons like falling off a cliff or during natural disasters like blizzards could have been scavenged by the predators which were wrongly considered as depredation by the respondents

(Liu & Jiang, 2003). More importantly, the loss reported in KCA appears unrealistic; the monetary values were possibly exaggerated to take advantage of the funding provided by WWF Nepal. Unfortunately, we could not access any recorded data to validate the reports to account for the recall bias of the respondents. However these potential errors of self-reporting do not invalidate the importance of our findings that are reflective of the locals' perceptions (Li et al., 2015). One might expect a general hostility between stockmen and carnivores, and certainly the negative attitudes we report are mirrored in those of ranchers in the USA (Agarwala et al., 2010), Norway (Bjerke et al., 2001), and Brazil (Marchini & Macdonald, 2012).

Men were more positive than women in their attitudes towards the carnivores. A possible explanation is that men generally migrate from our study villages for seasonal work in nearby cities (in TAR) during the summer herding season. Consequently, they may a) have less first-hand experience (plus responsibility and threat) than women of livestock depredation (Bickley et al., 2019) and b) be more exposed to a wider spectrum of attitudes to nature. An additional factor may be that in the Himalayas of Nepal women have less contact with conservation agencies compared to men (Gillingham & Lee, 1999). Insofar as this is detrimental to women's attitudes to conservation, Byers & Sainju (1994) make the proposal that women's education is a priority.

Of our three study areas, respondents from KCA had the most positive attitudes towards carnivores. Management and ownership of this conservation area belongs to the local communities which are adequately supported by both governmental and non-governmental organizations. Features of the human-carnivore relationship conspicuously present in KCA are a community-based livestock insurance scheme, and promotion of conservation awareness. These interventions appeared to have fostered recovery of snow leopards and their wild prey, blue sheep (Gurung et al., 2011), together with an increase in carnivore diversity. Wolves reappeared in the area in 2013 after an absence of 25 years (Subba et al., 2017), while in 2017, brown bear, never recorded previously in the area, were caught on camera-traps set by local citizen scientists (Tseten Sherpa, pers. comm. 2017).

Farhadinia et al., (2017) mention that dislike for wolves in Iran has the effect of eroding goodwill for the threatened Persian leopard *Panthera pardus saxicolor*. This suggestion arose because people losing livestock to wolves tended to have negative attitudes towards leopards, although wolves were responsible for greater economic loss in that study. Our findings offer a different perspective insofar as across the Himalayan landscape most carnivore conservation activities focus solely on snow leopards and thus it is only snow leopards that bring benefits to offset damage. Indeed, respondents in KCA stated that they would protect recolonising wolves only if the KCA Management Council altered the livestock insurance scheme to provide compensation for livestock depredation by wolves comparable to that for snow leopards.

Carnivore conservation interventions focused on a single species are not beneficial for the protection of an entire ecosystem, particularly if they fail to educate people about the interrelatedness of different species and the importance of biodiversity for ecosystem resilience. A snow leopard focused conservation approach as practiced currently, has motivated local people to protect snow leopards and blue sheep only, while ignoring the rest of the wildlife community. Clearly, conservation of the entire carnivore guild is essential for the functionality of the natural community, and the ecosystem services it delivers (Wolf & Ripple, 2017). Building on our earlier studies (Werhahn et al., 2017a; Kusi et al., 2018b), we re-emphasise that damage relief payments should urgently be extended to all carnivores depredating livestock. A legal framework (GON, 2015) for this is already in place in Nepal but awaits implementation.

Livestock depredation is worsened in our study areas because husbandry is often lax during herding seasons (Suryawanshi et al., 2013) which, exacerbating the threats, coincide with the breeding seasons of the Himalayan wolf (Feb-Jun) (Werhahn, et al., 2017a) and snow leopard (Jan-Jun) (McCarthy et al., 2017). Our respondents offered suggestions for improving herding practices, such as keeping livestock in larger herds attended by sufficient herders and avoiding known predation hotspots; that these obvious measures are in the villagers' minds, and yet not implemented is a symptom of the cognitive dissonance characteristic of human-wildlife conflict (see also Mijiddorj et al., (2018)). Herders' declared need for training can be met by demonstration of the construction of robust, durable, predator-proof, livestock corrals used to protect herds at night using locally available stones to build high walls in combination with wire netting especially for the open top (see Jackson & Wangchuk, 2001). Evidence based and, increasingly, experimentally validated, livestock corrals customised to local circumstances are now widely described and implemented worldwide (Loveridge et al., 2017) and there is a widespread and growing practical literature on the various interventions (flashing lights, sound deterrents, sirens, livestock guarding dogs) that may further mitigate risk. Insofar as no solution is infallible, it is often the case that a combination of methods is likely to be more effective than any one by itself (Espuno et al., 2004; Zabel & Roe, 2009). Irrespective of the availability of financial incentives, lax livestock guarding needs to be appropriately handled because a better vigilance by livestock owners and herders is required to sustainably live with carnivores. An integrated system of incentives and disincentives, that involves co-responsibility between pastoralists and conservation practitioners, is likely to better address the root causes of depredation. Participatory engagement of villagers by strengthening their empowerment, skills and sense of pride in living with the carnivores, can help bridge this gap (Jackson et al., 2010; Jackson & Lama, 2016; Mishra et al., 2017).

Livestock insurance schemes can provide a strong incentive to improve husbandry (Mishra et al., 2003), but so far are uncommon in the Nepalese Himalaya (Chetri et al., 2019). Where they have been trialled, the monetary compensation has often been insufficient, as indicated in KCA where many herders were unwilling to participate in the local livestock insurance scheme because the compensation offered for the loss to a snow leopard of a yak worth USD 923 (in 2016) was as little as 7.6 % (= USD 70) of the animal’s market value. Unsurprisingly, herders expect compensation equivalent at least to the market cost of a replacement calf. Overall compensation payments (especially those that do not involve community investments), have proven ineffective in changing behaviour or attitudes (Naughton-Treves et al., 2003; Agarwala et al., 2010) which ask all conservation interventions to work towards improving community investments. Payments to Encourage Coexistence (PEC) are more promising (Dickman et al., 2011). Compelling examples come from Sweden where villages are paid for every certified carnivore reproduction in the reindeer grazing grounds (Zabel & Uller, 2008) and from Mexico where ranchers are paid for recording large carnivores in camera traps placed on their lands (Nelson, 2009). Combining PEC methods with incentives such as sales of handicrafts (Mishra et al., 2003) or home stays (Jackson & Wangchuk, 2004) and livestock vaccination should be considered for the Himalayas as they may be better suited to improve attitudes towards carnivores in the region.

There is no hope of eradicating livestock depredation unless wild carnivores have an adequate supply of wild prey (Werhahn et al., 2019). Furthermore, there is an interaction between the densities of wild and domestic prey, with the former facing competitive exclusion by the latter (Berger et al., 2013; Karimov et al., 2018). Conservation is an increasingly holistic matter, requiring transdisciplinary knowledge (Macdonald, 2019). So the coexistence of large carnivores, domestic stock and wild prey requires understanding from biology, sociology and agriculture, for example at the interface of ensuring the continuation of the traditional practices of rotational grazing (Kusi et al., 2018b). Indeed, wildlife conservation in the Himalayas, as elsewhere, should develop an integrated multi-species plan, engaging with and mindful of local human communities and the fostering of coexistence.

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Eurasian lynx and Pallas's cat in Dolpa district of Nepal: genetics, distribution and diet

The Eurasian lynx *Lynx lynx* and the Pallas's cat *Otocolobus manul* are elusive felids which are little studied in the Himalayas of Nepal where they seem to occur at low densities. We present mtDNA genomic and dietary data of six Eurasian lynx scats and one Pallas's cat scat collected in Dolpa district of Nepal. The Eurasian lynx scats were found in alpine shrub lands at elevations from 4,738 - 4,818 m. Dietary analysis based on microscopic frequency of occurrence revealed that the six Eurasian lynx scats contained 56 % woolly hare *Lepus oiostolus*, 17.7 % pika *Ochotona* sp. and rodent *Alticola* sp., 9.7 % Himalayan marmot *Marmota himalayana*, 7 % vegetation and 3.3 % domestic goat *Capra aegagrus hircus*. The Pallas's cat scat found in a rocky boulder field contained 76 % pika hair, 18 % woolly hare hair and 4 % vegetation. This study provides the first scientific record of Eurasian lynx in Shey-Phoksundo National Park SPNP. The finding on Pallas's cat represents an expansion of the IUCN distribution range in Nepal, presents the second locality record of the species in the country, and with the sample collected at 5,539 m is the highest elevation record for the species to date. Further researches into population status, ecology, genetics and conservation of these elusive felids in the Himalayas are recommended.

The field work for this study was conducted in the arid high Himalayan landscape of upper Dolpa (29°-29°34’48’’ N / 82°53’24’’-83°26’24’’ E) at elevations ranging from 3,600 m - 5,600 m. The study area comprised alpine grassland and alpine steppe habitats (Miehe et al. 2016). In search of predator signs an area of approximately 1,088 km^2 was covered by walking 810 km between May to July 2016. The genetic analyses of the collected predator scats were conducted at the Center for Molecular Dynamics-Nepal with the genetics protocol developed by The Royal Zoological Society of Scotland WildGenes Laboratory (Werhahn et al. 2017). For species identification, samples were amplified and sequenced at the cytochrome *b* region of the mitochondrial DNA (generic MCB primer pairs MCB_398_F:TACCATGAGGACAAATATCATTCTG and MCB_869_R:CCTCCTAGTTTGTTAGGGATTGATCG; Verma & Singh 2003). The lynx samples were also amplified and sequenced at the D-loop region of the mitochondrial DNA (mtU: CTTTGGTCTTGTAAACCAAAAAA; and R3: TAAGAACCAGATGCCAGGTA; Rueness et al. 2003). The sequences were then examined with Geneious version 9.0.5 and compared with available sequences on the NCBI GenBank database using the Basic Local Alignment Search Tool BLAST to identify the species.

The microscopic diet analysis was conducted with a microscope (Bresser Science TRM-301,40x-1000x) applying a modified point-frame method: 50 hairs closest to the intersections in a gridded tray per scat were identified based on cuticular cell arrangements, medullary patterns and relative lengths with a reference collection and literature (Teerink 1991, Ciucci et al. 2004, Bahuguna et al. 2010, Klare et al. 2011). We then used the method frequency of occurrence per food item to provide qualitative dietary results (Klare et al. 2011).

Eurasian lynx: Results and Discussion

Six genetically verified scats of Eurasian lynx were found in alpine shrub and grasslands in two areas in upper Dolpa: three scats were found southwest of Bhijer village (29°23’42’’ - 29°26’9.6’’ N / 82°56’45.6’’-82°59’2.4’’ E; 4738 - 4818 m), and three scats east of Koma village (29°24’25.2’’-29°26’56.4’’ N / 83°7’22.8’’ - 83°8’24’’ E; 4330 - 4645 m; Fig. 1 & 2). The generated Eurasian lynx cytochrome *b* mtDNA sequences were 311 bp long, and all six scat sequences belonged to the same Eurasian lynx cytochrome *b* haplotype already deposited on NCBI GenBank, i.e. identity overlap of 100% with accessions KR132581 and

Fig. 1. Eurasian lynx (light blue) and Pallas's cat (dark blue) scat collection locations in Dolpa district, Nepal (modified from Himalayan Map House (2010)).

Fig. 2. Eurasian lynx habitat in the Himalayas of Dolpa, Nepal. Alpine shrubland at ≥ 4400 m above sea level (Photo G. Werhahn).

KM982549 (Paijmans et al. 2016), KF990332 (unpublished, originating from Mongolia) and KP202283 (Li et al. 2016). The 466 bp long D-loop mtDNA sequence of the five successfully amplified samples presents a new haplotype with 99.6 % identity overlap with accessions EU818861, EU818858 and EU818856 (which all belong to the South clade in Rueness et al. 2014); and 99.4% identity overlap with accession KR919624 (Ning et al. 2016). This Eurasian lynx D-loop haplotype newly found in Dolpa of western Nepal is deposited in GenBank under the NCBI accession MF997606.

The frequency of occurrence analysis of these six Eurasian lynx scats revealed a dietary content of 56% woolly hare hair, 17.7% pika and rodent Alticola sp. (hair, bones, jaw, and claw), 9.7% Himalayan marmot hair, 7% vegetation, 3.3% debris, 3.3% domestic goat hair and 3% unidentifiable bone parts.

Globally the Eurasian lynx is classified as Least Concern by the IUCN Red List and the subspecies reported for the Himalayas is *Lynx lynx isabellinus* (Kitchener et al. 2017). The IUCN national Red List series of Nepal classifies the Eurasian lynx as Vulnerable in the country due to indications for decreasing population trends (Jnawali et al. 2011). Only infrequent Eurasian lynx observations and reports are available, and very little is known about population size, distribution and the genetics of this elusive species in Nepal (Jnawali et al. 2011, Breitenmoser et al. 2015). Major threats to the Eurasian lynx in the Himalayas are persecution and retaliatory killings in response to livestock depredation and poaching for its fur, and these threats are likely to be also in effect across the border in China (Din & Nawaz 2010, Jnawali et al. 2011). Local people around Koma and Bhijer village have reported occasional livestock depredation incidences by lynx during social surveys conducted for this study. We recommend to include the Eurasian lynx in the legal framework of the wildlife damage relief guidelines of Nepal (GoN 2015) and in other conservation payment schemes which are locally already in place for snow leopards *Panthera uncia*, to encourage coexistence with predators in the Himalayan high-altitude habitats (Dickman et al. 2011, GoN 2015). We also recommend further researches into the population status, ecology, conservation status, and phylogeny of the Eurasian lynx in the Himalayas (Jnawali et al. 2011, Rueness et al. 2014).

Pallas's cat: Results and Discussion

A genetically verified Pallas's cat scat was collected at 5,593 m (29°18'43.2'' N / 83°25'33.6'' E) in barren rocky habitat (Figs. 1, 3, 4) towards northeast of Tinkyu village of upper Dolpa. The generated 352 bp long mtDNA sequence matched with mitochondrial DNA sequences of *Otocolobus manul* deposited in GenBank (identity overlap of 99.7% with KR132585 published in Paijmans et al. (2016), 99.0% with KP202295 originating from Mongolia and published in Li et al. (2016), and 99.0% with KF990330 (unpublished, originating from Mongolia). Matches to other felid species were at much lower identity overlap (i.e. the next closest thereafter was < 92% identity overlap with Bay cat *Catopuma badia*). This Pallas's cat sample represents a new haplotype, not previously submitted to NCBI GenBank, and is available under the NCBI accession MF997607. The revised taxonomy of felids mentions the Pallas's cat subspecies *Otocolobus manul nigripectus* to be found in the Himalayas (Kitchener et al. 2017) which our haplotype is likely to represent.

The optimal Pallas's cat habitat is reported to be a mix of grassland and shrub steppe with rocky cover, ravines and hill-slopes (Ross et al. 2016). Hence in addition to the genetic verification, habitat and prey availability were also supportive of the scat originating from a Pallas's cat: we found the Pallas's cat scat in a very rocky hillslope within montane grassland steppe, with pikas regularly sighted among the rocks (Fig. 4). This Pallas's cat scat contained 76% pika hair, 18% woolly hare hair, 4% vegetation, and 2% debris.

The presence of the Pallas's cats in the Himalayas of Nepal was documented for the first time in 2014 through camera trap images from the Annapurna Conservation Area in Manang at two locations, namely Thorkya (4,200 m) and Angumila Lapche (4,650 m; Shrestha et al. 2014). The historical presence of the species in the area is suggested by a pelt photographed in the year 1987 in a household in Nyesyang valley in Manang district (Lama et al. 2016) . Our record of the species from Dolpa district holds significance for Pallas's cat conservation in Nepal in that it provides the second locality record situated 90 km northeast of the first documented location in Manang. This implies the existence of a population, presumably at low density, across a larger range than previously known in the country and it also extends the known IUCN global distribution for the species westwards in Nepal (Ross et al. 2016; Fig. 3).

The Pallas's cat is globally classified by the IUCN Red List as Near Threatened. The global population trend is decreasing with main threats being habitat degradation, hunting for fur and depletion of prey base (Ross et al. 2016). The Pallas's cat is known to inhabit montane grassland and shrub land steppe of Central Asia with records up to 5,050 m in the northwestern Tibetan Plateau of China (Fox & Dorji 2007, Ross et al. 2016). This Pallas's cat record at 5,539 m provides the new highest elevation record of the species to our knowledge. However climate, habitat and prey base appear to be the main determinants for its presence (Ross et al. 2016).

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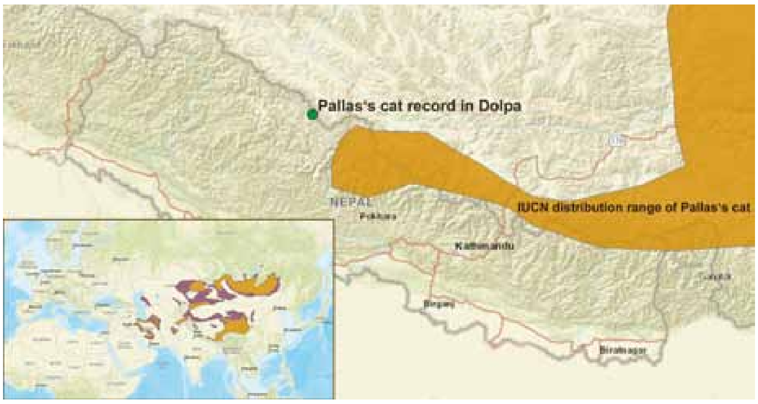

Fig. 3. Pallas's cat scat location in Dolpa district of Nepal (green dot) and the current IUCN distribution (orange: range; violet: possible range; map modified from Ross et al. (2016)).

Fig. 4. Pallas's cat habitat in rocky landscape at 5,593 m above sea level in Dolpa, Nepal (Photo G. Werhahn).

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Monogamy: cause, consequence or corollary of success in wild canids?

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Abstract

The Canidae are successful, being a widespread, abundant, speciose, and adaptable family. Several canids in particular have recently experienced rapid expansions in range and abundance, with similar situations mirrored on several continents by different species. Despite extreme behavioural diversity between and within species, monogamy is a common denominator in canid societies. In this review, we ask why canids are monogamous and how monogamy is related to their success. We begin with an overview of canid social monogamy, describing the pair bonding, paternal care, and often alloparental care that is characteristic of the family, and discuss theories on the evolution of mammalian social monogamy. We discuss why and how monogamy is maintained in canids, either voluntarily or enforced, and how ecological conditions influence either the functional advantages of monogamy or ability for enforcement and thus whether social monogamy is maintained. Social monogamy does not necessitate exclusive mating and many canids exhibit extra-pair paternity. We consider the costs and benefits of extra-pair mating for male and female canids and how ecological conditions can shift this cost/benefit balance and thus affect its prevalence. Monogamy may be responsible for many of the unusual canid reproductive characteristics through facilitating alloparental care and monogamy enforcement, and the domestic dogs' departure from monogamy supports our interpretation that it is an adaptation to resource availability. In asking whether monogamy is responsible, at least in part, for their success, we propose the *monogamy as pro-cooperative hypothesis*, suggesting four characteristics have con-

tributed to canid success: 1) ecological flexibility, 2) high mobility, 3) high reproductive rates, and 4) sociality/cooperation, with the latter two being consequences of monogamy. These four interconnected traits enhance one another and it is their combination, with monogamy at its foundation enabling cooperative sociality and thereby enhanced reproduction and survival, that together comprise the formula of canid success.

Introduction

Of their many notable attributes, three stand out about the Canidae: first, they are remarkably similar; second, they are remarkably different; and third, they are remarkably successful.

First, the similarity lies in the anatomical and behavioural traits that makes all 37 species of the family – from fennec fox (*Vulpes zerda*) to grey wolf (*Canis lupus*) – immediately recognizable as dogs (for a *dramatis personae* see Macdonald and Sillero-Zubiri, 2004). Not only are the largest species essentially morphologically inflated identikits of the smaller ones, but their expressions and demeanours are similar, as are their societies, all built around monogamy (Macdonald et al., 2004). Social monogamy is unusual amongst mammals, adopted by only 3-9% of Mammalia species and 16% of Carnivora species (Kleiman, 1977; Lukas and Clutton-Brock, 2013), yet is found in all canid species studied to date. In no other mammalian family is the pair bond so ubiquitous. In an overview of carnivore societies, Macdonald (1992) emphasized the phylogenetic descent that weaves canidness and monogamy throughout the family (as it does similarly for felidness and polygyny throughout the Felidae) (Macdonald and Kays, 2005).

Second, the canid family is highly diverse. Body sizes range from the 0.8 kg fennec fox to the 60+ kg grey wolf (Nowak 2005). Their diets range from the almost exclusively insectivorous (e.g. bat-eared fox [Otocyon megalotis]) to almost exclusively carnivorous (e.g. African wild dog [Lycaon pictus], bush dog [Speothos venaticus], Ethiopian wolf [Canis simensis], Marino et al., 2010), with a full spectrum of omnivory between (e.g. red fox [Vulpes vulpes], golden jackal [C. aureus], African golden wolf [C. lupaster]). Such extremes in diets are reflected in their dentition, because while most canids have 42 teeth well suited for generalist diets (e.g., carnassials for shearing flesh and molars for omnivory), bat-eared foxes have up to 50 less-specialised teeth (the most of any land mammal) for extreme insectivory (Klare et al., 2011), whereas the dholes, bush dogs, and African wild dogs have reduced or absent molars and enhanced carnassials for hypercarnivory (Van Valkenburgh, 1991). Canids are found in nearly all terrestrial habitats, including such extremes as Arctic tundra (Arctic fox [Vulpes lagopus]), desert (fennec fox), tropical forest (dhole [Cuon alpinus]), high-altitude environments (e.g. Himalayan wolf [C. [lupus] himalayensis], Werhahn et al., 2017, 2018; Ethiopian wolf, Marino, 2003) and human cities (e.g. coyote [C. latrans]). Some even partially exploit aquatic (e.g. short-eared dog [Atelocynus microtis], Oliviera, 2009; British Columbia coastal wolf, Darimont and Pacquet, 2002; Stronen et al., 2014) and arboreal (e.g. gray fox [Urocyon cinereoargenteus], Trapp and Hallberg, 1975) habitats. Canid social systems range from generally solitary species like the maned wolf (Chrysocyon brachyurus), where pairs share a territory but associate only during the mating season (Dietz, 1984), through the spatial groups of red and Arctic foxes (e.g. Hersteinsson and Macdonald, 1982), to species forming large, complex packs (e.g. African wild dogs, Creel et al., 2004; Ethiopian wolves, Sillero-Zubiri and Gottelli, 1995a; bush dogs, Macdonald, 1996; grey wolves, Jędrzejewski et al., 2005). Even within species, canids exhibit substantial variation (Macdonald and Moehlman, 1982; Moehlman, 1989; Creel and Macdonald, 1995; Geffen et al., 1996; Moehlman and Hofer, 1997) and variability within a species may be larger than that between species (Macdonald and Moehlman, 1982; Creel and Macdonald, 1995). For example, grey wolves, coyotes, black-backed jackals (Lupulella mesomelas) and red foxes may live solitarily, in pairs, or in large groups (Kleiman and Brady, 1978; Messier and Barrette, 1982; Mech and Boitani, 2003; Baker and Harris, 2004; Kamler et al., 2019). Red fox home ranges vary between populations by three orders of magnitude, their societies varying between ubiquitous socially monogamous pairs and spatial groups of six adults (Macdonald, 1981), while grey wolves occupy ranges varying from 75-2,500 km2 with groups varying from pairs to packs of up to 42 (Mech and Boitani, 2003). Arctic foxes can exist as a “coastal” ecotype, feeding on temporally stable seabirds and marine resources and consequently living a moderate lifestyle with litters averaging five cubs produced yearly. Alternatively, arctic foxes can exist as a “lemming” ecotype, feeding on rodents with extreme cyclic population variations and consequently mirroring this extreme lifestyle, producing litters of up to 18 cubs during rodent peaks and rarely reproducing during years of low prey availability (Tannerfeldt and Angerbjörn, 1998). Though they often exist as a single breeding pair, they may form large social groups of up to 31 individuals, referred to as “fox towns” (Elmhagen et al., 2014). Ethiopian wolves, Afroalpine specialists, also display such plasticity: pairs with large territories dominate in low productivity environments, while packs of up to 18 adults/subadults defend small territories in optimal habitats (Sillero-Zubiri et al., 2004, Marino et al., 2014).

Third, canids are successful, both over evolutionary and modern times. Evolutionarily, canids usurped Hyaenidae from the dog-niche in the Pliocene (Macdonald, 1992). Nowadays, many species of canids flourish alongside humanity. A canid currently claims the title of the world's most widely distributed non-domestic terrestrial mammal: the red fox (Macdonald and Sillero-Zubiri, 2004), who usurped this title from another canid, the grey wolf (Mech, 1995; Macdonald and Sillero-Zubiri, 2004), while free-ranging domestic dogs are found across the globe (Lord et al., 2013). Several canids have recently rapidly expanded their ranges: coyotes have become ubiquitous across North and Central America over the past two centuries (Gompper, 2002; Macdonald and Sillero-Zubiri, 2004), while golden jackals (Tóth et al., 2009; Rutkowski et al., 2015) and raccoon dogs (Helle and Kauhala, 1991; Kauhala and Saeki, 2004; Sutor, 2007; Kauhala and Kowalczyk, 2011) mirror this same rapid expansion across Europe. Following introductions of red foxes to Australia and eastern North America, they rapidly spread throughout most of continental Australia and USA (Kamler and Ballard, 2002). Despite intensive human efforts to control populations of red foxes, coyotes, golden jackals and free-ranging domestic dogs, these species continue to survive and thrive. Even grey wolves, driven to the brink of extinction in the 1800s, are now returning to their former range in North America and Europe (Mech 1995; Breitenmoser, 1998; Wydeven et al., 1998; Phillips et al., 2004; Mech, 2017). That these expansions are mirrored across several parts of the world raises the question of what has allowed these canids to achieve such wide distributions and high abundances, rapid expansion, colonization and biological invasion, and resilience to human population control.

Together, these canid attributes—similarity resulting from evolutionary conservatism and differences from behavioural flexibility—prompts the question of what are the limits to variation in each species, and if those limits differ between species, what evolutionary constraints have set them? And since the unusual common denominator of canid societies is monogamy, why are they monogamous and what role has this played in the family's success, both in evolutionary time and the Anthropocene?

Monogamy has long been an area of interest for evolutionary and behavioural ecologists (Orians, 1969; Emlen and Orling, 1977) and numerous early hypotheses attempted to explain its evolution and maintenance (e.g. Orians, 1969; Emlen and Orling, 1977; Wittenberger and Tilson, 1980). Decades of empirical and theoretical research suggest factors affecting monogamy's origin and maintenance can be complex, differ among taxa, and are subject of a constant evolutionary interplay between monogamy and associated traits (Klug, 2018; Lambert et al., 2018). However, most research on monogamy has focused on birds (Reichard and Boesch, 2003), unsurprisingly since approximately 90% of bird species exhibit social monogamy. In contrast, 95-97% of studied mammals are polygamous (i.e. polygynous, polygynandrous, and, less commonly, polyandrous) (Kleiman, 1977; Lukas and Clutton-Brock, 2012) – Canidae are one of the main exceptions (Kleiman, 2011). There are variations on every theme, and some cases in wild canids of polygyny, polyandry, polygynandry, plural breeding, communal breeding, cooperative breeding and promiscuity exist. Nonetheless, we are aware of no study of any canid species that has not revealed a mated pair at its nucleus. Sexual dimorphism generally correlates with mating system (Weckerly, 1998), and amongst canids monogamous mating and paternal care are associated with little or no sexual dimorphism (Kleimen, 1977; Bekoff et al., 1981; Kleimen, 2011; Johnson et al., 2017). The heavy investment by females in internal fertilization, gestation and lactation leaves females committed to much of the burden of parental care and provides considerable opportunity for males to desert their partners to seek additional mating opportunities (Orians, 1969; Trivers, 1972; Maynard Smith, 1977; Clutton-Brock, 1989). Why then would canids form prolonged, year-long pair bonds, maintained not only outside of the breeding season, but often for many years? And what variations of monogamy are exhibited among and within canid species and what causes these variations?

To begin this review, we first consider social monogamy, then reveal differences between canid social and genetic mating systems, i.e. social monogamy does not dictate exclusive mating. We consider canid-specific reproductive traits that may have developed from social and genetic monogamy and, following a detour around the anomalous case of domestic dogs, we reflect on whether monogamy, and the flexible social systems built around it, is a factor in the success of members of the canid family.

CANID SOCIAL MONOGAMY

1.1 Characteristics of Canid Social Monogamy

The fundamental canid social unit, irrespective of group size, is the socially monogamous pair. The primary defining characteristic of social monogamy is spatial congruence of a single breeding male and female. However, this basic criterion is generally exceeded in canid pairs by an affiliative social pair bond, including high rates of social interaction, cooperative territorial defence, mutual offspring care, den sharing, and intrasexual aggression directed at individuals outside the pair bond (Lord et al., 2013). Box 1 provides an overview of variations of social monogamy in canids.

1.1.1 Pair Bonding

Most socially monogamous animals practice serial seasonal monogamy, short-term pairing that lasts only a single breeding season, replaced by a new monogamous bond the following year (e.g. ducks of the *Anas* genus, Mock et al., 1985). Canids, in contrast, often maintain long-term affiliative and cooperative pair bonds and typically remain with the same partner, unless mortality intervenes (e.g. swift fox [*Vulpes velox*], Kitchen et al., 2005; Island fox [*Urocyon littoralis*], Roemer et al., 2001; kit fox [*V. macrotis*], Ralls et al., 2007; cape fox [*V. chama*], Kamler and Macdonald, 2014). For example, high mortality rates were responsible for serial monogamy in populations of red foxes (Zabel, 1986), swift foxes (Kamler et al., 2004a) and in intensely hunted grey wolves (Jędrzejewski et al., 2005). Amongst Ethiopian wolves, the dominant female's position changes only with her death, though male turn-over is more frequent (Sillero-Zubiri et al., 1996a, 2004a). Pairings as long as 8 years have been reported in coyotes (Hennessy, 2007) and black-backed jackals (Moehlman, 1989) and up to 9 years in grey wolves (Doug Smith, oral communication). The degree to which a pair associates outside the breeding season differs between species. For many large canids, such as grey wolves and African wild dogs, the mated pair remain closely associated year-round, coordinating their behaviour and hunting together (Creel and Creel, 1995; Mech and Boitani, 2003). For small species, such as cape foxes, swift foxes, and kit foxes, mated pairs share a territory throughout the year but hunt solitarily; they share dens and closely associate only during the breeding and cub-rearing seasons whereas other times of the year they use different dens and associate with each other less frequently (Kitchen et al., 2005a; Ralls et al., 2007; Kamler and Macdonald, 2014). The maned wolf may be an extreme example of this, as mated pairs apparently do not associate with each other at all outside of the breeding and pup-rearing season (Dietz, 1984), although intraspecific differences among populations might occur. Medium-sized canids, such as coyotes and jackals, may exhibit variations in year-around associations of mated pairs, possibly related to group size or prey size.

1.1.2 Paternal Care

Although paternal care occurs in only 5-10% of mammalian species (Kleiman and Malcolm, 1981; Clutton-Brock, 1991; Woodroffe and Vincent, 1994), it is nearly ubiquitous in canids (Malcolm, 1985; Asa and Valdespino, 1998; Kleiman, 2011).

Kleiman and Malcolm (1981) categorized mammalian parental care into indirect care, which does not require physical contact with young, and direct, which does. Indirect care includes territory acquisition, maintenance and defence, shelter or den construction, anti-predator defence, and mate care through guarding and provisioning. Direct care includes huddling, grooming, transporting, feeding, active defence against predators or conspecifics and playing and socializing. In some species, indirect paternal care predominates (e.g. Blanford's fox [*Vulpes cana*], corsac fox [*V. corsac*], Geffen and Macdonald, 1992; Asa and Valdespino, 1998; Kleiman, 2011); in others, females spend more time with the pups while males provide food (e.g. swift fox, Poessel and Gese, 2013; grey fox, Nicholson et al., 1985); in yet others, males spend more time with pups than the mother and exhibit every care-giving behaviour except lactation (e.g. bat-eared fox, Malcolm, 1986; Maas, 1993; Maas and Macdonald, 2004; Wright, 2006; raccoon dog [*Nyctereutes procyonoides*], Kauhala et al., 1998; African wild dog, Asa and Valdespino 1998). At an extreme, Kleiman (2011) reports that captive female bush dogs call their mate while giving birth, and the male helps remove pups from the birthing canal, grooms the neonates, and may help in removing the placenta (see also Macdonald, 1996).

Provisioning both the pups and lactating mother is widely documented paternal care amongst canids (Asa and Valdespino, 1998). As Macdonald (1992) noted, regurgitation of partially digested food is widely described in the lupine canid lineage (present in all species in the genera *Canis*, *Lycaon*, *Cuon*, *Chrysocyon* and *Speothos*; Biben, 1982; Johnsignh, 1982; Rasmussen and Tilson, 1984; Asa and Valdespino, 1998; Lord et al., 2013) but absent in the vulpine lineage (though see Poessel and Gese, 2013). In these more carnivorous canids, this economical means of transporting prey to the den without the risk of kleptoparasitism is clearly advantageous (e.g. van Lawick and van Lawick, 1970). African wild dogs can carry an estimated three days' worth of food in their stomachs to the pups and mothers (Reich, 1981; Creel and Creel, 1995), which allows males to successfully raise pups if the mother dies (Estes and Goddard, 1967). Species feeding on medium-sized prey can carry prey to the den, allowing both parents to provision young once they are old enough to be left alone at the den (e.g. red fox, Macdonald, 1977; arctic fox, Cameron et al., 2011), but for largely insectivorous canids, this is unfeasible. In largely termitivorous bat-eared fox (Klare et al., 2011), nursing mothers must spend >85% of the night foraging (Wright, 2003), leaving males primarily responsible for guarding, huddling and grooming cubs (Lamprecht, 1979; Malcolm, 1986; Maas, 1993; Maas and Macdonald, 2004; Wright, 2006). As bat-eared fox cubs begin foraging, the male accompanies them (Wright, 2006), acting as both protector and teacher, indicating patches of food to the cubs and occasionally pre-chewing larger beetles (Maas and Macdonald, 2004). A similar division of labour is reported amongst other insectivorous canids (e.g. hoary fox [*Lycalopex vetulus*], Courtenay et al., 2006; raccoon dog, Kauhala et al., 1998), where males compensate for their inability to directly feed cubs by guarding them.

An early theory by Moehlman (1986) posited that requirements for paternal investment in canids, along with other life-history traits, relate to body size. She argued that large canids have relatively smaller infants in larger litters, requiring heavy, prolonged post-partum parental (and alloparental) investment, whereas smaller canids have relatively larger young in smaller litters, requiring less post-partum parental investment. However, other studies concluded female weight was not a strong predictor of canid litter size (Bekoff et al., 1981; Geffen et al., 1996; but see Johnson et al., 2017) and that there was either no correlation between litter size and neonate weight (Geffen et al.

1996) or a positive correlation (Bekoff et al., 1981). Instead of body size, the need and capacity for paternal care is likely determined by diet. The greater energy requirements of large canids mean they must rely more heavily on carnivorous diets and thus larger prey, whereas smaller canids can be more omnivorous (Carbone et al., 1999; Slater, 2015). Large prey not only allow males to directly feed pups, but are also more difficult for young to learn to acquire, thus requiring a longer period of dependency and greater parental investment to ensure pups are fed; offspring even older than one year may be directly provisioned by adults by regurgitation in grey wolves (Mech et al., 1999) and black-baked jackals (Moehlman, 1986). With smaller omnivorous species there is less capacity and less need for males to provision young. For example, Blanford’s fox males cannot economically carry insects to the cubs so they are entirely reliant on the mother’s milk (Geffen and Macdonald, 1992). Diet, and more specifically prey size, therefore probably determines variations in paternal care (Kauhala et al., 1998).

1.1.3 Alloparental Care

Alloparental care by non-breeding adult “helpers” is widespread across canids (see Macdonald et al., 2004). Helpers are usually, but not invariably, related to the pups (e.g. Zabel, 1986, Sillero-Zubiri et al., 2004a; Jędrzejewski et al., 2005). There are many examples of “helpers” benefiting pup survival. Cases of alloparenting allowing litter survival despite the mother’s death have been documented in red fox (Macdonald, 1979a; von Schantz, 1984) and African wild dogs (Estes and Goddard, 1967). In black-backed jackals, the presence of one additional helper tended to result in survival of one additional pup ($R^2 = 0.89$, Moehlman, 1979). Similar but much weaker associations between pup survival and the number of helpers are found in coyotes (Bekoff and Wells, 1982) and African wild dogs (Malcolm, 1979). The presence of helpers can lead to larger litter sizes in African wild dogs (Gusset and Macdonald, 2010; Angulo et al., 2013), perhaps by increased provisioning of pregnant females. In red wolves (*Canis rufus*) and Ethiopian wolves, helpers increased female lifetime reproductive success by extending the female’s reproductive lifespan (i.e. age of last reproduction), thereby increasing the number of reproductive events and thus lifetime reproductive success (Sillero-Zubiri et al., 2004a; Sparkman et al., 2011a). Helpers reduce time pups are left unattended at the den in African wild dogs (Courchamp et al., 2002) and Ethiopian wolves (Sillero-Zubiri et al., 2004a) and can actively defend against predators (Macdonald, 1979a; Bekoff and Wells, 1982; Malcolm and Marten, 1982; Creel and Creel, 1995; Kamler and Gipson, 2000; Kamler et al., 2013a).

Other studies, however, have not found helpers to be beneficial. In Blanford's foxes, non-breeding adults were not observed providing direct care to the young (Geffen and Macdonald, 1992), though perhaps they provide indirect care by territory defence or pup guarding. Helpers did not affect offspring production and/or survival in studies of Arctic foxes (Kruchenkova et al., 2009), red foxes (Baker, 1998; Zabel and Taggart, 1998) and Ethiopian wolves (Sillero-Zubiri et al., 2004a; Marino et al., 2012). It is, however, possible that helpers provided other benefits, such as acting as insurance if a parent dies by adopting the litter (as seen in red foxes: Macdonald, 1979a; von Schantz, 1984), or lightening the work load for the parents (as in Ethiopian wolves: Sillero-Zubiri et al., 2004a), which may increase the breeding pair's future reproductive success (Marino et al., 2012, 2013). The benefit provided by helpers likely depends on ecological conditions. In grey wolves (Harrington et al., 1983) and African wild dogs (Malcolm and Marten, 1982), older siblings were observed feeding pups and thereby increasing pup survival only when there was a food availability surplus (Malcolm and Marten, 1982; Harrington et al., 1983). Furthermore, when food is scarce, not only do helpers not feed pups but will even steal food from them (Malcolm and Martin, 1982). Consequently, in lean years, pup survival can be negatively affected by competition with non-breeders (Harrington et al., 1983). Similarly, in red wolves, pup mass and survival positively correlated with the presence of helpers at low population densities, but negatively correlated with pup mass (though not survival) at high population densities. Furthermore, whilst the presence of helpers increases the breeding females' lifetime reproductive success, it can decrease the males' (Sparkman et al., 2011a). In African wild dogs, yearlings and pack size can increase pup survival (Malcolm and Marten, 1982) and there seems to be a minimum pack size threshold below which packs face an increasing probability of extinction due to the need of helpers for hunting, defence, and reproduction (i.e. an Allee effect) (Courchamp and Macdonald, 2001; Angulo et al., 2013). However, there also seems to be an optimal upper limit to pack size above which the increased competition can decrease pup survival (Macdonald and Carr, 1989; Creel and Creel, 1995). Theoretical research shows that if subordinates are related to the dominant pair, they do not need to have a positive effect to be accepted as group members and can even have a slight negative effect, if ecological constraints are such that they are unlikely to survive or reproduce if expelled from the group (Kokko et al., 2002). Dominants can increase their net fitness by allowing unhelpful or even damaging subordinates to remain in a group if it means they survive and can later reproduce (Kokko et al., 2002). Retaining subordinates in a group is often more for their benefit than that of the dominant pair (Kokko et al., 2002).

1.2 Evolution of Social Monogamy

Phylogenetic studies suggest that social monogamy has evolved independently perhaps as many as 61 times in mammals (Lukas and Clutton-Brock, 2013). Historically, suggestions for factors influencing its evolution include the need for biparental care, infanticide protection, and male mate guarding, each of which is discussed below.

1.2.1 Need for Biparental Care

The association between paternal care, social monogamy, and pair bonding led to an early emphasis amongst scholars on the need for biparental offspring care as the adaptive significance of monogamy (e.g. Orians, 1969; Kleiman, 1977; Wittenberger and Tilson, 1980; Kleiman and Malcolm, 1981; Clutton-Brock, 1989). These early hypotheses suggested that if females cannot successfully rear young without help and males cannot successfully divide care between multiple litters, both would benefit from social monogamy with biparental care (Kleiman, 1977; Wittenberger and Tilson, 1980; Kleiman and Malcolm, 1981; Malcolm, 1985; Birkhead and Møller, 1996). Canids typically have a single, large litter each year (modal litter size: 3-6, Hayssen et al., 1993) and young are altricial with a long dependency period, relative to other mammals (Asa and Valdespino, 1998; Lord et al., 2013). For example, it takes as long as 8 months for pups to reach independence in black-backed jackals, side-striped jackals (*Lupulella adusta*), African golden wolves and grey wolves (reviewed in Lord et al., 2013). The commonness of canid paternal care and the prolonged post-partum parental investment required thus supported these early views that the need for male help favoured the evolution of canid monogamy (e.g. Kleiman, 1977).

However, several more recent phylogenetic analyses suggest mammalian paternal care likely evolved after monogamy and that paternal care is a consequence of social monogamy, not the cause (Komers and Brotherton, 1997; Brotherton and Komers, 2003; Lukas and Clutton-Brock, 2013; Opie et al., 2013). If both sexes are monogamous for other reasons, paternal care may be the best option to improve fitness (Emlen and Oring, 1977; Lukas and Clutton-Brock, 2013), particularly as in this situation there is high paternity assurance (Trivers, 1972; Queller, 1997; Kvarnemo 2005; Fromhage and Jennions, 2016). Similarly, rather than large litter sizes requiring paternal care, litter size and paternal care likely coevolved (Stockley and Hobson, 2016). Canid litter sizes can be highly variable and dependent on food availability (Geffen et al., 1992; Marino et al., 2006, 2012), supported by field experiments in Arctic foxes showing provisioning increases litter sizes (Angerbjörn et al., 1991, 1995). Coevolution of paternal care and litter size resulted in larger litters which require paternal and even alloparental care to survive (Stockley and Hobson, 2016). If the need for bi-parental care was not what caused social monogamy to evolve, it is likely crucial to its maintenance (Klug, 2018).

Female choice likely contributed to the evolution of paternal care (Kvarnemo, 2005; Lukas and Clutton-Brock, 2013; Lambert et al., 2018). Kvarnemo (2005) suggested that if females select males that care for young, this can explain the correlation often observed between paternity and male care but in the opposite direction of causation to that often suggested (i.e. caring males are more likely to sire offspring, rather than males that sire offspring are more likely to provide care). This hypothesis has received far less attention than the reverse direction of causation and has been little investigated in mammals (but see Freeman-Gallant, 1996, Kvarnemo, 2005, and Alonzo, 2012 for support from invertebrates, fish, and birds). Nonetheless, there is evidence in at least one mammal: in a group-living monkey where males exhibit extreme degrees of care often towards unrelated young (Campbell, 2019), males that provide more care experience greater future mating success the following breeding season through female choice (Ménard et al., 1999). A male would therefore directly benefit from providing care, regardless of whether he cares for his own offspring or not. Sexual selection can therefore better explain cases where males care for unrelated young (e.g. wolves: Cassidy et al., 2016; bat-eared foxes: Wright et al., 2010; red foxes: Baker et al., 2004) than natural selection (Kvarnemo, 2005; Alonzo, 2012). However, this process would only be possible where females are able to assess males' investment in young before mating and bias mating towards those males (Alonzo, 2012). While this may not apply to the majority of socially monogamous taxa that display seasonal serial monogamy, finding new partners each year (see Section 1.1.1), the long-term partnerships of canids could allow females to bias paternity based on male care provided to the previous year's litter. Male canids can vary in the quality of paternal care bestowed (e.g. bat-eared foxes, Wright 2006), making this trait subject to sexual selection. Furthermore, even with large litters, a female can compensate for the male's work when he is absent or reduces investment (although at apparent cost to her health and survival, therefore compromising potential future reproductive success) (Sacks and Neale, 2001; Cameron et al., 2011) and females may adjust effort according to litter size (Mech et al., 1999), supporting that male care can be a female preference, rather than pure necessity. Lambert et al. (2018) suggested that monogamy and paternal care co-evolved when selection initially favoured affiliative males, which subsequently evolved into paternal care.

1.2.2 Protection Against Male Infanticide

It had been hypothesized that infanticide may select for social monogamy in mammals. If females deter infanticide by mating promiscuously to confuse paternity, males may counter by guarding mates to ensure paternity and protect offspring (e.g. Wolff and Macdonald, 2004; Lukas and Huchard, 2014). However, phylogenetic analysis across mammals concluded that social monogamy did not evolve from high infanticide levels and that social monogamy and infanticide seemingly evolved independently (Lukas and Clutton-Brock, 2013; Lukas and Huchard, 2014).

1.2.3 Male Mate Guarding

Several studies concluded, based on phylogenetic and empirical evidence, that mammalian social monogamy evolved where males could not defend multiple females (Komers and Brotherton, 1997; Brotherton and Komers, 2003; Lukas and Clutton-Brock, 2013), such as when females are solitary and occupy exclusive ranges at low density (Emlen and Oring, 1977, but also see Dobson et al., 2010). High-quality but scarce or patchy resources likely provided the selective pressures leading to social monogamy by increasing female feeding competition, resulting in female territoriality and intolerance. If breeding is also seasonal and synchronized, as with most canids (Asa and Valdespino, 1998), the temporal availability of oestrous females is also limited, such that males cannot effectively guard more than one (Lukas and Clutton-Brock, 2013). This combination of factors, making it more beneficial for males to monopolize their current partner than seeking others, is a powerful explanation for the evolution of mammalian social monogamy (Komers and Brotherton, 1997; Brotherton and Komers, 2003; Lukas and Clutton-Brock, 2013).

Maintenance of Social Monogamy

Social monogamy should be maintained only if (a) it is the optimal strategy for both the male and female, or (b) it is the optimal strategy for one sex and enforcement mechanisms are employed to prevent their mate from adopting other strategies, referred to as “voluntary” and “enforced” monogamy, respectively (Kvarnemo, 2018).

1.3.1 Voluntary Monogamy: Monogamy as the Optimal Strategy

If bi-parental care is highly beneficial or necessary for offspring survival, social monogamy may be the optimal strategy for both sexes. Because most canids are seasonal breeders (Asa and Valdespino, 1998; Lord et al., 2013), investment in one female limits the investment males can make in another (Kleiman and Malcolm, 1981). In bat-eared foxes, the best predictor of the number and proportion of surviving young is the amount of male den attendance (rather than parental age/size, territory quality, and maternal den attendance) (Wright, 2006). Males spend 30-57% of their time at the den, and a 10% increase in attendance corresponds with a 16% increase in cub survival, with complete litter survival when a male spends 49% of his time at the den (Wright, 2006; Wright et al., 2010). Males therefore cannot care for litters at two dens without severely sacrificing offspring survival at one or both. Similarly, male raccoon dogs may be required to keep pups warm while the female forages (Kauhala et al., 1998) and in black-baked jackals, an entire litter died following the male’s death (Moehlman, 1986).

1.3.2 Enforced Monogamy: Reproductive Suppression of Subordinates

In group-living canids, social monogamy is commonly enforced by reproductive suppression of subordinates (Moehlman, 1989; Creel and Creel, 1991; Creel and Macdonald, 1995; Moehlman and Hofer, 1997; Asa and Valdespino, 1998). In a review of 25 canid species, Moehlman and Hofer (1997) found reproductive suppression in 44%. The mechanism often involves copulation interference and aggression towards same-sex individuals attempting to breed (e.g. grey wolf, Rabb et al., 1967; Derix et al., 1993; African wild dog: Malcolm, 1979; red fox: Macdonald, 1979a) and infanticide, either by directly killing subordinates' pups (dingo [C. familiaris dingo]: Corbett, 1988; African wild dog: van Lawick, 1973) or indirectly, such as interfering with provisioning of subordinates' pups (African wild dog: Frame et al., 1979) or causing subordinate mothers to become so excessively anxious that their offspring die from the mother's fretfulness (red fox: Macdonald, 1979a; bush dog: Macdonald, 1996).

Physiological mechanisms of reproductive suppression are less documented in canids, but there is evidence for them (e.g. coyote: Moehlman and Hofer, 1997; African wild dog: Creel et al., 1997; Spiering et al., 2010; Ethiopian wolf: van Kesteren et al., 2012, 2013). In Ethiopian wolves, typically only the dominant females breed during a short mating season (Sillero-Zubiri et al., 1998). A study of adjacent Ethiopian wolf packs demonstrated increased oestradiol concentration in faeces from eleven dominant females but not in the nine subordinates sampled and no aggression from the dominant female preventing subordinate breeding was documented, indicating hormonal suppression of subordinate females (van Kesteren, 2013). Although female grey wolves can breed as yearlings (Medjo and Mech, 1976), they rarely do in the wild before age three, suggesting subordinate females may experience delayed maturation or suppressed oestrus. In African wild dogs, subordinate females were hormonally suppressed, preventing ovulation likely by elevated oestrogen and oestrogen/progestin ratios (Creel et al., 1997). However, the detailed physiological mechanisms involved in reproductive suppression remain unclear in many species; increased glucocorticoids from social stress is a mechanism of reproductive suppression in some other taxa (e.g. Hackländer et al., 2003), but no evidence of this has been found in canids (African wild dog: Creel et al., 1997; van Kesteren et al., 2013). Glucocorticoid levels in female African wild dogs did not differ according to dominance status (average faecal glucocorticoid concentration for dominant breeding females during the mating season was 207.47 ± 43.69 (SE) ng/g while for subordinate non-breeding females it was 202.5 ± 52.3 ng/g), suggesting other mechanisms were responsible for reproductive suppression (van Kesteren et al., 2013).

Reproductive suppression of subordinates may not be solely for the benefit of the dominant pair. Packard et al. (1983) suggest that deferred reproduction in grey wolves could have evolved by individual selection, as future reproductive fitness may be enhanced by remaining longer in a juvenile role in the native pack. Similarly, delayed dispersal in red wolf males lowers mortality, thereby increasing the chances of becoming reproductive (Sparkman et al., 2011b). Kokko and Johnstone (1999) showed that the delayed benefits of acquiring dominant status in the future ("social queuing") can provide enough incentive for subordinates to remain peacefully in a group without themselves breeding. Additionally, if larger groups experience greater survival (e.g. African wild dogs, Carbone et al., 1999), individuals may experience greater benefits by remaining as a non-breeding subordinate and helping to raise new group members, rather than dispersing to breed alone (Kokko et al., 2001). Furthermore, subordinates increase their inclusive fitness by helping to raise and improve the survival of their parents' next litter, since they are on average as closely related to their siblings as they would be to their own offspring (Moehlman, 1983, 1986).

Social dominance may play a role in reproductive suppression. Macdonald (1979a, 1987) reported that, although normally only the alpha red fox female bred, when the dominance status of the previously-alpha female waned until becoming equal with another vixen in the group, both conceived the following year. Similarly, Zabel (1986) observed that although a clear dominance relationship existed between dominant breeding females and submissive non-related helpers, social groups with two breeding females had no obvious female dominance hierarchy.

Subordinate reproduction can also be thwarted by expelling them from the group (Jungwirth and Johnstone, 2018) or not allowing subordinates to join (e.g. male bat-eared foxes are aggressively territorial towards young males that intrude on the territory, Maas and Macdonald, 2004; females "floating" on the periphery of Ethiopian wolf groups, Sillero-Zubiri et al., 1996a). Intrasexual aggression is common in canids (e.g. Rabb et al., 1967; Kleiman, 1981; Zabel, 1986), as is the expulsion of same-sex subordinates. The pros and cons of tolerating additional group members are explored by Macdonald and Carr (1989). In Ethiopian wolf packs with more than one subordinate female, the mother expelled the lowest-ranking female at 18-28 months old, with assistance from the dominant sister (Sillero-Zubiri et al., 1996a). Similarly, pregnancy in subordinate Ethiopian wolves can result in pack splitting, thus restoring social monogamy (Marino et al., 2013).

Interestingly, one strategy for subordinate females to avoid reproductive suppression is to raise a litter at the edge of their parent's territory, typically with a subordinate male from a neighboring group, seen in crab-eating foxes (Macdonald and Courtenay, 1996), red foxes (Baker et al., 2000), black-backed jackals (Kamler et al., 2019) and grey wolves (Mech and Boitani, 2003). In this way, subordinates may get the best of both worlds by avoiding risky dispersal into unknown areas yet allowing reproduction. However, the tolerance of alphas letting betas raise litters and use their territory edges might vary according to food abundance and dispersion and kinship.

1.4 Ecological Correlates of Social Monogamy

Social monogamy is maintained because either it is the optimal strategy for both sexes, or because polygamy (incl. polygyny, polyandry, polygynandry) is restricted due to monogamy enforcement mechanisms or ecological conditions. Ecological conditions can shift monogamy to other social systems, either by affecting the benefits (motivations) of social monogamy itself or by affecting the ability to enforce it (summarized in Table 1).

1.4.1 Resource Availability: Paternal Care and the Polygyny Threshold Model

One of the primary benefits of social monogamy in canids is benefits to offspring survival due to biparental care (Moehlman, 1989), though resource availability affects the degree to which male care is both necessary and feasible: when resources are plentiful, females may successfully raise pups with less male input (Maas, 1993) and males are able to provide more paternal care (Wright 2006). The polygyny threshold model (Verner 1964, Verner and Willson 1966, Orians 1969) posits that there is a threshold at which a female can raise as many young sharing a male and territory of higher quality (in a polygynous system) as she could being the sole female with an inferior male/territory (in a monogamous system). Thus, if male help is required and a male cannot share care between multiple litters without decreasing the quality and offspring survival below what could be achieved with his full attention on a single litter, monogamy would be the optimal strategy. If, however, resources are such that a female can raise as many offspring in a polygynous system, either because male help is less needed or because a male can provision multiple litters as well as he could one, the “polygyny threshold” can be crossed. Supporting this, Zabel and Taggart (1989) report that when food availability was high, 71% of the island population of red foxes they studied were bigamous, i.e. a single male cared for the litters of two females, and bigamous females had equal or greater (1.4 times) reproductive success than monogamous females when considering offspring survival in the first year (mean litter size of 4.3 ± 0.29 in bigamous vs 4.0 ± 1.0 in monogamous females, Zabel and Taggart, 1989). However, when the food supply crashed, the population shifted entirely to monogamy. Red foxes in Sweden also displayed polygyny with multiple breeding females when prey availability was high but a single breeding female when prey availability was low (von Schantz, 1984, see also Iossa et al., 2008). Similarly, food availability influenced the probability of forming larger groups across four populations of arctic fox, with variations including polygyny, plural breeding and communal breeding where food abundance differed substantially between years, whereas social monogamy is found in areas of stable resources (Angerbjörn et al., 2004; Elmhagen et al., 2014) and in marginal habitats with low food availability (Norén et al., 2012; Elmhagen et al., 2014).

1.4.2 Social Structure

As described in Box 1, canid social monogamy is not restricted to pair-living individuals but also includes group-living variations where social groups contain a single breeding male and female, in addition to non-breeding group members. Alternatively, canid groups may exhibit polygyny, polyandry, or polygynandry with multiple breeding adults. Social monogamy is, unsurprisingly, most common in smaller social groups (Clutton-Brock and Isvaran, 2006; Spiering et al., 2010). A greater availability of potential breeders is more difficult for the dominant pair to suppress (Marino et al., 2013). Spiering et al. (2010) found that many packs of African wild dogs contain only one adult female and thus inevitably only one breeding female. However, in the 30% of groups containing subordinate females, only half were socially monogamous: beta females also bred in 54.5% of years, though theta females never bred. In contrast, subordinate males always secured some paternity, but were only present in 47% of groups. Similarly, in bat-eared foxes studied by Maas and Macdonald (2004), social monogamy depended on the number of females in the group – additional females invariably bred, and in only 1 of 65 breeding events was there a non-breeding adult male in the group. In Ethiopian wolves, packs recovering from disease outbreak can become unusually large and contain more than two subordinate females, increasing the likelihood of pregnancy in subordinate females (Marino et al., 2013).

The mechanisms shaping sociality in carnivores, and in canids specifically, have been reviewed elsewhere (e.g. Macdonald, 1983; Creel and Macdonald, 1995; Macdonald et al., 2004; Macdonald and Sillero-Zubiri 2004). Canid groups generally form by retention of offspring that do not disperse (e.g. black-backed jackal: Moehlman, 1979, 1983; Kamler et al., 2019; hoary fox: Courtenay et al., 2006; kit fox: Ralls et al., 2001; red fox: Macdonald, 1980; arctic fox: Hersteinsson and Macdonald, 1982; bat-eared fox: Maas and Macdonald, 2004; Kamler et al., 2013b; Cape fox: Kamler and Macdonald, 2014) and thereby avoid dispersal costs (Bekoff and Wells, 1982; Lucas et al., 1994; Macdonald and Carr, 1989; Kamler et al., 2019). This results in family groups, though unrelated individuals can sometimes join existing groups (e.g. grey wolf: Jędrzejewski et al., 2005; red fox: Zabel and Taggart, 1989). Macdonald and Carr (1989), drawing heavily on canid examples, presented a profit and loss account of tolerating additional group members. A primary cost is food competition (Schmidt and Mech, 1997; Creel and Creel, 2002), but the list also includes increased risk of infectious disease (e.g. rabies, Macdonald and Bacon, 1982) and parasite transmission (Hoogland, 1979), and mate sharing (Zabel and Taggart, 1989; Spiering et al., 2010).

The adaptive functions of canid groups include greater hunting success (e.g. African wild dogs: Fanshawe and Fitzgibbon, 1993; Creel and Creel, 1995) and capacity to tackle larger prey (Fanshawe and Fitzgibbon, 1993). Larger African wild dog groups better defend food against spotted hyenas (*Crocuta crocuta*, Fanshawe and Fitzgibbon, 1993; Carbone et al., 2005), larger golden jackal groups can steal food from smaller groups (Macdonald, 1979b) and packs of dholes can steal prey from leopards (Venkataraman and Johnsignh, 2004). Larger groups also benefit territory defence, as victory in intergroup contests generally goes to the larger group (e.g. Ethiopian wolves: Sillero-Zubiri and Macdonald, 1998; Marino et al., 2012; grey wolves: Cassidy et al., 2017).

Grouping can also increase breeding success through alloparental care (e.g. Moehlman, 1979) and decreased predation vulnerability (Kamler et al., 2013a). Sociality can also provide thermoregulatory, energetic and physiological benefits through social thermoregulation (Campbell et al., 2018). Though social thermoregulation is little studied in canids, Hennemann et al. (1983) found that crab-eating foxes reduced heat loss and oxygen consumption (a measure of basal metabolic rate) by 5-18% when huddling with a partner, suggesting huddling can significantly impact daily energy expenditure in this and other canid species (Hennemann et al., 1983).

Large prey can favour cooperative hunting and larger groups (e.g. coyotes: Bowen, 1981). Intense intraspecific and interspecific competition and predation may favour group formation for strength in numbers: recolonizing grey wolves, displaying intra-guild aggression towards coyotes, led coyotes to form larger groups (Arjo and Pletscher, 1999) and higher jackal numbers increased bat-eared fox group sizes (Kamler et al., 2013a). Similarly, arctic foxes tend to form complex groups when facing greater predation pressure from red foxes (Norén et al., 2012) and a mother-daughter pair merged their litters into one den when facing red fox predation, despite low food availability at the time (B. Elmhagen, unpublished data, from Norén et al., 2012).

The costs of dispersal increase when the journey is hazardous and/or the availability of vacancies is low (Ballard et al., 1987; Norén et al., 2012). Therefore, population density, likely linked to food availability, affects the advantages of group formation such that polygamy can be associated with high population density (e.g. swift foxes, Kamler et al., 2004a; red foxes, Baker et al., 2004; Iossa et al., 2008a; grey foxes, Weston Glenn et al., 2009). Iossa et al. (2008a) found 60% of red fox groupings were socially monogamous at low population density but 23% at high population density. Similarly, in swift foxes at high population density from low predation pressure, 30% of social groups exhibited polygyny with communal denning and 40% included non-breeding females (in 10 social groups), whereas in low density/high predation pressure, only monogamy was observed, with no non-breeding helpers (16 groups, Kamler et al., 2004a).

Finally, group formation may occur not only when groups are beneficial or dispersal is costly, but rather when grouping carries little cost (Macdonald and Carr, 1989; Macdonald and Johnson, 2015). The resource dispersion hypothesis (RDH, Macdonald, 1981, 1983; Carr and Macdonald, 1986) posits that when resources are dispersed heterogeneously, the minimum territory needed to meet a breeding pair's resource requirements can often support additional group members with little or no cost to the dominant pair. Greater heterogeneity leads to larger group sizes. Macdonald (1980, 1987) reviewed the early literature to show that monogamous red fox pairs are associated with spatio-temporally homogeneous resources (e.g. farmlands of USA Midwestern states, Storm et al., 1976), often at low population density, whereas groups more commonly form where food availability is more spatio-temporally heterogeneous and foxes that exploit cyclic rodent populations may accommodate additional group members in peak rodent years (Macdonald, 1984; von Schantz, 1984; Elmhagen et al., 2014; see also Macdonald et al., 2016). Similarly, if the cost of tolerance is low, its benefits may be minimal: Geffen and Macdonald (1992) report dominant Blanford's fox pairs tolerating non-breeding subordinate vixens, but found no evidence that they act as helpers.

The balance of these costs and benefits of group formation, determined by local ecological conditions, will therefore influence the social structure of canids and thus whether they exist as a socially monogamous pair, one of the variations on social monogamy described above, or depart from monogamy altogether.

2. CONTRASTING CANID SOCIAL AND GENETIC MATING SYSTEMS:

Potential functions and ecological correlates of extra-pair mating in canids

Social monogamy is no guarantee of genetic monogamy, i.e. exclusive mating (Klug 2018; Lambert et al., 2018), and, indeed, almost every genetically studied canid species has revealed extra-pair paternities (EPP) (see Hennessy, 2007 for an exception). For example, extra-pair males sired 25% of 16 offspring in Island foxes (Roemer et al., 2001), 52% of 19 offspring from 15 litters in swift foxes (Kitchen et al., 2006), 31% of 176 offspring in arctic foxes (Cameron et al., 2011) and in red foxes from 38% of 38 offspring (Iossa et al., 2008b) to as much as 80% of 30 offspring (Baker et al., 2004). Many of these examples are drawn from pairs, rather than larger social groups.

In group-living canids, extra-pair mating can occur both within-pack and with extra-pack individuals. In Ethiopian wolves, despite the dominant pair's apparent social monogamy, extra-pair copulations (EPC) happen both within (rarely) and outside (more commonly) the pack (Gottelli et al., 1994): Sillero-Zubiri et al. (1996a) observed that 70% of copulations were between a female and male in adjoining packs and Randall et al. (2007) found that 50% of litters had offspring sired by an extra-pack male, with 28% of offspring with resolved paternities sired by extra-pack males. Though red wolves were found to be highly genetically monogamous, with only 4 of 174 litters (2%) showing EPP, these rare cases included extra-pair mating within and outside of the pack (Sparkman et al., 2012). In contrast, two studies in African wild dogs found that, although extra-pair mating was common, extra-pack males never sired offspring (of 226 offspring, Spiering et al., 2010, and 39 offspring, Moueix, 2006); when subordinate males existed in a pack, levels of mixed paternity in litters were 53% (of 15 litters, Spiering et al., 2010) and 100% (of 5 litters, Moueix, 2006).

2.1 Benefits and Costs of Genetic Polygyny

For males, whose reproductive success is generally limited by access to females, the benefit of extra-pair mating is obvious: mating with additional females can directly increase reproductive success by producing more offspring, and especially when these are cared for by another male. For example, male red foxes studied by Baker et al. (2004) sired more offspring with extra-pair females than with their social mate and travelled as far as 2.7 territories away during extra-territorial forays; consequently, they could have sired offspring in as many as 32 neighbouring groups (Baker et al., 2004). Such males benefit doubly, genetically and from the parental investment of cuckolded males, thus extra-territorial forays are widely recorded amongst canids during the courtship and mating periods (e.g. Macdonald, 1981; Zoellick and Smith, 1992; Baker et al., 2004; Deuel et al., 2017; Kamler et al., 2017, 2019). However, the costs of male philandering include leaving their mate unguarded and therefore increasing their own risk of being cuckolded, increased exposure to sexually transmitted disease and parasites (Poiani and Wilks, 2000; McLeod and Day, 2014), and risks of mortality, predation, intraspecific conflict and stress when travelling in unfamiliar areas (Harris and Smith, 1987; Young and Monfort, 2009).

2.2 Benefits of Genetic Polyandry

For females, whose reproductive output is limited, the benefits of extra-pair mating are less obvious. Various hypotheses have been proposed to explain why females engage in extra-pair mating (summarized in Table 2).

2.2.1 Increase Genetic Quality

A favoured explanation for extra-pair mating in birds is increased genetic fitness of offspring by mating with the highest quality males (Birkhead and Møller, 1992; Jennions and Petrie, 2000; Westneat and Stewart, 2003). In monogamous social systems where most individuals are paired, mate choice is constrained and thus most females would be partnered with suboptimal males. The majority of females would therefore benefit from seeking EPC with superior males. This may be the motivation for extra-pair mating by female red foxes studied by Iossa et al. (2008a,b). Red fox females typically engaged in EPC with dominant males from adjoining territories (Iossa et al., 2008a) and extra-pair males that sired offspring were always larger than the female's cuckolded social partner (Iossa et al., 2008b). By mating with males of higher quality than their partner, females can increase the genetic quality of their offspring. Similarly, three of four cases of EPP observed in Island foxes were by the two largest males in the study (Roemer, 1999, 2004), suggesting females engaged in extra-pair mating with high-quality males. Furthermore, multiple mating may also increase genetic quality of offspring by inciting sperm competition and allowing cryptic female choice (e.g. Kvarnemo and Simmons, 2013; Annaviet al., 2014).

Genetic quality, however, is not the only factor, as illustrated by female Ethiopian wolves that mate outside their pack being notably unselective about the dominance status of these mates (Sillero-Zubiri et al., 1996a; Randall et al., 2007) and similar observations of other red foxes being unselective outside, but selective within, the group in regards to male dominance status (Baker et al., 2004). While it is possible that females select for genetic quality using indicators other than dominance status (which is often influenced by size, health, strength), these studies suggest that, at times, other explanations are involved besides quality of the extra-pair mates (especially considering females are likely well informed of their neighbours' social status). The case of Bristol's urban red foxes is revealing in showing how motivations for extra-pair mating can change: in 1992-1994, females appeared to be unselective in the quality of extra-group males, mating with both dominant and subordinate males (Baker et al., 2004). In 1994-1996, the population declined by 80% due to mange (Baker et al., 2000; Iossa et al., 2008a). Subsequently, in 2002-2004, females became highly selective, reducing the frequency of extra-pair mating and mating only with males that appeared to be of higher quality than their social mate (Iossa et al., 2008b). Furthermore, rates of mixed paternity dropped from 38-69% pre-outbreak to 0% post-outbreak (Baker et al., 2004; Iossa et al., 2008b). Thus it seems that following substantial pressure from disease, females changed their reproductive strategy to emphasize genetic quality, which may increase the probability of their offspring surviving disease.

2.2.2 Increase Genetic Diversity

Canid litters can be sired by multiple males. Thus, polyandry might function to increase within-litter genetic diversity (Yasui, 1998; Jennions and Petrie, 2000; Slatyer et al., 2012). This was proposed to explain polyandry in arctic foxes, where 26% of litters were sired by multiple males (Cameron et al., 2011). In fluctuating environments such as the harsh arctic, the fittest genes may be unpredictable and thus increased within-litter genetic diversity may increase the probability that at least some offspring survive (Yasui, 1998; Jennions and Petrie, 2000). The majority of cases where female canids engage in EPC result in mixed paternity litters (e.g. Baker et al., 2004; Moueix, 2006; Randall et al., 2007; Spiering et al., 2010; Cameron et al., 2011; Converse 2012, but see Iossa et al., 2008a,b and Cameron et al., 2011 for exceptions). Increased within-litter genetic diversity may therefore be a common motivation for extra-pair mating in canids.

2.2.3 Inbreeding Avoidance

Extra-pair mating may function to prevent inbreeding (Stockley et al., 1993; Jennions and Petrie, 2000; Tregenza and Wedell, 2002; Annavi et al., 2014; Arct et al., 2015). This may be particularly important where territories are inherited by successive generations or there is a lack of dispersal (e.g. Ethiopian wolf, Sillero-Zubiri et al., 1996a; bat-eared fox, Maas and Macdonald, 2004), leading to highly related groups. However, in most canids that have been genetically investigated, mated pairs are generally unrelated (e.g. African wild dog: McNutt 1996; Girman et al., 1997 [average relatedness of social pairs: 0.05 ± 0.11, N=5]; grey wolf: Smith et al., 1997 [0.01 ± 0.14, N=16]; arctic fox: Cameron et al., 2011 [0.01 ± 0.14, N=13]; kit fox: Ralls et al., 2001 [-0.07 ± 0.07, N=10]; coyote: Hennessy et al., 2007 [0.00 ± 0.14, N=7]; swift fox: Kitchen et al., 2006 [-0.01 ± 0.23, N=48]; red wolf: Sparkman et al., 2012, $R < 0.50$ for 95% of 174 mating events). Although there are occasional instances where social pairs are closely related (e.g. Hennessy, 2007: 1/7 coyote pairs, R=0.26; Kitchen et al., 2006: 1/48 swift fox pairs, R=0.48; Weston Glenn et al., 2009: one grey fox pair, R=0.36; Roemer, 1999: 4/15 Island fox pairs, R=0.19, 0.35, 0.35, 0.52; Jędrzejewski et al., 2005: one grey wolf half-sibling pair), there are also cases where extra-pair mating occurs between relatives. For example, Baker et al. (2004) found four incestuous EPPs between close relatives with experiential histories (mother-son for two years, father-daughter, and half-brother-half-sister) and three additional pairings between more distantly related individuals (e.g. R=0.13) in red foxes, Cameron et al. (2011) found one incestuous mother-son case of EPP (of 13 mated pairs, 7.6%) between arctic foxes that were socially paired with non-relatives, and Sparkman et al. (2012) found 4 parent-offspring and 4 full-sibling matings (of 174 mated pairs, 9%). There may be greater risk of this where there is neighbourhood settlement by dispersers, as in crab-eating foxes (Macdonald and Courtenay, 1996), bat-eared foxes (Kamler et al., 2013b), swift foxes (Kitchen et al., 2005b) and black-backed jackals (Kamler et al., 2019). When it has been investigated, relatedness between social mates and extra-pair mates did not differ (e.g. Cameron et al., 2011: mean ± SD relatedness between social mates: 0.05 ± 0.12, N=9; between extra-pair mates: -0.09 ± 0.11, N=4). It therefore seems that canids achieve inbreeding avoidance by other mechanisms, such as avoiding mating within ones' natal pack, sex-biased dispersal and adult dispersal (Kamler et al., 2004c; Geffen et al., 2011; Sparkman et al., 2012; Kamler et al., 2013b; Kamler and Macdonald, 2014).

One exception, however, could be in Ethiopian wolves. A lack of dispersal opportunities from shrinking habitat, coupled with male philopatry, results in highly related packs (Sillero-Zubiri et al., 1996a; Randall et al., 2007). Sillero-Zubiri et al. (1996a) observed that the majority (70%) of copulations by female Ethiopian wolves were with males from adjoining packs, rather than her own pack, and thus extra-pack mating was suggested to be an inbreeding avoidance strategy. Females rejected advances from all males within their packs except those from the alpha male, yet were unselective concerning the status of extra-pack males with which they mated, suggesting outbreeding was of importance rather than mate quality (Sillero-Zubiri et al., 1996a). However, subsequent research found that, although packs are indeed highly related (mean pairwise relatedness within packs was 0.39) and there is a high prevalence of incestuous pairing (22% of mating pairs within packs were closely related [R=0.18-0.44]), members of neighbouring packs were also closely related so incestuous pairing occurred with both within-pack and extra-pack mating (33% of extra-pack mating pairs were closely related [R=0.42-0.44]). In this case, female dispersal appears to contribute more than extra-pack mating to reduce inbreeding (Randall et al., 2007). Was this an artefact of the unusual, modern, circumstances of these wolves? Perhaps extra-pack mating evolved as an inbreeding avoidance strategy, but modern conditions, exacerbated by recurrent rabies outbreaks (Randall et al., 2004; Marino et al., 2006), changes in dispersal and demographic events led to neighbouring packs and breeding pairs being more closely related than during evolutionary time (Randall et al., 2007).

2.2.4 Infanticide Protection by Paternity Confusion

Based on evidence across 33 mammal families, Wolff and Macdonald (2004) concluded that the most convincing explanation for polyandry across mammals is paternity confusion to deter infanticide. This hypothesis, originally proposed by Hrdy (1974, 1979), relies on female promiscuity being an effective counterstrategy against male infanticide (Lukas and Huchard, 2014) and predicts that females mate with many males. However, a complication is that, in contrast to some taxa (notably felids, see Macdonald et al., 2010), male infanticide in canids would not hasten female oestrus because most canids are seasonal breeders (Asa and Valdespino, 1998; Valdespino et al., 2002; Lord et al., 2013), notwithstanding some possible exceptions in African wild dogs (Frame et al., 1979), bat-eared foxes (Rosenberg, 1971), and bush dogs (Porton et al., 1987). This raises the question of what male canids could gain by infanticide. Indeed, amongst canids infanticide appears most commonly practiced by females (e.g. grey wolf: McLeod, 1990; Ethiopian wolf: Sillero-Zubiri et al., 1996a; African wild dog: van

Lawick, 1973; Girman et al., 1997; dingo: Corbett, 1988; coyote: Camenzind, 1978), either as suppression of subordinate breeding attempts by the dominant female (Corbett, 1988; McLeod, 1990; Sillero-Zubiri et al., 1996a; Girman et al., 1997) or perhaps to increase availability of breeding territories or dens (see Zabel, 1986). Although examples of male infanticide are numerous amongst ursids and felids (e.g. Loveridge et al., 2007), we know of none in canids. The closest reports seem to be a description of a lone female red fox being harassed by males who intruded on her den and eventually the entire litter died (Zabel, 1986; Zabel and Taggart, 1988) and Latham and Boutin (2011) suggested that the death of a grey wolf pup may have been infanticide by a male, though the evidence was inconclusive and might best be explained by intergroup resource competition. Furthermore, in contrast to infanticidal male takeovers in other taxa (e.g. Loveridge et al., 2007), quite the opposite has been reported in grey wolves: when a new and unrelated alpha wolf takes over a pre-existing pack, he provisions and cares for pups that are not his own, which may increase his acceptance by the pack (Cassidy et al., 2016). Thus paternity confusion to prevent infanticide is an unconvincing explanation for extra-pair mating in canids.

2.2.5 Fertilization Assurance

Females may engage in EPC for fertilization assurance to guard against male infertility (Wetton and Parkin, 1991; Hoogland, 1998; Hasson and Stone, 2009). Canids are unusual among Carnivora in that they are monoestrous, having only one ovulation event each season (Asa and Valdespino, 1998; see Section 3.2.2), making the stakes high if a female's mate is infertile. Multiple mating may guard against this possibility.

2.2.6 Alloparental Care from Paternity Confusion/Dilution

In communal or cooperative breeding situations, selective female promiscuity with group members could be beneficial by confusing or diluting paternity and thus potentially increasing offspring care, particularly when paternal care is indivisible. This would not apply to most cases of EPC in canids where mating occurs outside of the social group (e.g. Sillero-Zubiri et al., 1996a; Baker et al., 2004). However, in African wild dogs, despite previous beliefs that only the alpha pair breeds, research found females frequently mate with subordinate males and documented high levels of paternity sharing, though none of the offspring analysed (226 pups, Spiering et al., 2010; 39 pups, Moueix, 2006) were sired by extra-pack males. Spiering et al. (2010) found that the three top-ranking males always sired pups, or, if there were only two males in a pack, they shared the litter's paternity equally (similarly see Moueix, 2006). Male African wild dogs invest heavily in offspring care (Creel et al., 2004) so by mating with multiple males in a group, females may dilute paternity and increase the amount of care for her offspring while also increasing within-litter genetic diversity.

2.3 Costs of Genetic Polyandry

Like males, females engaging in extra-pair mating risk increased exposure to sexually transmitted disease and parasites (Poiani and Wilks, 2000; McLeod and Day, 2014) and increased stress (Young and Monfort, 2009) and mortality (Harris and Smith, 1987). Additionally, females may lose investment in her offspring by her mate or other group members.

2.3.1 Loss of Paternal Care

If males adjust investment according to confidence in paternity (Trivers, 1972; Møller and Birkhead, 1993; Sheldon, 2002), females should be less likely to seek EPCs when paternal care is important (Mulder et al., 1994; Westneat and Stewart, 2003; Lambert et al., 2018). Such reduced paternal investment by males with unfaithful partners is observed in arctic foxes: Cameron et al. (2011) found that, in faithful partnerships, den attendance rates were similar for males and females, whereas cuckolded males showed a 56% reduction in den attendance compared to non-cuckolded males and a non-significant 52% reduction in food provisioning. Overall rates of food provisioning did not differ between litters, meaning greater burden of care was placed on unfaithful females. The potential costs to males were substantial: 11% of litters were cared for by a male that did not sire any of the offspring. In contrast, in bat-eared foxes, cuckolded males did not invest less than other males (Wright et al., 2010). One explanation for this difference is that, because canids can have mixed paternity litters, when male care is indivisible among pups, such as vigilance against predators, females may be able to get away with some EPP without reducing male investment. Amongst bat-eared foxes, male den attendance is important and cannot be split amongst the young, whether sired by that male or not; conversely, food provisioning by male arctic foxes could be preferentially directed towards their own progeny, although this is untested (Wright, 2010; Cameron et al., 2011). However, this presupposes males can recognize their own offspring, which may be unlikely considering success of cross-fostering in coyotes (Kitchen and Knowlton, 2006), red wolves (Gese et al., 2015), grey wolves (Goodman, 1990; US Fish and Wildlife Service 2004) and dingoes and African wild dogs (Kitchen and Knowlton, 2006) and cases where cuckolded male foxes care for litters sired entirely by other males (Baker et al., 2004; Cameron et al., 2011). An alternative explanation for this difference between arctic and bat-eared foxes relates to the potential risk and costs. Comparative research across taxa suggests males reduce investment when cuckolded only when there is high cost and high risk of cuckoldry (Griffin et al., 2013). EPP was twice as frequent in arctic foxes as in bat-eared foxes and arctic males faced high potential costs of caring for litters sired entirely by other males (Wright, 2010; Cameron et al., 2011). A third explanation relates to female choice: if females preferentially mate with males that provide care, males can increase future breeding success by caring even for unrelated young (Kvarnemo, 2005; Alonzo, 2012; see Section 1.2.1). If females adjust the amount of extra-pair mating according to levels of male care (as in some birds: Freeman-Gallant 1996), this can even paradoxically result in greater male investment when cuckolded to avoid losing future breeding opportunities.

2.3.2 Loss of Alloparental Care

A similar potential cost to engaging in extra-pair mating is the possible loss of alloparental care by philopatric young (who might otherwise be assumed to be related to new pups as full sibs). However, although there is little research on how EPP affects alloparental care, increased inclusive fitness is not the only motivation for alloparenting. Helpers are not always related to pups (Zabel, 1986) and may receive other benefits, such as inheritance of dens/territories (Lindström, 1986; Zabel, 1986; Kokko et al., 2002; Converse, 2012; Marino et al., 2012, 2013) or dominance status (Baker et al., 1998;

Kokko and Johnstone, 1999), or where individuals achieve greater fitness by being in larger groups (e.g. African wild dogs: Carbone et al., 1999), thus making alloparental care beneficial even when helpers are unrelated to the young (“group augmentation”; Kokko et al., 2001).

2.4 Ecological Correlates of Extra-Pair Mating

Ecological conditions can affect the balance of these costs and benefits of extra-pair mating and thus its prevalence (summarized in Table 1).

2.4.1 Resource Availability and Reliance on Paternal Care

When resources are abundant, offspring survival may be less dependent on male care and thus the potential costs of EPCs may be outweighed by potential benefits (Norén et al., 2012). Though desirable, there are cases where paternal care is not essential and females can at least sometimes raise litters without male assistance (e.g. swift fox: Kamler et al., 2004b; cape fox: Kamler and Macdonald, 2014; bat-eared fox: Maas, 1993; coyote: Sacks and Neale, 2001). This leads to the prediction that in socially monogamous species with biparental care, EPCs should increase with increased resource availability; a prediction supported for birds (Griffith et al., 2002; Møller 2000). A comparative analysis of 15 mammal species (including three canids) found that rates of EPP correlated with levels of paternal care (Huck et al., 2014; see also Dillard and Westneat, 2016). Similarly, EPCs may be more common in mammals than in birds because paternal care is more common in birds (Isvaran and Clutton-Brock, 2006).

Within canids, among the lowest reported values of EPP thus far found is in bat-eared foxes (9.8-15.6%, Wright, 2010), a species wherein male care can be highly beneficial for offspring survival (Wright, 2006). The frequency of EPP in arctic foxes studied by Cameron et al. (2011) was argued to reflect variations in the need for paternal care: EPP correlated with spatial variation in food availability, being more frequent when closer to a goose colony. However, this study did not control for effects of population density, which often correlates with resource availability (e.g. Clark, 1972; White and Garrott, 1997) and thus could have been responsible for greater EPP closer to the goose colony. However, this hypothesis was not supported in a study of urban coyotes, wherein despite optimal food availability, pairs were strictly genetically monogamous (96 offspring from 18 litters, Hennessy et al., 2012).

2.4.2 Diet (Foraging Strategy and Time Budget)

Mate guarding is a common strategy to prevent EPCs, though the feasibility is affected by mate proximity during foraging and/or foraging time budgets. Solitary foragers (e.g. Island fox: Roemer et al., 1999; Arctic fox: Cameron et al., 2011; red fox: Baker et al., 2004; Iossa et al., 2008a; swift fox: Kitchen et al., 2006) may have greater opportunity to engage in clandestine EPCs, allowing relatively high levels of EPP (Island foxes: 25%, Roemer et al., 1999; Arctic foxes: 31%, Cameron et al., 2011; red fox: 38-80%, Baker et al., 2004; Iossa et al., 2008b; swift fox: 52%, Kitchen et al., 2006). These typically solitary foragers apparently try to reduce EPCs by spending more time closer to their partners during the breeding season (Kitchen et al., 2005a). In contrast, the relatively low levels of EPP in bat-eared foxes (9.8-15.6%, Wright et al., 2010) may reflect their insectivorous diet which enables partners to forage together (Wright, 2003). Additionally, insectivorous species with high foraging requirements may not have time to search for extra-pair mates: bat-eared foxes spend 80% of the night feeding (Wright et al., 2010), leaving little free time to search for extra-pair mates.

2.4.3 Population Density

Population density and female dispersion can be key factors affecting levels of EPC (Iossa et al., 2008a). High population density decreases the potential risks of embarking on extra-territorial forays to search for mating opportunities by decreasing distances between individuals, increasing encounter rates between males and females seeking EPCs and allowing males to assess the reproductive conditions of neighbouring females (Gorman and Trowbridge, 1989). High population densities are associated with high levels of EPP in red foxes (up to 80% of cubs sired by extra-pair males at a density of 19.6-27.6 adults/km^2; Baker et al., 2004) and Island foxes (25% of 16 offspring sired by extra-group males with population density of 2.4-15.9 foxes/km^2; Roemer et al., 2001).

Particularly compelling evidence of the effect of population density on extra-pair mating comes from a population of red fox that experienced severe population declines while food availability remained constant. In a shift from high density (19.6-27.6 adults/km^2) to low (4.0-5.5 adults/km^2) from mange outbreak, EPP rates decreased from 80% (30 offspring) to 38% (38 offspring) and multiple paternity rates of litters decreased from 38-69% (16 litters) to 0% (10 litters, Baker et al., 2004; Iossa et al., 2008a). Fox body mass did not differ between the high and low density periods, indicating the population was not resource-limited at high densities (Soulsbury et al., 2008), suggesting food availability was not responsible for these differences.

However, population density did not seem to affect EPP in swift foxes studied by Kitchen et al. (2006) and urban coyotes living at high density with high resource availability were entirely genetically monogamous (Hennessy et al., 2012).

2.4.4 Social Structure

The number of potential breeders in a group influences the probability of extra-pair mating in canids (Spiering et al., 2010) and in mammals more generally (Clutton-Brock and Isvaran, 2006; Isvaran and Clutton-Brock, 2006; Lambert et al., 2018). Thus, the factors affecting group formation (described in Section 1.4.2) can influence EPC.

THE UNUSUAL CANID REPRODUCTIVE SYSTEM

Anomalous canid reproductive traits and their potential relationship with monogamy

Canids exhibit a suite of reproductive and physiological features that are unusual or even unique among mammals (Asa and Valdespino, 1998). The ultimate cause of these unusual traits could relate to the monogamous social system of canids by facilitating alloparental care and enforcing monogamy.

3.1 Facilitation of Alloparental Care

Long-term monogamous mating results in high levels of kinship between group members, an important factor in the evolution of mammalian alloparental care and cooperative breeding (Lukas and Clutton-Brock, 2012). Phylogenetic research shows that mammalian cooperative breeding evolved from social monogamy (Lukas and Clutton-Brock, 2012; Dillard and Westneat, 2016). Canids have developed several physiological characteristics that facilitates caring of offspring by individuals other than the mother, including the ability to provide food through regurgitation, obligate pseudopregnancy with potential spontaneous lactation, and seasonal prolactin surges. The conflict between whether to help raise siblings or breed is minimized since older offspring of a monogamous pair are equally related to their full-siblings as they would be to their own offspring.

3.1.1 Regurgitation

An innovation in canids is the ability to directly feed both pups and mother by regurgitation of partially digested food. This ability is found in all wolf-like canids (Canis, Cuon and Lycaon genera, Johnsignh, 1982; Lord et al., 2013) as well as maned wolves (Rasmussen and Tilson, 1984) and bush dogs (Biben, 1982). Regurgitation is generally absent from vulpine canids, although it was recently reported in the swift fox (Poessel and Gese, 2013). Regurgitation may be seen as an evolutionary adaptation facilitating paternal care, alloparental care and cooperative breeding, which would be advantageous in a closely-related monogamous social system.

3.1.2 Hormonal Priming of Alloparental Care

Canid ovulation that does not result in pregnancy is followed by a remarkably long dioestrous phase of nearly the same duration as pregnancy (2 months, Asa and Valdespino, 1998), during which time progesterone and prolactin are elevated, similar to pregnancy. This is therefore called pseudopregnancy. Spontaneous ovulation followed by obligate pseudopregnancy with hormonally-primed allomaternal care and the potential for additional lactating females has clear benefits for helping the pack and caring for the dominants’ offspring.

All canid species whose reproductive physiology have thus far been studied exhibit obligate pseudopregnancy, including grey wolves, coyotes, arctic foxes, red foxes, Ethiopian wolves and culpeos (*Lycalopex culpaeus*, Asa, 1996; Asa and Valdespino, 1998; van Kesteren et al., 2013). The endocrine similarity of obligate pseudopregnancy to true pregnancy hormonally primes all females that have ovulated for maternal behaviour, regardless of whether they conceived, thereby encouraging allomaternal care by non-breeding subordinate females. The hormonal similarity of pseudopregnancy and pregnancy can even cause spontaneous lactation, providing the possibility for females aside from the mother to nurse pups (Jöchle, 1997; Asa and Valdespino, 1998; van Kesteren et al., 2013). Allonursing has been reported in all *Canis* species except golden jackals (Lord et al., 2013) and can increase pup survival (Sillero-Zubiri et al., 2004a). The potential for allo-suckling may be an adaptive function of pseudopregnancy (Macdonald, 1992; Jöchle, 1997). In grey wolves, all pack members experience seasonal peaks in prolactin coinciding with pup birth. All adult wolves, even gonadectomized individuals, experience this prolactin peak, which is identical for males and females (Kreeger et al., 1991). Prolactin is associated with parental care in other species (reviewed by Angelier and Chastel, 2009) and thus this is likely related to the parental care exhibited by all pack members, including males (Kreeger et al., 1991; Jöchle, 1997).

3.2 Monogamy Enforcement

3.2.1 Post-Copulatory Lock

A post-copulatory tie has been reported for all canid species where copulation was observed, though of varying duration (Asa and Valdespino, 1998). In African wild dogs, the lock can be very brief (Asa and Valdespino, 1998), while in fennec foxes, locks can last as long as 2.75 hours (average 1.8 hours, Valdespino et al., 2002). The function may be to increase the probability of fertilization and enhance sperm transport. It has also been suggested to be an anatomical adaptation to encourage monogamy as a form of post-copulatory mate-guarding, since no other males can access the female during the period of sperm transport (Gomendio et al., 1998).

3.2.2 Monoestrum

The only carnivores to exhibit monoestrum, the restriction of seasonal reproduction to a single ovulatory cycle (Asa and Valdespino, 1998), are canids and their close relatives, ursids (Hayssen et al., 1993; Agnarsson et al., 2010). Polyoestrum, which is typical of most other mammals, is characterized by successive cycles of oestrus and ovulation without an intervening period of anoestrus (reproductive quiescence), which can be seasonal or year-round. Thus, if a female fails to conceive at one ovulation she has additional opportunities. Seasonal monoestrum limits females to a single conception opportunity per year – potentially a very risky reproductive strategy.

In ursids, the risk of monoestrous leading to missed mating opportunities is reduced by induced ovulation, meaning females only ovulate in the presence of appropriate stimuli (e.g. a male or copulation), similar to many other Carnivora species (e.g. felids, mustelids, Hayysen et al., 1993). Canids, however, not only have a single ovulatory cycle per season, but also exhibit spontaneous ovulation (Conaway, 1971; Asa and Valdespino, 1998), seemingly a derived trait as there is evidence of induced oestrus and ovulation in the most basal canid genus, *Urocyon* (Lindblad-Toh et al., 2005): Island foxes (*U. littoralis*) ovulate only in the presence of males (Asa et al., 2007) (whether there is induced oestrus and ovulation in the other member of the *Urocyon* genus [e.g., grey fox, *U. cinereoargenteus*] is unknown). Canids are thus unique in that they exhibit both spontaneous ovulation and monoestrum. This combination may increase the value of long-term pair bonding. Although the risks of monoestrous are reduced by the long oestrous period in canids (lasting approximately one week, contrasting with the one-day oestrous of many mammals, Asa and Valdespino, 1998), with only a single spontaneous oestrous cycle per year, there would be considerable risk if a female does not find a partner during the limited window of reproductive opportunity or if a female unknowingly paired with an infertile or genetically incompatible mate; long-term successful pairing may provide assurance against these possibilties.

Asa and Valdespino (1998) argue that the ultimate cause for monoestrum could be the canid social system, facilitating social monogamy and cooperative breeding through reproductive suppression. Monoestrum eliminates the opportunity for additional periods of oestrus in subordinates, which could cause social tension. They argue that if canids were polyoestrous, the dominant female would likely conceive on the first cycle but subordinates would continue cycling. However, due to canids’ long oestrous period (Asa and Valdespino 1998), the duration of time in oestrus may be equivalent between monoestrum and polyoestrum, resulting in the same amount of effort needed for reproductive suppression, regardless if over several cycles or one.

4. The exception proves the rule

Social and Mating System of the Domestic Dog

The domestic dog presents an interesting case because its social and mating system differs from all other members of the *Canis* genus. Domestic dogs derived from the grey wolf an estimated 11,000 to 40,000 years ago (Wang et al., 2013; Frantz et al., 2016; Botigué et al., 2017; see reviews by Driscoll et al., 2009; Driscoll and Macdonald, 2010). Despite their close evolutionary history, the general *Canis* pattern of social monogamy, pair bonding, extended paternal and alloparental care and monoestrous seasonal reproduction is conspicuously absent from dogs (Lord et al., 2013).

Free-living dogs generally exhibit a promiscuous mating system with no breeding hierarchy (Lord et al., 2013), though they can exhibit a range of mating systems (Pal, 2011). All adults can have the opportunity to breed and thus dog social groups can contain multiple lactating females with litters, in addition to other male and female group members (Macdonald and Carr, 1995; Pal, 2011; Paul et al., 2014). Though free-living dogs often live in groups, they do not always form a structured pack (Macdonald and Carr, 1995; Kamler et al., 2003a; Majumder et al., 2014). A social group may defend a territory together, but groups can be dynamic in composition, influenced by mating interests, resource availability, and closeness to source populations (Macdonald and Carr, 1995; Kamler et al., 2003a; Majumder et al., 2014). Some groups hunt cooperatively (Kamler et al., 2003b; Fleming et al., 2006) while others seemingly do not (Macdonald and Carr, 1995). Care is predominantly provided by the mother (Macdonald and Carr, 1995; Lord et al., 2013), though there are some observations of paternal (Pal, 2005) and alloparental (Paul et al., 2014) care. Although regurgitation and provisioning of offspring by males and helpers is characteristic of all other *Canis* species, it is rare in domestic dogs and mainly exhibited by the mother (Malm, 1995; Lord et al., 2013; though see Pal, 2005). Dogs reach independence much earlier than other Canis species, at 10-13 weeks, compared to an approximate average age of six months for other members of the genus (Pal, 2005; Lord et al., 2013). Dog pups do not receive extended parental care. After weaning, dog pups are independent of parenting and no longer directly fed; they instead must find their own food and compete with adults and juveniles. Wild *Canis* all exhibit reproductive seasonality, including dingoes, with births coinciding with seasonal increases in food availability; dogs are the only exception, with females coming into oestrous approximately every seven months and males always being capable of reproducing, though there can be concentrations of breeding during certain times of the year (Lord et al., 2013). Thus, dogs exhibit approximately two oestrus per year, unlike all other monoestrous *Canis* (Asa and Valdespino, 1998; Lord et al., 2013). This vastly different social and mating behaviour of domestic dogs compared to wild Canis is associated with different ecological conditions, providing an opportunity for understanding the ecological conditions that shape monogamy in other canids.

Are these differences adaptive?

Although Macdonald and Carr (1995) cautioned against interpreting the behaviour of a domesticated species as adaptive, Lord et al. (2013) argue that humans have had little reproductive control over the vast majority of dogs because most are free-ranging. They argue that the differences in the reproductive systems and behaviour of dogs compared to other *Canis* are adaptations to a new ecological niche created by the permanent and stationary settlement of humans and the associated food resource availability, rather than by artificial selection or reduced natural selection. The proposition that their behaviour is adaptive is supported by findings that two different dog communities behaved very differently when exposed to contrasting ecological circumstances (Macdonald and Carr, 1995).

Dogs tend to cluster in areas of human waste and the diet of most free-ranging dogs originates from humans, either directly through provisioning or indirectly from scavenging (Kamler et al., 2003a; Vanak and Gomper, 2009). Dogs are therefore released from seasonal fluctuations in resource availability, avoid high costs of having to hunt and generally experience high resource abundance (Macdonald and Carr, 1995; Pal, 2008; Kamler et al., 2003a; Lord et al., 2013). The ease of finding and processing food decreases the necessity for energetically-expensive parental care behaviours, making male care less valuable for domestic dogs than for wild canids. The reliable, year-round availability of human-derived food likely favoured the loss of reproductive seasonality (Lord et al., 2013). Freed from seasonality in resource availability, dogs can breed continuously throughout the year, avoiding competition with other dog litters even within the same social group. This also allows early age at first reproduction as dogs can breed as soon as they come into maturity, rather than waiting for the next breeding season (Lord et al., 2013). By avoiding energetically-costly parenting behaviour, dog parents can redirect energy into breeding year-round and multiple times per year, thereby increasing fecundity (Lord et al., 2013).

Although for wild canids the optimal strategy for maximizing reproductive success is often monogamy with biparental care, for domestic dogs paternal and alloparental care is not necessary due to more stable resource availabilities and thus they benefit from adopting an entirely different strategy. The genus-atypical reproductive and parental behaviour of domestic dogs supports the hypothesis that monogamy in canids is largely an adaptation allowing wild canids to make the most of fluctuations in resource availability (Lord et al., 2013).

5. Canid Success: Cause, Corollary or Consequence of Monogamy, the pro-cooperative Hypothesis

5.1 Canid Success

This essay was prompted by the question of whether monogamy is a cause, consequence, or correlate of Canidae success, as individuals, species and family. Unlike many other carnivore families, canids have thrived in the rapidly changing conditions of the Anthropocene (Wang and Tedford, 2007, 2008). The world’s most widely distributed wild terrestrial mammal is a canid: the red fox, found from the Arctic Circle to North Africa, North America and Eurasia and introduced and now widespread in Australia and USA (Macdonald and Sillero-Zubiri, 2004). Prior to the ascent of red foxes, this title was held by another canid, the grey wolf, originally distributed throughout the Northern Hemisphere in every habitat large ungulates were found (Mech, 1995; Macdonald and Sillero-Zubiri, 2004), until widespread human persecution caused their near-complete extirpation by the late 1800s (Mech, 1995; Phillips et al., 2004). But canids are resilient and grey wolves are now returning to their former range in both North America and

Europe (Mech, 1995; Breitenmoser, 1998; Wydeven et al., 1998; Phillips et al., 2004; Mech, 2017). Coyotes have dramatically increased their range over the past two centuries. Previously found only in the prairies and deserts of western North America, they are now ubiquitous in every country and state from Alaska to Panama, found in nearly all available habitats, including forest, prairie, desert, mountain, tropical habitats, and cities (Gompper, 2002; Macdonald and Sillero-Zubiri, 2004). Coyote range expansion was likely catalysed by the extermination of grey wolves, thus reducing intra-guild competition (Macdonald and Sillero-Zubiri, 2004), and land conversion through logging and agriculture which opened up additional habitat (Méndez-Carvajal and Moreno, 2014). The rapid expansion of coyotes in North and Central America is mirrored by that of golden jackals in Europe. Native to the Middle East and southern Asia, golden jackals arrived at the southern edge of Central and Eastern Europe around 8,000 years ago and began slowly expanding in the 19th century, but since the 1950's their expansion has accelerated into the north and west of Europe (Tóth et al., 2009; Rutkowski et al., 2015). They are now found as far north as Finland, four degrees below the Arctic Circle (Banea and Giannatos, 2019), and as far west as Switzerland (Arnold et al., 2012; Trouwborst et al., 2015) and their continued expansion, for reasons paralleling those for coyotes, seems likely (Arnold et al., 2012). In addition to these natural rapid expansions, introductions of canids by humans allowed several to thrive as invasive species. Raccoon dogs, originally from Siberia, East Asia and Japan, were introduced as a furbearing species in the Soviet Union from 1928-1955 and within 50 years had colonized 1.4 million km^2 of northern and eastern Europe (Helle and Kauhala, 1991; Kauhala and Saeki, 2004; Sutor, 2007; Kauhala and Kowalczyk, 2011). The reintroduction and subsequent rapid spread of red foxes in mainland Australia and USA is another classic example of biological invasion (Kamler and Ballard, 2002; Fleming et al., 2006). The domestic dog, a few genes adrift from grey wolves, is one of the world's most successful mammal: population estimates range from 700 million (Hughes and Macdonald, 2013) to over one billion (Lord et al., 2013), roughly 80% of which are estimated to be free-ranging (Lord et al., 2013). Despite considerable efforts, humans have been unable to control the populations of these canids.

What has allowed these canids – red fox, grey wolf, coyote, golden jackal, raccoon dog, and domestic dog – to be so successful as to rapidly colonize new areas, dramatically increase in abundance, and continue to do so despite human efforts to control populations, and could their monogamous lifestyle be partially to blame? We suggest that canid success may be attributed to four main characteristics: 1) their generalist nature, adaptability, flexibility, and intelligence, allowing them to adapt to diverse habitats, diets and circumstances; 2) their high mobility and capacity for long-distance travel, facilitating fast colonization, expansive gene flow and genetic diversity, creating a selective advantage in changing environments and minimising the risk of inbreeding from founder effects (Reed and Frankham, 2003); 3) their high reproductive rate, allowing them to quickly increase in number and recover following population declines from disease and persecution; and 4) their sociality and ability to cooperate, which can provide numerous benefits (see Section 1.4.2; Macdonald and Carr 1989; Macdonald et al., 2004). We argue that while the first and second attributes on this list are conserved traits that arose early in canids' phylogenetic history, the third and fourth are consequences of monogamy, and that it is the combination of these four characteristics that contribute to canids' success.

5.2 Canid Success Traits that are Consequences of Monogamy

5.2.1 High Reproductive Rates

Co-evolution of paternal care and litter size resulted in the large litters that are characteristic of canids, thus increasing reproductive output (Stockley and Hobson, 2016). Canids can also reproduce in their first year and breed annually (Lord et al., 2013). In comparison to other omnivorous Carnivores, canids are distinguished by the platform provided by monogamy for benefiting from paternal care, cooperative breeding and allopaternal care, all of which can enhance lifetime reproductive success (Lukas and Clutton-Brock, 2013; Opie et al., 2013). Rapid reproduction allows canids to withstand high mortality rates (from human persecution and disease, itself often anthropogenic; Rhodes et al., 1998) and quickly colonize new areas. Despite widespread persecution, red foxes, coyotes, golden and black-backed jackals and raccoon dogs are able to thrive, while wolves continue to recover in North America and Europe despite ongoing illegal killings (Mech, 1995, 2017).

High reproductive rates may also allow for rapid phenotypic and genotypic adaptations to cope with new or changing environments and prevents inbreeding depression (Reed and Frankham, 2003). Furthermore, socially monogamous mating systems are predicted to produce greater reproductive output and genetic diversity compared to polygynous or polyandrous systems, leading to larger effective population sizes (Parker and Waite, 1997; Waite and Parker, 1997).

5.2.2 Sociality and Cooperation

The complex, cooperative social systems of canids that evolved from monogamy (Lukas and Clutton-Brock, 2013; Dillard and Westneat, 2016) provides numerous benefits discussed in Section 1 (e.g. cooperative hunting, food defence, reproductive success). Furthermore, the cooperation that first developed between members of the monogamous pair can spill over to other individuals, generally kin, when resource dispersion facilitates cohabitation by a spatial group (see Macdonald and Johnson 2015). While this might originally be focused on young, it is a small step to cooperating with, and even assisting, other adult group members (e.g. adult red foxes caught in traps may be fed by other foxes [Garcelon et al., 1999]).

Canids have among the largest relative brain sizes in Carnivora (Gittleman, 1986; Swanson et al., 2012) and an enlarged prefrontal cortex compared to felids and other carnivores (Radinsky, 1969), associated with increased intelligence and behavioural complexity and flexibility. Across carnivore species, experiments show that greater relative brain size is associated with greater problem solving (Benson-Amram et al., 2016) and across mammals, larger brain sizes are associated with the ability to successfully adapt to, colonize, and invade novel habitats (Sol et al., 2008). The sociality and cooperation that evolved in canids from monogamy may have increased canid brain size through influence on diet. Cooperative hunting allows canids to tackle larger vertebrate prey and increases hunting success (Fanshawe and Fitzgibbon, 1993; Creel and Creel, 1995). Carnivore species that consume vertebrates have the largest brains, omnivores intermediate, and insectivores the smallest (Swanson et al., 2012). This could be because hunting vertebrate prey is more cognitively demanding than omnivory or insectivory, and particularly when synchronizing hunting behavior with pack mates, or because consuming higher-energy foods allows evolution of metabolically expensive brain tissue (Swanson et al., 2012).

Increased brain size is also argued to be a consequence of the complex social relationships that monogamy requires. Shultz and Dunbar (2007) found that larger relative brain size is correlated with socially monogamous pair bonding in Carnivora, other mammalian orders, and birds. Furthermore, bird species with long-term monogamous pair bonds (like that of most canids) have larger brains than species with short-term seasonal monogamy (Schultz and Dunbar, 2007; West, 2013). Shultz and Dunbar (2010) concluded that increased brain size evolved in birds as a result of long-term pair bonding, not that larger brains allowed long-term pair bonding. Three hypotheses have been proposed for why monogamous pair bonding may select for larger brains. Shultz and Dunbar (2007) and Dunbar (2009) argue that the cognitive demands of behavioural coordination, synchronization, and negotiation necessary for navigating and maintaining stable pair bonded partnerships is responsible for increased brain sizes seen with monogamy. Alternatively, the potentially high costs of selecting an unreliable or infertile mate, particularly in species forming long-term monogamous pair bonds where there is reduced availability of alternative mates, may have selected for cognitively-demanding mate selection processes (Dunbar, 2009). A third hypothesis relates to the pressures of mate guarding and procuring fitness-increasing extra-pair copulations while maintaining social partnerships (West, 2013). This is supported in birds, where larger brains not only correlate with social monogamy but also with extra-pair paternity: as rates of extra-pair paternity increase, so does brain size. This suggests there is an intersexual co-evolutionary arms race with both sexes trying to outsmart each other in trying to sneak extra-pair copulations while preventing their mate from doing the same, leading to larger brains in both sexes (West, 2013).

Sociality beyond the pair bond may also contribute to increased brain sizes. Swanson et al. (2012) found that carnivoran social complexity is positively correlated with relative cerebrum volume (but not total brain volume), in line with the social brain hypothesis in primates which posits that larger brains evolved due to the cognitive demands posed by complex social systems (Dunbar, 1992; Schultz and Dunbar 2007; but see DeCasien et al., 2017) (studies on the relationship between total relative brain volume and carnivore sociality have found conflicting results, see Gittleman, 1986; Dunbar and Bever, 1998; Perez-Barberia et al., 2007; Schultz and Dunbar, 2007; Finarelli and Flynn, 2009; Swanson et al., 2012).

Thus, the sociality and cooperation that evolved in canids as a result of monogamy also likely made them more intelligent and adaptable compared to other carnivore families, which would have further facilitated the relative dominance of canids and allowed several canid species to prosper even during the Anthropocene.

In this sense, monogamy primed pro-cooperative, pro-social behaviours in ancestral canids that were, and largely remain, facultative rather than obligate. This facility for cooperation offers canids a selective advantage to maximise opportunities more readily than would have been the case without the pro-cooperative bonus brought by monogamy.

5.3 Conserved Traits for Success Amongst the Canidae

5.3.1 Intra-Specific Ecological Flexibility

Canids are highly flexible and adaptable in their ecology, able to exploit a wide range of diets, habitats, and social structures. Although the central theme of canid social and mating systems is monogamy, their extreme flexibility leads to intraspecific variation in social behaviour as adaptations to varying ecological conditions, allowing them to take advantage of superior strategies when opportunity permits. Consider Robertson's (2016) finding that in areas with low resource availability, female coyotes delay reproduction and instead bide at home as helpers, more than half breeding only after their third year. In areas with high resource availability, almost half bred as yearlings, and almost all did so by their third birthday. This flexibility allows rapid increases in numbers, contributing to resilience to intense hunting pressure (Berger, 2006). Interspecifically, consider the domestic dog, which abandoned the *Canis* pattern of monogamy and its associated social and reproductive traits to take advantage of the abundant resources in its niche, allowing domestic dogs to become the most abundant carnivore on earth (Hughes and Macdonald, 2013; Lord et al., 2013). The remarkable intra-specific flexibility of canids means that while monogamy, and the cooperation it facilitates, is their norm, they can survive and even sometimes thrive alone when needed (much like omnivorous viverrids and musteloids) or adopt polygamy when ecological conditions present that as a superior strategy. They can be flexible as necessity or circumstance requires or permits.

5.3.2 High Mobility

Canids are capable of fast and wide-ranging movements. Movements of 86 km in a little over a month and 230 km in approximately 3 months have been recorded in the African golden wolf (Karssene 2018), grey wolves disperse as far as 1000 km in search of new territories and mates (Mech et al., 1995; Ciucci et al., 2009) and minimum dispersal distances documented for coyotes are 94 km for females and 113 km for males (Harrison 1992). The interplay of this high mobility with their ecological flexibility allows canids to quickly move to new areas and adjust to local conditions.

5.4 Formula for Canid Success: the monogamy as pro-cooperative hypothesis

The four attributes presented above may be the keys to the formula of canid success among carnivores. This hypothesis might be termed the monogamy as pro-cooperative hypothesis (Figure 1). In short, monogamy appears to have arisen when females are dispersed between sharable territories that arise as a result of the dispersion of available resources (Macdonald and Johnson 2015), preventing males from defending multiple females but nonetheless allowing them to cohabit with one, such that guarding a single female is the most efficient male strategy (Komers and Brotherton, 1997; Brotherton and Komers, 2003; Lukas and Clutton-Brock, 2013), emerging from the ancestral canids' ecological circumstances. Monogamy together with biparental and alloparental care allowed for high reproductive output and cooperative sociality and intelligence. This perspective is congruent with Lukas and Clutton-Brock's (2013) conclusion, based on phylogenetic analysis, that biparental care evolved from social monogamy, and cooperative breeding systems evolved from that. Their high mobility allows canids to quickly expand to new areas, their intelligence and generalist nature allows them to adapt to new diets and habitats in these new areas, and together with their high reproductive output and cooperative sociality allows them to quickly increase in numbers and successfully colonize.

5.4.1 Comparison within Canidae

To consider further the role these four traits play in canid success, we explore how some of the most successful wild canids, as outlined above – red fox, golden jackal, coyote, raccoon dog – differ from those species that are not faring so well. Of the 37 extant Canidae species, one is listed by the IUCN as Critically Endangered (red wolf) and four as Endangered (Ethiopian wolf, African wild dog, dhole and Darwin's fox); no species are listed as Vulnerable and only one canid has gone extinct in historical times (Falkland islands wolf, *Dusicyon australis*, in 1876). How do these species differ? For the Falkland islands wolf and Darwin's fox, their small and isolated ranges, was and is their main downfall. For the other species mentioned here, they lack a critical piece of the puzzle for canid success – their generalist, adaptable nature. African wild dogs and dholes are hypercarnivorous, with specialized dentition for a diet primarily of large vertebrate prey. Their large body size coincides with high energy requirements (Carbone et al., 1999; Slater, 2015), meaning they must consume large prey for foraging to be energetically economical. This results in a greater reliance on cooperative hunting, such that, unlike other canids that can adjust social structure and thrive alone, as pairs, or in groups, these species may have a minimum pack size threshold for successful hunting and breeding (Carbone et al., 1999). The African wild dog and dhole are also among the least monogamous canids – dholes tend to exhibit communal breeding, living in large clans and more often with multiple breeding females in a group (Fox, 1984), while African wild dogs are often polyandrous or polygynandrous with multiple litters in a pack (see Section 2.4). African wild dogs deviate from other canid patterns – they exhibit the shortest recorded post-copulatory lock (Frame 1979; Asa and Valdespino, 1998), which we suggested could be a monogamy enforcement adaptation (Section 3.2.1). The Ethiopian wolf has also increased dietary specialization, but in the opposite direction – rather than specializing on large prey, this species specializes on Afroalpine rodents, which can comprise as much as 97% of prey volume of their diet (Sillero-Zubiri and Gottelli, 1995b). Afroalpine rodent communities can therefore limit the distribution of Ethiopian wolves (Sillero-Zubiri et al., 1995a,b).

5.4.2 Comparison among Carnivora

To isolate the importance of monogamy within the canid syndrome of breeding fast and cooperating as opportunity allows requires solving the algebra of alternative evolutionary pathways that led other generalist, omnivorous carnivores to life histories that do not involve monogamy. How do they fare?

Consider the feliform solution to the same evolutionary problem, the Viverridae (genets and civets). Although viverrid biology is not well known (e.g. Ross et al., 2017) they too excel at omnivory, but exhibit classic carnivorean polygyny, produce small litters, and lack the pioneering adaptability and cooperative tendencies of canids. The same might be said of most omnivorous musteloids and ursids, themselves carrying much of the caniform phylogenetic baggage shared by canids (Koepfli et al., 2017), but with societies conspicuously lacking monogamy (Macdonald and Newman, 2017). The comparison is not flawless, but sufficiently compelling that the role of monogamy in the syndrome of canid attributes is part of their particular success. Perhaps the extinction of the tenth family of caniforme Carnivores, the Amphicyonidae (the “bear-dogs”), 2.6 million years ago, probably due to competition with true dogs, lay in the trump card of monogamy. The point might even be stretched to explain how, five million years ago, canids ousted the dog-like Hyeanidae that once outnumbered them (Macdonald, 1992). Amongst the Caniforme suborder of Carnivora, despite their close phylogenetic relationships, Canidae are the only family to exhibit monogamy. Of the 37 extant canid species, only 5 – 13.5% – are listed as threatened (Critically Endangered, Endangered, or Vulnerable) on the IUCN Red List. This is the lowest proportion among all Caniforme families. An avenue worth exploring may be associations between the conservation status of species in a family and the number of the critical traits identified here that they exhibit (amongst Caniforme families we think that none other than canids display all four).

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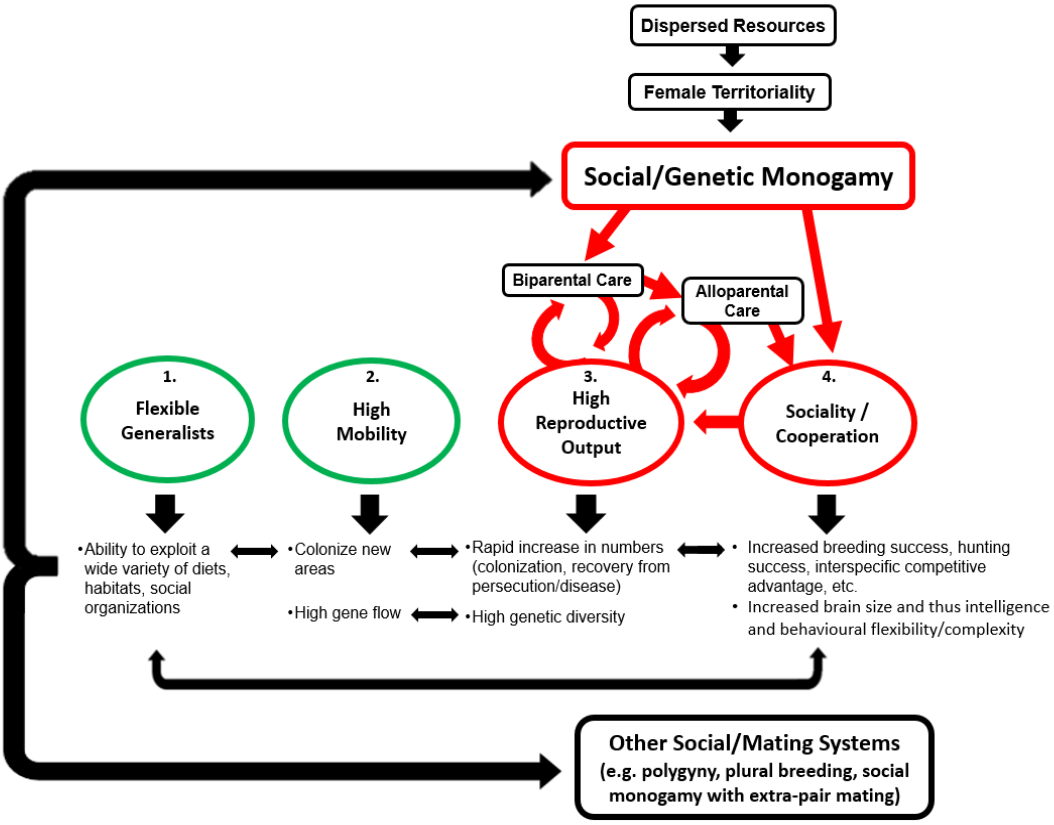

Figure 1. The *monogamy as pro-cooperative hypothesis*: Canid success may be attributed to four characteristics: 1) their flexible, generalist, adaptable nature, 2) high mobility, 3) high reproductive rates, 4) sociality and cooperation. Traits 1 and 2 appear to be early traits of canids' phylogenetic history, while 3 and 4 arose from monogamy. Dispersed resources, insufficient to support multiple breeding females but sufficient for a female and male within a territory, seem to have led to the evolution of social monogamy, which in turn led to the evolution of biparental care and, thus, where ecological circumstances allow, alloparental care and sociality/cooperation (trait 4) and high reproductive rates (trait 3). These create feedback loops, where biparental/alloparental care, sociality and cooperation lead to higher reproductive output, which, in turn require biparental/alloparental care, sociality and cooperation.

These four canid traits are interconnected and enhance one another. For example, high mobility allows canids to move to new areas, their generalist flexible nature allows them to adapt to these new areas, their ability to quickly increase in number through high reproductive rates allows them to establish in these new areas, with survival and reproduction further enhanced by their sociality and cooperation. The interplay of rapid reproduction and high mobility, allowing gene flow and enhancing genetic diversity, contributes to their adaptability and flexibility, and sociality further contributes to their flexibility through its association with enhanced intelligence, in line with the social brain hypothesis.

This flexibility means that canids can adapt social and mating systems to suit local ecological conditions, which may result in social monogamy, or, if ecological conditions are such that superior strategies exist, canids can exploit alternative social/mating strategies yet retain the benefits of high reproductive rates and sociality/cooperation that monogamy afforded (see Section 1: Ecological Correlates of Social Monogamy for ecological conditions affecting maintenance of social monogamy vs. alternative strategies such as polygyny, plural breeding, etc., and Section 2: Ecological Correlates of Extra-Pair Mating for ecological conditions affecting genetic monogamy vs. extra-pair mating).

It is thus the combination of these four traits, with monogamy at its foundation, that together make up the formula of canid success, explaining the rapid expansion, colonization, and invasion of multiple canid species in recent years.

Social Monogamy: social structure involving a single breeding male and female, which in canids typically involves an affiliative pair bond, shared territory with mutual territory defense, and biparental offspring care. Group-living canids may exhibit social monogamy, with a single breeding male and female pair and additional non-breeding group members (often their offspring).

Genetic Monogamy: exclusive reproduction between one male and one female (i.e. no extra-pair paternity).

Pair Bonding: affiliative bond between a breeding male and female, which in canids is generally maintained for several years, often until the death of one of the pair. In some canids, the mated pair maintains close associations year-round, whereas in maned wolves and many small canids, although a pair shares and defends a territory year-round, close associations and den sharing occur primarily during the breeding and pup-rearing seasons (Dietz, 1984; Kitchen et al., 2005a; Ralls et al., 2007; Kamler and Macdonald, 2014).

Canid Variations of Social Monogamy

Pairs: a single pair-bonded mating male and female share a territory year-round and young disperse. Example: swift fox (Kitchen et al., 2006).

Trios: a single pair-bonded dominant mating male and female, plus an additional subordinate non-breeding adult. The additional adult is usually the offspring of at least one of the pair from a previous year, though not always. The third adult may or may not actively help in raising young, and trios may be stable over several years. Example: kit fox (White and Ralls, 1993).

Groups: a single pair-bonded dominant male and female, plus additional subordinate non-breeding adults. Additional adults are usually offspring of at least one of the pair from previous years, though not always. In some cases, additional adults may actively help in raising pups and groups may cooperatively hunt and defend resources, in which case it can be considered **cooperative breeding** (though cooperatively breeding groups could also be polygamous) Example: grey wolves (Bekoff and Wells, 1982). In other cases, additional adults do not actively help in raising pups and group members do not coordinate behavior. Example: Blanford foxes (Geffen and Macdonald, 1992).

Double litters/Plural breeding: multiple (usually two) non-interbreeding pairs of males and females share a den and territory and produce litters. Two litters may be born in the same den (e.g. coyote: Hennessy, 2007), or two litters may later merge (e.g. arctic fox: see Norén et al., 2012). The two females are often close relatives (e.g. mother-daughter). Note that these terms have also been used to describe polygynous/polygynandrous systems. Although double litters are commonly reported in coyotes, more genetic research is needed to distinguish cases where these are in fact multiple litters or large litters with size differences between pups, or whether this represents polygamous systems, though one study has confirmed two genetically monogamous pairs (Hennessy, 2007). If alloparental care is provided to the other litter (e.g. allo-nursing between females can be common), this represents **communal breeding** (i.e. not social monogamy).

Deviations from social monogamy: Group-living canids may instead exhibit social polygyny (e.g. bigamous red foxes: Zabel and Taggart 1988), social polyandry (e.g. African wild dogs, Spiering et al., 2010), polygynandry/communal breeding (e.g. African wild dogs, Spiering et al., 2010).

Box 1. Variations of social monogamy in canids.

Table 1. Correlates of social and genetic monogamy in canids.

Correlate	**Summary**
Social Monogamy vs. Alternative Strategies	
1.4.1 Resource availability	High resource availability reduces the probability of social monogamy by either reducing reliance on male care of infants and thus the benefits of monogamy or by allowing males to provision multiple litters.
1.4.2 Social Structure	The costs and benefits of canid group formation are influenced by many ecological factors (e.g. resource availability/dispersion, prey size, inter- and intraspecific competition, predation pressure, population density, territory availability). Larger social groups are less likely to exhibit social monogamy.
Genetic Monogamy vs. Extra-Pair Mating	
2.4.1 Resource availability	High resource availability reduces reliance on male care of infants and thus the potential cost of reduced male investment from engaging in extra-pair mating.
2.4.2 Diet: Foraging strategy	Canids that can forage with their mate can more effectively mate guard, whereas canids that must forage solitarily to reduce food competition have more opportunity to engage in extra-pair mating.
2.4.2 Diet: Time budget	Canids that must allocate more time to foraging (e.g. insectivorous species) have less time available to seek extra-pair mates.
2.4.3 Population density	High population density increases the availability of extra-pair mates and reduces potential costs of seeking extra-pair mating (reducing distances between individuals, increasing encounter rate and the probability of finding extra-pair mate).
2.4.4 Social structure	More potential breeders in a group increases the probability of extra-pair mating. As with population density, the costs of embarking on extra-territorial forays are reduced if extra-pair mating is within the group.

Table 2. Potential functional explanations for extra-pair mating by female canids.

Functional Hypothesis	**Support in Canids**
2.2.1 Increase genetic quality	Some support. Extra-pair mating is biased towards more dominant or larger males in some canids; may depend on circumstances/ species.
2.2.2 Increase genetic diversity	Could be a common motivation. Extra-pair mating is generally associated with multiple paternity in canids.
2.2.3 Inbreeding avoidance	Little support. Social pairs are generally unrelated so other mechanisms may be responsible for inbreeding avoidance, and breeding with close relatives can occur both with social mates and extra-pair mates in canids.
2.2.4 Infanticide protection by paternity confusion	Little support, unlikely. Infanticide does not increase males' breeding opportunities because canids breed seasonally and annually; little evidence that male canids engage in infanticide.
2.2.5 Fertilization assurance	Could be a common motivation due to canid monoestrum, but has not been studied.
2.2.6 Increased alloparental care by paternity confusion/dilution	Unlikely to apply to most cases where extra-pair mating with individuals outside the social group, but may be relevant when extra-pair mating is within the group.

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Appendix B.

SUPPLEMENTARY MATERIALS OF GENETIC STUDIES

Appendix B-1. Supplementary information for: Phylogenetic evidence for the ancient Himalayan wolf: towards a clarification of its taxonomic status based on genetic sampling from western Nepal

Geraldine Werhahn, Helen Senn, Jennifer Kaden, Jyoti Joshi, Susmita Bhattarai, Naresh Kusi, Claudio Sillero-Zubiri and David W. Macdonald

METHODS

Genetic analysis procedure

DNA extraction

A mixture of commercially available Isohelix and Qiagen Stool kit parts were used for DNA extraction. Samples were centrifuged for one minute at 4000rpm followed by pipetting the supernatant into clean 1.5ml flip-top tube (~450μl). 250μl InhibitEX solution was added after which it was vortexed for a minute. Then the sample solution was left for one minute at room temperature to allow the inhibitors to be absorbed. This was followed by centrifugation for one minute at full speed (14.5x1000rpm) to pellet remaining stool particles. The supernatant was then pipetted into a 1.5ml flip-top tube and 25μl of proteinase K solution was added. This was placed on a thermoblock at 60˚C, and mixed for approximately one hour. Then 500μl CT solution was added and the tube inverted several times to mix, followed by briefly centrifuging to remove liquid from the lid. Of this 600µl was pipetted into a spin column collection tube and centrifuged for one minute at full speed. This was repeated with new collection tubes until all the lysate was through the spin column. The spin column was then put into a clean collection tube and spun through 500µ Qiagen l AW1 wash buffer for one minute at full speed. The spin column was then put into a clean collection tube and spun through 500µl Qiagen AW2 wash buffer for one minute at full speed. The spin column was then put into a clean collection tube and spun for two minutes at 14.5x1000rpm to remove any residual ethanol from the cartridge. Then it was transferred to a labelled 1.5ml flip-top tube and 75μl AE elution buffer, which was heated to ~55˚C on the thermoblock. It was then pipetted on to the cartridge and left to settle for one minute followed by spinning for one minute at full speed. The DNA sample was then quantified and stored at -20°C.

DNA sequencing

Cytochrome *b* and D-loop sequences of the mtDNA, and X- and Y-linked zinc-finger protein gene (ZFX and ZFY) sequences were generated from 104 samples collected in 2015.

Primers for both D-loop and cytochrome *b* sequences were designed at RZSS WildGenes laboratory. For the X- and Y-linked zinc-finger protein gene (ZFX and ZFY) sequences were amplified using the protocol and primers used by (Koepfli et al., 2015; Nakagome et al., 2008; Tsubouchi et al., 2012) (for the primer sequences see supplementary material Table S4).

The D-loop control region primers amplified a 296bp long section (forward primer WolfJack dloop: GCACCCAAAGCTGAAATTCT, reverse primer WolfJack dloop: ATGGGCCCGGAGCGAGAAGAG). Sequencing primers were diluted to 10μM and Taq master mix (Thermo Fisher Scientific Inc.) and were run with the following PCR program: Amplification performed with an initial denaturation step of 95˚C for 5 minutes, followed with 40 cycles for 30 seconds at 95˚C (denaturation), 61˚C for 30 seconds (primer annealing) and 72˚C for 60 seconds (elongation), and ending with 72˚C extension for 10 minutes. Samples were viewed on a 1.5% agarose gel to check if they have amplified.

For cytochrome *b*, primers amplifying at the 353bp section were used (forward primer WolfJackCytb1: TTGTATTTCAACTATAAGAACAT, reverse primer WolfJackCytb: GCAAAGAATCGTGTTAGGGTTG).

The amplification procedure for the cytochrome *b* was as for the D-loop but with the following PCR thermocycling conditions: 95˚C for 5 minutes, then 35 cycles of 95˚C for 30 seconds, then 50˚C for 90 seconds and 72˚C for 30 seconds, followed by 60˚C for 30 minutes. As positive controls, 8 samples were repeated with D-loop forward, 27 with D-loop reverse, and 33 were repeated for both forward and reverse cytochrome *b*.

Table S1. Haplotypes of Himalayan wolves and dogs found in the study area in Humla (Nepal) with NCBI GenBank accessions.

Region	**Species**	**Haplotype Name**	**GenBank Accession**
D-loop	*C. himalayensis*	Himalayan wolf D-loop 1	KY996529
D-loop	*C. himalayensis*	Himalayan wolf D-loop 2	KY996530
D-loop	*C. himalayensis*	Himalayan wolf D-loop 3	KY940301
D-loop	*C. lupus familiaris*	Domestic dog D-loop Nepal 1	KY996526
cytochrome *b*	*C. himalayensis*	Himalayan wolf Cytochrome B 1	KY996533
cytochrome *b*	*C. himalayensis*	Himalayan wolf Cytochrome B 2	KY996534
cytochrome *b*	*C. lupus familiaris*	Domestic dog Cytochrome B Nepal 1	KY996532
ZFY	*C. himalayensis*	Himalayan wolf Nepal ZFY	MF101862
ZFX	*C. himalayensis*	Himalayan wolf Nepal ZFX	MF101863

Table S2. Overview of the D-loop data used in the phylogenetic analysis. Listed are the new haplotypes of Himalayan wolf 1-3 and one domestic dog found in the study area in Humla (Nepal), and all reference sequences obtained from NCBI GenBank. Marked with an asterisk* are sequences that are unpublished and have been directly submitted to NCBI GenBank by the respective authors. Reference sequences from the following publications were included: (Aggarwal et al., 2007; Björnerfeldt et al., 2006; Gaubert et al., 2012; Gottelli et al., 2004; İbiş et al., 2015; Ishiguro et al., 2009; Koepfli et al., 2015; Rueness et al., 2011; Sharma et al., 2004; Thalmann et al., 2013; Waters et al., 2015).

Haplotype	Name abbreviated	Unique Haplotypes Accession	Identical Sequences Accessions	Publication
Side-striped Jackal 1		JQ088674.1		Gaubert et al. (2012)
Side-striped Jackal 2		JQ088669.1		Gaubert et al. (2012)
			JQ088670.1	Gaubert et al. (2012)
			JQ088671.1	Gaubert et al. (2012)
			JQ088672.1	Gaubert et al. (2012)
			JQ088673.1	Gaubert et al. (2012)
African Wild Dog		KT448283.1		Koepfli et al. (2015)
Asian Dhole		KT448282.1		Koepfli et al. (2015)
Coyote 1		DQ480509		Björnerfeldt et al. (2006)
			DQ480511	Björnerfeldt et al. (2006)
Coyote 2		DQ480510		Björnerfeldt et al. (2006)
Red Fox		KY996531		This study
Himalayan wolf 1 Humla Nepal	HW1_Humla Nepal	KY996529		This study
			AY333740	Sharma et al. (2004)
			AY289986	Aggarwal et al. (2007)
			AY289995	Aggarwal et al. (2007)
			AY289985	Aggarwal et al. (2007)
			AY289994	Aggarwal et al. (2007)
			AY289977	Aggarwal et al. (2007)
			AY289993	Aggarwal et al. (2007)
			AY289992	Aggarwal et al. (2007)
			AY289991	Aggarwal et al. (2007)
			AY289978	Aggarwal et al. (2007)
			AY289990	Aggarwal et al. (2007)
			AY289979	Aggarwal et al. (2007)
			AY289980	Aggarwal et al. (2007)
			AY289989	Aggarwal et al. (2007)
			AY289981	Aggarwal et al. (2007)
			AY289988	Aggarwal et al. (2007)
			AY289982	Aggarwal et al. (2007)
			AY289983	Aggarwal et al. (2007)
			AY289987	Aggarwal et al. (2007)
			JX415352	unpublished*
			JX415350	unpublished*

Himalayan wolf 2 Humla Nepal	HW2_Humla Nepal	KY996530		This study
			JX415351	unpublished*
			EU442884.2	unpublished*
Himalayan wolf 3 Humla Nepal	HW3_Humla Nepal	KY940301		This study
			JX415343	unpublished*
Himalayan wolf 4 Ladhak India	HW4_Ladhak	AY333741		Sharma et al. (2004)
Himalayan wolf 5 Qinghai Lake China	HW5_Qinghai_Lake_China	JX415347		unpublished*
Himalayan wolf 6 Qinghai Lake China	HW6_Qinghai_Lake_China	JX415345		unpublished*
Himalayan wolf 7 Museum Tibet	HW7_Museum Tibet	AY333739		Sharma et al. (2004)
Himalayan wolf 8 Tibet	HW8_Tibet	KF573616		unpublished*
			AB480742	Ishiguro et al. (2009)
Himalayan wolf 9 Museum Nepal	HW9_Museum Nepal	AY333738		Sharma et al. (2004)
			JX415344	unpublished*
Himalayan wolf 10 Museum Tibet	HW10_Museum Tibet	AY333742		Sharma et al. (2004)
Himalayan wolf 11 Qinghai Lake China	HW11_Qinghai_Lake_China	JX415348		unpublished*
Indian grey wolf 1	IW 1	AY333745		Sharma et al. (2004)
Indian grey wolf 2	IW 2	AY289973		Aggarwal et al. (2007)
Indian grey wolf 3	IW 3	AY333746		Sharma et al. (2004)
Indian grey wolf 4	IW 4	AY333743		Sharma et al. (2004)
Indian grey wolf 5	IW 5	AY289974		Aggarwal et al. (2007)
			AY289975	Aggarwal et al. (2007)
			AY289976	Aggarwal et al. (2007)
			AY289984	Aggarwal et al. (2007)
			AY333746	Sharma et al. (2004)
			AY333744	Sharma et al. (2004)
Grey wolf_China 1	GW China 1	KF661041		Thalmann et al. (2013)
			KF661053	Thalmann et al. (2013)
Grey wolf_Mongolia 1	GW Mongolia 1	KY996527		WildGenes collection
Grey wolf_Mongolia 2	GW Mongolia 2	KY996528		WildGenes collection
Grey wolf_Russia	GW Russia	KF661046		Thalmann et al. (2013)
Grey wolf_Poland	GW Poland	KF661045		Thalmann et al. (2013)
Grey wolf_India	GW India	KF661043		Thalmann et al. (2013)
			KF661054	Thalmann et al. (2013)
Grey wolf_Egypt	GW Egypt	JQ088677.1		Gaubert et al. (2012)
			KF661055	Thalmann et al. (2013)
Grey wolf_Saudi Arabia	GW Saudi Arabia	DQ480506		Björnerfeldt et al. (2006)

Grey wolf_Israel	GW Israel	KF661042		Thalmann et al. (2013)
			AY333733	Sharma et al. (2004)
Grey wolf_Oman		KF661050		Thalmann et al. (2013)
Grey wolf_Iran		KF661051		Thalmann et al. (2013)
Grey wolf_Finnland	GW Finnland	KF661038		Thalmann et al. (2013)
			KF661039	Thalmann et al. (2013)
			DQ480503	Björnerfeldt et al. (2006)
Grey wolf_Sweden	GW Sweden	KF661040		Thalmann et al. (2013)
			KF661044	Thalmann et al. (2013)
			KF661049	Thalmann et al. (2013)
			KF661052	Thalmann et al. (2013)
			DQ480504	Björnerfeldt et al. (2006)
Grey wolf_Italy	GW Italy	KF661048		Thalmann et al. (2013)
Grey wolf_Spain	GW Spain	DQ480505		Björnerfeldt et al. (2006)
Grey wolf_USA	GW USA	KF661064		Thalmann et al. (2013)
			KF661068	Thalmann et al. (2013)
			KF661069	Thalmann et al. (2013)
			KF661072	Thalmann et al. (2013)
Grey wolf_Canada 1	GW Canada 1	KF661074		Thalmann et al. (2013)
Grey wolf_Canada 2	GW Canada 2	KF661061		Thalmann et al. (2013)
			KF661062	Thalmann et al. (2013)
			KF661063	Thalmann et al. (2013)
			KF661056	Thalmann et al. (2013)
			DQ480508	Björnerfeldt et al. (2006)
Grey wolf_Alaska 1	GW Alaska 1	KF661058		Thalmann et al. (2013)
Grey wolf_Alaska 2	GW Alaska 2	KF661066		Thalmann et al. (2013)
Grey wolf_Alaska 3	GW Alaska 3	KF661071		Thalmann et al. (2013)
			KF661059	Thalmann et al. (2013)
			KF661073	Thalmann et al. (2013)
			KF661057	Thalmann et al. (2013)
Grey wolf_Mexico	GW Mexico	KF661060		Thalmann et al. (2013)
			KF661065	Thalmann et al. (2013)
Domestic dog 12	DD 12	DQ480491		Björnerfeldt et al. (2006)
Domestic dog 2	DD 2	DQ480497		Björnerfeldt et al. (2006)
			KF661036	Thalmann et al. (2013)
			DQ480498	Björnerfeldt et al. (2006)
Domestic dog 3 Himachal India	DD 3	AY333736		Sharma et al. (2004)
			DQ480499	Björnerfeldt et al. (2006)
			KF661037	Thalmann et al. (2013)
Domestic dog 4 Arunachal Pradesh India	DD 4	AY333731		Sharma et al. (2004)
Domestic dog 5	DD 5	DQ480496		Björnerfeldt et al. (2006)
Domestic dog 6 Tibetan Mastiff	DD 6	EU408300		unpublished*

Domestic dog 7 Arunachal Pradesh India	DD 7	AY333728		Sharma et al. (2004)
			DQ480493	Björnerfeldt et al. (2006)
			DQ480501	Björnerfeldt et al. (2006)
			AY333727	Sharma et al. (2004)
Domestic dog 8	DD 8	DQ480490		Björnerfeldt et al. (2006)
Domestic dog 9 Arunachal Pradesh	DD 9	AY333735		Sharma et al. (2004)
Domestic dog 10	DD 10	DQ480500		Björnerfeldt et al. (2006)
			KF661047	Thalmann et al. (2013)
			DQ480494	Björnerfeldt et al. (2006)
			AY333730	Sharma et al. (2004)
		KF661050		Thalmann et al. (2013)
			DQ480507	Björnerfeldt et al. (2006)
		KF661051		Sharma et al. (2004)
Domestic dog 11 Arunachal Pradesh	DD 11	AY333732		Sharma et al. (2004)
Domestic dog Nepal 1	DD 1 Nepal	KY996526		This study
			AY333737	Sharma et al. (2004)
			DQ480495	Björnerfeldt et al. (2006)
Domestic dog 13 Gujarat India	DD 13	AY333729		Sharma et al. (2004)
Domestic dog 14	DD 14	DQ480492		Björnerfeldt et al. (2006)
Domestic dog 15	DD 15	DQ480502		Björnerfeldt et al. (2006)
African Wolf 1	AW1	HQ845259		Rueness et al. (2011)
African Wolf 2	AW2	JQ088675.1		Gaubert et al. (2012)
			JQ088676.1	Gaubert et al. (2012)
African Wolf 3	AW 3	JQ088678.1		Gaubert et al. (2012)
African Wolf 4	AW 4	JQ088684.1		Gaubert et al. (2012)
African Wolf 5	AW 5	JQ088679.1		Gaubert et al. (2012)
African Wolf 6	AW 6	JQ088680.1		Gaubert et al. (2012)
			KM670012	Waters et al. (2015)
African Wolf 7	AW 7	JQ088683.1		Gaubert et al. (2012)
African Wolf 8	AW 8	JQ088681.1		Gaubert et al. (2012)
African Wolf 9	AW 9	JQ088682.1		Gaubert et al. (2012)
Ethopian Wolf 1		AY551930.1		Gottelli et al. (2004)
Ethopian Wolf 2		KT448281.1		Koepfli et al. (2015)
Golden Jackal 1		KT988009.1		İbiş et al. (2015)
Golden Jackal 2		KT988007.1		İbiş et al. (2015)
Golden Jackal 3		KT343802.1		İbiş et al. (2015)
			KT988006.1	İbiş et al. (2015)
Golden Jackal 4		KT343803.1		İbiş et al. (2015)
			HQ845260	Rueness et al. (2011)
			KT268319.1	İbiş et al. (2015)
Golden Jackal 5		AY289996		Aggarwal et al. (2007)
			AY289997	Aggarwal et al. (2007)

Table S3. Overview of the cytochrome *b* sequences used in the phylogenetic analysis. Two unique Himalayan wolf cytochrome *b* haplotypes (i.e. Himalayan wolf Cytochrome B 1, Himalayan wolf Cytochrome B 2) and one unique domestic dog haplotype (i.e. Domestic dog Humla Nepal B 1) were found in the samples collected in the study area in Humla (Nepal). Marked with an asterisk* are sequences that are unpublished and have been directly submitted to NCBI GenBank by the respective authors. Reference sequences from the following publications were included: (Aggarwal et al., 2007; Björnerfeldt et al., 2006; Gaubert et al., 2012; Gottelli et al., 2004; İbiş et al., 2015; Ishiguro et al., 2009; Koepfli et al., 2015; Rueness et al., 2011; Sharma et al., 2004; Thalmann et al., 2013; Waters et al., 2015).

Haplotype	**Unique Haplotypes Accession No.**	**Identical sequences Accession No.**	**Publication**
Himalayan wolf Cytochrome B 1	KY996533		This study
Himalayan wolf Cytochrome B 2	KY996534		This study
		AY291431	Aggarwal et al. (2007)
Domestic dog Humla Nepal B 1	KY996532		This study
		KT447685	Koepfli et al. (2015)
		DQ480497	Björnerfeldt et al. (2006)
		DQ480491	Björnerfeldt et al. (2006)
		DQ480498	Björnerfeldt et al. (2006)
		DQ480495	Björnerfeldt et al. (2006)
		DQ480496	Björnerfeldt et al. (2006)
Indian grey wolf 1	AY291432		Aggarwal et al. (2007)
Domestic dog 1	KT447684		Koepfli et al. (2015)
		DQ480490	Björnerfeldt et al. (2006)
		DQ480494	Björnerfeldt et al. (2006)
		DQ480500	Björnerfeldt et al. (2006)
Grey wolf Israel 1	KT447705		Koepfli et al. (2015)
		KT447706	Koepfli et al. (2015)
		KT447700	Koepfli et al. (2015)
		KT447702	Koepfli et al. (2015)
		KT447709	Koepfli et al. (2015)
		KT447710	Koepfli et al. (2015)
		KT447707	Koepfli et al. (2015)
Grey wolf Ukraine	KT447701		Koepfli et al. (2015)
Grey wolf Israel 2	KT447708		Koepfli et al. (2015)
		DQ480504	Björnerfeldt et al. (2006)
		DQ480505	Björnerfeldt et al. (2006)
Grey wolf Canada 1	KT447699		Koepfli et al. (2015)
Grey wolf Oman	KT447703		Koepfli et al. (2015)
Grey wolf Saudi Arabia 1	KT447704		Koepfli et al. (2015)
Domestic dog 2	DQ480499		Björnerfeldt et al. (2006)
Grey wolf Saudi Arabia 2	DQ480506		Björnerfeldt et al. (2006)
Grey wolf Canada 2	DQ480508		Björnerfeldt et al. (2006)
Grey wolf Russia	DQ480503		Björnerfeldt et al. (2006)
Grey wolf Saudi Arabia 3	DQ480507		Björnerfeldt et al. (2006)
Domestic dog 3	DQ480492		Björnerfeldt et al. (2006)

		DQ480493	Björnerfeldt et al. (2006)
		DQ480502	Björnerfeldt et al. (2006)
		DQ480501	Björnerfeldt et al. (2006)
Tibetean Fox Cytochrome B	identical with KT033906		Werhahn et al. (2016)
Coyote 1	KT447695		Koepfli et al. (2015)
Coyote 2	DQ480511		Björnerfeldt et al. (2006)
		DQ480509	Björnerfeldt et al. (2006)
		DQ480510	Björnerfeldt et al. (2006)
		KT447697	Koepfli et al. (2015)
		KT447698	Koepfli et al. (2015)
		KT447696	Koepfli et al. (2015)
Ethopian wolf	KT447693		Koepfli et al. (2015)
		KT447694	Koepfli et al. (2015)
		KT447692	Koepfli et al. (2015)
		KT447691	Koepfli et al. (2015)
Side-striped Jackal	KT447687		Koepfli et al. (2015)
African Wild dog	KT447689		Koepfli et al. (2015)
Black-backed jackal	KT447688		Koepfli et al. (2015)
Sechuran fox	KT447686		Koepfli et al. (2015)
Red fox	KY996535		This study
Dhole	KT447690		Koepfli et al. (2015)
Golden jackal 1	AY291433		Aggarwal et al. (2007)
Golden jackal 2	KT447713		Koepfli et al. (2015)
		KT447729	Koepfli et al. (2015)
		KT447732	Koepfli et al. (2015)
		KT447715	Koepfli et al. (2015)
		KT447731	Koepfli et al. (2015)
		KT447725	Koepfli et al. (2015)
		KT447718	Koepfli et al. (2015)
		KT447724	Koepfli et al. (2015)
		KT447719	Koepfli et al. (2015)
		KT447730	Koepfli et al. (2015)
		KT447757	Koepfli et al. (2015)
		KT447758	Koepfli et al. (2015)
		KT447756	Koepfli et al. (2015)
		KT447755	Koepfli et al. (2015)
		KT447754	Koepfli et al. (2015)
		KT447752	Koepfli et al. (2015)
		KT447751	Koepfli et al. (2015)
		KT447750	Koepfli et al. (2015)
		KT447749	Koepfli et al. (2015)
		KT447753	Koepfli et al. (2015)
		KT447726	Koepfli et al. (2015)
		KT447728	Koepfli et al. (2015)
Golden jackal 3	KT447748		Koepfli et al. (2015)
Golden jackal 4	KT447712		Koepfli et al. (2015)
Golden jackal 5	KT447727		Koepfli et al. (2015)
African wolf 1	KT447762		Koepfli et al. (2015)
		KT447761	Koepfli et al. (2015)
African wolf 2	KT447720		Koepfli et al. (2015)
		KT447723	Koepfli et al. (2015)
		KT447714	Koepfli et al. (2015)
		KT447716	Koepfli et al. (2015)
		KT447721	Koepfli et al. (2015)
		KT447722	Koepfli et al. (2015)
African wolf 3	KT447759		Koepfli et al. (2015)
		KT447760	Koepfli et al. (2015)

African wolf 4	KT447733		Koepfli et al. (2015)
		KT447745	Koepfli et al. (2015)
		KT447746	Koepfli et al. (2015)
		KT447747	Koepfli et al. (2015)
			Koepfli et al. (2015)
African wolf 5	KT447735		Koepfli et al. (2015)
		KT447736	Koepfli et al. (2015)
		KT447739	Koepfli et al. (2015)
		KT447740	Koepfli et al. (2015)
		KT447741	Koepfli et al. (2015)
		KT447742	Koepfli et al. (2015)
		KT447743	Koepfli et al. (2015)
		KT447744	Koepfli et al. (2015)
		KT447738	Koepfli et al. (2015)
		KT447734	Koepfli et al. (2015)
African wolf 6	KT447717		Koepfli et al. (2015)
African wolf 7	KT447737		Koepfli et al. (2015)

Table S4. Primer sequences with references as used in the analysis of the Zinc-finger X-chromosomal (ZFX) and Y-chromosomal (ZFY) protein gene sequences.

Oligo Name	Sequence (5' to 3')	Reference
Zinc-Finger X-chromosomal (ZFX) and Y-chromosomal (ZFY)		
U-ZF-2F	GACCTGAWTCCARRCAGTAC	(Nakagome et al., 2008)
U-ZF-2R	SCCACAAAWCATGCAAGG	
C-ZFX-F	GTTCCCTTAAGGCAGGCATA	(Tsubouchi et al., 2012)
C-ZFX-R	AAAGCTTAAATCCACCTATGGAAA	
C-ZFY-F	CAAGTTAGCATAAATTTGGTTTG	
C-ZFY-R	TGTCTCTGCCTCTCTGTGTCTC	
Canidpost-ZFYR	AAATTTCTTCACTCAGATGAAATAACA	(Koepfli et al., 2015)

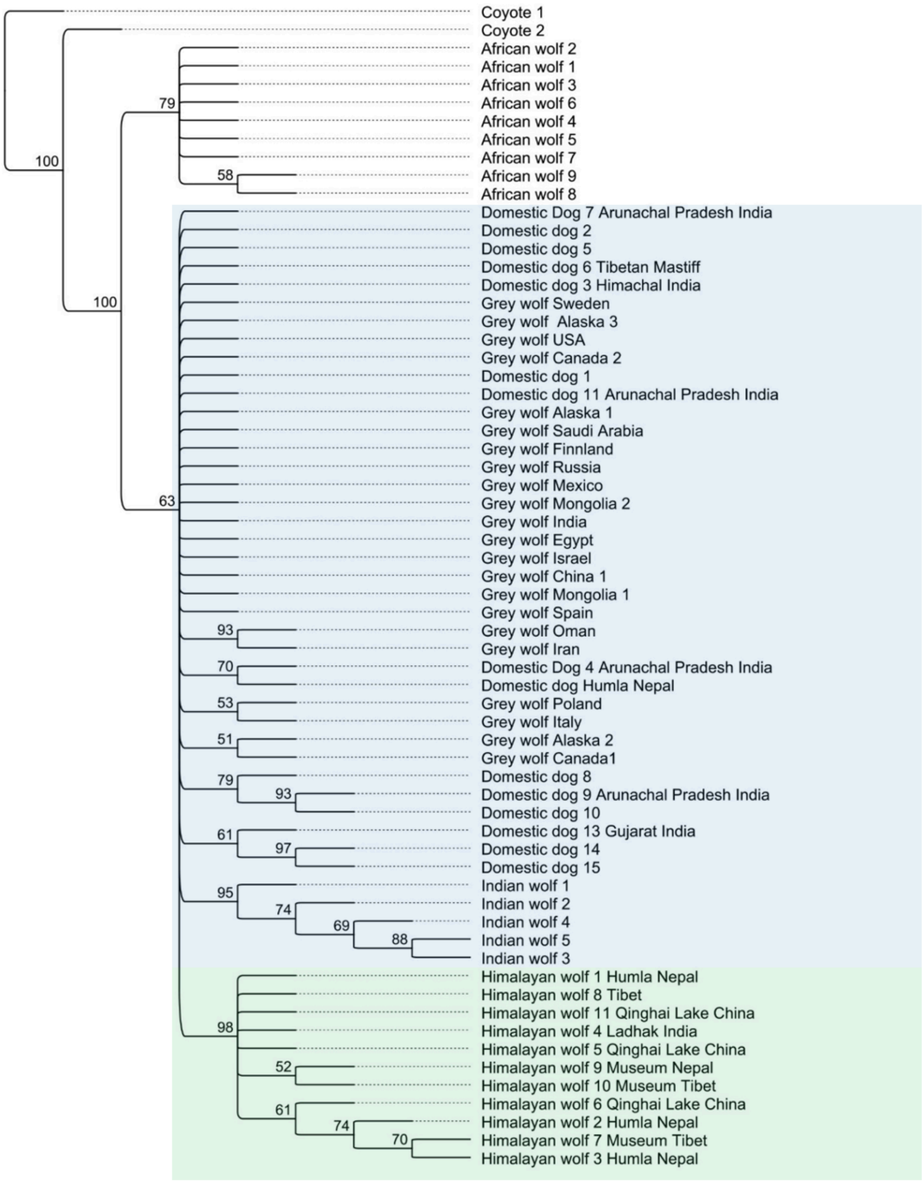

Figure S1. Phylogeny of 242bp D-loop mtDNA based on Neighbourhood joining with consensus support given in the branch nodes. The Himalayan wolf (green) is a monophyletic clade within the grey wolf-dog clade (blue) which includes also domestic dogs.

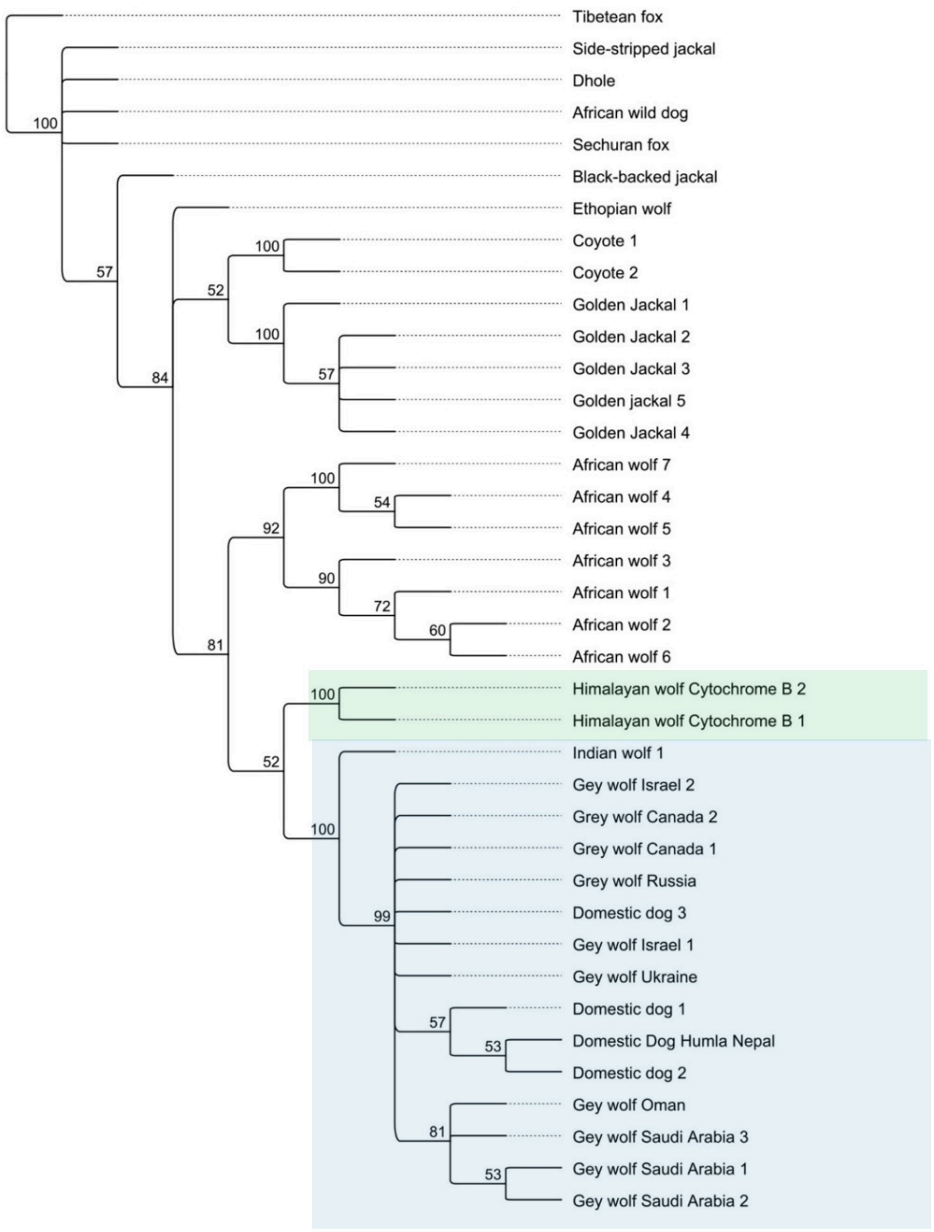

Figure S2. Phylogeny of 508bp cytochrome *b* mtDNA based on Neighbourhood joining with consensus support given in the branch nodes. The Himalayan wolf (green) is a monophyletic clade basal to the grey wolf-dog clade (blue) which includes also domestic dogs.

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Appendix B-2. Supplementary information for: The unique genetic adaptation of the Himalayan wolf to high-altitudes and the consequences for conservation

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METHODS

Genetic analysis procedure

DNA extraction

Refer to Werhahn et al. 2017 supplementary material.

DNA sequencing

Refer to Werhahn et al. 2017 supplementary material.

Genetic analysis procedure for sex identification

We used two rounds of PCR. The first round used the NON-LABELLED primers (DBY_R, DBY_Fext, AHTx40_F, AHTx40_Rext), and the second round used the LABELLED primers (DBY_R, DBY_F (FAM labelled), AHTx40_F, AHTx40_R (VIC labelled)). Please see Table S4 for the primers.

Microsatellite analysis conditions

Microsatellite primers were diluted to 20µM**.** The mastermix (Type-it) was prepared with the following calculations:

5µl Qiagen Type-It * number of samples (plus error)

1µl Qiagen Q-Solution * number of samples (plus error)

2µl ddH2O * number of samples (plus error)

1µl Primer mix$_1$ * number of samples (plus error)

We pipetted the DNA in to 0.2ml plates and used 1µl of DNA per reaction. For our faecal DNA we used a 1:5 dilution to reduce the effect of inhibitors. We always used a positive and negative control. We then checked all the DNA containing wells and then added 9µl mastermix to each well. We then sealed and spun the plates to make sure all the solution is in the bottom of the well.

We used the following PCR conditions:

One cycle of 95˚C x 5 min. Then 40 cycles of 95˚C x 30 sec followed by 55˚C x 30 sec and then 72˚C x 60 sec. then once cycle of 60˚C x 30 min. After the PCR we checked the samples on Agarose gels.

Hypoxia sequencing PCR conditions

The hypoxia sequencing protocol is based on (Zhang et al., 2014). The Sequencing primers were diluted to 10µM.

We prepared the Maxima Hot start Taq mastermix using the following calculations for sequencing:

14µl Maxima Hot Start * number of samples (plus error)

2µl Forward primer [10µM] * number of samples (plus error)

2µl Reverse primer [10µM] * number of samples (plus error)

We then pipetted the DNA in to 0.2ml 8-strips in a plate. We used 2µl of DNA for each reaction.

For the faecal DNA samples we used a 1:5 dilution to reduce the effect of inhibitors. We always used a positive and negative control.

We then added 18µl of master mix to each plate.

Seal and briefly spin the plate to make sure all the solution is in the bottom of the well.

We then placed the plate in the PCR machine and to run the PCR with the following programs:

95˚C x 5 min once. Then 40 cycles of: 95˚C x 30 sec followed by 55˚C x 30 sec, and then 72˚C x 60 sec. Then once 72˚C x 10 min.

Table S1. Haplotypes of Himalayan wolves and domestic dogs (*Canis l. familiaris*) found in the study areas in Humla, Dolpo and KCA (Nepal) with NCBI GenBank accessions.

Region	**Species**	**Haplotype Name**	**GenBank Accession**	
D-loop	Himalayan wolf #	Himalayan wolf D-loop 1	KY996529	Werhahn et al. 2017
D-loop	Himalayan wolf #	Himalayan wolf D-loop 2	KY996530	Werhahn et al. 2017
D-loop	Himalayan wolf #	Himalayan wolf D-loop 3	KY940301	Werhahn et al. 2017
D-loop	Himalayan wolf #	Himalayan wolf D-loop 4	MK113901	This study
D-loop	Himalayan wolf #	Himalayan wolf D-loop 5	MK113902	This study
D-loop	*C. l. familiaris*	Domestic dog D-loop Nepal 1	KY996526	Werhahn et al. 2017
D-loop	*C. l. familiaris*	Domestic dog D-loop Nepal 2	MK113903	This study
D-loop	*C. l. familiaris*	Domestic dog D-loop Nepal 3	MK113904	This study
D-loop	*C. l. familiaris*	Domestic dog D-loop Nepal 4	MK113905	This study
D-loop	*C. l. familiaris*	Domestic dog D-loop Nepal 5	MK113906	This study
cyt *b*	Himalayan wolf #	Himalayan wolf Cytochrome B 1	KY996533	Werhahn et al. 2017
cyt *b*	Himalayan wolf #	Himalayan wolf Cytochrome B 2	KY996534	Werhahn et al. 2017
cyt *b*	*C. l. familiaris*	Domestic dog Cytochrome B Nepal 1	KY996532	Werhahn et al. 2017
cyt *b*	*C. l. familiaris*	Domestic dog Cytochrome B Nepal 2	MK113907	This study
cyt *b*	*C. l. familiaris*	Domestic dog Cytochrome B Nepal 3	MK113908	This study
cyt *b*	*C. l. familiaris*	Domestic dog Cytochrome B Nepal 4	MK113909	This study
cyt *b*	*C. l. familiaris*	Domestic dog Cytochrome B Nepal 5	MK113910	This study
ZFY	Himalayan wolf #	Himalayan wolf Nepal ZFY	MF101862	Werhahn et al. 2017
ZFX	Himalayan wolf #	Himalayan wolf Nepal ZFX	MF101863	Werhahn et al. 2017

[#] Formal taxonomic classification pending

Table S2. Overview of the D-loop data used in the phylogenetic analysis. Listed are the new haplotypes of Himalayan wolf 1-3 and one domestic dog found in the study area in Humla (Nepal), and all reference sequences obtained from NCBI GenBank. Marked with an asterisk* are sequences that are unpublished and have been directly submitted to NCBI GenBank by the respective authors. Reference sequences from the following publications were included: (Aggarwal et al., 2007; Björnerfeldt et al., 2006; Gaubert et al., 2012; Gottelli et al., 2004; R. Gundry et al., 2007; R. L. Gundry et al., 2007; İbiş et al., 2015; Ishiguro et al., 2009; Koblmüller, 2016; Koepfli et al., 2015; Meng et al., 2009; Rueness et al., 2011; Sharma et al., 2004; Thalmann et al., 2013; Waters et al., 2015; Zhang et al., 2015; Zhang and Chen, 2011).

Haplotype	**Abbreviated**	**Unique Haplotypes**	**Identical Sequences**	**Publication**
Side-striped jackal 1		JQ088674.1		Gaubert et al. (2012)
Side-striped jackal 2		JQ088669.1		Gaubert et al. (2012)
			JQ088670.1	Gaubert et al. (2012)
			JQ088671.1	Gaubert et al. (2012)
			JQ088672.1	Gaubert et al. (2012)
			JQ088673.1	Gaubert et al. (2012)
African wild dog		KT448283.1		Koepfli et al. (2015)
Asian dhole		KT448282.1		Koepfli et al. (2015)
Coyote 1		DQ480509		Björnerfeldt et al. (2006)
			DQ480511	Björnerfeldt et al. (2006)
Coyote 2		DQ480510		Björnerfeldt et al. (2006)
Red fox		KY996531		This study
Himalayan wolf 1 Nepal	HW1_ Nepal	KY996529		This study
			AY333740	Sharma et al. (2004)
			AY289986	Aggarwal et al. (2007)
			AY289995	Aggarwal et al. (2007)
			AY289985	Aggarwal et al. (2007)
			AY289994	Aggarwal et al. (2007)
			AY289977	Aggarwal et al. (2007)
			AY289993	Aggarwal et al. (2007)
			AY289992	Aggarwal et al. (2007)
			AY289991	Aggarwal et al. (2007)
			AY289978	Aggarwal et al. (2007)
			AY289990	Aggarwal et al. (2007)
			AY289979	Aggarwal et al. (2007)
			AY289980	Aggarwal et al. (2007)
			AY289989	Aggarwal et al. (2007)
			AY289981	Aggarwal et al. (2007)
			AY289988	Aggarwal et al. (2007)
			AY289982	Aggarwal et al. (2007)
			AY289983	Aggarwal et al. (2007)
			AY289987	Aggarwal et al. (2007)
			JX415352	unpublished*
			JX415350	unpublished*
			SRX1024624 (Tibet China)	Zhang et al. (2014)
Himalayan wolf 2	HW2_Nepal	KY996530 (Nepal)		Werhahn et al (2017)

			JX415351	unpublished*
			NC011218 Xining Zoo, Qinghai	Meng et al. (2009)
			EU442884.2 (Qinghai China)	unpublished*#
			FJ032363.2	Meng et al. (2009)
			SRX1024633 (Tibet China)	Zhang et a. (2014)
			SRX1024634 (Qinghai China)	Zhang et a. (2014)
Himalayan wolf 3	HW3_ Nepal	KY940301		Werhahn et al (2017)
			JX415343	unpublished*
Himalayan wolf 4	HW4_ Nepal	MK113901		This study
			SRX1024635 (Qinghai)	Zhang et a. (2014)
Himalayan wolf 5	HW5_ Nepal	MK113902		This study
Himalayan wolf 6 Qinghai Lake China	HW6_Qinghai_ Lake_China	JX415345		unpublished*
Himalayan wolf 7 Museum Tibet	HW7_Museum Tibet	AY333739		Sharma et al. (2004)
Himalayan wolf 8 Tibet	HW8_Tibet	KF573616		unpublished*
			AB480742	Ishiguro et al. (2009)
Himalayan wolf 9 Museum Nepal	HW9_Museum Nepal	AY333738		Sharma et al. (2004)
			JX415344	unpublished*
Himalayan wolf 10 Museum Tibet	HW10_Museum Tibet	AY333742		Sharma et al. (2004)
Himalayan wolf 11 Qinghai Lake China	HW11_Qinghai_ Lake_China	JX415348		unpublished*
Himalayan wolf 12 Ladhak India	HW12_Ladhak	AY333741		Sharma et al. (2004)
Himalayan wolf 13 Qinghai Lake China	HW13_Qinghai_ Lake_China	JX415347		unpublished*
Indian wolf 1	IW 1	AY333745		Sharma et al. (2004)
Indian wolf 2	IW 2	AY289973		Aggarwal et al. (2007)
Indian wolf 3	IW 3	AY333746		Sharma et al. (2004)
Indian wolf 4	IW 4	AY333743		Sharma et al. (2004)
Indian wolf 5	IW 5	AY289974		Aggarwal et al. (2007)
			AY289975	Aggarwal et al. (2007)
			AY289976	Aggarwal et al. (2007)
			AY289984	Aggarwal et al. (2007)
			AY333746	Sharma et al. (2004)
			AY333744	Sharma et al. (2004)
Grey wolf Xinjiang China 1	GW Xinjiang China 1	KC461238		Zhang et al. (2014)
Grey wolf Xinjiang China 2	GW Xinjiang China 2	SRX1024627		Zhang et al. (2014)
			KF661053	Thalmann et al. (2013)
Grey wolf Mongolia 1	GW Mongolia 1	KY996527		Werhahn et al. (2017)
Grey wolf Mongolia 2	GW Mongolia 2	KY996528		Werhahn et al. (2017)
Grey wolf Mongolia 3	GW Mongolia 3	KU696392		Koblmüller et al. (2016)
			SRX1024637 (Inner Mongolia China)	Zhang et al. (2014)
			GQ374438 (Inner Mongolia China)	Zhang and Chen (2011)

Grey wolf Mongolia 4	GW Mongolia 4	KU696393		Koblmüller et al. (2016)
			KF661041 (China)	Thalmann et al. (2013)
			KU696391 (China)	Koblmüller et al. (2016)
			SRX1024636 (Inner Mongolia China)	Zhang et al. (2014)
			SRX1024629 (Xinjiang China)	Zhang et al. (2014)
Grey wolf_Mongolia 5	GW Mongolia 6	KU696395.1		Koblmüller et al. (2016)
Grey wolf Mongolia 6	GW Mongolia 6	KC896375		Zhang et al. (2015)
Grey wolf Kyrgyzstan 1	GW Kyrgyzstan 1	MK113898		This study
Grey wolf Kyrgyzstan 2	GW Kyrgyzstan 2	MK113899		This study
Grey wolf Kyrgyzstan 3	GW Kyrgyzstan 3	MK113900		This study
		KC414578		unpublished
Grey wolf_Russia	GW Russia	KF661046		Thalmann et al. (2013)
Grey wolf_Poland	GW Poland	KF661045		Thalmann et al. (2013)
Grey wolf_India	GW India	KF661043		Thalmann et al. (2013)
			KF661054	Thalmann et al. (2013)
Grey wolf_Egypt	GW Egypt	JQ088677.1		Gaubert et al. (2012)
			KF661055	Thalmann et al. (2013)
Grey wolf_Saudi Arabia	GW Saudi Arabia	DQ480506		Björnerfeldt et al. (2006)
Grey wolf_Israel	GW Israel	KF661042		Thalmann et al. (2013)
			AY333733	Sharma et al. (2004)
Grey wolf_Oman	GW Oman	KF661050		Thalmann et al. (2013)
Grey wolf_Iran	GW Iran	KF661051		Thalmann et al. (2013)
Grey wolf_Finland	GW Finnland	KF661038		Thalmann et al. (2013)
			KF661039	Thalmann et al. (2013)
			DQ480503	Björnerfeldt et al. (2006)
Grey wolf_Sweden	GW Sweden	KF661040		Thalmann et al. (2013)
			KF661044	Thalmann et al. (2013)
			KF661049	Thalmann et al. (2013)
			KF661052	Thalmann et al. (2013)
			DQ480504	Björnerfeldt et al. (2006)
Grey wolf_Italy	GW Italy	KF661048		Thalmann et al. (2013)
Iberian wolf_North Spain 1	GW Spain 2		KY550008	This study
Iberian wolf_North Spain 2	GW Spain 3		KY550009	This study
Iberian wolf_North Spain 3	GW Spain 4		KY550010	This study
Iberian wolf_Spain	GW Spain	DQ480505		Björnerfeldt et al. (2006)
Grey wolf_USA	GW USA	KF661064		Thalmann et al. (2013)
			KF661068	Thalmann et al. (2013)
			KF661069	Thalmann et al. (2013)
			KF661072	Thalmann et al. (2013)
Grey wolf_Canada 1	GW Canada 1	KF661074		Thalmann et al. (2013)
Grey wolf_Canada 2	GW Canada 2	KF661061		Thalmann et al. (2013)
			KF661062	Thalmann et al. (2013)
			KF661063	Thalmann et al. (2013)
			KF661056	Thalmann et al. (2013)
			DQ480508	Björnerfeldt et al. (2006)
Grey wolf_Alaska 1	GW Alaska 1	KF661058		Thalmann et al. (2013)
Grey wolf_Alaska 2	GW Alaska 2	KF661066		Thalmann et al. (2013)
Grey wolf_Alaska 3	GW Alaska 3	KF661071		Thalmann et al. (2013)
			KF661059	Thalmann et al. (2013)
			KF661073	Thalmann et al. (2013)
			KF661057	Thalmann et al. (2013)

Grey wolf_Mexico	GW Mexico	KF661060		Thalmann et al. (2013)
			KF661065	Thalmann et al. (2013)
Grey wolf Texas USA		AY240073.1		Gundry et al. (2007)
Domestic dog 12	DD 12	DQ480491		Björnerfeldt et al. (2006)
Domestic dog 2	DD 2	DQ480497		Björnerfeldt et al. (2006)
			KF661036	Thalmann et al. (2013)
			DQ480498	Björnerfeldt et al. (2006)
			DQ480499	Björnerfeldt et al. (2006)
			KF661037	Thalmann et al. (2013)
Domestic dog 4 Arunachal Pradesh India	DD 4	AY333731		Sharma et al. (2004)
Domestic dog 5	DD 5	DQ480496		Björnerfeldt et al. (2006)
Domestic dog 6 Tibetan mastiff	DD 6	EU408300		unpublished*
Domestic dog 7 Arunachal Pradesh India	DD 7	AY333728		Sharma et al. (2004)
			DQ480493	Björnerfeldt et al. (2006)
			DQ480501	Björnerfeldt et al. (2006)
			AY333727	Sharma et al. (2004)
Domestic dog 9 Arunachal Pradesh	DD 9	AY333735		Sharma et al. (2004)
			KF661047	Thalmann et al. (2013)
			DQ480494	Björnerfeldt et al. (2006)
			AY333730	Sharma et al. (2004)
		KF661050		Thalmann et al. (2013)
			DQ480507	Björnerfeldt et al. (2006)
		KF661051		Sharma et al. (2004)
Domestic dog 11 Arunachal Pradesh	DD 11	AY333732		Sharma et al. (2004)
Domestic dog D-loop Nepal 1	DD 1 Nepal	KY996526		Werhahn et al (2017)
			AY333737	Sharma et al. (2004)
			DQ480495	Björnerfeldt et al. (2006)
Domestic dog D-loop Nepal 2	DD 2 Nepal	MK113903		This study
Domestic dog D-loop Nepal 3	DD 3 Nepal	MK113904		This study
		DQ480500		Björnerfeldt et al. (2006)
		DQ480490		Björnerfeldt et al. (2006)
Domestic dog D-loop Nepal 4	DD 4 Nepal	MK113905		This study
Domestic dog D-loop Nepal 5	DD 5 Nepal	MK113906		This study
Domestic dog 13 Gujarat India	DD 13	AY333729		Sharma et al. (2004)
Domestic dog 14	DD 14	DQ480492		Björnerfeldt et al. (2006)
Domestic dog 15	DD 15	DQ480502		Björnerfeldt et al. (2006)
African wolf 1	AW1	HQ845259		Rueness et al. (2011)
African wolf 2	AW2	JQ088675.1		Gaubert et al. (2012)
			JQ088676.1	Gaubert et al. (2012)
African wolf 3	AW 3	JQ088678.1		Gaubert et al. (2012)
African wolf 4	AW 4	JQ088684.1		Gaubert et al. (2012)
African wolf 5	AW 5	JQ088679.1		Gaubert et al. (2012)
African wolf 6	AW 6	JQ088680.1		Gaubert et al. (2012)
			KM670012	Waters et al. (2015)
African wolf 7	AW 7	JQ088683.1		Gaubert et al. (2012)
African wolf 8	AW 8	JQ088681.1		Gaubert et al. (2012)
African wolf 9	AW 9	JQ088682.1		Gaubert et al. (2012)
Ethopian wolf 1		AY551930.1		Gottelli et al. (2004)
Ethopian wolf 2		KT448281.1		Koepfli et al. (2015)
Dhole1		NC013445		Zhang et al. (2014)
Golden jackal 1		KT988009.1		İbiş et al. (2015)
Golden jackal 2		KT988007.1		İbiş et al. (2015)
Golden jackal 3		KT343802.1		İbiş et al. (2015)
			KT988006.1	İbiş et al. (2015)

Golden jackal 4		KT343803.1		İbiş et al. (2015)
			HQ845260	Rueness et al. (2011)
			KT268319.1	İbiş et al. (2015)
Golden jackal 5		AY289996		Aggarwal et al. (2007)
			AY289997	Aggarwal et al. (2007)

[#] Cited in (Zhang and Chen, 2011)

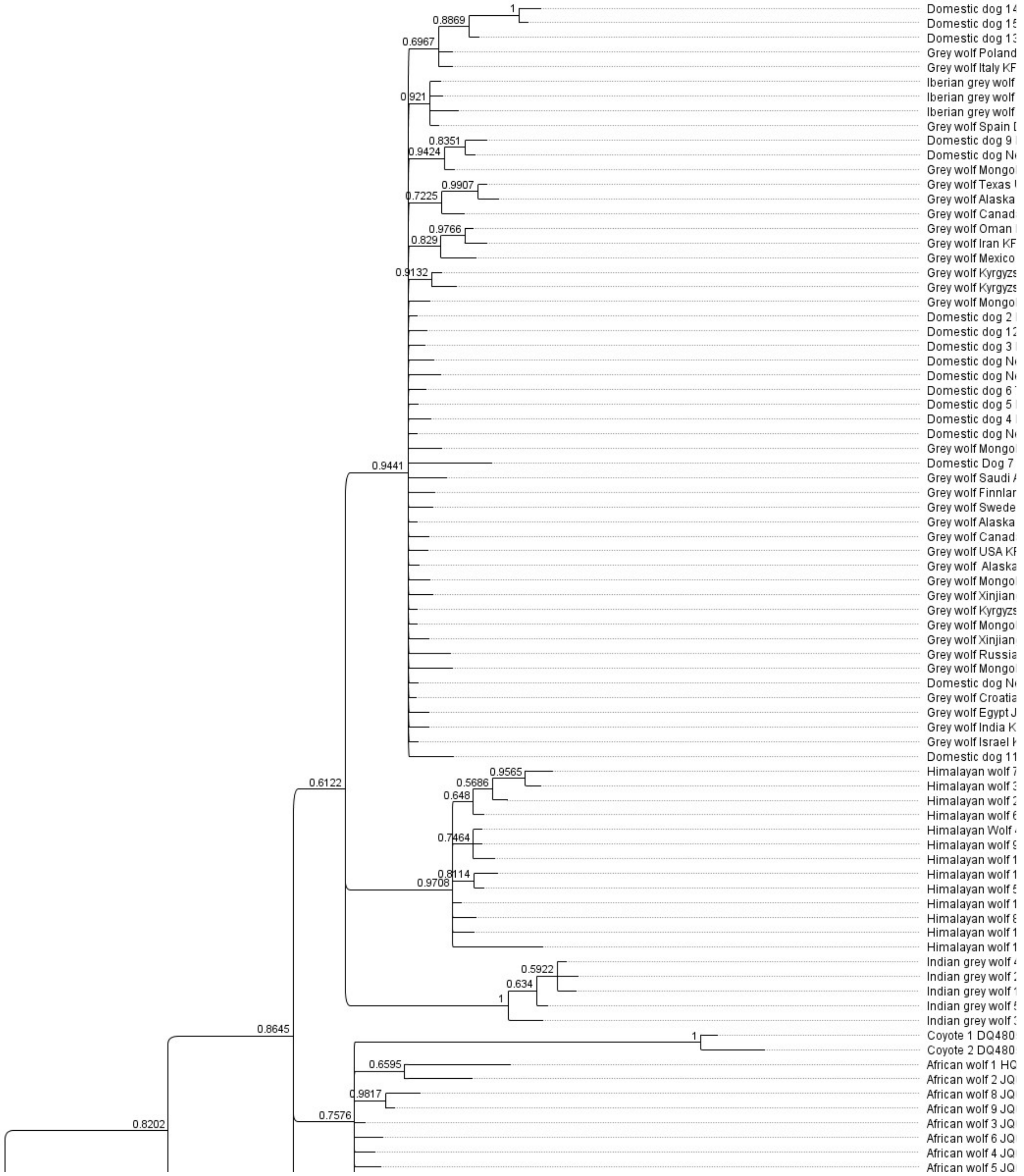

Figure S1. D-loop phylogenetic tree built with Bayesian tree building algorithms and posterior probabilities shown at nodes (Substitution model HKY85; chainlenght: 1’100’000; Burn-in: 100’000).

Table S3. Overview of the cytochrome *b* sequences used in the phylogenetic analysis. Two unique Himalayan wolf cytochrome *b* haplotypes (i.e. Himalayan wolf Cytochrome B 1, Himalayan wolf Cytochrome B 2) and one unique domestic dog haplotype (i.e. Domestic dog Humla Nepal B 1) were found in the samples collected in the study area in Humla (Nepal). Marked with an asterisk* are sequences that are unpublished and have been directly submitted to NCBI GenBank by the respective authors. Reference sequences from the following publications were included: (Aggarwal et al., 2007; Björnerfeldt et al., 2006; Gaubert et al., 2012; Gottelli et al., 2004; İbiş et al., 2015; Ishiguro et al., 2009; Koepfli et al., 2015; Meng et al., 2009; Rueness et al., 2011; Sharma et al., 2004; Thalmann et al., 2013; Waters et al., 2015; Zhang et al., 2013, 2014).

Haplotype	Unique Haplotypes Accession No.	Identical sequences Accession No. (location)	Publication
Himalayan wolf Cytochrome B 1	KY996533		Werhahn et al (2017)
		SRX1024633 (Tibet China)	Zhang et a. (2014)
		SRX1024635 (Qinghai China)	Zhang et a. (2014)
		SRX1024634 (Qinghai China)	Zhang et a. (2014)
		FJ032363.2 (Tibet China)	Meng et al. (2009)
		EU442884	unpublished*#
		NC011218 (Tibet)	Meng et al. (2009)
Himalayan wolf Cytochrome B 2	KY996534		Werhahn et al (2017)
		AY291431	Aggarwal et al. (2007)
		SRX1024624 (Tibet China)	Zhang et al. (2014)
Domestic dog Nepal 1	KY996532		Werhahn et al (2017)
		KT447685	Koepfli et al. (2015)
		DQ480497	Björnerfeldt et al. (2006)
		DQ480491	Björnerfeldt et al. (2006)
		DQ480498	Björnerfeldt et al. (2006)
		DQ480495	Björnerfeldt et al. (2006)
		DQ480496	Björnerfeldt et al. (2006)
		KU696394 (Grey wolf Mongolia)	Koblmüller et al. (2016)
Domestic dog Nepal 2	MK113907		This study
		KT447684	Koepfli et al. (2015)
Domestic dog Nepal 3	MK113908		This study
		KU644669.1 (China)	Koblmuller et al. (2016)
		KU696391 (China)	Koblmuller et al. (2016)
Domestic dog Nepal 4	MK113909		This study
Domestic dog Nepal 5	MK113910		This study
Indian wolf 1	AY291432		Aggarwal et al. (2007)
		DQ480490	Björnerfeldt et al. (2006)
		DQ480494	Björnerfeldt et al. (2006)
		DQ480500	Björnerfeldt et al. (2006)
Grey wolf Israel 1	KT447705		Koepfli et al. (2015)
		KT447706	Koepfli et al. (2015)
		KT447700	Koepfli et al. (2015)
		KT447702	Koepfli et al. (2015)
		KT447709	Koepfli et al. (2015)

		KT447710	Koepfli et al. (2015)
		KT447707	Koepfli et al. (2015)
		KU696393 (Mongolia)	Koblmüller et al. (2016)
Grey wolf_Mongolia 3	KU696392		Koblmüller et al. (2016)
		KU696396	Koblmüller et al. (2016)
		GQ374438	Zhang and Chen (2011)
		KU696395	Koblmüller et al. (2016)
	Grey wolf Inner Mongolia 2	SRX1024636 (Inner Mongolia)	Zhang et al. (2014)
		SRX1024627 (Xinjiang)	Zhang et al. (2014)
Grey wolf Xinjiang China 2		SRX1024629 (Xinjiang)	Zhang et al. (2014)
Grey wolf_Croatia	KU696398		Koblmüller et al. (2016)
Grey wolf Inner Mongolia 1	SRX1024637		Zhang et al. (2014)
Grey wolf Xinjiang China 1	KC461238		Zhang et al. (2014)
Grey wolf Ukraine	KT447701		Koepfli et al. (2015)
Grey wolf Kyrgyzstan			This study
		Iberian grey wolf (Spain)	This study
		DQ480505	Björnerfeldt et al. (2006)
		KC896375 (Mongolia)	Zhang et al. (2015)
		KT447708 (Israel)	Koepfli et al. (2015)
		DQ480504	Björnerfeldt et al. (2006)
Grey wolf Canada 1	KT447699		Koepfli et al. (2015)
Grey wolf Oman	KT447703		Koepfli et al. (2015)
Grey wolf Saudi Arabia 1	KT447704		Koepfli et al. (2015)
Domestic dog 2	DQ480499		Björnerfeldt et al. (2006)
Grey wolf Saudi Arabia 2	DQ480506		Björnerfeldt et al. (2006)
Grey wolf Canada 2	DQ480508		Björnerfeldt et al. (2006)
Grey wolf Russia	DQ480503		Björnerfeldt et al. (2006)
Grey wolf Saudi Arabia 3	DQ480507		Björnerfeldt et al. (2006)
Domestic dog 3	DQ480492		Björnerfeldt et al. (2006)
		DQ480493	Björnerfeldt et al. (2006)
		DQ480502	Björnerfeldt et al. (2006)
		DQ480501	Björnerfeldt et al. (2006)
Tibetan fox Cytochrome B	identical with KT033906		Werhahn et al. (2016)
Coyote 1	KT447695		Koepfli et al. (2015)
Coyote 2	DQ480511		Björnerfeldt et al. (2006)
		DQ480509	Björnerfeldt et al. (2006)
		DQ480510	Björnerfeldt et al. (2006)
		KT447697	Koepfli et al. (2015)
		KT447698	Koepfli et al. (2015)
		KT447696	Koepfli et al. (2015)
Ethopian wolf	KT447693		Koepfli et al. (2015)
		KT447694	Koepfli et al. (2015)
		KT447692	Koepfli et al. (2015)
		KT447691	Koepfli et al. (2015)
Side-striped jackal	KT447687		Koepfli et al. (2015)
African wild dog	KT447689		Koepfli et al. (2015)
Black-backed jackal	KT447688		Koepfli et al. (2015)
Sechuran fox	KT447686		Koepfli et al. (2015)
Red fox	KY996535		Werhahn et al. (2017)
Dhole	KT447690		Koepfli et al. (2015)
Dhole		NC013445	Zhang et al. (2014)
Golden jackal 1	AY291433		Aggarwal et al. (2007)
Golden jackal 2	KT447713		Koepfli et al. (2015)
		KT447729	Koepfli et al. (2015)
		KT447732	Koepfli et al. (2015)
		KT447715	Koepfli et al. (2015)
		KT447731	Koepfli et al. (2015)

		KT447725	Koepfli et al. (2015)
		KT447718	Koepfli et al. (2015)
		KT447724	Koepfli et al. (2015)
		KT447719	Koepfli et al. (2015)
		KT447730	Koepfli et al. (2015)
		KT447757	Koepfli et al. (2015)
		KT447758	Koepfli et al. (2015)
		KT447756	Koepfli et al. (2015)
		KT447755	Koepfli et al. (2015)
		KT447754	Koepfli et al. (2015)
		KT447752	Koepfli et al. (2015)
		KT447751	Koepfli et al. (2015)
		KT447750	Koepfli et al. (2015)
		KT447749	Koepfli et al. (2015)
		KT447753	Koepfli et al. (2015)
		KT447726	Koepfli et al. (2015)
		KT447728	Koepfli et al. (2015)
Golden jackal 3	KT447748		Koepfli et al. (2015)
Golden jackal 4	KT447712		Koepfli et al. (2015)
Golden jackal 5	KT447727		Koepfli et al. (2015)
African wolf 1	KT447762		Koepfli et al. (2015)
		KT447761	Koepfli et al. (2015)
African wolf 2	KT447720		Koepfli et al. (2015)
		KT447723	Koepfli et al. (2015)
		KT447714	Koepfli et al. (2015)
		KT447716	Koepfli et al. (2015)
		KT447721	Koepfli et al. (2015)
		KT447722	Koepfli et al. (2015)
African wolf 3	KT447759		Koepfli et al. (2015)
		KT447760	Koepfli et al. (2015)
African wolf 4	KT447733		Koepfli et al. (2015)
		KT447745	Koepfli et al. (2015)
		KT447746	Koepfli et al. (2015)
		KT447747	Koepfli et al. (2015)
			Koepfli et al. (2015)
African wolf 5	KT447735		Koepfli et al. (2015)
		KT447736	Koepfli et al. (2015)
		KT447739	Koepfli et al. (2015)
		KT447740	Koepfli et al. (2015)
		KT447741	Koepfli et al. (2015)
		KT447742	Koepfli et al. (2015)
		KT447743	Koepfli et al. (2015)
		KT447744	Koepfli et al. (2015)
		KT447738	Koepfli et al. (2015)
		KT447734	Koepfli et al. (2015)
African wolf 6	KT447717		Koepfli et al. (2015)
African wolf 7	KT447737		Koepfli et al. (2015)

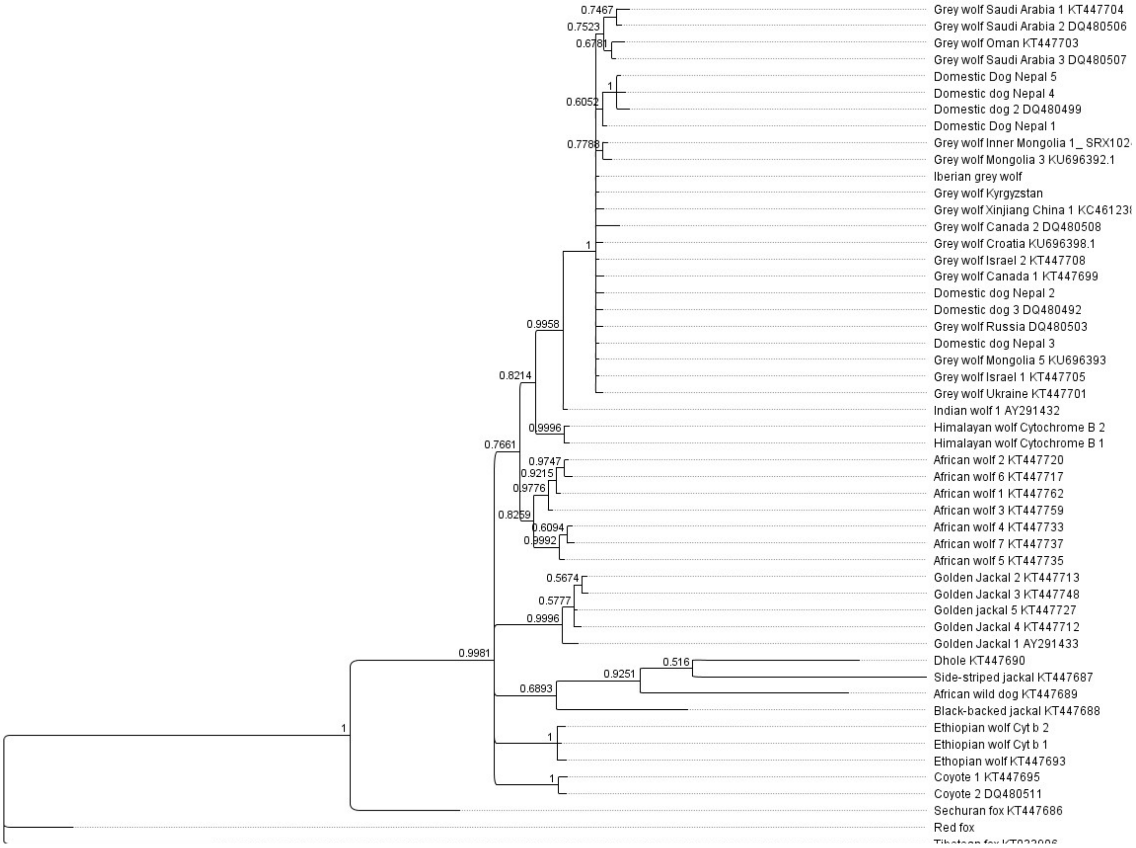

Figure S2. Cytochrome *b* tree built with Bayesian tree building algorithms and posterior probabilities shown at nodes (Substitution model HKY85; chainlenght: 1’100’000; Burn-in: 100’000).

Table S4. Microsatellite primers used for genotyping. Highlighted in green are the 10 loci recommended for Himalayan wolf monitoring and research.

Primer	**Sequence (5' to 3')**	**5'Dye**	**Reference**
P1:			
WLF_FH2088_F	CCCTCTGCCTACATCTCTGC	FAM	(Francisco et al., 1996)
WLF_FH2088_R		TAGGGCATGCATATAACCAGC	
WLF_FH2096_F	CCGTCTAAGAGCCTCCCA	VIC	
WLF_FH2096_R		GACAAGGTTTCCTGGTTCCA	
WLF_FH2001_F	TCCTCCTCTTCTTTCCATTGG	PET	
WLF_FH2001_R		TGAACAGAGTTAAGGATAGACACG	
WLF_FH2137_F	GCAGTCCCTTATTCCAACATG	NED	
WLF_FH2137_R		CCCCAAGTTTTGCATCTGTT	
WLF_CPH08_F	AGGCTCACAATCCCTCTCATA	FAM	
WLF_CPH08_R		TAGATTTGATACCTCCCTGAGTCC	
P2:			
WLF_C20.253_F	AATGGCAGGATTTTCTTTTGC	NED	(Ostrander et al., 1993)
WLF_C20.253_R		ATCTTTGGACGAATGGATAAGG	
WLF_CPH05_F	TCCATAACAAGACCCCAAAC	VIC	(Fredholm and Winterø, 1995)
WLF_CPH05_R		GGAGGTAGGGGTCAAAAGTT	
WLF_C09.250_F	TTAGTTAACCCAGCTCCCCCA	PET	(Ostrander et al., 1993)
WLF_C09.250_R		TCACCCTGTTAGCTGCTCAA	
WLF_CPH04_F	ACTGGAGATGAAAACTGAAGATTATA	FAM	(Fredholm and Winterø, 1995)
WLF_CPH04_R		TTACAGGGGAAAGCCTCATT	
WLF_CPH12_F	GGCATTACTTGGAGGGAGGAA	FAM	(Fredholm and Winterø, 1995)
WLF_CPH12_R		GATGATTCCTATGCTTCTTTGAG	
P3:			
WLF_C27.442_F	CCAAGAACAGCCTAAGCTGG	NED	(Ostrander et al., 1993)
WLF_C27.442_R		ACACATACACGCCCAATTCA	
WLF_FH2010_F	AAATGGAACAGTTGAGCAT	VIC	(Francisco et al., 1996)
WLF_FH2010_R		CCCCTTACAGCTTCATTTTCC	
WLF_FH2161_F	TCAGCAAGAAACCCTCCAGT	PET	(Francisco et al., 1996)
WLF_FH2161_R		CATTCCCAACGGAGGACTCT	
P4:			
WLF_C09.173_F	ATCCAGGTCTGGAATACCCC	FAM	(Ostrander et al., 1993)
WLF_C09.173_R		TCCTTTGAATTAGCACTTGGC	
WLF_CPH09_F	CAGAGACTGCCACTTTAAACACAC	VIC	(Fredholm and Winterø, 1995)
WLF_CPH09_R		AAAGTTCTCAAATACCATTGTGTTACA	
WLF_CPH14_F	GAAAGACAATCCCTGAAATGC	PET	(Fredholm and Winterø, 1995)
WLF_CPH14_R	ACCCCATTTATGAGAATCATGT		
WLF_C13.758_F	AAGCATCCAGAATCCCTGG	NED	(Mellersh et al., 1997)
WLF_C13.758_R	GTTGATTGGGAGATAATCCACA		

Table S5. Primers used for the Hypoxia sequencing panel based on (Zhang et al., 2014).

Name	**Gene**	**LOC**	**Forward**	**Reverse**	**Size (bp)**
ANGPT1_8141664	ANGPT1	chr13:8141664	CGAGTAACCAAGACTTGAAGA	TAAACTCAGGATCTAGTTGAGT	173
RYR2_2589113	RYR2-1	chr04:2589113	TCCAGCTCCTTCTGACTTGGAG	ATCTTCCACAATGAGGACTTGA	142
RYR2_2778722	RYR2-2	chr04:2778722	CAACTGTGCATGAAGGATGAG	CACTTGTCTTATGGCAACGGC	147
EPAS1_48630137	EPAS1-1	chr10:48630137	GAGACCAGCACCCTACTCTTG	CAGGTGTAGCCCATCTACCTG	179

Table S6. Allele frequency for each population and each locus.

Locus	Allele/n	Himalayan wolf	Domestic dogs Nepal	African wolf	Grey wolf Mongolia	Grey wolf Europe	Grey wolf Kyrg	Iberian wolf
FH2096	**N**	27	3	7	2	2	3	15
	94	0.648	0.667	0.000	0.000	0.000	0.000	0.100
	98	0.352	0.167	0.571	0.750	0.500	0.000	0.433
	102	0.000	0.167	0.429	0.250	0.250	0.500	0.467
	106	0.000	0.000	0.000	0.000	0.250	0.500	0.000
FH2088	**N**	30	3	7	2	2	4	15
	91	0.917	0.333	0.000	0.000	0.250	1.000	0.233
	95	0.000	0.000	0.929	0.000	0.000	0.000	0.000
	115	0.067	0.000	0.000	0.750	0.000	0.000	0.267
	119	0.000	0.000	0.000	0.000	0.250	0.000	0.133
	123	0.017	0.500	0.071	0.000	0.250	0.000	0.333
	127	0.000	0.167	0.000	0.250	0.250	0.000	0.000
	131	0.000	0.000	0.000	0.000	0.000	0.000	0.033
FH2001	**N**	17	3	7	2	2	1	15
	134	0.000	0.000	0.214	0.250	0.000	0.000	0.100
	138	0.000	0.167	0.000	0.000	0.000	0.000	0.100
	142	0.559	0.167	0.000	0.000	0.000	0.000	0.067
	146	0.235	0.333	0.000	0.500	0.500	1.000	0.567
	150	0.176	0.333	0.643	0.250	0.000	0.000	0.133
	154	0.029	0.000	0.143	0.000	0.500	0.000	0.000
	158	0.000	0.000	0.000	0.000	0.000	0.000	0.033
FH2137	**N**	24	3	3	2	2	2	15
	154	0.188	0.500	0.000	0.000	0.000	0.000	0.000
	158	0.042	0.000	0.000	0.000	0.000	0.000	0.000
	162	0.229	0.167	0.000	0.500	0.000	0.500	0.033
	164	0.000	0.000	0.000	0.000	0.000	0.000	0.033
	166	0.104	0.000	0.000	0.000	0.250	0.000	0.067
	170	0.125	0.000	1.000	0.250	0.750	0.000	0.067
	172	0.000	0.000	0.000	0.000	0.000	0.000	0.033
	174	0.104	0.000	0.000	0.000	0.000	0.000	0.300
	176	0.000	0.000	0.000	0.000	0.000	0.000	0.133
	178	0.208	0.333	0.000	0.250	0.000	0.000	0.000
	180	0.000	0.000	0.000	0.000	0.000	0.500	0.300
	184	0.000	0.000	0.000	0.000	0.000	0.000	0.033
CPH08	**N**	23	3	7	2	2	4	15
	197	0.022	0.333	0.000	0.000	0.000	0.500	0.033
	199	0.065	0.000	0.000	0.000	0.000	0.000	0.000
	201	0.000	0.000	0.000	0.000	0.250	0.000	0.000
	203	0.000	0.000	0.000	0.000	0.250	0.000	0.000
	205	0.783	0.333	1.000	0.500	0.000	0.000	0.667
	207	0.000	0.167	0.000	0.250	0.000	0.000	0.267
	209	0.000	0.000	0.000	0.250	0.000	0.125	0.000
	211	0.000	0.000	0.000	0.000	0.500	0.375	0.000
	213	0.065	0.167	0.000	0.000	0.000	0.000	0.033
	215	0.065	0.000	0.000	0.000	0.000	0.000	0.000
C20.253	**N**	28	3	7	2	2	4	15
	93	0.393	0.500	0.500	0.000	0.000	0.000	0.000
	95	0.107	0.000	0.000	0.000	0.250	0.000	0.100
	99	0.179	0.167	0.000	0.250	0.250	0.375	0.133
	101	0.000	0.000	0.500	0.000	0.250	0.125	0.033
	103	0.268	0.167	0.000	0.000	0.250	0.125	0.533
	105	0.000	0.000	0.000	0.000	0.000	0.375	0.000
	107	0.054	0.167	0.000	0.750	0.000	0.000	0.200
CPH05	**N**	30	3	7	2	2	4	15
	108	0.617	0.333	0.000	0.000	0.000	0.000	0.000
	110	0.300	0.167	0.000	0.750	0.500	0.500	0.133
	112	0.017	0.167	0.357	0.000	0.000	0.500	0.033
	114	0.017	0.000	0.000	0.000	0.250	0.000	0.567

	116	0.000	0.167	0.000	0.000	0.000	0.000	0.033
	118	0.000	0.167	0.000	0.250	0.000	0.000	0.000
	122	0.050	0.000	0.000	0.000	0.250	0.000	0.000
	124	0.000	0.000	0.000	0.000	0.000	0.000	0.233
	126	0.000	0.000	0.643	0.000	0.000	0.000	0.000
C09.250	**N**	22	3	7	2	2	4	15
	133	0.182	0.000	0.429	0.000	0.250	0.000	0.667
	135	0.682	0.667	0.286	0.750	0.250	0.500	0.033
	137	0.091	0.167	0.000	0.250	0.250	0.000	0.000
	139	0.000	0.000	0.000	0.000	0.000	0.500	0.100
	141	0.000	0.167	0.000	0.000	0.250	0.000	0.000
	143	0.045	0.000	0.000	0.000	0.000	0.000	0.200
	145	0.000	0.000	0.286	0.000	0.000	0.000	0.000
CPH04	**N**	6	2	6	2	2	4	15
	136	0.417	0.000	0.000	0.000	0.000	0.000	0.000
	138	0.000	0.000	0.833	0.000	0.000	0.000	0.000
	140	0.250	0.250	0.000	0.000	0.500	0.000	0.000
	142	0.167	0.750	0.000	0.250	0.500	0.000	0.967
	144	0.000	0.000	0.167	0.500	0.000	0.000	0.033
	148	0.167	0.000	0.000	0.250	0.000	1.000	0.000
CPH12	**N**	20	3	7	2	2	4	15
	186	0.000	0.000	0.286	0.000	0.000	0.000	0.000
	188	0.000	0.000	0.500	0.000	0.000	0.000	0.000
	190	0.000	0.000	0.000	0.000	0.000	0.000	0.033
	194	0.200	0.833	0.214	0.500	1.000	0.000	0.333
	196	0.275	0.000	0.000	0.500	0.000	0.000	0.367
	198	0.100	0.000	0.000	0.000	0.000	0.000	0.100
	200	0.100	0.000	0.000	0.000	0.000	0.000	0.000
	202	0.200	0.167	0.000	0.000	0.000	0.000	0.167
	204	0.050	0.000	0.000	0.000	0.000	1.000	0.000
	206	0.075	0.000	0.000	0.000	0.000	0.000	0.000
C27.442	**N**	26	3	7	2	2	3	15
	156	0.000	0.000	0.000	0.000	0.000	0.000	0.200
	158	0.019	0.000	1.000	0.000	0.250	0.000	0.033
	162	0.038	0.000	0.000	1.000	0.500	0.000	0.700
	164	0.942	1.000	0.000	0.000	0.000	1.000	0.067
	166	0.000	0.000	0.000	0.000	0.250	0.000	0.000
FH2010	**N**	27	3	7	2	2	4	15
	217	0.000	0.000	0.000	0.500	0.000	0.000	0.000
	221	0.000	0.000	0.000	0.000	0.250	0.000	0.000
	225	0.148	0.333	0.643	0.000	0.000	0.000	0.533
	229	0.315	0.167	0.000	0.250	0.500	0.625	0.467
	233	0.241	0.333	0.357	0.250	0.250	0.375	0.000
	237	0.296	0.167	0.000	0.000	0.000	0.000	0.000
FH2161	**N**	21	2	6	2	2	4	15
	232	0.000	0.000	0.000	0.000	0.000	0.500	0.000
	236	0.000	0.500	0.917	0.000	0.250	0.375	0.033
	240	0.071	0.000	0.000	0.250	0.000	0.125	0.367
	244	0.667	0.250	0.000	0.250	0.750	0.000	0.267
	248	0.095	0.250	0.083	0.500	0.000	0.000	0.167
	250	0.024	0.000	0.000	0.000	0.000	0.000	0.000
	252	0.095	0.000	0.000	0.000	0.000	0.000	0.067
	256	0.024	0.000	0.000	0.000	0.000	0.000	0.100
	260	0.024	0.000	0.000	0.000	0.000	0.000	0.000
C09.173	**N**	29	3	7	2	2	4	15
	101	0.155	0.000	0.000	0.750	0.000	0.000	0.000
	103	0.052	0.167	0.571	0.250	0.000	0.375	0.233
	105	0.000	0.000	0.000	0.000	0.750	0.500	0.367
	107	0.000	0.000	0.429	0.000	0.250	0.125	0.233
	109	0.034	0.167	0.000	0.000	0.000	0.000	0.067
	111	0.017	0.000	0.000	0.000	0.000	0.000	0.000
	113	0.414	0.167	0.000	0.000	0.000	0.000	0.100

	115	0.224	0.500	0.000	0.000	0.000	0.000	0.000
	117	0.103	0.000	0.000	0.000	0.000	0.000	0.000
CPH09	**N**	30	3	7	2	2	4	15
	141	0.050	0.167	0.071	0.000	0.250	0.000	0.367
	143	0.000	0.000	0.500	0.000	0.000	0.125	0.267
	145	0.067	0.000	0.214	0.250	0.250	0.000	0.000
	147	0.433	0.500	0.000	0.000	0.000	0.000	0.067
	149	0.383	0.167	0.214	0.000	0.000	0.000	0.000
	151	0.067	0.167	0.000	0.000	0.000	0.500	0.000
	153	0.000	0.000	0.000	0.000	0.250	0.375	0.000
	155	0.000	0.000	0.000	0.000	0.000	0.000	0.300
	157	0.000	0.000	0.000	0.500	0.000	0.000	0.000
	159	0.000	0.000	0.000	0.250	0.250	0.000	0.000
CPH14	**N**	27	1	7	2	2	4	15
	190	0.000	0.000	0.929	0.000	0.000	0.000	0.000
	194	0.000	0.000	0.000	0.250	0.000	0.500	0.300
	198	0.000	0.000	0.000	0.250	0.000	0.000	0.000
	200	0.000	0.000	0.071	0.000	0.250	0.000	0.300
	202	0.352	1.000	0.000	0.000	0.500	0.125	0.167
	204	0.481	0.000	0.000	0.000	0.250	0.375	0.233
	206	0.167	0.000	0.000	0.500	0.000	0.000	0.000
C13.758	**N**	9	1	6	2	2	3	15
	222	0.000	0.000	0.000	0.250	0.000	0.000	0.000
	224	0.000	0.500	0.750	0.000	0.250	0.000	0.400
	226	0.000	0.000	0.000	0.000	0.000	0.500	0.000
	228	0.000	0.000	0.083	0.500	0.000	0.000	0.267
	230	0.000	0.000	0.167	0.000	0.000	0.500	0.100
	234	0.167	0.500	0.000	0.000	0.250	0.000	0.033
	236	0.056	0.000	0.000	0.000	0.500	0.000	0.200
	238	0.167	0.000	0.000	0.000	0.000	0.000	0.000
	240	0.111	0.000	0.000	0.250	0.000	0.000	0.000
	242	0.500	0.000	0.000	0.000	0.000	0.000	0.000

Table S7. Overview of samples analysed in this study.

Geographic Origin	**Origin type**	**Year collected**	**Lineage**	**Samples No.**	**Sample type**
Humla, Nepal	Field collected	2015	Himalayan wolf*	104	scat and hair (non-invasive)
Dolpa, Nepal	Field collected	2016	Himalayan wolf*	139	scat and hair (non-invasive)
KCA, Nepal	Field collected	2016	Himalayan wolf*	44	scat (non-invasive)
Kyrgyzstan	Field collected	2017	Grey wolf *Canis lupus* sp.	4	scat (non-invasive)
Northern Spain	Field collected	2017	Iberian wolf *Canis lupus signatus*	30	scat and tissue
Ethiopia	Field collected	2016/2017	Ethiopian wolf *Canis simensis*	6	tissue
Africa	Field collected/ RZSS WildGenes Collection	2013	African wolf*	7	blood
Europe	Zoo Animal RZSS WildGenes	2015	Grey wolf *Canis lupus* sp.	6	blood
Mongolia	Zürich Zoo animal/RZSS WildGenes Collection	2015	Grey wolf *Canis lupus* sp.	6	tissue

*Formal taxonomic classification pending

Table S8. Informative positions found in the final intron sequences of the zinc-finger X-chromosomal (ZFX, 514bp) between the tested canid lineages. (Table augmented from Table 4 in (Werhahn et al., 2017).

Seq. Source	**Species**	**Sample size**	**ZFX final intron**			**Haplotype**
			328	**381**	**425**	
			1bp insertion	**T/A**	**A/G**	
NCBI GenBank	Golden jackal *C. aureus* Israel (KT448243.1)	1	G	A	A	**A**
	African wolf *C. aureus lupaster* Kenya (KT448251.1)	1	G	T	G	**B**
	Grey wolf *C. lupus* Canada (KT448225.1)	1	-	T	A	**C**
This study	Grey wolf *C. lupus* Europe	1	-	T	A	**C**
	Grey wolf *C. lupus* Mongolia	4	-	T	A	
	Grey wolf *C. lupus* Arabian	1	-	T	A	
	Dog *C. lupus* Nepal	2	-	T	A	
	Iberian wolf *C.l. signatus* Europe (Spain)	12	-	T	A	
	Grey wolf *C. lupus* Kyrgyzstan	3	G	T	G	**B**
	Himalayan wolf # Nepal	17	G	T	G	**B**

Formal taxonomic classification pending.

Table S9. Informative positions found in the final intron sequences of the zinc-finger Y-chromosomal (ZFY, 1176bp) of the tested canid lineages. (Table augmented from Table 3 in (Werhahn et al., 2017)).

Seq. Source	**Species**	**Sample size**	**ZFY final intron**									**Haplotype**
			23	**123**	**193-201**	**236-446**	**880-909**	**1010**	**1036-1037**	**1056**	**1094**	
			A/G	**1bp insertion**	**9bp insertion**	**210bp SINEII insertion**	**30bp deletion**	**T/G**	**2bp insertion**	**G/A**	**T/C**	
Seq. Source	Grey wolf *C. lupus* Canada (KT448254.1)	1	A	-	-	-	30bp	G	-	G	T	**C**
	Golden Jackal *C. aureus* Serbia (KT448259.1)	1	G	A	9bp	210bp	-	T	TA	A	T	**A**
	Golden Jackal *C. aureus* Israel (KT448266.1)	1	G	A	9bp	210bp	-	T	TA	A	T	
	African wolf *C. aureus lupaster* Kenya and Morocco (KT448270.1, KT448267.1)	2	A	-	-	-	-	T	-	G	T	**B**
This Study	African wolf *C. aureus lupaster* Morocco	2	A	-	-	-	-	T	-	G	T	
	Grey wolf *C. lupus* Europe and Mongolia (captive Zurich Zoo)	2	A	-	-	-	30bp	G	-	G	T	**C**
	Domestic Dog Nepal	3	A	-	-	-	30bp	G	-	G	T	**C**
	Iberian wolf *C. l. signatus* Europe (Spain)	7	A	-	-	-	30bp	G	-	G	T	**C**
	Grey wolf *C. lupus* Kyrgyzstan	4	A	-	-	-	30bp	G	-	G	T	**C**
	Himalayan wolf [#] Nepal	15	A	-	-	-	30bp	T	-	G	T	**D**

[#] Formal taxonomic classification pending.

Table S10. Result overview per sample across the different tested markes: D-loop and cyt B of the mitochondrial genome and on the nuclear the ZFX and ZFY, microsatellite results based on the STRUCTURE results with K=5 and the hypoxia related path way genes. ZFX/ZFY haplotype corresponds to Table S8 and S9.

Location	Sample ID	mtDNA D-loop	mtDNA cyt b	ZFX (haplotype)	ZFY (haplotype)	microsatellites (K=5)	ANGPT1	EPAS1-1		RYR2-1		RYR2-2
							73bp	6bp	22bp	19bp	22bp	35bp
Nepal (Humla)	WF226	HW D-loop 1	HW Cyt B2									
Nepal (Humla)	WF229	HW D-loop 2	HW Cyt B1									
Nepal (Humla)	WF230	HW D-loop 3	HW Cyt B1			Himalayan wolf (>95%)						
Nepal (Humla)	WF236	HW D-loop 2					C	fail	fail	C	T	G
Nepal (Humla)	WF237	HW D-loop 2					C/T	G	A	C	T	G
Nepal (Humla)	WF238	HW D-loop 2					C	G	A	C	T	fail
Nepal (Humla)	WF240	HW D-loop 2		Himalayan wolf (B)	Himalayan wolf (D)	Himalayan wolf (>95%)	C/T	G	A	C	T	fail
Nepal (Humla)	WF242	HW D-loop 2										
Nepal (Humla)	WF243	HW D-loop 2	HW Cyt B1									
Nepal (Humla)	WF244	HW D-loop 3	HW Cyt B1				C/T	G	A	C	T	G
Nepal (Humla)	WF245	HW D-loop 1	HW Cyt B2									
Nepal (Humla)	WF247	HW D-loop 2					C/T	fail	fail	C	T	G
Nepal (Humla)	WF248	HW D-loop 2										
Nepal (Humla)	WF249	HW D-loop 2				Himalayan wolf (>95%)	C/T	G	A	C	T	G
Nepal (Humla)	WF250	HW D-loop 2										
Nepal (Humla)	WF251	HW D-loop 2										
Nepal (Humla)	WF252	HW D-loop 2										
Nepal (Humla)	WF253	HW D-loop 1										
Nepal (Humla)	WF258	HW D-loop 2		Himalayan wolf (B)		Himalayan wolf (>95%)	C	G	A	C	T	G
Nepal (Humla)	WF260	HW D-loop 2										
Nepal (Humla)	WF261	HW D-loop 2			Himalayan wolf (D)	Himalayan wolf (>95%)	C/T	G	A	C	T	fail

Nepal (Humla)	WF263	HW D-loop 2		Himalayan wolf (B)		Himalayan wolf (>95%)	C/T	G	A	C	T	fail
Nepal (Humla)	WF264	HW D-loop 2	HW Cyt B1									
Nepal (Humla)	WF265	HW D-loop 2										
Nepal (Humla)	WF266	HW D-loop 2				Himalayan wolf (>95%)	C/T	G	A	C	T	fail
Nepal (Humla)	WF267	HW D-loop 2					C	G	A	C	T	fail
Nepal (Humla)	WF269	HW D-loop 2										
Nepal (Humla)	WF270	HW D-loop 2					C/T	G	A	C	T	G
Nepal (Humla)	WF272	HW D-loop 2		Himalayan wolf (B)			C	fail	fail	C	T	G
Nepal (Humla)	WF274	HW D-loop 2		Himalayan wolf (B)			C/T	G	A	C	T	G
Nepal (Humla)	WF278	HW D-loop 2					C	G	A	C	T	fail
Nepal (Humla)	WF279	HW D-loop 2										
Nepal (Humla)	WF280	HW D-loop 2		Himalayan wolf (B)		Himalayan wolf (>95%)	C	G	A	C	T	fail
Nepal (Humla)	WF281	HW D-loop 2										
Nepal (Humla)	WF283	HW D-loop 1										
Nepal (Humla)	WF284	HW D-loop 1										
Nepal (Humla)	WF285	HW D-loop 2	HW Cyt B1									
Nepal (Humla)	WF286	HW D-loop 3	HW Cyt B1									
Nepal (Humla)	WF288	HW D-loop 2					C/T	G	A	C	T	fail
Nepal (Humla)	WF289	HW D-loop 2					C	G/T	A/G	C	T	fail
Nepal (Humla)	WF290	HW D-loop 2	HW Cyt B1				T	G	A	C	T	fail
Nepal (Humla)	WF291	HW D-loop 3	HW Cyt B1		Himalayan wolf (D)	Himalayan wolf (>95%)	C	G	A	C	T	G
Nepal (Humla)	WF292	HW D-loop 2										
Nepal (Humla)	WF293	HW D-loop 2										
Nepal (Humla)	WF295	HW D-loop 2	HW Cyt B1									
Nepal (Humla)	WF296	HW D-loop 2										
Nepal (Humla)	WF297						C	G	A	C	T	G
Nepal (Humla)	WF298	HW D-loop 2										

Nepal (Humla)	WF300	HW D-loop 1		Himalayan wolf (B)	Himalayan wolf (D)	Himalayan wolf (84%)	C	G	A	C	T	G
Nepal (Humla)	WF301	HW D-loop 1	HW Cyt B2									
Nepal (Humla)	WF302	HW D-loop 2					C/T	G	A	C	T	fail
Nepal (Humla)	WF303	HW D-loop 2										
Nepal (Humla)	WF304	HW D-loop 2										
Nepal (Humla)	WF305	HW D-loop 2		Himalayan wolf (B)			C/T	G	A	C	T	G
Nepal (Humla)	WF306	HW D-loop 2										
Nepal (Humla)	WF308	HW D-loop 2					C/T	G	A	C	T	G
Nepal (Humla)	WF310	HW D-loop 1										
Nepal (Humla)	WF311	HW D-loop 1										
Nepal (Humla)	WF312	HW D-loop 1										
Nepal (Humla)	WF313	HW D-loop 1					C	G	A	C	T	fail
Nepal (Humla)	WF315	HW D-loop 1										
Nepal (Humla)	WF316			Himalayan wolf (B)		Himalayan wolf (>95%)	C	G	A	C	T	fail
Nepal (Humla)	WF318	HW D-loop 2										
Nepal (Humla)	WF319	HW D-loop 2										
Nepal (Humla)	WF320	HW D-loop 1			Himalayan wolf (D)	Himalayan wolf (>95%)	C	G	A	C	T	G
Nepal (Humla)	WF321	HW D-loop 2										
Nepal (Dolpa)	WF333	HW D-loop 4					C/T	G	A	C	T	G
Nepal (Dolpa)	WF334	HW D-loop 4		Himalayan wolf (B)			C/T	G	A	C	T	G
Nepal (Dolpa)	WF335	HW D-loop 4	HW Cyt B1			Himalayan wolf (>95%)	C/T	G	A	C	T	G
Nepal (Dolpa)	WF336	HW D-loop 4				Himalayan wolf (>95%)	C/T	fail	fail	C	T	fail
Nepal (Dolpa)	WF337	HW D-loop 4										
Nepal (Dolpa)	WF338	HW D-loop 4										
Nepal (Dolpa)	WF341	HW D-loop 3	HW Cyt B1		Himalayan wolf (D)		C	G	A	C	T	G
Nepal (Dolpa)	WF342	HW D-loop 4	HW Cyt B1									
Nepal (Dolpa)	WF343	HW D-loop 4					C	G	A	G	T	fail

Nepal (Dolpa)	WF347	HW D-loop 3										
Nepal (Dolpa)	WF348	HW D-loop 3					C	G	A	C	T	fail
Nepal (Dolpa)	WF350	HW D-loop 3	HW Cyt B1									
Nepal (Dolpa)	WF351	HW D-loop 4										
Nepal (Dolpa)	WF357	HW D-loop 3				Himalayan wolf (>95%)	C	G	A	C	T	C/G
Nepal (Dolpa)	WF359	HW D-loop 4				Himalayan wolf (>95%)	C/T	G	A	C/G	T	C/G
Nepal (Dolpa)	WF360	HW D-loop 3				Himalayan wolf (91%)	C	G	A	C	T	G
Nepal (Dolpa)	WF363	HW D-loop 4				Himalayan wolf (>95%)	C	G	A	C/G	T	C/G
Nepal (Dolpa)	WF365	HW D-loop 3				Himalayan wolf (>95%)	C	G	A	C	T	G
Nepal (Dolpa)	WF366	HW D-loop 3	HW Cyt B1									
Nepal (Dolpa)	WF367	HW D-loop 3										
Nepal (Dolpa)	WF368	HW D-loop 3		Himalayan wolf (B)	Himalayan wolf (D)		C	G	A	C	T	G
Nepal (Dolpa)	WF372	HW D-loop 4	HW Cyt B1	Himalayan wolf (B)			C/T	G	A	C	T	G
Nepal (Dolpa)	WF374	HW D-loop 3				Himalayan wolf (>95%)	C	G	A	C	T	G
Nepal (Dolpa)	WF377	HW D-loop 3										
Nepal (Dolpa)	WF378	HW D-loop 4				Himalayan wolf (88%)	C/T	G	A	C	T	fail
Nepal (Dolpa)	WF383	HW D-loop 4										
Nepal (Dolpa)	WF389	HW D-loop 3	HW Cyt B1				C	G	A	C	T	fail
Nepal (Dolpa)	WF390	HW D-loop 3										
Nepal (Dolpa)	WF391	HW D-loop 4					C	G	A	C	T	C
Nepal (Dolpa)	WF396	HW D-loop 4		Himalayan wolf (B)			C/T	G	A	C	T	G
Nepal (Dolpa)	WF397	HW D-loop 4										
Nepal (Dolpa)	WF398	HW D-loop 3										
Nepal (Dolpa)	WF399	HW D-loop 4	HW Cyt B1									
Nepal (Dolpa)	WF402	HW D-loop 4										
Nepal (Dolpa)	WF403	HW D-loop 4										
Nepal (Dolpa)	WF404	HW D-loop 4										

Nepal (Dolpa)	WF406	HW D-loop 3										
Nepal (Dolpa)	WF407	HW D-loop 3										
Nepal (Dolpa)	WF409	HW D-loop 4										
Nepal (Dolpa)	WF410	HW D-loop 4										
Nepal (Dolpa)	WF411	HW D-loop 1										
Nepal (Dolpa)	WF418	HW D-loop 4	HW Cyt B1			Himalayan wolf (>95%)	C	G	A	C/G	T	C
Nepal (Dolpa)	WF420	HW D-loop 4					C	fail	fail	C/G	T	C
Nepal (Dolpa)	WF422	HW D-loop 4					C	G	A	C/G	T	fail
Nepal (Dolpa)	WF423	HW D-loop 4				Himalayan wolf (>95%)	C	G	A	C/G	T	fail
Nepal (Dolpa)	WF424	HW D-loop 4										
Nepal (Dolpa)	WF426	HW D-loop 3	HW Cyt B1									
Nepal (Dolpa)	WF427	HW D-loop 4	HW Cyt B1									
Nepal (Dolpa)	WF428	HW D-loop 4										
Nepal (Dolpa)	WF429	HW D-loop 4										
Nepal (Dolpa)	WF430	HW D-loop 4										
Nepal (Dolpa)	WF431	HW D-loop 4										
Nepal (Dolpa)	WF433	HW D-loop 4										
Nepal (Dolpa)	WF434	HW D-loop 4										
Nepal (Dolpa)	WF436	HW D-loop 4			Himalayan wolf (D)	Himalayan wolf (>95%)	C	G	A	C/G	T	C
Nepal (Dolpa)	WF438	HW D-loop 4										
Nepal (Dolpa)	WF440	HW D-loop 3			Himalayan wolf (D)	Himalayan wolf (>95%)	C	G	A	C/G	T	C
Nepal (Dolpa)	WF442	HW D-loop 3										
Nepal (Dolpa)	WF443	HW D-loop 3										
Nepal (Dolpa)	WF444	HW D-loop 3										
Nepal (Dolpa)	WF445	HW D-loop 3										
Nepal (Dolpa)	WF446	HW D-loop 4										
Nepal (Dolpa)	WF449	HW D-loop 4					C/T	G	A	C	T	G

Nepal (Dolpa)	WF450	HW D-loop 4					C/T	G	A	C	T	G
Nepal (Dolpa)	WF451	HW D-loop 3	HW Cyt B1	Himalayan wolf (B)	Himalayan wolf (D)	Himalayan wolf (>95%)	C	G	A	C	T	C
Nepal (Dolpa)	WF454	HW D-loop 3		Himalayan wolf (B)	Himalayan wolf (D)		C	G	A	C	T	C/G
Nepal (Dolpa)	WF455	HW D-loop 3										
Nepal (Dolpa)	WF456	HW D-loop 3										
Nepal (Dolpa)	WF457	HW D-loop 5										
Nepal (Dolpa)	WF459	HW D-loop 3										
Nepal (Dolpa)	WF461	HW D-loop 3										
Nepal (Dolpa)	WF462	HW D-loop 3										
Nepal (Dolpa)	WF463	HW D-loop 3										
Nepal (Dolpa)	WF465	HW D-loop 4										
Nepal (Dolpa)	WF468	HW D-loop 4										
Nepal (Dolpa)	WF469	HW D-loop 4					C/T	G	A	C	T	G
Nepal (Dolpa)	WF470	HW D-loop 4					C/T	G	A	C	T	C
Nepal (Dolpa)	WF471	HW D-loop 3		Himalayan wolf (B)	Himalayan wolf (D)	Himalayan wolf (>95%)	C	G	A	C	T	G
Nepal (Dolpa)	WF472	HW D-loop 3				Himalayan wolf (>95%)	C	G	A	C	T	C/G
Nepal (Dolpa)	WF473	HW D-loop 3										
Nepal (Dolpa)	WF477	HW D-loop 3										
Nepal (KCA)	WF488	HW D-loop 5		Himalayan wolf (B)	Himalayan wolf (D)	Himalayan wolf (89%)	C	G/T	A/G	C	T	G
Nepal (KCA)	WF499	HW D-loop 5										
Nepal (KCA)	WF505	HW D-loop 5										
Nepal (KCA)	WF506	HW D-loop 5										
Nepal (KCA)	WF507	HW D-loop 5										
Nepal (KCA)	WF508	HW D-loop 5										
Nepal (KCA)	WF510	HW D-loop 5										
Nepal (KCA)	WF513	HW D-loop 5										
Nepal (KCA)	WF515	HW D-loop 5										

Nepal (KCA)	WF517	HW D-loop 5			Himalayan wolf (D)	Himalayan wolf (87%)	C	G	A	C	T	G
Nepal (KCA)	WF519	HW D-loop 5										
Nepal (KCA)	WF524	HW D-loop 5										
Nepal (KCA)	WF530	HW D-loop 3										
Domestic Dogs Nepal												
Dog Nepal (Humla)	WF228	DD 1	DD Cyt B1									
Dog Nepal (Humla)	WF246	DD 1	DD Cyt B1									
Dog Nepal (Dolpa)	WF384	DD 1										
Dog Nepal (Dolpa)	WF405	DD 3	DD Cyt B2		Grey wolf (C)	Grey wolf (>95%)	T	G	A	G	T	C
Dog Nepal (Dolpa)	WF425	DD 2	DD Cyt B1		Grey wolf (C)	Grey wolf (>95%)	T	G	A	G	T	fail
Dog Nepal (Dolpa)	WF437	DD 5	DD Cyt B3									
Dog Nepal (Dolpa)	WF466	DD 6	DD Cyt B5									
Dog Nepal (KCA)	WF487	DD 2			Grey wolf (C)	Grey wolf (>95%)	T	G	A	G	T	C
Iberian wolf												
Spain	WLF114	Iberian wolf	Grey wolf	Grey wolf (C)	Grey wolf (C)	Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF115	Iberian wolf	Grey wolf	Grey wolf (C)	Grey wolf (C)	Grey wolf (77%)/Iberian wolf (22%)	T	T	G	G	T	C
Spain	WLF116	Iberian wolf	Grey wolf			Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF117	Iberian wolf	Grey wolf			Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF118	Iberian wolf	Grey wolf			Grey wolf (59%)/Iberian wolf (40%)	T	T	G	G	T	C
Spain	WLF119	Iberian wolf	Grey wolf	Grey wolf (C)	Grey wolf (C)	Iberian wolf (>95%)	fail	T	G	G	T	C
Spain	WLF120	Iberian wolf	Grey wolf			Iberian wolf (>95%)	T	T	G	G	T	C

Spain	WLF121	Iberian wolf	Grey wolf	Grey wolf (C)	Grey wolf (C)	Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF122	Iberian wolf	Grey wolf			Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF123	Iberian wolf	Grey wolf	Grey wolf (C)	Grey wolf (C)	Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF124	Iberian wolf	Grey wolf	Grey wolf (C)		Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF125	Iberian wolf	Grey wolf	Grey wolf (C)	Grey wolf (C)	Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF126	Iberian wolf	Grey wolf	Grey wolf (C)		Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF127	Iberian wolf	Grey wolf	Grey wolf (C)		Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF128	Iberian wolf	Grey wolf	Grey wolf (C)	Grey wolf (C)	Iberian wolf (>95%)	T	T	G	G	T	C
Grey wolf (Kyrgyz Republic)												
Kyrgyzstan	WLF129	Grey wolf	Grey wolf	Himalayan wolf (B)	Grey wolf (C)	Grey wolf Kyrgyz Republic (72%)	T	G/T	A/G	G	T	C
Kyrgyzstan	WLF130	Grey wolf	Grey wolf	Himalayan wolf (B)	Grey wolf (C)	Grey wolf Kyrgyz Republic (>95%)	T	G/T	A/G	G	T	C
Kyrgyzstan	WLF131	Grey wolf	Grey wolf	Himalayan wolf (B)	Grey wolf (C)	Grey wolf Kyrgyz Republic (>95%)	T	G/T	A/G	G	T	fail
Kyrgyzstan	WLF132	Grey wolf	Grey wolf		Grey wolf (C)	Grey wolf Kyrgyz Republic (>95%)	T	T	G	fail	fail	fail
Grey wolf												
Europe	WF031						T	T	G	G	T	C
Europe	WF032					Grey wolf (>95%)	T	T	G	G	T	C
Europe	WF033						T	T	G	G	T	C
Europe	WF034			Grey wolf (C)	Grey wolf (C)	Grey wolf (>95%)	T	T	G	G	T	C
Mongolia	WF017			Grey wolf (C)	Grey wolf (C)	Grey wolf (>95%)						
Mongolia	WF014			Grey wolf (C)		Grey wolf (>95%)	T	T	G	G	T	C
African wolf/Golden Jackal												
Morocco	WLF027			Grey wolf (C)	African wolf (B)		T	T	G	G	T	C

Morocco	WLF028											
Morocco	WLF029						T	T	G	fail	fail	C
Morocco	WLF056						T	T	G	G	T	C
AE Al-Ain Zoo	WRF020					African wolf (>95%)						
AE Al-Ain Zoo	WRF021					African wolf (>95%)						
AE Al-Ain Zoo	WRF022					African wolf (>95%)						
AE Al-Ain Zoo	WRF023					African wolf (>95%)	T	T	G	fail	fail	C
AE Al-Ain Zoo	WRF024					African wolf (>95%)	T	T	G	fail	fail	C
AE Al-Ain Zoo	WRF025					African wolf (>95%)						
AE Al-Ain Zoo	WRF026					African wolf (>95%)						
Ethiopian wolf												
Ethiopia	WLF133	Etiopian wolf	Etiopian wolf				T	T	G	G	C	C
Ethiopia	WLF134	Etiopian wolf	Etiopian wolf				T	T	G	G	C	fail
Ethiopia	WLF135	Etiopian wolf	Etiopian wolf				T	T	G	G	C	C
Ethiopia	WLF136	Etiopian wolf	Etiopian wolf				T	T	G	fail	fail	C
Ethiopia	WLF137	Etiopian wolf	Etiopian wolf				T	T	G	G	C	C
Ethiopia	WLF138	Etiopian wolf	Etiopian wolf				T	T	G	G	C	fail

Spain	WLF121	Iberian wolf	Grey wolf	Grey wolf (C)	Grey wolf (C)	Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF122	Iberian wolf	Grey wolf			Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF123	Iberian wolf	Grey wolf	Grey wolf (C)	Grey wolf (C)	Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF124	Iberian wolf	Grey wolf	Grey wolf (C)		Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF125	Iberian wolf	Grey wolf	Grey wolf (C)	Grey wolf (C)	Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF126	Iberian wolf	Grey wolf	Grey wolf (C)		Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF127	Iberian wolf	Grey wolf	Grey wolf (C)		Iberian wolf (>95%)	T	T	G	G	T	C
Spain	WLF128	Iberian wolf	Grey wolf	Grey wolf (C)	Grey wolf (C)	Iberian wolf (>95%)	T	T	G	G	T	C
Grey wolf (Kyrgyz Republic)												
Kyrgyzstan	WLF129	Grey wolf	Grey wolf	Himalayan wolf (B)	Grey wolf (C)	Grey wolf Kyrgyz Republic (72%)	T	G/T	A/G	G	T	C
Kyrgyzstan	WLF130	Grey wolf	Grey wolf	Himalayan wolf (B)	Grey wolf (C)	Grey wolf Kyrgyz Republic (>95%)	T	G/T	A/G	G	T	C
Kyrgyzstan	WLF131	Grey wolf	Grey wolf	Himalayan wolf (B)	Grey wolf (C)	Grey wolf Kyrgyz Republic (>95%)	T	G/T	A/G	G	T	fail
Kyrgyzstan	WLF132	Grey wolf	Grey wolf		Grey wolf (C)	Grey wolf Kyrgyz Republic (>95%)	T	T	G	fail	fail	fail
Grey wolf												
Europe	WF031						T	T	G	G	T	C
Europe	WF032					Grey wolf (>95%)	T	T	G	G	T	C
Europe	WF033						T	T	G	G	T	C
Europe	WF034			Grey wolf (C)	Grey wolf (C)	Grey wolf (>95%)	T	T	G	G	T	C
Mongolia	WF017			Grey wolf (C)	Grey wolf (C)	Grey wolf (>95%)						
Mongolia	WF014			Grey wolf (C)		Grey wolf (>95%)	T	T	G	G	T	C
African wolf/Golden Jackal												
Morocco	WLF027			Grey wolf (C)	African wolf (B)		T	T	G	G	T	C

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Appendix B-3. Supplementary information for: Himalayan wolf distribution and admixture based on multiple genetic markers

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Table S1. New mtDNA haplotypes found in this study with NCBI GenBank accessions.

Region	Species	Haplotype Name	GenBank Accession	
D-loop	Himalayan wolf [#]	Himalayan wolf D-loop 14 (China)	*To follow*	This study
D-loop	Himalayan wolf [#]	Himalayan wolf D-loop 15 (China)	*To follow*	This study
D-loop	*C. l. familiaris*	Domestic dog D-loop China 1	*To follow*	This study
D-loop	*C. l. familiaris*	Domestic dog D-loop China 2	*To follow*	This study
D-loop	*C. l. familiaris*	Domestic dog D-loop China 3	*To follow*	This study
D-loop	*C. l. familiaris*	Domestic dog D-loop China 4	*To follow*	This study
D-loop	*C. l. familiaris*	Domestic dog D-loop China 5	*To follow*	This study
D-loop	*C. l. familiaris*	Domestic dog D-loop China 6	*To follow*	This study
D-loop	*C. lupus*	Grey wolf Tajikistan 1	*To follow*	This study
D-loop	*C. lupus*	Grey wolf Tajikistan 2	*To follow*	This study
D-loop	*C. lupus*	Grey Wolf China 1	*To follow*	This study
D-loop	*C. lupus*	Grey wolf Xinjiang 5	*To follow*	This study
Cyt b	*C. lupus*	Grey wolf Tajikistan 1	*To follow*	This study
Cyt b	*C. lupus*	Grey wolf Tajikistan 2	*To follow*	This study
Cyt b	*C. lupus*	Grey wolf Tajikistan 3	*To follow*	This study
Cyt b	*C. lupus*	Grey wolf Tajikistan 4	*To follow*	This study
Cyt b	*C. lupus*	Grey wolf Tajikistan 5	*To follow*	This study
Cyt b	*C. lupus*	Grey wolf China 1 (QHW6)	*To follow*	This study
Cyt b	*C. lupus*	Grey wolf China Xinjiang 3	*To follow*	This study
cyt *b*	Himalayan wolf [#]	Himalayan wolf Cytochrome B 3 (China)	*To follow*	This study
cyt *b*	Himalayan wolf [#]	Himalayan wolf Cytochrome B 4 (China)	*To follow*	This study
cyt *b*	Himalayan wolf [#]	Himalayan wolf Cytochrome B 5 (China)	*To follow*	This study
cyt *b*	*C. l. familiaris*	Domestic Dog China 1	*To follow*	This study

[#] Formal taxonomic classification pending. Currently recommended as *Canis lupus chanco* (Alvares et al. 2019).

Table S2. Overview of the D-loop data used in the phylogenetic analysis. Reference sequences from the following publications were included: (Aggarwal, Kivisild, Ramadevi, & Singh, 2007; Björnerfeldt, Webster, & Vilà, 2006; Gaubert et al., 2012; Gottelli, Marino, Sillero-Zubiri, & Funk, 2004; R. Gundry et al., 2007; R. L. Gundry et al., 2007; İbiş, Aksöyek, Özcan, & Tez, 2015; Ishiguro, Inoshima, & Shigehara, 2009; Koblmüller, 2016; Koepfli et al., 2015; Meng, Zhang, & Meng, 2009; Rueness et al., 2011; Sharma, Maldonado, Jhala, & Fleischer, 2004; Thalmann et al., 2013; Waters, El Harrad, Amhouch, Taiqui, & Senn, 2015; H. Zhang & Chen, 2011; H. Zhang et al., 2015). Marked with an asterisk* are sequences that are unpublished and have been directly submitted to NCBI GenBank by the respective authors.

Haplotype	Unique Haplotypes Accession	Identical Sequences Accessions	Publication
Himalayan wolf 1 Nepal	KY996529		Werhahn et al. (2017)
		AY333740	Sharma et al. (2004)
		AY289986	Aggarwal et al. (2007)
		AY289995	Aggarwal et al. (2007)
		AY289985	Aggarwal et al. (2007)
		AY289994	Aggarwal et al. (2007)
		AY289977	Aggarwal et al. (2007)
		AY289993	Aggarwal et al. (2007)
		AY289992	Aggarwal et al. (2007)
		AY289991	Aggarwal et al. (2007)
		AY289978	Aggarwal et al. (2007)
		AY289990	Aggarwal et al. (2007)
		AY289979	Aggarwal et al. (2007)
		AY289980	Aggarwal et al. (2007)
		AY289989	Aggarwal et al. (2007)
		AY289981	Aggarwal et al. (2007)
		AY289988	Aggarwal et al. (2007)
		AY289982	Aggarwal et al. (2007)
		AY289983	Aggarwal et al. (2007)
		AY289987	Aggarwal et al. (2007)
		JX415352	unpublished*
		JX415350	unpublished*
		SRX1024624 (Tibet China)	Zhang et al. (2014)
Himalayan wolf 2	KY996530 (Nepal)		Werhahn et al (2017)
		JX415351	unpublished*
		NC011218 Xining Zoo, Qinghai	Meng et al. (2009)
		EU442884.2 (Qinghai China)	unpublished*#
		FJ032363.2	Meng et al. (2009)
		SRX1024633 (Tibet China)	Zhang et a. (2014)
		SRX1024634 (Qinghai China)	Zhang et a. (2014)
Himalayan wolf 3	KY940301		Werhahn et al (2017)
		JX415343	unpublished*

Himalayan wolf 4	MK113901		Werhahn et al (2018)
		SRX1024635 (Qinghai)	Zhang et a. (2014)
Himalayan wolf 5	MK113902		Werhahn et al (2018)
Himalayan wolf 6 Qinghai Lake China	JX415345		unpublished*
Himalayan wolf 7 Museum Tibet	AY333739		Sharma et al. (2004)
Himalayan wolf 8 Tibet	KF573616		unpublished*
		AB480742	Ishiguro et al. (2009)
Himalayan wolf 9 Museum Nepal	AY333738		Sharma et al. (2004)
		JX415344	unpublished*
Himalayan wolf 10 Museum Tibet	AY333742		Sharma et al. (2004)
Himalayan wolf 11 Qinghai Lake China	JX415348		unpublished*
Himalayan wolf 12 Ladhak India	AY333741		Sharma et al. (2004)
Himalayan wolf 13 Qinghai Lake China	JX415347		unpublished*
Himalayan wolf 14 (China)	To follow		This study
Himalayan wolf 15 (China)	To follow		This study
Indian wolf 1	AY333745		Sharma et al. (2004)
Indian wolf 2	AY289973		Aggarwal et al. (2007)
Indian wolf 3	AY333746		Sharma et al. (2004)
Indian wolf 4	AY333743		Sharma et al. (2004)
Indian wolf 5	AY289974		Aggarwal et al. (2007)
		AY289975	Aggarwal et al. (2007)
		AY289976	Aggarwal et al. (2007)
		AY289984	Aggarwal et al. (2007)
		AY333746	Sharma et al. (2004)
		AY333744	Sharma et al. (2004)
Grey wolf Xinjiang China 1	KC461238		Zhang et al. (2014)
Grey wolf Xinjiang China 2	SRX1024627		Zhang et al. (2014)
		KF661053	Thalmann et al. (2013)
Grey wolf Xinjiang China 3			This study
		MG818351	
Grey wolf Xinjiang 4			This study
		KY549998	
Grey wolf Xinjiang 5	to follow		This study
Grey wolf Mongolia 1	KY996527		Werhahn et al. (2017)
Grey wolf Mongolia 2	KY996528		Werhahn et al. (2017)
Grey wolf Mongolia 3	KU696392		Koblmüller et al. (2016)
		SRX1024637 (Inner Mongolia China)	Zhang et al. (2014)
		GQ374438 (Inner Mongolia China)	Zhang and Chen (2011)
Grey wolf Mongolia 4	KU696393		Koblmüller et al. (2016)
		KF661041 (China)	Thalmann et al. (2013)
		KU696391 (China)	Koblmüller et al. (2016)
		SRX1024636 (Inner Mongolia China)	Zhang et al. (2014)
		SRX1024629 (Xinjiang China)	Zhang et al. (2014)
Grey wolf_Mongolia 5	KU696395.1		Koblmüller et al. (2016)
Grey wolf Mongolia 6	KC896375		Zhang et al. (2015)
Grey wolf Kyrgyzstan 1	MK113898		Werhahn et al (2018)
Grey wolf Kyrgyzstan 2	MK113899		Werhahn et al (2018)
Grey wolf Kyrgyzstan 3	MK113900		Werhahn et al (2018)

	KC414578		unpublished
Grey wolf Tajikistan 1	*To follow*		This study
Grey wolf Tajikistan 2	*To follow*		This study
Grey wolf Tajikistan3	*To follow*		This study
Grey wolf Tajikistan4	*To follow*		This study
Grey wolf Tajikistan4	*To follow*		This study
Grey wolf_Russia	KF661046		Thalmann et al. (2013)
Grey wolf_Poland	KF661045		Thalmann et al. (2013)
Grey wolf_India	KF661043		Thalmann et al. (2013)
		KF661054	Thalmann et al. (2013)
Grey wolf_Egypt	JQ088677.1		Gaubert et al. (2012)
		KF661055	Thalmann et al. (2013)
Grey wolf_Saudi Arabia	DQ480506		Björnerfeldt et al. (2006)
Grey wolf_Israel	KF661042		Thalmann et al. (2013)
		AY333733	Sharma et al. (2004)
Grey wolf Oman	KF661050		Thalmann et al. (2013)
Grey wolf_Iran	KF661051		Thalmann et al. (2013)
Grey wolf_Finland	KF661038		Thalmann et al. (2013)
		KF661039	Thalmann et al. (2013)
		DQ480503	Björnerfeldt et al. (2006)
Grey wolf_Sweden	KF661040		Thalmann et al. (2013)
		KF661044	Thalmann et al. (2013)
		KF661049	Thalmann et al. (2013)
		KF661052	Thalmann et al. (2013)
		DQ480504	Björnerfeldt et al. (2006)
Grey wolf_Italy	KF661048		Thalmann et al. (2013)
Iberian wolf_North Spain 1		KY550008	Werhahn et al (2018)
Iberian wolf_North Spain 2		KY550009	Werhahn et al (2018)
Iberian wolf_North Spain 3		KY550010	Werhahn et al (2018)
Iberian wolf_Spain	DQ480505		Björnerfeldt et al. (2006)
Grey wolf_USA	KF661064		Thalmann et al. (2013)
		KF661068	Thalmann et al. (2013)
		KF661069	Thalmann et al. (2013)
		KF661072	Thalmann et al. (2013)
Grey wolf_Canada 1	KF661074		Thalmann et al. (2013)
Grey wolf_Canada 2	KF661061		Thalmann et al. (2013)
		KF661062	Thalmann et al. (2013)
		KF661063	Thalmann et al. (2013)
		KF661056	Thalmann et al. (2013)
		DQ480508	Björnerfeldt et al. (2006)
Grey wolf_Alaska 1	KF661058		Thalmann et al. (2013)
Grey wolf_Alaska 2	KF661066		Thalmann et al. (2013)
Grey wolf_Alaska 3	KF661071		Thalmann et al. (2013)
		KF661059	Thalmann et al. (2013)
		KF661073	Thalmann et al. (2013)
		KF661057	Thalmann et al. (2013)
Grey wolf_Mexico	KF661060		Thalmann et al. (2013)
		KF661065	Thalmann et al. (2013)
Grey wolf Texas USA	AY240073.1		Gundry et al. (2007)
Domestic dog 12	DQ480491		Björnerfeldt et al. (2006)
Domestic dog 2	DQ480497		Björnerfeldt et al. (2006)
		KF661036	Thalmann et al. (2013)
		DQ480498	Björnerfeldt et al. (2006)
		DQ480499	Björnerfeldt et al. (2006)
		KF661037	Thalmann et al. (2013)
Domestic dog 4 Arunachal Pradesh India	AY333731		Sharma et al. (2004)

Domestic dog 5	DQ480496		Björnerfeldt et al. (2006)
Domestic dog 6 Tibetan mastiff	EU408300		unpublished*
Domestic dog 7 Arunachal Pradesh India	AY333728		Sharma et al. (2004)
		DQ480493	Björnerfeldt et al. (2006)
		DQ480501	Björnerfeldt et al. (2006)
		AY333727	Sharma et al. (2004)
Domestic dog 9 Arunachal Pradesh	AY333735		Sharma et al. (2004)
		KF661047	Thalmann et al. (2013)
		DQ480494	Björnerfeldt et al. (2006)
		AY333730	Sharma et al. (2004)
	KF661050		Thalmann et al. (2013)
		DQ480507	Björnerfeldt et al. (2006)
	KF661051		Sharma et al. (2004)
Domestic dog 11 Arunachal Pradesh	AY333732		Sharma et al. (2004)
Domestic dog D-loop Nepal 1	KY996526		Werhahn et al (2017)
		AY333737	Sharma et al. (2004)
		DQ480495	Björnerfeldt et al. (2006)
Domestic dog D-loop Nepal 2	MK113903		Werhahn et al (2018)
Domestic dog D-loop Nepal 3	MK113904		Werhahn et al (2018)
	DQ480500		Björnerfeldt et al. (2006)
	DQ480490		Björnerfeldt et al. (2006)
Domestic dog D-loop Nepal 4	MK113905		Werhahn et al (2018)
Domestic dog D-loop Nepal 5	MK113906		Werhahn et al (2018)
Domestic dog 13 Gujarat India	AY333729		Sharma et al. (2004)
Domestic dog 14	DQ480492		Björnerfeldt et al. (2006)
Domestic dog 15	DQ480502		Björnerfeldt et al. (2006)
Domestic dog D-loop China 1	To follow		This study
Domestic dog D-loop China 2	To follow		This study
Domestic dog D-loop China 3	To follow		This study
Domestic dog D-loop China 4	To follow		This study
African wolf 1	HQ845259		Rueness et al. (2011)
African wolf 2	JQ088675.1		Gaubert et al. (2012)
		JQ088676.1	Gaubert et al. (2012)
African wolf 3	JQ088678.1		Gaubert et al. (2012)
African wolf 4	JQ088684.1		Gaubert et al. (2012)
African wolf 5	JQ088679.1		Gaubert et al. (2012)
African wolf 6	JQ088680.1		Gaubert et al. (2012)
		KM670012	Waters et al. (2015)
African wolf 7	JQ088683.1		Gaubert et al. (2012)
African wolf 8	JQ088681.1		Gaubert et al. (2012)
African wolf 9	JQ088682.1		Gaubert et al. (2012)
Dhole1	NC013445		Zhang et al. (2014)
Golden jackal 1	KT988009.1		İbiş et al. (2015)
Golden jackal 2	KT988007.1		İbiş et al. (2015)
Golden jackal 3	KT343802.1		İbiş et al. (2015)
		KT988006.1	İbiş et al. (2015)
Golden jackal 4	KT343803.1		İbiş et al. (2015)
		HQ845260	Rueness et al. (2011)
		KT268319.1	İbiş et al. (2015)
Golden jackal 5	AY289996		Aggarwal et al. (2007)
		AY289997	Aggarwal et al. (2007)

[#] Cited in (H. Zhang & Chen, 2011)

Table S3. Overview of the cytochrome *b* sequences used in the phylogenetic analysis. Reference sequences from the following publications were included: (Aggarwal et al., 2007; Björnerfeldt et al., 2006; Gaubert et al., 2012; Gottelli et al., 2004; İbiş et al., 2015; Ishiguro et al., 2009; Koepfli et al., 2015; Meng et al., 2009; Rueness et al., 2011; Sharma et al., 2004; Thalmann et al., 2013; Waters et al., 2015; H. Zhang, Zhang, Chen, & Liu, 2013; W. Zhang et al., 2014). Marked with an asterisk* are sequences that are unpublished and have been directly submitted to NCBI GenBank by the respective authors.

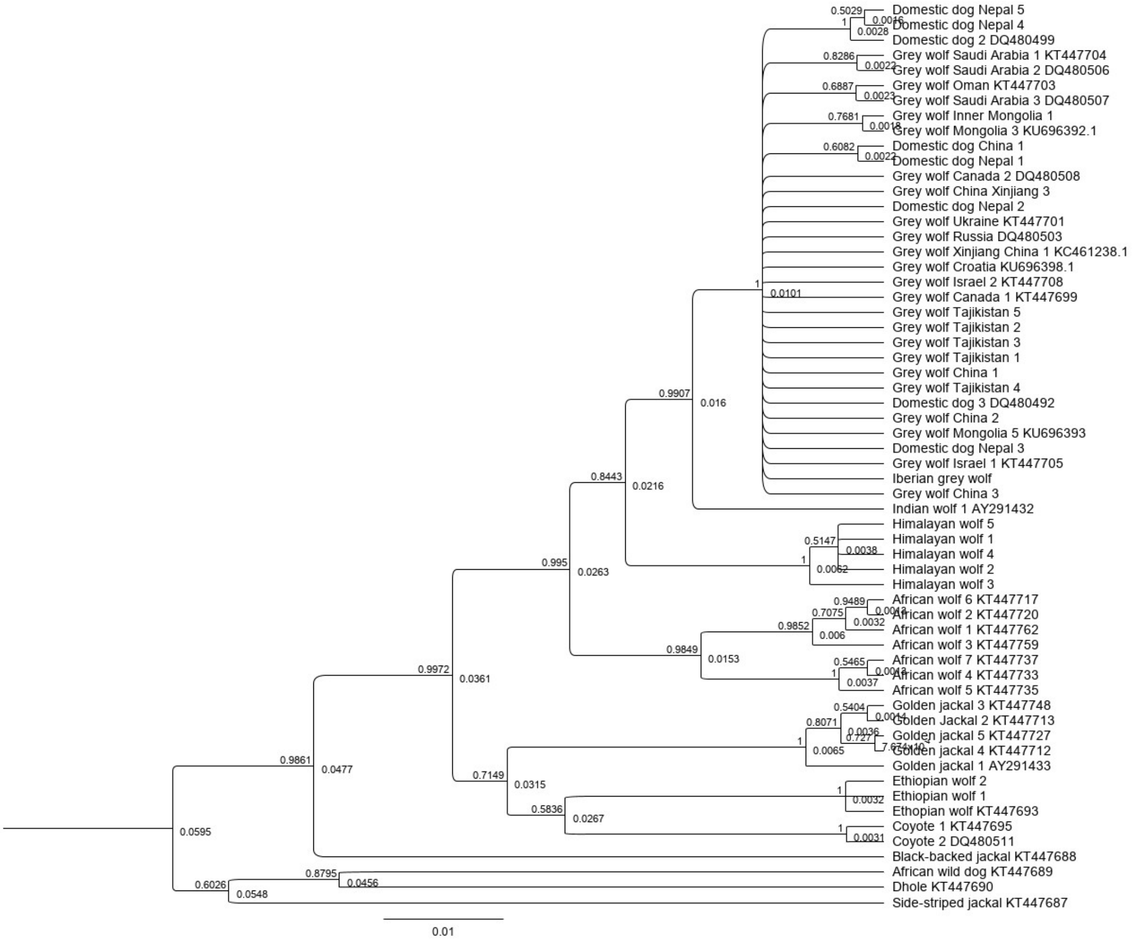
Haplotype	Unique Haplotypes Accession No.	Identical sequences Accession No. (location)	Publication
Himalayan wolf Cytochrome B 1	KY996533		Werhahn et al (2017)
		SRX1024633 (Tibet China)	Zhang et a. (2014)
		SRX1024635 (Qinghai China)	Zhang et a. (2014)
		SRX1024634 (Qinghai China)	Zhang et a. (2014)
		FJ032363.2 (Tibet China)	Meng et al. (2009)
		EU442884	unpublished*#
		NC011218 (Tibet)	Meng et al. (2009)
Himalayan wolf Cytochrome B 2	KY996534		Werhahn et al (2017)
		AY291431	Aggarwal et al. (2007)
		SRX1024624 (Tibet China)	Zhang et al. (2014)
Himalayan wolf Cytochrome B 3	To follow		This study
Himalayan wolf Cytochrome B 4	To follow		This study
Himalayan wolf Cytochrome B 5	To follow		This study
Domestic dog Nepal 1	KY996532		Werhahn et al (2017)
		KT447685	Koepfli et al. (2015)
		DQ480497	Björnerfeldt et al. (2006)
		DQ480491	Björnerfeldt et al. (2006)
		DQ480498	Björnerfeldt et al. (2006)
		DQ480495	Björnerfeldt et al. (2006)
		DQ480496	Björnerfeldt et al. (2006)
		KU696394 (Grey wolf Mongolia)	Koblmüller et al. (2016)
Domestic dog Nepal 2	MK113907		This study
		KT447684	Koepfli et al. (2015)
Domestic dog Nepal 3	MK113908		This study
		KU644669.1 (China)	Koblmuller et al. (2016)
		KU696391 (China)	Koblmuller et al. (2016)
Domestic dog Nepal 4	MK113909		Werhahn et al. (2018)
Domestic dog Nepal 5	MK113910		Werhahn et al. (2018)
Domestic dog China 1	To follow		This study
Indian wolf 1	AY291432		Aggarwal et al. (2007)
		DQ480490	Björnerfeldt et al. (2006)
		DQ480494	Björnerfeldt et al. (2006)
		DQ480500	Björnerfeldt et al. (2006)
Grey wolf China 1	To follow		This study
Grey wolf China 2		KU696393	Koblmüller et al. (2016)

Grey wolf China 3		KU696411 (Alaska)	Koblmüller et al. (2016)
Grey wolf Israel 1	KT447705		Koepfli et al. (2015)
		KT447706	Koepfli et al. (2015)
		KT447700	Koepfli et al. (2015)
		KT447702	Koepfli et al. (2015)
		KT447709	Koepfli et al. (2015)
		KT447710	Koepfli et al. (2015)
		KT447707	Koepfli et al. (2015)
		KU696393 (Mongolia)	Koblmüller et al. (2016)
Grey wolf_Mongolia 3	KU696392		Koblmüller et al. (2016)
		KU696396	Koblmüller et al. (2016)
		GQ374438	Zhang and Chen (2011)
		KU696395	Koblmüller et al. (2016)
	Grey wolf Inner Mongolia 2	SRX1024636 (Inner Mongolia)	Zhang et al. (2014)
		SRX1024627 (Xinjiang)	Zhang et al. (2014)
Grey wolf Xinjiang China 2		SRX1024629 (Xinjiang)	Zhang et al. (2014)
Grey wolf China Xinjiang 3	To follow		This study
Grey wolf_Croatia	KU696398		Koblmüller et al. (2016)
Grey wolf Inner Mongolia 1	SRX1024637		Zhang et al. (2014)
Grey wolf Xinjiang China 1	KC461238		Zhang et al. (2014)
Grey wolf Ukraine	KT447701		Koepfli et al. (2015)
Grey wolf Kyrgyzstan			This study
		Iberian grey wolf (Spain)	This study
		DQ480505	Björnerfeldt et al. (2006)
		KC896375 (Mongolia)	Zhang et al. (2015)
		KT447708 (Israel)	Koepfli et al. (2015)
		DQ480504	Björnerfeldt et al. (2006)
Grey wolf Tajikistan 1	*To follow*		This study
Grey wolf Tajikistan 2	*To follow*		This study
Grey wolf Tajikistan 3	*To follow*		This study
Grey wolf Tajikistan 4	*To follow*		This study
Grey wolf Tajikistan 5	*To follow*		This study
Grey wolf Canada 1	KT447699		Koepfli et al. (2015)
Grey wolf Oman	KT447703		Koepfli et al. (2015)
Grey wolf Saudi Arabia 1	KT447704		Koepfli et al. (2015)
Domestic dog 2	DQ480499		Björnerfeldt et al. (2006)
Grey wolf Saudi Arabia 2	DQ480506		Björnerfeldt et al. (2006)
Grey wolf Canada 2	DQ480508		Björnerfeldt et al. (2006)
Grey wolf Russia	DQ480503		Björnerfeldt et al. (2006)
Grey wolf Saudi Arabia 3	DQ480507		Björnerfeldt et al. (2006)
Domestic dog 3	DQ480492		Björnerfeldt et al. (2006)
		DQ480493	Björnerfeldt et al. (2006)
		DQ480502	Björnerfeldt et al. (2006)
		DQ480501	Björnerfeldt et al. (2006)
Coyote 1	KT447695		Koepfli et al. (2015)
Coyote 2	DQ480511		Björnerfeldt et al. (2006)
		DQ480509	Björnerfeldt et al. (2006)
		DQ480510	Björnerfeldt et al. (2006)
		KT447697	Koepfli et al. (2015)
		KT447698	Koepfli et al. (2015)
		KT447696	Koepfli et al. (2015)
Ethopian wolf	KT447693		Koepfli et al. (2015)
		KT447694	Koepfli et al. (2015)
		KT447692	Koepfli et al. (2015)
		KT447691	Koepfli et al. (2015)
Side-striped jackal	KT447687		Koepfli et al. (2015)
African wild dog	KT447689		Koepfli et al. (2015)

Black-backed jackal	KT447688		Koepfli et al. (2015)
Dhole	KT447690		Koepfli et al. (2015)
Dhole		NC013445	Zhang et al. (2014)
Golden jackal 1	AY291433		Aggarwal et al. (2007)
Golden jackal 2	KT447713		Koepfli et al. (2015)
		KT447729	Koepfli et al. (2015)
		KT447732	Koepfli et al. (2015)
		KT447715	Koepfli et al. (2015)
		KT447731	Koepfli et al. (2015)
		KT447725	Koepfli et al. (2015)
		KT447718	Koepfli et al. (2015)
		KT447724	Koepfli et al. (2015)
		KT447719	Koepfli et al. (2015)
		KT447730	Koepfli et al. (2015)
		KT447757	Koepfli et al. (2015)
		KT447758	Koepfli et al. (2015)
		KT447756	Koepfli et al. (2015)
		KT447755	Koepfli et al. (2015)
		KT447754	Koepfli et al. (2015)
		KT447752	Koepfli et al. (2015)
		KT447751	Koepfli et al. (2015)
		KT447750	Koepfli et al. (2015)
		KT447749	Koepfli et al. (2015)
		KT447753	Koepfli et al. (2015)
		KT447726	Koepfli et al. (2015)
		KT447728	Koepfli et al. (2015)
Golden jackal 3	KT447748		Koepfli et al. (2015)
Golden jackal 4	KT447712		Koepfli et al. (2015)
Golden jackal 5	KT447727		Koepfli et al. (2015)
African wolf 1	KT447762		Koepfli et al. (2015)
		KT447761	Koepfli et al. (2015)
African wolf 2	KT447720		Koepfli et al. (2015)
		KT447723	Koepfli et al. (2015)
		KT447714	Koepfli et al. (2015)
		KT447716	Koepfli et al. (2015)
		KT447721	Koepfli et al. (2015)
		KT447722	Koepfli et al. (2015)
African wolf 3	KT447759		Koepfli et al. (2015)
		KT447760	Koepfli et al. (2015)
African wolf 4	KT447733		Koepfli et al. (2015)
		KT447745	Koepfli et al. (2015)
		KT447746	Koepfli et al. (2015)
		KT447747	Koepfli et al. (2015)
			Koepfli et al. (2015)
African wolf 5	KT447735		Koepfli et al. (2015)
		KT447736	Koepfli et al. (2015)
		KT447739	Koepfli et al. (2015)
		KT447740	Koepfli et al. (2015)
		KT447741	Koepfli et al. (2015)
		KT447742	Koepfli et al. (2015)
		KT447743	Koepfli et al. (2015)
		KT447744	Koepfli et al. (2015)
		KT447738	Koepfli et al. (2015)
		KT447734	Koepfli et al. (2015)
African wolf 6	KT447717		Koepfli et al. (2015)
African wolf 7	KT447737		Koepfli et al. (2015)

Appendix B3.

A)



B)

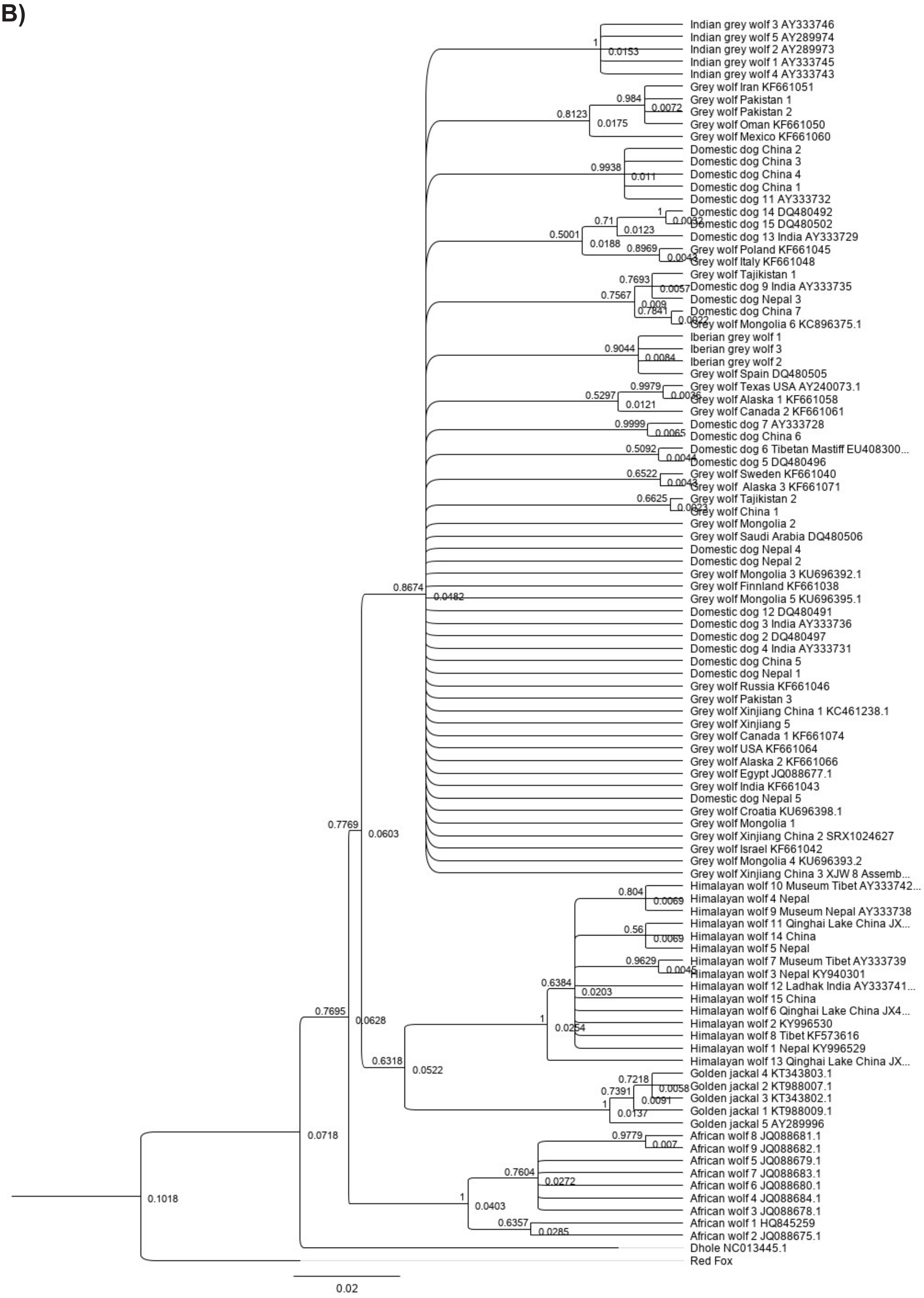


Figure S1. Bayesian phylogeny based on A) 508bp Cytochrome *b* and B) 242bp of D-loop gene sequences.

Table S4. Information of all the samples included in this analysis based on non-invasive wolf scats (except WLF179/WLF180 derive from blood samples). Information provided is geographic origin, lineage based on mtDNA, lineage based on mtDNA and microsatellites combined, ZF sex linked genes and the hypoxia pathway related SNPs.

Lab ID	**Province**	**Species mtDNA**	**mtDNA and microsats**	ZFX	ZFY	**ANGPT1**	**EPAS1-1**		**RYR2-1**		**RYR2-2**
SL0007	Sichuan	**Himalayan wolf**				T	G	A	C	T	G
SL0008	Sichuan	**Himalayan wolf**				C/T	G	A	C/G	T	
SL0010	Sichuan	**Himalayan wolf**				C	G	A	C/G	T	C
SL0012	Sichuan	**Himalayan wolf**				T	G	A	C/G	T	C
SL0015	Sichuan	**Himalayan wolf**					fail	fail			fail
SL0018	Sichuan	**Himalayan wolf**				C	G	A	C	T	C
SL0022	Sichuan	**Himalayan wolf**				C	G	A			G
SL0037	Sichuan	**Himalayan wolf**									fail
SL0039	Sichuan	**Himalayan wolf**					fail	fail			fail
SL0042	Sichuan	**Himalayan wolf**					fail	fail	C	T	fail
SL0146	Qinghai	**Grey wolf**	Grey wolf								
SL0194	Qinghai	**Himalayan wolf**	Admixed								
SL0206	Qinghai	**Himalayan wolf**									
SL0207	Qinghai	**Himalayan wolf**									
SL0209	Qinghai	**Himalayan wolf**	Admixed								
SL0213	Qinghai	**Grey wolf**	Grey wolf	B							
SL0214	Qinghai	**Himalayan wolf**	Admixed								
SL0215	Qinghai	**Grey wolf**	Grey wolf								
SL0357	Qinghai	**Himalayan wolf**	Himalayan wolf								
SL0418	Qinghai	**Grey wolf**	Grey wolf (but see Structure K=4 its Hwig)								
SL0436	Qinghai	**Himalayan wolf**	Admixed								
SL0438	Qinghai	**Himalayan wolf**	Himalayan wolf	B		C	G	A	C/G	T	G
SL0439	Qinghai	**Himalayan wolf**	Himalayan wolf	B	**B**	C/T	G	A	C/G	T	G
SL0490	Qinghai	**Himalayan wolf**									
SL0497	Qinghai	**Himalayan wolf**									
SL0701	Qinghai	**Himalayan wolf**									
SL0702	Qinghai	**Himalayan wolf**									
SL0705	Qinghai	**Dog**									
SL0706	Qinghai	**Dog**	Dog	C		T	G	A	G	T	fail
SL0707	Qinghai	**Dog**	Dog	C		T	G	A	G	T	C
SL0708	Qinghai	**DD Nepal 5**	Dog	B		C	G	A	C	T	fail
SL0709	Qinghai	**DD Nepal 5**									
SL0710	Qinghai	**Himalayan wolf**									
SL0711	Qinghai	**Himalayan wolf**									
SL0712	Qinghai	**DD Nepal 5**									
SL0713	Qinghai	**Dog**	Dog	C	**C**	T	G	A	G	T	C
SL0714	Qinghai	**Dog**									

SL0716	Qinghai	**Dog**									
SL0717	Qinghai	**Dog**	Dog	C		T	G	A	G	T	C
SL0719	Qinghai	**Himalayan wolf**									
SL0722	Qinghai	**DD Nepal 5**									
SL0726	Qinghai	**Himalayan wolf**									
SL0728	Qinghai	**Himalayan wolf**									
SL0730	Qinghai	**Himalayan wolf**									
SL0732	Qinghai	**Himalayan wolf**									
SL0733	Qinghai	**Dog**		B		C/T	G	A	C	T	G
SL0734	Qinghai	**Himalayan wolf**									
SL0735	Qinghai	**Himalayan wolf**									
SL0736	Qinghai	**Himalayan wolf**		B							
SL0738	Qinghai	**Dog**									
SL0739	Qinghai	**Himalayan wolf**									
SL0742	Qinghai	**Himalayan wolf**	Himalayan wolf	B		T	G/T	A/G	C/G	T	C/G
SL0743	Qinghai	**DD Nepal 5**									
SL0744	Qinghai	**Himalayan wolf**									
SL0749	Qinghai	**Dog**									
SL0754	Qinghai	**Dog**	Dog	C		T	G	A	G	T	C
SL0755	Qinghai	**Himalayan wolf**									
sl759	Qinghai	**Himalayan wolf**									
sl763	Qinghai	**Himalayan wolf**									
sl764	Qinghai	**Himalayan wolf**									
sl766	Qinghai	**Himalayan wolf**									
sl767	Qinghai	**Himalayan wolf**									
sl770	Qinghai	**Himalayan wolf**									
sl774	Qinghai	**Himalayan wolf**									
sl782	Qinghai	**Dog**									
sl783	Qinghai	**Himalayan wolf**									
sl785	Qinghai	**Himalayan wolf**									
sl786	Qinghai	**Himalayan wolf**									
sl788	Qinghai	**Himalayan wolf**	Himalayan wolf	B		C	G	A	C	T	fail
sl789	Qinghai	**Himalayan wolf**		C							
sl793	Qinghai	**Dog**									
sl798	Qinghai	**Dog**		C	**C**	T	G	A	G	T	C
sl799	Qinghai	**Himalayan wolf**	Himalayan wolf	B	**C**	C	G	A	C	T	fail
sl800	Qinghai	**Himalayan wolf**	Himalayan wolf	C		C	G	A	C	T	G
sl802	Qinghai	**Himalayan wolf**	Himalayan wolf			C	G	A	C	T	G
sl803	Qinghai	**Himalayan wolf**		B		C	G	A	C	T	fail
sl805	Qinghai	**Himalayan wolf**									
sl806	Qinghai	**Himalayan wolf**									
sl807	Qinghai	**Himalayan wolf**									
sl808	Qinghai	**Dog**	Dog	C		T	G	A	G	T	C
XL02	Sichuan	**Himalayan wolf**	Admixed	C		C/T	G/T	A/G	C/G	T	fail
XL03A	Sichuan	**Himalayan wolf**							C	T	fail

XL11	Sichuan	**Himalayan wolf**	Admixed			C	G	A	C/G	T	
XL19	Sichuan	**Himalayan wolf**									
XL30	Sichuan	**Himalayan wolf**									
YJ-02	Sichuan	**Himalayan wolf**									
YJ-03	Sichuan	**Himalayan wolf**	Admixed			T	G	A	fail	fail	
YJ-05	Sichuan	**Himalayan wolf**	Admixed			C	G	A	G	T	fail
YJ-07	Sichuan	**Himalayan wolf**							G	T	C
YJ-54	Sichuan	**Himalayan wolf**	Admixed			T	G	A	C/G	T	C
YJ-67	Sichuan	**Himalayan wolf**									
YJ-68	Sichuan	**Himalayan wolf**									
YJ-69	Sichuan	**Himalayan wolf**	Admixed			T	G	A	G	T	C
GHW01	Gansu Yanchiwan	**Grey wolf**									
GHW02	Gansu Yanchiwan	**Grey wolf**	**Grey wolf**						C	T	fail
GHW03	Gansu Yanchiwan	**Dog**									
GHW04	Gansu Yanchiwan	**Grey wolf**									
GHW05	Gansu Yanchiwan	**Dog**									
GHW06	Gansu Yanchiwan	**Grey wolf**									
GHW07	Gansu Yanchiwan	**Grey wolf**									
GHW08	Gansu Yanchiwan	**Grey wolf**									
GHW10	Gansu Yanchiwan	**Grey wolf**	**Grey wolf**	B		fail	T	G	G	T	C
GHW11	Gansu Yanchiwan	**Himalayan wolf**	Admixed				G	A	C	T	fail
GHW12	Gansu Yanchiwan	**Himalayan wolf**				C	G	A	C/G	T	C
GHW13	Gansu Yanchiwan	**Himalayan wolf**	Admixed	B		C	G	A	C/G	T	C/G
GHW14	Gansu Yanchiwan	**Himalayan wolf**	Admixed			C	G	A	fail	fail	G
GHW15	Gansu Yanchiwan	**Himalayan wolf**									
GHW16	Gansu Yanchiwan	**Himalayan wolf**	Admixed	B	**B**	C	G	A	C	T	fail
GHW17	Gansu Yanchiwan	**Himalayan wolf**									
GHW18	Gansu Yanchiwan	**Grey wolf**	**Grey wolf**			C/T	T	G	C	T	fail
GHW19	Gansu Yanchiwan	**Grey wolf**									
GHW20	Gansu Yanchiwan g	**Dog**	**Dog**		**B**		G	A	G	T	
GHW21	Gansu Yanchiwan	**Grey wolf**									
GHW22	Gansu Yanchiwan	**Himalayan wolf**	Admixed	C		C	G	A	C	T	G
GHW23	Gansu Yanchiwan	**Himalayan wolf**	Admixed	C			G	A	C/G	T	fail
GHW24	Gansu Yanchiwan	**Himalayan wolf**	Admixed	C	**C**	fail	G/T	A/G	C/G	T	C/G
GHW25	Gansu Yanchiwan	**Himalayan wolf**									
GHW26	Gansu Yanchiwan	**Dog/Grey wolf**									
GHW27	Gansu Yanchiwan	**Grey wolf**									
GHW28	Gansu Yanchiwan	**Grey wolf**									
GHW29	Gansu Yanchiwan	**Grey wolf**									
GHW30	Gansu Yanchiwan	**Grey wolf**									

GHW31	Gansu Yanchiwan	**Grey wolf**									
GHW32	Gansu Qilianshan 2017 Spring	**Grey wolf**									
GHW33	Gansu Qilianshan 2017 Spring	**Himalayan wolf**									
GHW34	Gansu Qilianshan 2017 Spring	**Himalayan wolf**	-	-	-						
GHW35	YCW 2017 Dec	**Grey wolf**									
QHW01	Qinghai Qilianshan	**Himalayan wolf**									
QHW02	Qinghai Qilianshan	**Grey wolf**	**Grey wolf**								
QHW03	Qinghai Qilianshan	**Grey wolf**									
QHW04	Qinghai Qilianshan	**Grey wolf**	-	-	-						
QHW05	Qinghai Qilianshan	**Grey wolf**									
QHW06	Qinghai Qilianshan	**Grey wolf**									
QHW07	Qinghai Qilianshan	**Himalayan wolf**									
QHW08	Qinghai Qilianshan	**Grey wolf**									
QHW09	Qinghai Sanjiangyuan	**Himalayan wolf**									
QHW10	Qinghai Sanjiangyuan	**Himalayan wolf**									
QHW11	Qinghai Sanjiangyuan	**Himalayan wolf**	Admixed								
QHW12	Qinghai Sanjiangyuan	**Himalayan wolf**	Admixed								
QHW13	Qinghai Sanjiangyuan	**Himalayan wolf**	Admixed								
QHW14	Qinghai Sanjiangyuan	**Himalayan wolf**									
SHW01	Sichuan Gonggashan	**Himalayan wolf**									
SHW02	Sichuan Gonggashan	**Himalayan wolf**									
SHW03	Sichuan Gonggashan	**Himalayan wolf**									
SHW04	Sichuan Gonggashan	**Himalayan wolf**	**Himalayan wolf**								
THW01	Tibet Qomolangma	**Himalayan wolf**	**Himalayan wolf**	B		C	G	A			C/G
THW02	Tibet Qomolangma	**Himalayan wolf**									
THW03	Tibet Qomolangma	**Himalayan wolf**	**Himalayan wolf**	B			G	A			
THW04	Tibet Qomolangma	**Himalayan wolf**									
THW05	Tibet Qomolangma	**Himalayan wolf**									
THW07	Tibet Qomolangma	**Himalayan wolf**					G	A			
THW08	Tibet Qomolangma	**Himalayan wolf**									
THW09	Tibet Qomolangma	**NA**									
THW10	Tibet Qomolangma	**Himalayan wolf**	**Himalayan wolf**				G	A			fail
THW11	Tibet Qomolangma	**Dog**									
THW12	Tibet Qomolangma	**Himalayan wolf**		B		C					fail
THW13	Tibet Qomolangma	**Himalayan wolf**	**Himalayan wolf**	B		C	G	A			
THW14	Tibet Qomolangma	**Himalayan wolf**									
THW15	Tibet Qomolangma	**Himalayan wolf**		B		C	G	A			

THW16	Tibet Qomolangma	**Himalayan wolf**	**Himalayan wolf**		**B**	C	G	A	C	T	fail
THW17	Tibet Qomolangma	**Himalayan wolf**									
THW18	Tibet Qomolangma	**Himalayan wolf**									
THW19	Tibet Qomolangma	**NA**									
THW20	Tibet Qomolangma	**Himalayan wolf**	**Himalayan wolf**	B		T	G	A	C/G	T	
THW21	Tibet Qomolangma	**Himalayan wolf**									
THW22	Tibet Qomolangma	**Himalayan wolf**									
THW23	Tibet Qomolangma	**Himalayan wolf**									
THW24	Tibet Qomolangma	**Himalayan wolf**									
THW25	Tibet Qomolangma	**Himalayan wolf**									
THW26	Tibet Qomolangma	**Himalayan wolf**									
THW27	Tibet Qomolangma	**Himalayan wolf**									
THW28	Tibet Qomolangma	**Himalayan wolf**									
THW29	Tibet Qomolangma	**Himalayan wolf**									
THW30	Tibet Qomolangma	**Himalayan wolf**									
THW31	Tibet Qomolangma	**Himalayan wolf**									
THW32	Tibet Qomolangma	**Himalayan wolf**									
THW33	Tibet Qomolangma	**Himalayan wolf**									
THW34	Tibet Qomolangma	**Himalayan wolf**									
THW35	Tibet Qomolangma	**Himalayan wolf**									
THW36	Tibet Qomolangma	**Himalayan wolf**									
THW37	Tibet Qomolangma	**Himalayan wolf**									
THW38	Tibet Qomolangma	**Himalayan wolf**									
THW39	Tibet Qomolangma	**Himalayan wolf**									
THW40	Tibet Qomolangma	**Himalayan wolf**									
THW41	Tibet Qomolangma	**Dog**									
THW42	Tibet Qomolangma	**Himalayan wolf**									
THW43	Tibet Qomolangma	**Himalayan wolf**									
THW44	Tibet Qomolangma	**Himalayan wolf**									
THW45	Tibet Qomolangma	**Himalayan wolf**									
THW46	Tibet Qomolangma	**Himalayan wolf**									
THW47	Tibet Qomolangma	**Dog**									
THW48	Tibet Qomolangma	**Himalayan wolf**									
THW49	Tibet Qomolangma	**Himalayan wolf**	**Himalayan wolf**								
THW50	Tibet Qomolangma	**Himalayan wolf**									
THW51	Tibet Qomolangma	**Himalayan wolf**									
THW52	Tibet Qomolangma	**Himalayan wolf**	**Himalayan wolf**	B			G	A	C	T	
THW54	Tibet Qomolangma	**Himalayan wolf**									

THW55	Tibet Qomolangma	**Himalayan wolf**									
THW56	Tibet Qomolangma	**Himalayan wolf**									
THW57	Tibet Qomolangma	**Himalayan wolf**									
THW58	Tibet Qomolangma	**Dog**									
THW59	Tibet Qomolangma	**Himalayan wolf**									
THW60	Tibet Qomolangma	**Himalayan wolf**									
THW61	Tibet Qomolangma	**Himalayan wolf**	**Himalayan wolf**	B	**B**	C	G	A	C	T	fail
THW62	Tibet Qomolangma	**Himalayan wolf**	**Himalayan wolf**				G	A	C	T	
THW63	Tibet Qomolangma	**Himalayan wolf**									
THW64	Tibet Qomolangma	**Himalayan wolf**									
THW65	Tibet Qomolangma	**Himalayan wolf**									
THW66	Tibet Qomolangma	**Himalayan wolf**	Admixed	B							G
THW67	Tibet Qomolangma	**Himalayan wolf**	**Himalayan wolf**			C					
THW68	Tibet Qomolangma	**Dog**									
THW69	Tibet Qomolangma	**Dog**									
THW70	Tibet Qomolangma	**Himalayan wolf**									
THW71	Tibet Qomolangma	**Himalayan wolf**									
THW72	Tibet Qomolangma	**Himalayan wolf**									
THW73	Tibet Qomolangma	**Himalayan wolf**									
THW74	Tibet Qomolangma	**Himalayan wolf**									
THW75	Tibet Qomolangma	**Dog**									
THW76	Tibet Qomolangma	**Himalayan wolf**									
THW77	Tibet Qomolangma	**Himalayan wolf**	**Himalayan wolf**	B	**B**	C	G	A	C	T	
THW78	Tibet Qomolangma	**Dog**									
THW79	Tibet Qomolangma	**Dog**									
THW80	Tibet Qomolangma	**Himalayan wolf**									
THW81	Tibet Qomolangma	**Dog**									
THW82	Tibet Qomolangma	**Dog**									
THW83	Tibet Qomolangma	**Dog**									
THW84	Tibet Qomolangma	**Dog**									
THW85	Tibet Qomolangma	**Dog**									
THW86	Tibet Qomolangma	**Dog**	**Dog**	C		T			G	T	
THW87	Tibet Qomolangma	**Dog**									
THW88	Tibet Qomolangma	**Dog**									
THW89	Tibet Qomolangma	**Dog**									
THW90	Tibet Qomolangma	**Dog**									
THW91	Tibet Qomolangma	**Himalayan wolf**									
THW92	Tibet Qomolangma	**Dog**									
THW93	Tibet Qomolangma	**Himalayan wolf**									

THW94	Tibet Qomolangma	**Dog**									
XJW01	Xinjiang Bortala	**Grey wolf**	**Grey wolf**								
XJW02	Xinjiang Bortala	**Grey wolf**									
XJW03	Xinjiang Bortala	**Dog**									
XJW05	Xinjiang Bortala	**Grey wolf**									
XJW06	Xinjiang Bortala	**Grey wolf**									
XJW07	Xinjiang Bortala	**Grey wolf**									
XJW08	Xinjiang Bortala	**Grey wolf**									
WLF129	Sarychat-Ertash, Kyrgyzstan	**Grey wolf**	**Grey wolf**	Himalayan wolf (B)	GrBy wolf (C)	T	G/T	A/G	G	T	C
WLF130	Sarychat-Ertash, Kyrgyzstan	**Grey wolf**	**Grey wolf**	Himalayan wolf (B)	GrBy wolf (C)	T	G/T	A/G	G	T	C
WLF131	Sarychat-Ertash, Kyrgyzstan	**Grey wolf**	**Grey wolf**	Himalayan wolf (B)	GrBy wolf (C)	T	G/T	A/G	G	T	fail
WLF132	Sarychat-Ertash, Kyrgyzstan	**Grey wolf**	**Grey wolf**		GrBy wolf (C)	T	T	G	fail	fail	fail
WLF140	Zorkul, TJK	**Grey wolf**	**Grey wolf**			fail	fail	fail	G	T	fail
WLF141	Zorkul, TJK	**Grey wolf**									
WLF142	Zorkul, TJK	**Grey wolf**	**Grey wolf**			fail	fail	fail	G	T	fail
WLF143	Zorkul, TJK	**Grey wolf**	**Grey wolf**			fail	fail	fail	G	T	fail
WLF144	Zorkul, TJK	**Grey wolf**	**Grey wolf**								
WLF145	Zorkul, TJK	**Grey wolf**	**Grey wolf**								
WLF146	Zorkul, TJK	**Grey wolf**	**Grey wolf**								
WLF147	Zorkul, TJK	**Grey wolf**	**Grey wolf**								
WLF148	Zorkul, TJK	**Grey wolf**	**Grey wolf**			T	G	A	G	T	fail
WLF149	Zorkul, TJK	**Grey wolf**	**Grey wolf**								
WLF150	Zorkul, TJK	**Grey wolf**	**Grey wolf**			T	G	A	G	T	C
WLF151	Zorkul, TJK	**Grey wolf**	**Grey wolf**			T	G	A	G	T	fail
WLF152	Zorkul, TJK	**Grey wolf**	**Grey wolf**			fail	fail	fail	G	T	fail
WLF153	Zorkul, TJK	**Grey wolf**	**Grey wolf**			fail	fail	fail	G	T	fail
WLF154	Zorkul, TJK	**Grey wolf**	**Grey wolf**			T	G	A	G	T	C
WLF 156	Zorkul, TJK	**Grey wolf**									
WLF 157	Zorkul, TJK	**Grey wolf**									
WLF158	Zorkul, TJK	**Grey wolf**	**Grey wolf**								
WLF162	Zorkul, TJK	**Grey wolf**	**Grey wolf**			fail	fail	fail	G	T	C
WLF163	Zorkul, TJK	**Grey wolf**									
WLF164	Zorkul, TJK	**Grey wolf**	**Grey wolf**			T	G	A	fail	fail	C
WLF165	Zorkul, TJK	**Grey wolf**	**Grey wolf**								
WLF166	Zorkul, TJK	**Grey wolf**									
WLF167	Zorkul, TJK	**Grey wolf**									
WLF170	Zorkul, TJK	**Grey wolf**	**Grey wolf**			fail	T	G	fail	fail	fail
WLF171	Zorkul, TJK	**Grey wolf**									
WLF172	Zorkul, TJK	**Grey wolf**									
WLF173	Zorkul, TJK	**Grey wolf**	**Grey wolf**								
WLF178	Zorkul, TJK	**Grey wolf**									
WLF179	Pikertyk, KGZ	**Grey wolf**	**Grey wolf**			T	G/T	A/G	G	T	C
WLF180	Sarychat, KGZ	**Grey wolf**	**Grey wolf**			T	T	G	G	T	C

Table S5. Hypoxia results from this study and (Geraldine Werhahn et al., 2018) condensed with green colour indicating alleles typical of Himalayan wolf and blue colour indicating alleles typical of grey wolf.

Geographic Origin of Sample	ANGPT1	EPAS1-1	RYR2-1	RYR2-2	Lineage	Published
Nepalese Himalayas	C	Fail	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	Fail	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	Fail	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	T	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	Fail	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	G	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018

Nepalese Himalayas	C	**G-A**	C	C/G	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C/G	C/G	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C/G	C/G	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	C	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C/G	C	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	Fail	C/G	C	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C/G	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C/G	Fail	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C/G	C	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C/G	C	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	C	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	C/G	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C/T	**G-A**	C	C	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	C/G	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	T	**G-A**	G	Fail	dog	Werhahn et al. 2018
Nepalese Himalayas	T	**G-A**	G	C	dog	Werhahn et al. 2018
Nepalese Himalayas	T	**G-A**	G	C	dog	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Nepalese Himalayas	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	Werhahn et al. 2018
Tibet	C	**G-A**	Fail	C/G	**Himalayan wolf (mtDNA and msat)**	This study
Tibet	C	**G-A**	Fail	Fail	**Himalayan wolf (mtDNA and msat)**	This study
Tibet	C	**G-A**	Fail	Fail	**himalayan wolf mtDNA only)**	This study
Tibet	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	This study
Tibet	T	**G-A**	C/G	Fail	**Himalayan wolf (mtDNA and msat)**	This study
Tibet	Fail	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	This study
Tibet	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	This study
Tibet	Fail	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	This study
Tibet	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	This study
Qinghai Tibetan Plateau	C/T	**G-A**	C/G	**G**	**Himalayan wolf (mtDNA and msat)**	This study
Qinghai Tibetan Plateau	T	**G-A**	C/G	C/G	**Himalayan wolf (mtDNA and msat)**	This study

Qinghai Tibetan Plateau	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	This study
Qinghai Tibetan Plateau	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	This study
Qinghai Tibetan Plateau	C	**G-A**	C	Fail	**Himalayan wolf (mtDNA and msat)**	This study
Qinghai Tibetan Plateau	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	This study
Qinghai Tibetan Plateau	C	**G-A**	C	**G**	**Himalayan wolf (mtDNA and msat)**	This study
Qinghai Tibetan Plateau	T	**G-A**	G	Fail	**Admixed (mtDNA and msat)**	This study
Qinghai Tibetan Plateau	C	**G-A**	C/G	**G**	**Admixed (mtDNA and msat)**	This study
Qinghai Tibetan Plateau	T	**G-A**	G	C	**dog**	This study
Qinghai Tibetan Plateau	T	**G-A**	G	C	**dog**	This study
Qinghai Tibetan Plateau	T	**G-A**	G	Fail	**dog**	This study
Qinghai Tibetan Plateau	T	**G-A**	G	C	**dog**	This study
Qinghai Tibetan Plateau	C	**G-A**	C	Fail	**dog**	This study
Qinghai Tibetan Plateau	T	**G-A**	G	C	**dog**	This study
Qinghai Tibetan Plateau	T	**G-A**	G	C	**dog**	This study
Qinghai Tibetan Plateau	C/T	**G-A**	C	**G**	**dog**	This study
Qinghai Tibetan Plateau	T	**G-A**	G	Fail	**dog**	This study
Qinghai Tibetan Plateau	T	**G-A**	G	C	**dog**	This study
Sichuan	T	**G-A**	C	**G**	**Himalayan wolf (mtDNA only)**	This study
Sichuan	C/T	**G-A**	C/G	Fail	**Himalayan wolf (mtDNA only)**	This study
Sichuan	C	**G-A**	C/G	C	**Himalayan wolf (mtDNA only)**	This study
Sichuan	T	**G-A**	C/G	C	**Himalayan wolf (mtDNA only)**	This study
Sichuan	C	**G-A**	C	C	**Himalayan wolf (mtDNA only)**	This study
Sichuan	C	**G-A**	Fail	**G**	**Himalayan wolf (mtDNA only)**	This study
Sichuan	C/T	**G-A**	C/G	Fail	**Admixed (mtDNA and msat)**	This study
Sichuan	C	**G-A**	C/G	Fail	**Admixed (mtDNA and msat)**	This study
Sichuan	T	**G-A**	Fail	Fail	**Admixed (mtDNA and msat)**	This study
Sichuan	C	**G-A**	G	Fail	**Admixed (mtDNA and msat)**	This study
Sichuan	T	**G-A**	C/G	C	**Admixed (mtDNA and msat)**	This study
Sichuan	T	**G-A**	G	C	**Admixed (mtDNA and msat)**	This study
Tajikistan	T	**G-A**	G	Fail	GW (mtDNA and msat)	This study
Tajikistan	T	**G-A**	G	C	GW (mtDNA and msat)	This study
Tajikistan	T	**G-A**	G	Fail	GW (mtDNA and msat)	This study
Tajikistan	T	**G-A**	G	C	GW (mtDNA and msat)	This study
Tajikistan	Fail	Fail	G	C	GW (mtDNA and msat)	This study
Tajikistan	T	**G-A**	Fail	C	GW (mtDNA and msat)	This study
Kyrgyzstan	T	**G-A**	G	C	GW (mtDNA and msat)	This study
Kyrgyzstan	T	**T-G**	G	C	GW (mtDNA and msat)	This study
Kyrgyzstan	T	**G-A**	G	C	GW (mtDNA and msat)	Werhahn et al. 2018
Kyrgyzstan	T	**G-A**	G	C	GW (mtDNA and msat)	Werhahn et al. 2018
Kyrgyzstan	T	**G-A**	G	Fail	GW (mtDNA and msat)	Werhahn et al. 2018

Kyrgyzstan	T	**T-G**	Fail	Fail	GW (mtDNA and msat)	Werhahn et al. 2018
Qilianshan Mountains (Yanchiwan)	Fail	**T-G**	G	C	GW (mtDNA and msat)	This study
Qilianshan Mountains (Yanchiwan)	C/T	**T-G**	C	Fail	GW (mtDNA and msat)	This study
Qilianshan Mountains (Yanchiwan)	Fail	**G-A**	G	Fail	**dog**	This study
Qilianshan Mountains (Yanchiwan)	C	**G-A**	C/G	C	**Himalayan wolf (mtDNA only)**	This study
Qilianshan Mountains (Yanchiwan)	Fail	**G-A**	C	Fail	**Admixed (mtDNA and msat)**	This study
Qilianshan Mountains (Yanchiwan)	C	**G-A**	C/G	C/G	**Admixed (mtDNA and msat)**	This study
Qilianshan Mountains (Yanchiwan)	C	**G-A**	fail	**G**	**Admixed (mtDNA and msat)**	This study
Qilianshan Mountains (Yanchiwan)	C	**G-A**	C	Fail	**Admixed (mtDNA and msat)**	This study
Qilianshan Mountains (Yanchiwan)	C	**G-A**	C	**G**	**Admixed (mtDNA and msat)**	This study
Qilianshan Mountains (Yanchiwan)	Fail	**G-A**	C/G	Fail	**Admixed (mtDNA and msat)**	This study
Qilianshan Mountains (Yanchiwan)	Fail	**G-A**	C/G	C/G	**Admixed (mtDNA and msat)**	This study

Table S6. Final intron for ZFY and ZFX.

	ZFY final intron (1176bp)				Haplotype	Reference
Species (Origin, N)	**880-909**	**1010**	**1036-1037**	**1056**		
	30bp deletion	**T/G**	**2bp insertion**	**G/A**		
Coyote (North America)	30bp	T	-	G	**A**	
Golden Jackal (Israel)	-	T	TA	A		
African wolf (Kenya)	-	T	-	G		
Grey wolf (North America)	30bp	G	-	G	**C**	
Grey wolf (Europe and Mongolia)	30bp	G	-	G		
Iberian wolf Europe (n=12)	30bp	G	-	G		
Grey wolf Kyrgyzstan (n=4)	30bp	G	-	G		
Dog (Nepal, n=1)	30bp	G	-	G		
Dog (Qinghai Tibetan Plateau, n=3)	30bp	G	-	G		
Himalayan wolf (Zhadoi, Qinghai, n=1)	30bp	G	-	G		
Himalayan wolf (Nepal, n=15)	30bp	T	-	G	**B**	Werhahn et al. 2018
Himalayan wolf (Zhadoi, Qinghai, n=1)	30bp	T	-	G		
Himalayan wolf (Tibet, n=3)						
Admixed (Qilianshan, n=1)	30bp	T	-	G		
Admixed (Qilianshan, n=1)	30bp	G	-	G	**C**	
Grey wolf Tajikistan (n=4)	30bp	G	-	G		
Species (Origin, N)	**ZFX final intron (514bp)**				**Haplotype**	
		328	**381**	**425**		
		1bp insertion	**T/A**	**A/G**		
Golden Jackal (Israel)		G	A	A	**A**	KT448243.1
African wolf (Kenya)		G	T	G	**B**	KT448251.1
Grey wolf (Europe and Mongolia)		-	T	A	**C**	
Himalayan wolf (Nepal, n=17)		G	T	G	**B**	Werhahn et al. 2018
Iberian wolf (Spain, n=12)		-	T	A	**C**	Werhahn et al. 2018
Grey wolf Kyrgyzstan (n=3)		G	T	G	**B**	
Dog (Qinghai, n=9)		-	T	A	**C**	
Himalayan wolf (Zhadoi, Qinghai, n=1)		-	T	A		
Admixed (Sichuan, n=1)		-	T			
Admixed (Sichuan, n=1)		G	T	G	**B**	
Himalayan wolf (Qinghai, n=5)		G	T	G		
Admixed (Qinghai, n=1)		G	T	G		
Dog (Qinghai , n=2)		G	T	G		
Grey wolf (Qinghai, n=1)		G	T	G		
Grey wolf (Qilianshan, n=1)			T	G	**B**	
Admixed (Qilianshan, n=2)		G	T			
Himalayan wolf (Tibet, n=10)		G	T	G		
Dog (Tibet, n=1)		-	T	A	**C**	
Admixed Qilianshan, n=3)		-	T	A		
Grey wolf (Tajikistan, n=14)		-	T	A		

Table S7. Allelic richness in the different geographic populations based on 17 microsatelite loci and 2 genes.

Lineage (Geographic population)	**Allelic Richness**	**Private Allelic Richness**
Himalayan wolf (Nepalese Himalayan & Tibetan Plateau)	1.69	0.16
Dogs (Tibetan mastiff from Himalayan & Tibetan Plateau)	1.62	0.16
Grey wolf (Mongolia & Xinjiang)	2.45	0.14
Grey wolf (Europe)	2.53	0.18
Grey wolf (Kyrgyzstan)	3.57	0.22
Grey wolf (Tajikistan)	1.75	0.35
Grey wolf and Admixed (Qilianshan)	2.64	0.21
Himalayan wolf and Admixed (Qinghai)	1.75	0.16
Admixed Wolf (Sichuan)	1.72	0.36

Table S8. Allele frequency per population of the microsatellite data.

Locus	Allele/n	Himalayan Wolf (Nepal&Tibet)	Dog	Grey wolf (Mongolia& Xinjiang)	Grey wolf (Europe)	Grey wolf (Kyrgyzstan)	Wolf Qilianshan	Wolf Qinghai	Wolf Sichuan	Grey wolf Tajikistan
FH2088	**N**	41	3	2	0	1	7	20	6	18
	91	0.780	0.667	0.500	0.000	1.000	0.500	0.450	0.417	0.389
	95	0.000	0.000	0.000	0.000	0.000	0.500	0.100	0.000	0.000
	103	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000
	111	0.000	0.000	0.000	0.000	0.000	0.000	0.150	0.083	0.028
	115	0.085	0.167	0.500	0.000	0.000	0.000	0.200	0.000	0.111
	119	0.037	0.000	0.000	0.000	0.000	0.000	0.050	0.250	0.278
	123	0.061	0.167	0.000	0.000	0.000	0.000	0.000	0.250	0.167
	127	0.037	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.028
CPH08	**N**	35	5	2	2	4	6	19	3	14
	197	0.043	0.000	0.000	0.250	0.000	0.000	0.184	0.000	0.429
	199	0.043	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	201	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.667	0.036
	203	0.143	0.300	0.000	0.000	0.000	0.667	0.132	0.000	0.000
	205	0.571	0.600	0.750	0.500	0.375	0.167	0.211	0.167	0.071
	207	0.029	0.000	0.000	0.000	0.000	0.167	0.053	0.000	0.143
	209	0.014	0.000	0.000	0.250	0.375	0.000	0.132	0.167	0.036
	211	0.029	0.000	0.250	0.000	0.125	0.000	0.211	0.000	0.250
	213	0.071	0.100	0.000	0.000	0.000	0.000	0.079	0.000	0.036
	215	0.043	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	217	0.000	0.000	0.000	0.000	0.125	0.000	0.000	0.000	0.000
FH2096	**N**	40	7	3	2	2	5	20	7	17
	94	0.500	0.500	0.833	0.500	0.500	0.600	0.300	0.214	0.294
	98	0.350	0.500	0.167	0.000	0.500	0.200	0.225	0.357	0.088
	100	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000
	102	0.138	0.000	0.000	0.500	0.000	0.100	0.300	0.357	0.500
	106	0.013	0.000	0.000	0.000	0.000	0.100	0.175	0.000	0.118
FH2137	**N**	32	2	2	1	0	7	20	4	15
	154	0.188	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	156	0.000	0.000	0.250	1.000	0.000	0.071	0.150	0.000	0.000
	158	0.031	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	160	0.000	0.000	0.250	0.000	0.000	0.000	0.050	0.125	0.000
	162	0.219	0.500	0.250	0.000	0.000	0.286	0.050	0.000	0.100
	164	0.000	0.000	0.000	0.000	0.000	0.071	0.350	0.500	0.200
	166	0.094	0.000	0.000	0.000	0.000	0.071	0.025	0.125	0.033
	168	0.000	0.500	0.250	0.000	0.000	0.143	0.100	0.125	0.033
	170	0.156	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.033
	172	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.033
	174	0.078	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.033
	176	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.100
	178	0.203	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.033

	180	0.016	0.000	0.000	0.000	0.000	0.286	0.025	0.000	0.233
	182	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.167
	184	0.016	0.000	0.000	0.000	0.000	0.000	0.075	0.000	0.000
	186	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000
FH2001	**N**	27	5	2	1	1	0	8	5	11
	134	0.019	0.100	0.000	0.000	0.000	0.000	0.125	0.000	0.045
	138	0.019	0.000	0.000	0.000	0.000	0.000	0.125	0.100	0.000
	140	0.000	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	142	0.389	0.100	0.250	0.500	0.500	0.000	0.250	0.300	0.045
	144	0.000	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	146	0.296	0.400	0.750	0.500	0.000	0.000	0.313	0.000	0.409
	150	0.167	0.200	0.000	0.000	0.500	0.000	0.188	0.300	0.500
	154	0.111	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	158	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.300	0.000
CPH04	**N**	13	5	1	2	2	1	9	7	16
	134	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.357	0.344
	136	0.192	0.000	0.500	0.000	0.000	0.000	0.111	0.000	0.000
	138	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.031
	140	0.231	0.700	0.000	0.250	0.250	1.000	0.389	0.286	0.031
	142	0.308	0.000	0.500	0.000	0.000	0.000	0.389	0.000	0.125
	144	0.115	0.300	0.000	0.250	0.000	0.000	0.056	0.071	0.063
	146	0.000	0.000	0.000	0.250	0.500	0.000	0.056	0.286	0.031
	148	0.154	0.000	0.000	0.000	0.250	0.000	0.000	0.000	0.375
	150	0.000	0.000	0.000	0.250	0.000	0.000	0.000	0.000	0.000
CPH12	**N**	33	6	0	2	1	1	15	4	17
	192	0.061	0.083	0.000	0.000	0.000	0.000	0.100	0.000	0.088
	194	0.364	0.833	0.000	0.500	1.000	0.500	0.267	0.125	0.206
	196	0.212	0.000	0.000	0.250	0.000	0.000	0.067	0.000	0.176
	198	0.061	0.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000
	200	0.061	0.000	0.000	0.000	0.000	0.000	0.033	0.000	0.000
	202	0.152	0.083	0.000	0.000	0.000	0.500	0.333	0.375	0.118
	204	0.045	0.000	0.000	0.000	0.000	0.000	0.200	0.000	0.294
	206	0.045	0.000	0.000	0.250	0.000	0.000	0.000	0.250	0.118
CPH05	**N**	43	11	2	2	5	7	18	7	18
	106	0.000	0.182	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	108	0.535	0.455	0.250	0.750	0.600	0.286	0.389	0.357	0.056
	110	0.337	0.318	0.750	0.250	0.400	0.429	0.333	0.214	0.444
	112	0.023	0.045	0.000	0.000	0.000	0.214	0.222	0.143	0.139
	114	0.023	0.000	0.000	0.000	0.000	0.000	0.028	0.071	0.111
	116	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167
	118	0.023	0.000	0.000	0.000	0.000	0.071	0.028	0.000	0.056
	122	0.047	0.000	0.000	0.000	0.000	0.000	0.000	0.214	0.000
	124	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.028
C20.253	**N**	41	10	1	1	5	4	11	6	17
	93	0.317	0.250	1.000	1.000	0.100	0.375	0.318	0.083	0.059
	95	0.085	0.000	0.000	0.000	0.200	0.000	0.000	0.000	0.118

	99	0.183	0.050	0.000	0.000	0.000	0.000	0.000	0.250	0.176
	101	0.012	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.029
	103	0.220	0.250	0.000	0.000	0.200	0.250	0.182	0.167	0.265
	105	0.073	0.300	0.000	0.000	0.200	0.125	0.182	0.083	0.118
	107	0.098	0.150	0.000	0.000	0.200	0.000	0.273	0.083	0.059
	109	0.012	0.000	0.000	0.000	0.100	0.125	0.000	0.000	0.029
	113	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.250	0.147
	133	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.083	0.000
C09.250	**N**	33	10	2	2	5	7	18	5	19
	119	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.105
	125	0.000	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	129	0.000	0.000	0.000	0.250	0.000	0.000	0.000	0.000	0.000
	131	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.026
	133	0.152	0.050	0.000	0.250	0.100	0.000	0.056	0.000	0.026
	135	0.636	0.500	0.500	0.000	0.500	0.929	0.528	0.000	0.342
	137	0.121	0.100	0.500	0.500	0.300	0.000	0.222	0.800	0.184
	139	0.015	0.000	0.000	0.000	0.000	0.071	0.139	0.100	0.132
	141	0.030	0.050	0.000	0.000	0.000	0.000	0.028	0.100	0.132
	143	0.045	0.100	0.000	0.000	0.100	0.000	0.028	0.000	0.053
	145	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	147	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000
FH2010	**N**	40	9	2	2	4	1	19	4	14
	217	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	221	0.025	0.000	0.000	0.000	0.000	0.000	0.026	0.125	0.107
	223	0.013	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	225	0.138	0.444	0.750	0.500	0.625	0.000	0.105	0.375	0.071
	229	0.325	0.278	0.000	0.000	0.375	0.500	0.579	0.375	0.571
	233	0.225	0.000	0.000	0.000	0.000	0.500	0.158	0.125	0.250
	237	0.225	0.278	0.250	0.500	0.000	0.000	0.132	0.000	0.000
C27.442	**N**	38	11	3	2	6	7	21	5	14
	154	0.000	0.000	0.000	0.000	0.083	0.071	0.000	0.000	0.000
	158	0.026	0.182	0.000	0.000	0.250	0.071	0.048	0.000	0.036
	160	0.013	0.000	0.000	0.000	0.000	0.071	0.071	0.000	0.000
	162	0.132	0.000	0.000	0.000	0.000	0.071	0.167	0.000	0.179
	164	0.816	0.773	0.833	0.750	0.583	0.500	0.667	1.000	0.786
	166	0.013	0.045	0.000	0.250	0.083	0.214	0.048	0.000	0.000
	168	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.000	0.000
FH2161	**N**	30	6	3	2	2	5	19	3	14
	228	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.071
	232	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.333	0.250
	234	0.000	0.000	0.000	0.000	0.000	0.000	0.053	0.167	0.000
	236	0.050	0.000	0.167	0.000	0.000	0.000	0.000	0.000	0.321
	238	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000
	240	0.100	0.000	0.000	0.250	0.500	0.200	0.079	0.000	0.071
	244	0.583	0.750	0.333	0.250	0.000	0.300	0.263	0.000	0.107
	248	0.150	0.250	0.167	0.000	0.500	0.500	0.421	0.167	0.179

	250	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	252	0.067	0.000	0.000	0.250	0.000	0.000	0.158	0.000	0.000
	256	0.017	0.000	0.333	0.250	0.000	0.000	0.026	0.000	0.000
	260	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
CP09.173	**N**	42	11	2	2	6	11	21	6	19
	101	0.214	0.500	0.000	0.250	0.000	0.136	0.167	0.083	0.000
	103	0.060	0.045	0.000	0.250	0.250	0.136	0.071	0.000	0.158
	105	0.036	0.136	0.000	0.000	0.083	0.091	0.119	0.500	0.289
	107	0.036	0.045	0.000	0.000	0.000	0.091	0.071	0.333	0.447
	109	0.036	0.000	0.000	0.500	0.333	0.000	0.143	0.083	0.053
	111	0.036	0.000	0.000	0.000	0.167	0.091	0.119	0.000	0.026
	113	0.321	0.227	0.250	0.000	0.167	0.182	0.048	0.000	0.026
	115	0.190	0.045	0.500	0.000	0.000	0.182	0.190	0.000	0.000
	117	0.071	0.000	0.250	0.000	0.000	0.091	0.000	0.000	0.000
	119	0.000	0.000	0.000	0.000	0.000	0.000	0.024	0.000	0.000
	121	0.000	0.000	0.000	0.000	0.000	0.000	0.024	0.000	0.000
	123	0.000	0.000	0.000	0.000	0.000	0.000	0.024	0.000	0.000
CPH09	**N**	43	11	2	2	6	10	19	6	16
	139	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063
	141	0.058	0.045	0.000	0.250	0.167	0.000	0.184	0.250	0.094
	143	0.000	0.091	0.000	0.000	0.000	0.050	0.079	0.000	0.063
	145	0.070	0.000	0.000	0.000	0.083	0.150	0.000	0.417	0.188
	147	0.372	0.636	0.500	0.000	0.167	0.350	0.368	0.000	0.063
	149	0.326	0.182	0.500	0.750	0.250	0.150	0.237	0.083	0.063
	151	0.058	0.000	0.000	0.000	0.000	0.000	0.132	0.000	0.156
	153	0.012	0.000	0.000	0.000	0.083	0.050	0.000	0.000	0.125
	155	0.000	0.000	0.000	0.000	0.000	0.200	0.000	0.083	0.000
	157	0.047	0.045	0.000	0.000	0.250	0.050	0.000	0.083	0.188
	159	0.058	0.000	0.000	0.000	0.000	0.000	0.000	0.083	0.000
C13.758	**N**	20	9	2	2	6	3	18	4	13
	220	0.075	0.056	0.000	0.000	0.000	0.000	0.000	0.000	0.154
	222	0.050	0.167	0.000	0.000	0.000	0.000	0.028	0.125	0.000
	224	0.100	0.000	0.000	0.000	0.167	0.000	0.000	0.125	0.077
	226	0.075	0.056	0.000	0.250	0.083	0.000	0.111	0.500	0.346
	228	0.050	0.222	0.000	0.250	0.167	0.000	0.139	0.125	0.231
	230	0.000	0.000	0.750	0.250	0.167	0.000	0.278	0.000	0.154
	232	0.000	0.056	0.000	0.000	0.167	0.000	0.056	0.125	0.000
	234	0.125	0.111	0.000	0.000	0.083	0.000	0.028	0.000	0.038
	236	0.125	0.167	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	238	0.100	0.000	0.250	0.250	0.000	0.167	0.056	0.000	0.000
	240	0.075	0.167	0.000	0.000	0.167	0.667	0.222	0.000	0.000
	242	0.225	0.000	0.000	0.000	0.000	0.167	0.083	0.000	0.000
CPH14	**N**	37	11	2	2	6	6	17	5	15
	188	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.200	0.233
	192	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000	0.000
	194	0.054	0.000	0.000	0.000	0.000	0.000	0.000	0.200	0.167

	196	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.000
	198	0.041	0.000	0.000	0.000	0.250	0.000	0.029	0.000	0.167
	200	0.014	0.000	0.000	0.000	0.000	0.333	0.029	0.000	0.000
	202	0.311	0.136	0.250	0.250	0.167	0.167	0.382	0.200	0.167
	204	0.432	0.500	0.500	0.500	0.417	0.250	0.471	0.300	0.233
	206	0.149	0.364	0.250	0.250	0.000	0.250	0.088	0.000	0.033

Table S9. New ZFY and ZFX primers targeting the gene regions of interest more specifically and improved from (G. Werhahn et al., 2017; Geraldine Werhahn et al., 2018) for better success rates on non-invasive samples.

Primer name	Sequence (5' > 3')
WLF_ZFX_F	TGAAGTTTTCAGACCAGGGTTC
WLF_ZFX_R	TCCACGTTGGTTTCTTCAAGC
WLF_ZFY_F	TCCCAGAATCCCAGAATCAG
WLF_ZFY_R	TGCTTTGGTTCTTCAACCACT

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Appendix C.

MICROSCOPIC HAIR REFERENCE COLLECTION

Appendix C-1. Supplementary Material for Himalayan wolf foraging ecology and the importance of wild prey

Geraldine Werhahn*[1,5], Naresh Kusi[1], Xiaoyu Li[2], Cheng Chen[2,3], Lu Zhi[2], Raquel Lázaro Martín[4], Claudio Sillero-Zubiri[1,5], and David W. Macdonald[1,5]

Table S1. Prey abundance and prey use by wolves during summer as estimated by the Jacobs Index (from − 1, total avoidance, to + 1, max. selection) per study area and overall mean. Also given are ratios of livestock versus wild prey (based on biomass), and elevation and average humans sighted during distance sampling in the respective study months. *Wolf scat sample size for this study area was less than N=10.

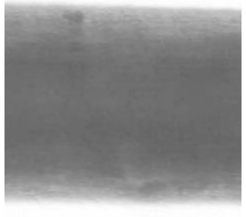
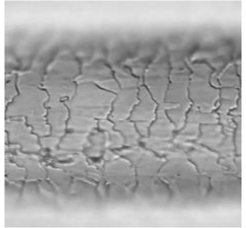
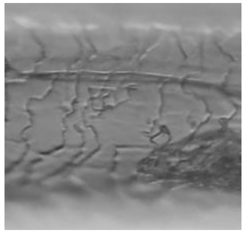
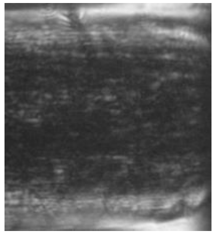
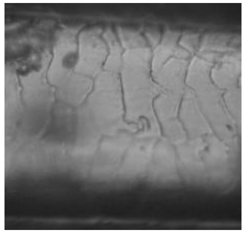
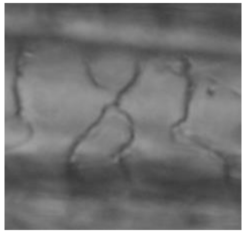
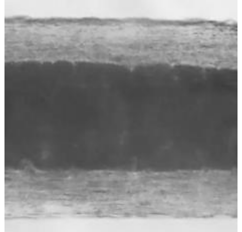
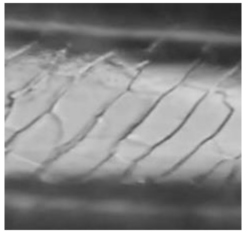
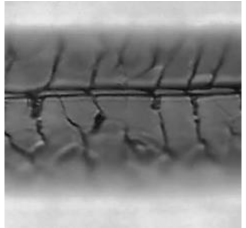
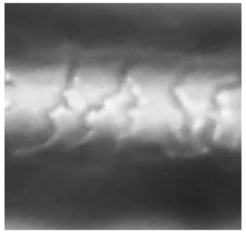
Study area/ Species	Density per km² (±SE)	Prey biomass (kg/km²)	Rel. prey biomass available (%)	Rel. biomass eaten (%)	Jacobs Index	Livestock vs. wild prey	Elevation	Human presence (study months)
Humla						3.5	4820m	0.77 (Jul/Aug)
Yak/Cow	4.91 (±0.32)	1166.13	62.73	17.3	-0.78			
Horse/Mule	0.68 (±0.12)	238.35	12.82	0	-1.00			
Goat	1.50 (±0.26)	41.25	2.22	0	-1.00			
Kiang	1.13 (±0.16)	367.25	19.76	52.9	0.64			
Naur	0.83 (±0.09)	45.87	2.47	5.2	0.37			
Tibetan gazelle	NR#	NA	NA	0.8	1			
Marmot¥	1.56*	NA	NA	22.8	NA			
Dolpa						41.7	4560m	2.7 (May/Jun/Jul)
Yak Cow	9.20 (±0.30)	2185.00	60.37	66.2	0.13			
Horse/Mule	2.12 (±0.15)	742.00	20.50	10.1	-0.39			
Goat	22.10 (±0.40)	607.75	16.79	2.2	-0.80			
Naur	1.54 (±0.12)	84.70	2.34	11.0	0.68			

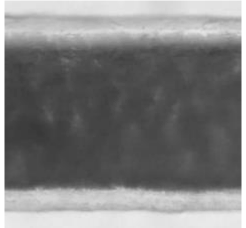
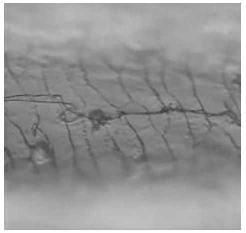
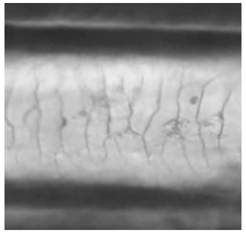
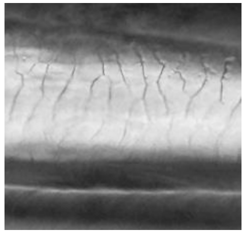
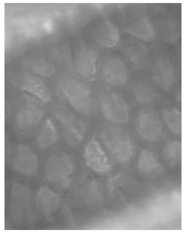
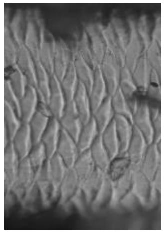
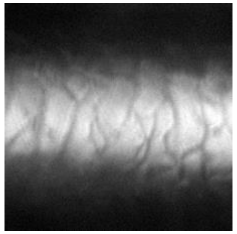
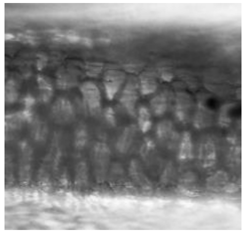
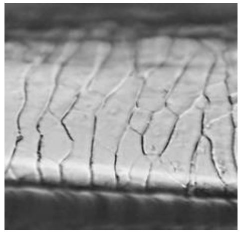
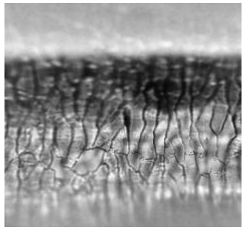
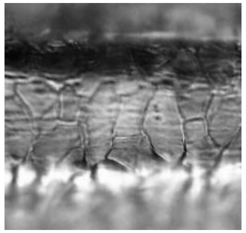
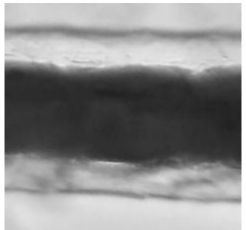
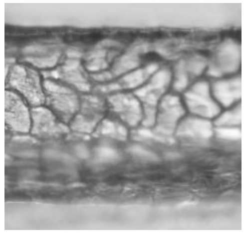
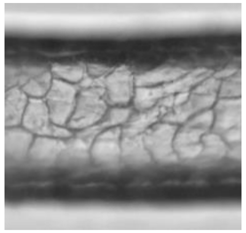
Tibetan gazelle	NR#	NA	NA	0.3	1			
Marmot¥	0.76*	NA	NA	8.5	Na			
KCA						12.7	4130m	1 (Sept)
Yak/Cow	8.50 (±0.46)	2018.75	87.36	63.5	-0.60			
Horse/Mule	0.21 (±0.08)	74.55	3.23	6.7	0.37			
Goat	1.79 (±0.29)	49.23	2.13	0	-1.00			
Naur	3.06 (±0.27)	168.30	7.28	25.7	0.63			
Marmot¥	0.02*	NA	NA	0.9				
Namsai*						3.8	4330m	5.5 (Aug)
Yak/Cow	20.80 (±1.95)	4940.00	79.09	29.00	-0.81			
Naur	19.00 (±1.42)	1045.00	16.73	0.50	-0.95			
White-lipped deer	1.58 (±0.40)	260.70	4.17	0.00	-1.00			
Tibetan gazelle	0.00	0.00	0.00	23.00	1.00			
Marmot*	1.25*	NA	NA	45.50	NA			
Musk deer	NA	NA	Opportunistically observed	0.00	NA			
Zhaqing*						18.3	4560m	1.33 (Aug)
Yak/Cow	20.80 (±1.95)	4940.00	94.81	92.90	-0.17			

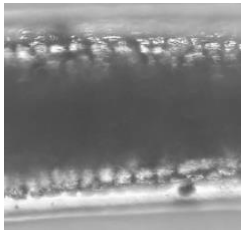
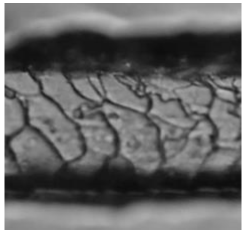
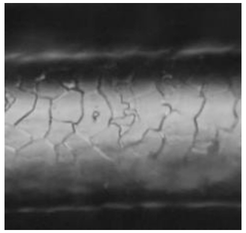
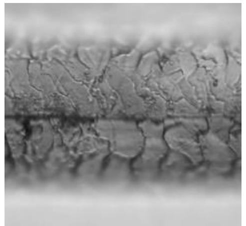
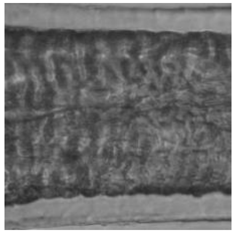
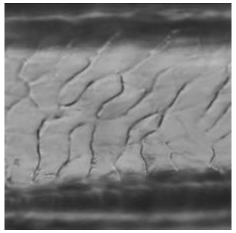
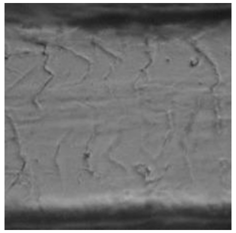
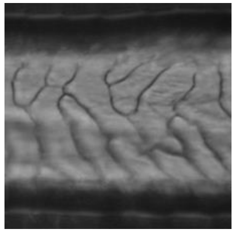
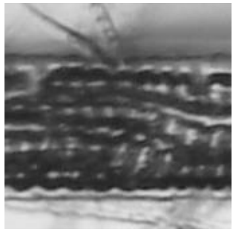
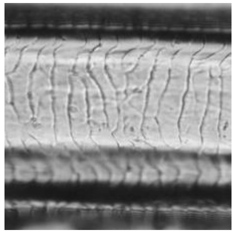
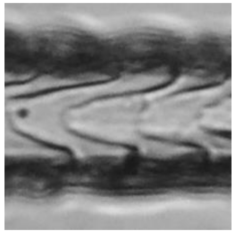
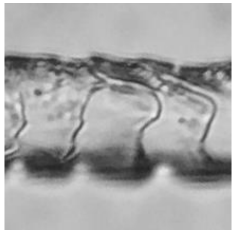
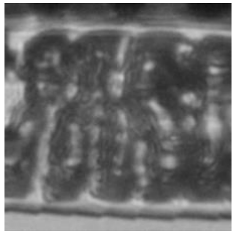
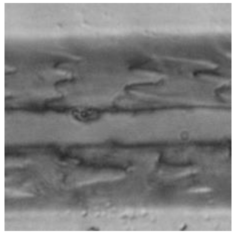
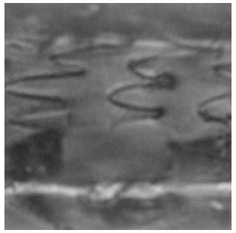
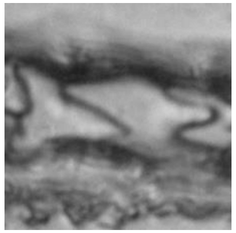
White-lipped deer	0.76 (±0.16)	125.57	2.41	0.00	-1.00			
Tibetan gazelle	0.79 (±0.27)	11.11	0.21	1.43	0.74			
Naur	2.43 (±0.27)	133.65	2.57	0.00	-1.00			
Marmot[¥]	0.97*	NA	NA	5.60	NA			
Mean								
Yak/Cow			77 (±13)	54 (±27)	-0.44 (±0.37)			
Horse/Mule			12 (±7)	6 (±4)	-0.34 (±0.47)			
Goat			7 (±7)	1 (±1)	-0.93 (±46)			
Naur			6 (±6)	8 (±9)	-0.06 (±0.76)			
Kiang			20 (±0)	53 (±26)	0.64 (±26)			
White-lipped deer			2 (±2)	0	-1.0 (±0.47)			
Tibetan gazelle			0	33 (±26)	0.94 (±39)			

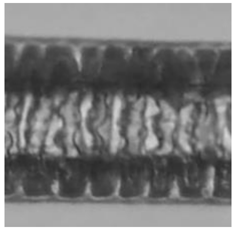
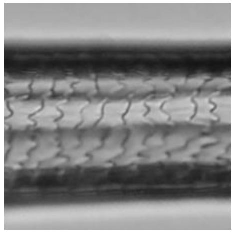
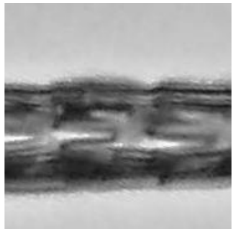
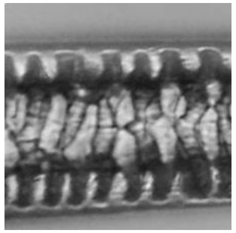
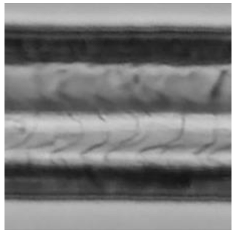
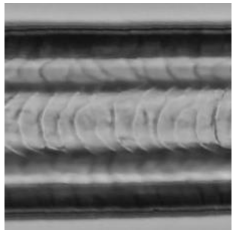
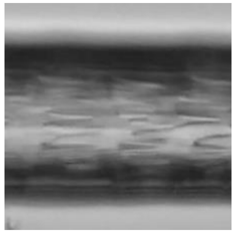
[¥] The marmot index was used to compare the relative marmot abundance among study areas based on sightings of marmots and counting theirs burrows but the method does not allow to estimate individuals/km^2 for marmots. [#] NR: Not recorded during the DS survey transects but found in the wolf diet. In Humla Tibetan gazelles were observed opportunistically (Werhahn et al., 2015).

Table C-1. Microscopic reference images of prey species guard hairs. The figure shows the medullar and cuticular characteristics necessary for identification. Domestic species are marked with *.

Species	**Medulla** (medial)	**Cuticula** (basal)	**Cuticula** (medial)	**Cuticula** (apical)
Cow* *Bos taurus*				
Yak* *Bos grunniens*				
Horse* *Equus ferus caballus*				

Kiang *Equus kiang*				
Tibetan Gazelle *Procapra picticaudata*				
Blue sheep *Pseudois nayaur*				
Goat* *Capra aegagrus hircus*				

Sheep* *Ovis aries*				
Himalayan Marmot *Marmot himalayana*				
Woolly hare *Lepus oiostolus*				
Large-eared pika *Ochotona macrotis*				

Nubra Pika *Ochotona nubrica*				
Plateu pika *Ochotona curzoniae*				
Tibetan Dwarf Hamster *Cricetulus alticola*