Original Research Article

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

Geraldine Werhahn [a, *], Helen Senn [b], Muhammad Ghazali [b],
Dibesh Karmacharya [c], Adarsh Man Sherchan [c], Jyoti Joshi [c], Naresh Kusi [a],
José Vincente López-Bao [d], Tanya Rosen [e], Shannon Kachel [f, g], Claudio Sillero-
Zubiri [a, h], David W. Macdonald [a]

[a] Wildlife Conservation Research Unit, Department of Zoology, University of Oxford, The Recanati-Kaplan Centre, Tubney House, Tubney, OX13 5QL, UK
[b] WildGenes Laboratory, Royal Zoological Society of Scotland, Edinburgh, EH12 6TS, UK
[c] Centre for Molecular Dynamics Nepal, GPO Box 21049, Kathmandu, Nepal
[d] Research Unit of Biodiversity (UO/CSIC/PA), Oviedo University, 33600, Mieres, Spain
[e] Panthera Foundation, Kyrgystan
[f] Panthera, New York, NY, USA
[g] School of Environmental and Forest Sciences, University of Washington, Seattle, WA, USA
[h] IUCN SSC Canid Specialist Group, Oxford, UK

ARTICLE INFO

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.

* Corresponding author.
   E-mail addresses: geraldine.werhahn@zoo.ox.ac.uk (G. Werhahn), tanya@iisd.org (T. Rosen).

Please cite this article as: Werhahn, G et al., The unique genetic adaptation of the Himalayan wolf to high-altitudes and consequences for conservation, Global Ecology and Conservation, https://doi.org/10.1016/j.gecco.2018.e00455

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.
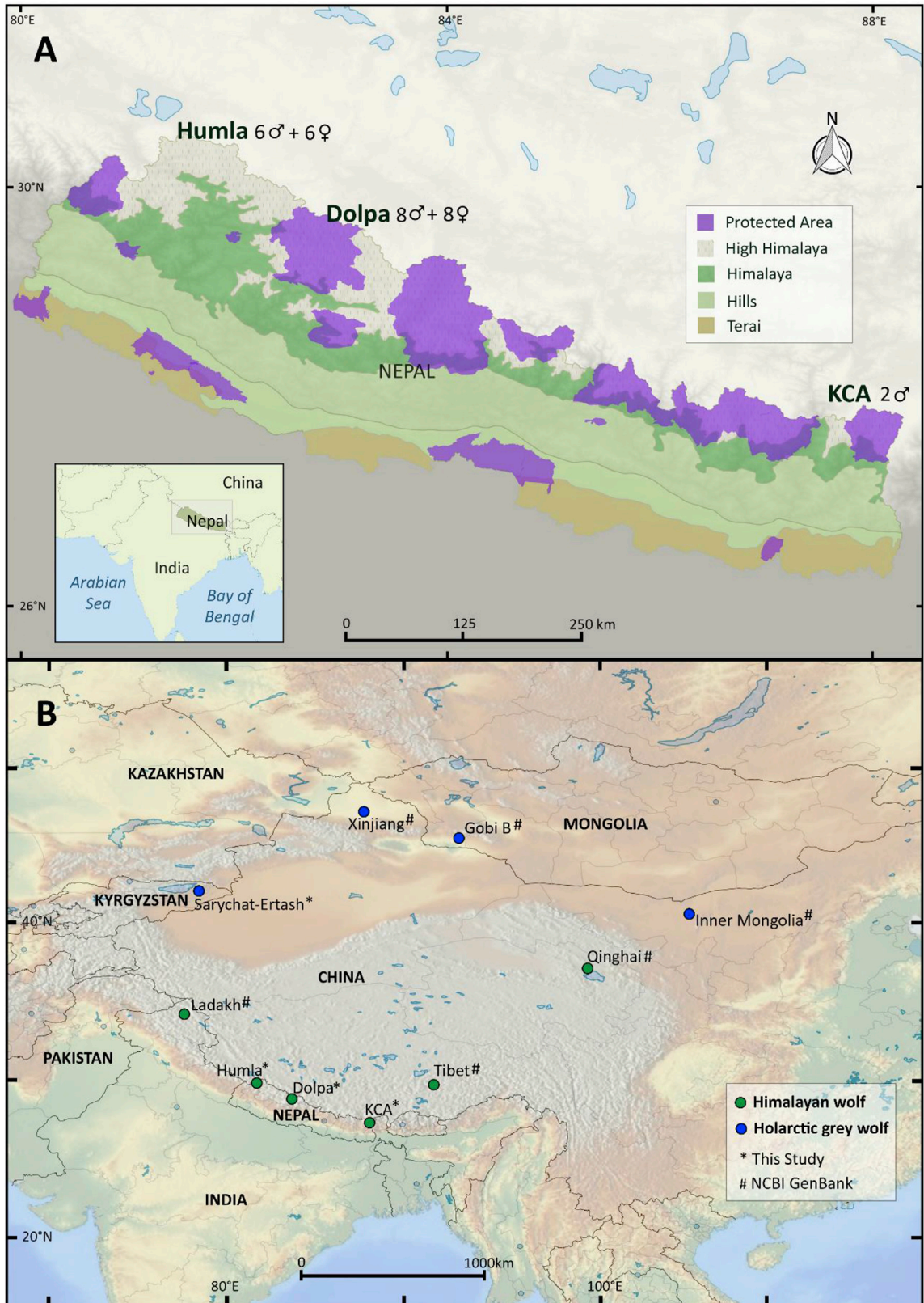
2.2. Samples and analysis of genetic material

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).

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
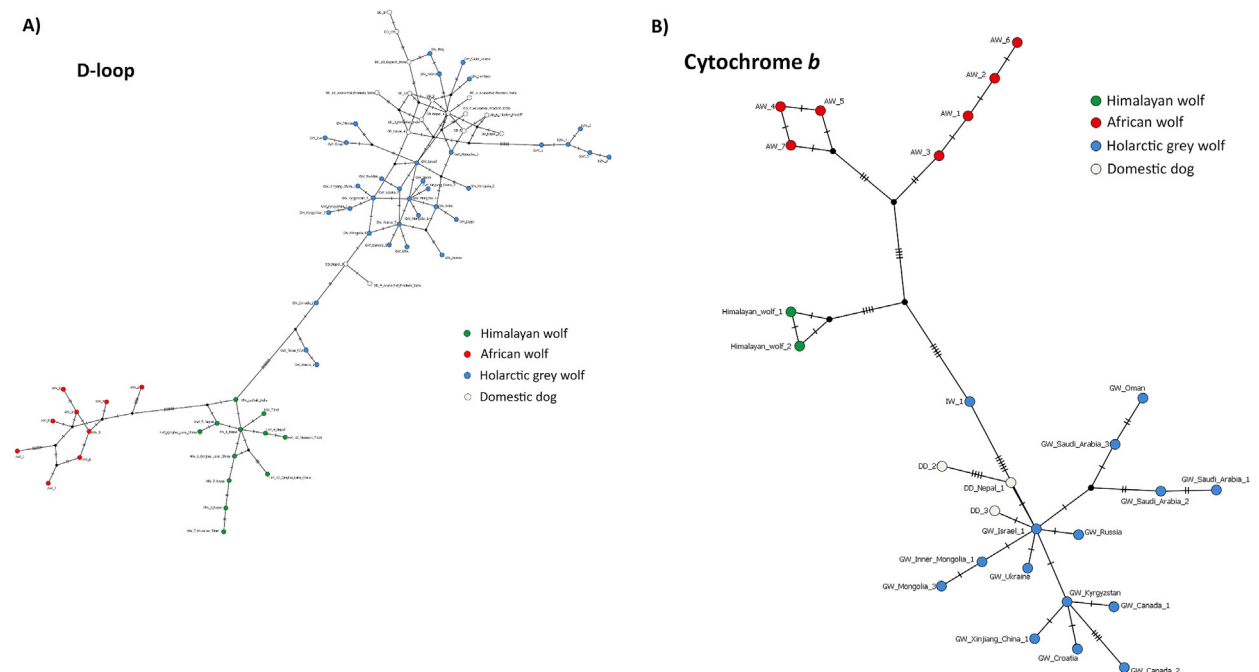
Fig. 3. Haplotype networks for A) D-loop, and B) cytochrome *b*. Himalayan wolf is shown in green, African wolf in red (AW), and Holarctic grey wolf in blue (GW for grey wolf, IW for Indian wolf *Canis lupus pallipes*), and DD for domestic dog shown in white. For the corresponding phylogenetic trees 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 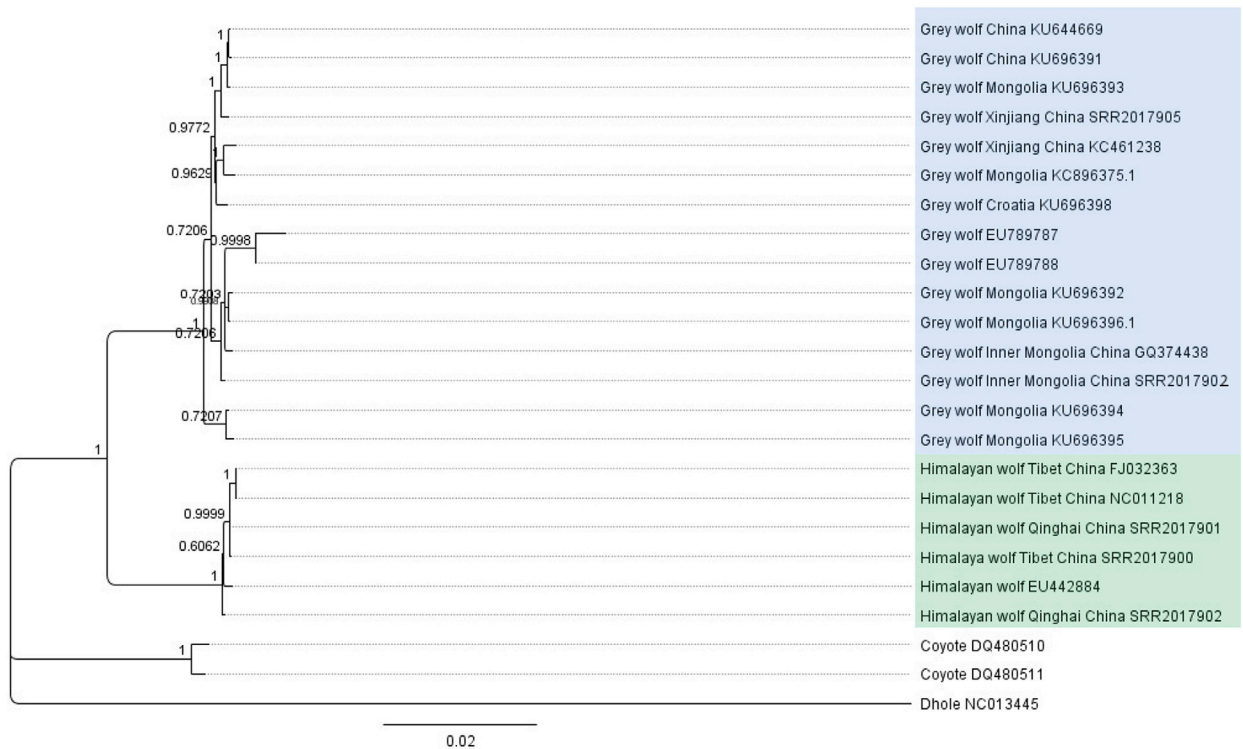wolf complex at 691,000–740,000 years before present (691,000 years before present using the Tree prior 'Coalescent: Exponential growth', 740,000 years before present using the Tree prior 'Bayesian SkyGrid') (Fig. 4). Divergence estimates based on this full mtDNA dataset calculated in MEGA are shown 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
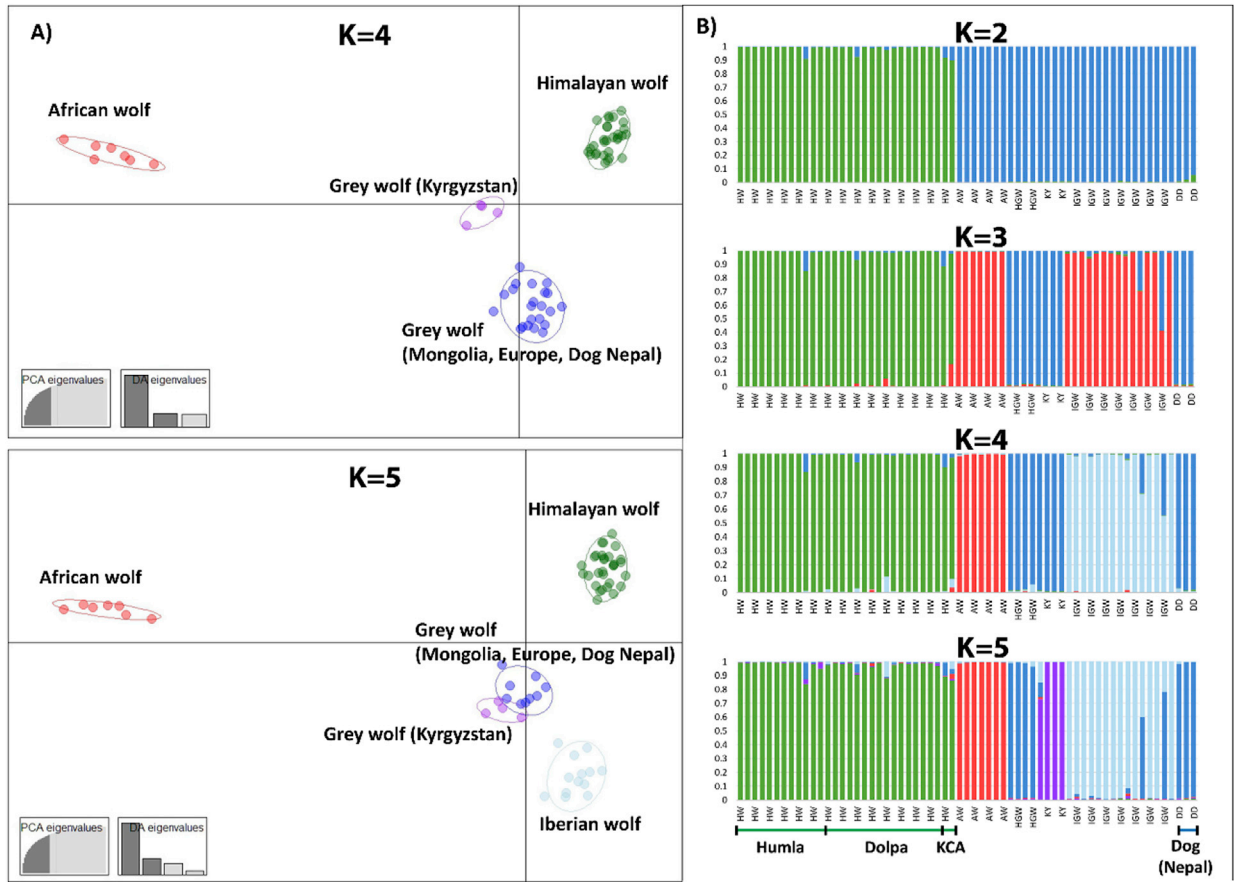
Fig. 5. A) DAPC with four and five populations (K = 4 and K = 5) showing the Himalayan wolf, the African wolf, grey wolf including wolves from Europe and Mongolia, the Iberian wolf, and Kyrgyz wolf samples. **B)** STRUCTURE plots with Himalayan wolf (HW from Humla, Dolpa, and KCA) splitting at K = 2; 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.
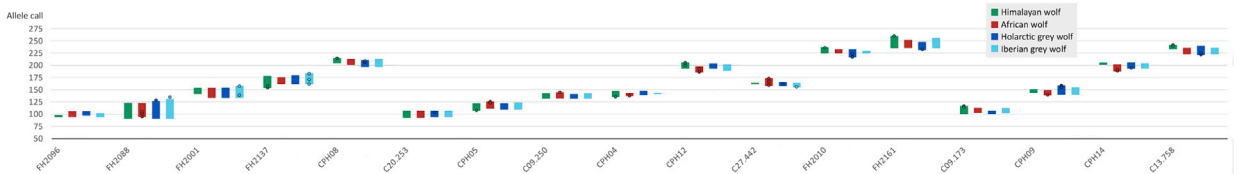

Fig. 6. Allele range and private alleles per population as indicated by the circles. The Holarctic grey wolf includes samples from Europe, Mongolia and Kyrgyzstan (For full details on allele frequencies see Supplementary Table S6.

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.

Role of the funding source

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.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gecco.2018.e00455.

References

Aggarwal, R.K., Kivisild, T., Ramadevi, J., Singh, L., 2007. Mitochondrial DNA coding region sequences support the phylogenetic distinction of two Indian wolf species. J. Zool. Syst. Evol. Res. 45 (2), 163–172.

Baker, R.J., Bradley, R.D., 2006. Speciation in mammals and the genetic species concept. J. Mammal. 87 (4), 643–662. https://doi.org/10.1644/06-MAMM-F-038R2.1.

Bandelt, H.J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16 (1), 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036.

Beall, C.M., 2007. Two routes to functional adaptation: Tibetan and Andean high-altitude natives. Proc. Natl. Acad. Sci. Unit. States Am. 104 (Suppl. 1), 8655–8660. https://doi.org/10.1073/pnas.0701985104.

Björnerfeldt, S., Webster, M.T., Vilà, C., 2006. Relaxation of selective constraint on dog mitochondrial DNA following domestication. Genome Res. 16 (8), 990–994. https://doi.org/10.1101/gr.5117706.

Bocci, A., Lovari, S., Khan, M.Z., Mori, E., 2017. Sympatric snow leopards and Tibetan wolves: coexistence of large carnivores with human-driven potential competition. Eur. J. Wildl. Res. 63 (92). https://doi.org/10.1007/s10344-017-1151-0.

Bolnick, D.I., Fitzpatrick, B.M., 2007. Sympatric speciation: models and empirical evidence. Annu. Rev. Ecol. Evol. Syst. 38 (1), 459–487. https://doi.org/10.1146/annurev.ecolsys.38.091206.095804.

Bradley, R.D., Baker, R.J., 2001. A test of the genetic species concept: cytochrome-b sequences and mammals. J. Mammal. 82 (4), 960–973. https://doi.org/10.1644/1545-1542(2001)082<0960:ATOTGS>2.0.CO;2.

Chetri, M., Jhala, Y.V., Jnawali, S.R., Subedi, N., Dhakal, M., Yumnam, B., 2016. Ancient himalayan wolf (Canis lupus chanco) lineage in upper mustang of the annapurna conservation area, Nepal. ZooKeys 582, 143–156. https://doi.org/10.3897/zookeys.582.5966.

Chetri, M., Odden, M., Wegge, P., 2017. Snow leopard and himalayan wolf: food habits and prey selection in the central Himalayas, Nepal. PloS One. https://doi.org/10.1371/journal.pone.0170549.

R Core Team, 2018. R: a Language and Environment for Statistical Computing. Vienna, Austria.

Dieckmann, U., Doebeli, M., 1999. On the origin of species by sympatric speciation. Nature 400 (6742), 354. https://doi.org/10.1038/22521.

Doebeli, M., Dieckmann, U., 2000. Evolutionary branching and sympatric speciation caused by different types of ecological interactions. Am. Nat. 156 (S4), S77–S101. https://doi.org/10.1086/303417.

Doebeli, M., Dieckmann, U., 2003. Speciation along environmental gradients. Nature 421 (6920), 259. https://doi.org/10.1038/nature01274.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. (29), 1969–1973.

Earl, D.A., vonHoldt, B.M., 2012. Structure harvester: a website and program for visualizing structure output and implementing the Evanno method. Conserv. Genet. Res. 4 (2), 359–361. https://doi.org/10.1007/s12686-011-9548-7.

Ersmark, E., Klütsch, C.F.C., Chan, Y.L., Sinding, M.-H.S., Fain, S.R., Illarionova, N.A., et al., 2016. From the past to the present: wolf phylogeography and demographic history based on the mitochondrial control region. Front. Ecol. Evol. 4 https://doi.org/10.3389/fevo.2016.00134.

Fan, Z., Silva, P., Gronau, I., Armero, A.S., Schweizer, R.M., Ramirez, O., et al., 2016. Worldwide Patterns of Genomic Variation and Admixture in Gray Wolves, pp. 163–173. https://doi.org/10.1101/gr.197517.115.

Filchner, 1903. Wissenschaftliche Ergebnisse der Expedition Filchner nach Tibet und China. Retrieved from. https://www.biodiversitylibrary.org/item/53427#page/7/mode/1up.

Fox, J.L., Chundawat, R.S., 1995. Wolves in the Transhimalayan region of India: the continued survival of a low-density population. In: Carbyn, L.N., Fritts, S.H. , Seip, D.R. (Eds.), Ecology and Conservation of Wolves in a Changing World. Canadian Circumpolar Institute, Edmonton, pp. 95–103.

Frankham, R., Ballou, J.D., Dudash, M.R., Eldridge, M.D.B., Fenster, C.B., Lacy, R.C., et al., 2012. Implications of different species concepts for conserving biodiversity. Biol. Conserv. 153, 25–31. https://doi.org/10.1016/j.biocon.2012.04.034.

Frankham, R., Ballou, J.D., Ralls, K., Eldridge, M.D.B., Dudash, M.R., Fenster, C.B., et al., 2017. Genetic Management of Fragmented Animal and Plant Populations. Oxford University Press, Oxford, New York.

Galpern, P., Manseau, M., Hetting, P., Smith, K., Wilson, P., 2012. allelematch: an R package for identifying unique multilocus genotypes where genotyping error and missing data may be present. Mol. Ecol. Res.s 771–778.

Gaubert, P., Bloch, C., Benyacoub, S., Abdelhamid, A., Pagani, P., Djagoun, C.A.M.S., et al., 2012. Reviving the African Wolf Canis lupus lupaster in North and West Africa: a Mitochondrial Lineage Ranging More than 6,000 km Wide. PloS One 7 (8), e42740. https://doi.org/10.1371/journal.pone.0042740.

Geffen, E., Anderson, M.J., Wayne, R.K., 2004. Climate and habitat barriers to dispersal in the highly mobile grey wolf. Mol. Ecol. 13 (8), 2481–2490. https://doi.org/10.1111/j.1365-294X.2004.02244.x.

Ghazali, M., Kaden, J., Senn, H., 2016. RZSS WildGenes Laboratory Training Guide: Himalayan Wolf Zinc-Finger Testing. Royal Zoological Society of Scotland, Edinburgh.

Gillespie, D., Fill, M., 2013. Pernicious attrition and inter-RyR2 CICR current control in cardiac muscle. J. Mol. Cell. Cardiol. 58 (Suppl. C), 53–58. https://doi.org/10.1016/j.yjmcc.2013.01.011.

Gou, X., Wang, Z., Li, N., Qiu, F., Xu, Z., Yan, D., et al., 2014. Whole-genome sequencing of six dog breeds from continuous altitudes reveals adaptation to high-altitude hypoxia. Genome Res. 24 (8), 1308–1315. https://doi.org/10.1101/gr.171876.113.

Gray, 1863. Chanco. Proc. Zool. Soc. Lond. 94. Retrieved from. https://www.biodiversitylibrary.org/item/96445.

Hassanin, A., Ropiquet, A., Couloux, A., Cruaud, C., 2009. Evolution of the mitochondrial genome in mammals living at high altitude: new insights from a study of the tribe Caprini (Bovidae, antilopinae). J. Mol. Evol. 68 (4), 293–310. https://doi.org/10.1007/s00239-009-9208-7.

Hausdorf, B., 2011. Progress toward a general species concept. Evolution 65 (4), 923–931. https://doi.org/10.1111/j.1558-5646.2011.01231.x.

Hennelly, L., Habib, B., Lyngdoh, S., 2015. Himalayan wolf and feral dog displaying mating behaviour in Spiti Valley, India, and potential conservation threats from sympatric feral dogs. Canid Biol. Conserv. 18 (7), 27–30.

Hennelly, L., Habib, B., Root-Gutteridge, H., Palacios, V., Passilongo, D., 2017. Howl Variation across Himalayan, North African, Indian, and Holarctic Wolf Clades: Tracing Divergence in the World's Oldest Wolf Lineages Using Acoustics.

Hodgson, 1847. Wolf of Tibet. Calcutta journal of natural history, and miscellany of the arts and sciences in India. Retrieved from. https://www.biodiversitylibrary.org/item/124837.

Huelsenbeck, J., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees, 17 (8), 754–755.

Huerta-Sanchez, E., 2014. Altitude adaptation in Tibetans caused by introgression of Denisovan-like DNA. Nature 512, 194–197. Retrieved from. https://www.nature.com/articles/nature13408.

Jombart, T., 2008. adegenet: a R package for the multivariate analysis of genetic markers. Bioninformatics (24), 1403–1405. https://doi.org/10.1093/bioinformatics/btn129.

Jombart, T., Ahmed, I., 2011. Adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. Bioinformatics. https://doi.org/10.1093/bioinformatics/btr521.

Kawecki, T.J., 1997. Sympatric speciation via habitat specialization driven by deleterious mutations. Evolution 51 (6), 1751–1763. https://doi.org/10.1111/j.1558-5646.1997.tb05099.x.

Koblmüller, 2016. Whole mitochondrial genomes illuminate ancient intercontinental dispersals of grey wolves (Canis lupus). J. Biogeogr. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/jbi.12765/full.

Koepfli, K.-P., Pollinger, J., Godinho, R., Robinson, J., Lea, A., Hendricks, S., et al., 2015. Genome-wide evidence reveals that african and eurasian golden jackals are distinct species. Curr. Biol. 25 (16), 2158–2165. https://doi.org/10.1016/j.cub.2015.06.060.

Kumar, S., Stecher, G., Tamura, K., 2015. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets (submitted).

Large Carnivore Initiative for Europe, 2007. Canis lupus, (The IUCN Red List of Threatened Species 2007).

Leigh, J.W., Bryant, D., 2015. PopART: full-feature software for haplotype network construction. Methods Ecol Evol. 6 (9), 1110–1116.

Leonard, J.A., 2014. Ecology drives evolution in grey wolves. Evol. Ecol. Res. (16), 461–473.

Leonard, A., Vilà, C., Fox-Dobbs, K., Koch, P.L., Wayne, R.K., Van Valkenburgh, B., 2007. Megafaunal extinctions and the disappearance of a specialized wolf ecomorph. Curr. Biol. 17 (13), 1146–1150. https://doi.org/10.1016/j.cub.2007.05.072.

Li, Y., Wu, D.-D., Boyko, A.R., Wang, G.-D., Wu, S.-F., Irwin, D.M., Zhang, Y.-P., 2014. Population variation revealed high-altitude adaptation of Tibetan mastiffs. Mol. Biol. Evol. 31 (5), 1200–1205. https://doi.org/10.1093/molbev/msu070.

Loog, L., Thalmann, O.L., Sinding, M.S., Schuenemann, V.J., Perri, A., Germonpré, M., et al., 2018. Modern wolves trace their origin to a late Pleistocene expansion from Beringia. BioRxiv. Retrieved from. https://www.biorxiv.org/content/early/2018/07/18/370122.

Marino, J., 2003. Threatened Ethiopian wolves persist in small isolated Afroalpine enclaves. Oryx 37 (1), 62–71. https://doi.org/10.1017/S0030605303000139.

Matsumura, S., Inoshima, Y., Ishiguro, N., 2014b. Reconstructing the colonization history of lost wolf lineages by the analysis of the mitochondrial genome. Mol. Phylogenet. Evol. 80, 105–112. https://doi.org/10.1016/j.ympev.2014.08.004.

Matsumura, S., Inoshima, Y., Ishiguro, N., 2014a. Reconstructing the colonization history of lost wolf lineages by the analysis of the mitochondrial genome. Retrieved. http://www.sciencedirect.com/science/article/pii/S1055790314002711. (Accessed 28 November 2017).

Mayr, E., 1942. Systematics and the Origin of Species. Columbia University Press, New York.

McNeely, J.A., Miller, K.R., Reid, W.V., Mittermeier, R.A., Werner, T.B., 1990. Conserving the world's biological diversity. In: Conserving the World's Biological Diversity, pp. 17–23. Retrieved from. https://www.cabdirect.org/cabdirect/abstract/19911620843.

Mech, L.D., Boitani, L., 2003. Wolves: Behavior, Ecology, and Conservation. University of Chicago Press.

Meng, C., Zhang, H., Meng, Q., 2009. Mitochondrial genome of the Tibetan wolf. Mitochondrial DNA 20 (2–3), 61–63. https://doi.org/10.1080/19401730902852968.

Miehe, G., Pendry, C., Chaudhary, R. (Eds.), 2016. Nepal: an Introduction to the Natural History, Ecology and Human Environment of the Himalayas.

Nakagome, S., Pecon-Slattery, J., Masuda, R., 2008. Unequal rates of Y chromosome gene divergence during speciation of the family Ursidae, 25 (7), 1344–1356.

Pang, J.-F., Kluetsch, C., Zou, X.-J., Zhang, A., Luo, L.-Y., Angleby, H., et al., 2009. mtDNA data indicate a single origin for dogs south of Yangtze river, less than 16,300 Years ago, from numerous wolves. Mol. Biol. Evol. 26 (12), 2849–2864. https://doi.org/10.1093/molbev/msp195.

Patel, S.A., Simon, M.C., 2008. Biology of hypoxia-inducible factor-2α in development and disease. Cell Death Differ. 15, 628–634. Retrieved from. https://www.nature.com/articles/cdd200817.

Peakall, R., Smouse, P.E., 2006. Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes 6 (1), 288–295. https://doi.org/10.1111/j.1471-8286.2005.01155.x.

Peakall, R., Smouse, P.E., 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28 (19), 2537–2539. https://doi.org/10.1093/bioinformatics/bts460.

Pennell, M.W., Miller, C.R., 2012. Capwire, Version 1.1.4.

Pilot, M., Branicki, W., Jędrzejewski, W., Goszczyński, J., Jędrzejewska, B., Dykyy, I., et al., 2010. Phylogeographic history of grey wolves in Europe. BMC Evol. Biol. 10, 104. https://doi.org/10.1186/1471-2148-10-104.

Prabhakar, N.R., Semenza, G.L., 2012. Adaptive and maladaptive cardiorespiratory responses to continuous and intermittent hypoxia mediated by hypoxia-inducible factors 1 and 2. Physiol. Rev. 92 (3), 967–1003. https://doi.org/10.1152/physrev.00030.2011.

Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155 (2), 945–959.

Rueness, E.K., Trosvik, P. al, Atickem, A., Sillero-Zubiri, C., Trucchi, E., 2015. The African wolf is a missing link in the wolf-like canid phylogeny. BioRxiv, 017996. https://doi.org/10.1101/017996.

Rutledge, L.Y., Devillard, S., Boone, J.Q., Hohenlohe, P.A., White, B.N., 2015. RAD sequencing and genomic simulations resolve hybrid origins within North American Canis. Biol. Lett. 11 (7), 20150303. https://doi.org/10.1098/rsbl.2015.0303.

Sastre, N., Francino, O., Lampreave, G., Bologov, V.V., López-Martín, J.M., Sánchez, A., Ramírez, O., 2009. Sex identification of wolf (Canis lupus) using non-invasive samples. Conserv. Genet. 10 (3), 555–558. https://doi.org/10.1007/s10592-008-9565-6.

Sharma, D.K., Maldonado, J.E., Jhala, Y.V., Fleischer, R.C., 2004. Ancient wolf lineages in India. Proc. R. Soc. Lond. B Biol. Sci. 271 (Suppl. 3), S1–S4.

Shrotriya, S., Lyngdoh, S., Habib, B., 2012. Wolves in Trans-Himalayas: 165 years of taxonomic confusion. Curr. Sci. 103 (8), 885.

Sillero-Zubiri, C., Hoffmann, M., Macdonald, D., 2004. Canids: Foxes, Wolves, Jackals and Dogs. Status Survey and Conservation Action Plan. IUCN/SSC Canid Specialist Group, Gland, Switzerland and Cambridge.

Smith, H., 1840. Dogs. The naturalist's library. Retrieved from. https://www.biodiversitylibrary.org/item/60173.

Sotnikova, M., Rook, L., 2010. Dispersal of the canini (mammalia, canidae: caninae) across Eurasia during the late miocene to early Pleistocene. Quat. Int. 212 (2), 86–97. https://doi.org/10.1016/j.quaint.2009.06.008.

Swofford, D.L., 2004. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for Inferring Very Large Phylogenies by Using the Neighbor-joining Method, pp. 11030–11035.

Tedford, R.H., Wang, X., Taylor, B.E., 2009. Phylogenetic systematics of the north american fossil caninae (carnivora: canidae). Bull. Am. Mus. Nat. Hist. 1–218 https://doi.org/10.1206/574.1.

Thalmann, O., Shapiro, B., Cui, P., Schuenemann, V.J., Sawyer, S.K., Greenfield, D.L., et al., 2013. Complete mitochondrial genomes of ancient canids suggest a european origin of domestic dogs. Science 342 (6160), 871–874. https://doi.org/10.1126/science.1243650.

Tsubouchi, A., Fukui, D., Ueda, M., Tada, K., Toyoshima, S., Takami, K., et al., 2012. Comparative molecular phylogeny and evolution of sex chromosome DNA sequences in the family Canidae (Mammalia: Carnivora), 29 (3), 151–161.

Vasemägi, A., Primmer, R., 2005. Challenges for identifying functionally important genetic variation: the promise of combining complementary research strategies - VASEMÄGI - 2005 - molecular ecology - Wiley Online Library. Mol. Ecol. (14), 3623–3642.

Verma, S.K., Singh, L., 2003. Novel universal primers establish identity of an enormous number of animal species for forensic application. Mol. Ecol. Notes 3 (1), 28–31. https://doi.org/10.1046/j.1471-8286.2003.00340.x.

Vilà, C., Amorim, I.R., Leonard, J.A., Posada, D., Castroviejo, J., Petrucci-Fonseca, F., et al., 1999. Mitochondrial DNA phylogeography and population history of the grey wolf Canis lupus. Mol. Ecol. 8 (12), 2089–2103. https://doi.org/10.1046/j.1365-294x.1999.00825.x.

vonHoldt, B.M., Cahill, J.A., Fan, Z., Gronau, I., Robinson, J., Pollinger, J.P., et al., 2016. Whole-genome sequence analysis shows that two endemic species of North American wolf are admixtures of the coyote and gray wolf. Science Adv. 2 (7), e1501714 https://doi.org/10.1126/sciadv.1501714.

Werhahn, G., Senn, H., Kaden, J., Joshi, J., Bhattarai, S., Kusi, N., et al., 2017a. Phylogenetic evidence for the ancient Himalayan wolf: towards a clarification of its taxonomic status based on genetic sampling from western Nepal. Royal Soc. Open Sci. 4 (170186).

Werhahn, G., Kusi, N., Sillero-Zubiri, C., Macdonald, D.W., 2017b. Conservation Implications for the Himalayan Wolf Canis (Lupus) Himalayensis Based on Observations of Their Packs and Home Sites in Nepal. Oryx.

Wilson, P.J., Grewal, S., Lawford, I.D., Heal, J.N., Granacki, A.G., Pennock, D., et al., 2000. DNA profiles of the eastern Canadian wolf and the red wolf provide evidence for a common evolutionary history independent of the gray wolf. Can. J. Zool. 78 (12), 2156–2166. https://doi.org/10.1139/z00-158.

Zachos, F.E., 2018. Mammals and meaningful taxonomic units: the debate about species concepts and conservation. Mamm Rev. Retrieved from https://onlinelibrary.wiley.com/doi/abs/10.1111/mam.12121.

Zachos, F.E., Cutton-Brock, T.H., Festa-Bianchet, M., Lovari, S., Macdonald, D.W., 2013, February 6. Taxonomy: Species Splitting Puts Conservation at Risk [Comments and Opinion]. https://doi.org/10.1038/494035c.

Zhang, H., Chen, L., 2011. The complete mitochondrial genome of dhole Cuon alpinus: phylogenetic analysis and dating evolutionary divergence within canidae. Mol. Biol. Rep. 38 (3), 1651–1660. https://doi.org/10.1007/s11033-010-0276-y.

Zhang, W., Fan, Z., Han, E., Hou, R., Zhang, L., Galaverni, M., et al., 2014. Hypoxia adaptations in the grey wolf (Canis lupus chanco) from Qinghai-tibet plateau. PLoS Genet. 10 (7), e1004466 https://doi.org/10.1371/journal.pgen.1004466.