Genomic analysis of wolves from Pakistan clarifies boundaries among three divergent wolf lineages

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Abstract

Among the three main divergent lineages of gray wolf (*Canis lupus*), the Holarctic lineage is the most widespread and best-studied, particularly in North America and Europe. Less is known about Tibetan (also called Himalayan) and Indian wolf lineages in southern Asia, especially in areas surrounding Pakistan where all three lineages are thought to meet. Given the endangered status of the Indian wolf in neighboring India and unclear southwestern boundary of the Tibetan wolf range, we conducted mitochondrial and genome-wide sequencing of wolves from Pakistan and Kyrgyzstan. Sequences of the mitochondrial D-loop region of 81 wolves from Pakistan indicated contact zones between Holarctic and Indian lineages across the northern and western mountains of Pakistan. Reduced-representation genome sequencing of 8 wolves indicated an east-to-west cline of Indian to Holarctic ancestry, consistent with a contact zone between these two lineages in Pakistan. The western boundary of the Tibetan lineage corresponded to the Ladakh region of India's Himalayas with a narrow zone of admixture spanning this boundary from the Karakoram Mountains of northern Pakistan into Ladakh, India. Our results highlight the conservation significance of Pakistan's wolf populations, especially the remaining populations in Sindh and Southern Punjab that represent the highly endangered Indian lineage.

Keywords: *Canis lupus pallipes;* contact zones, genomics, gray wolf; Pakistan

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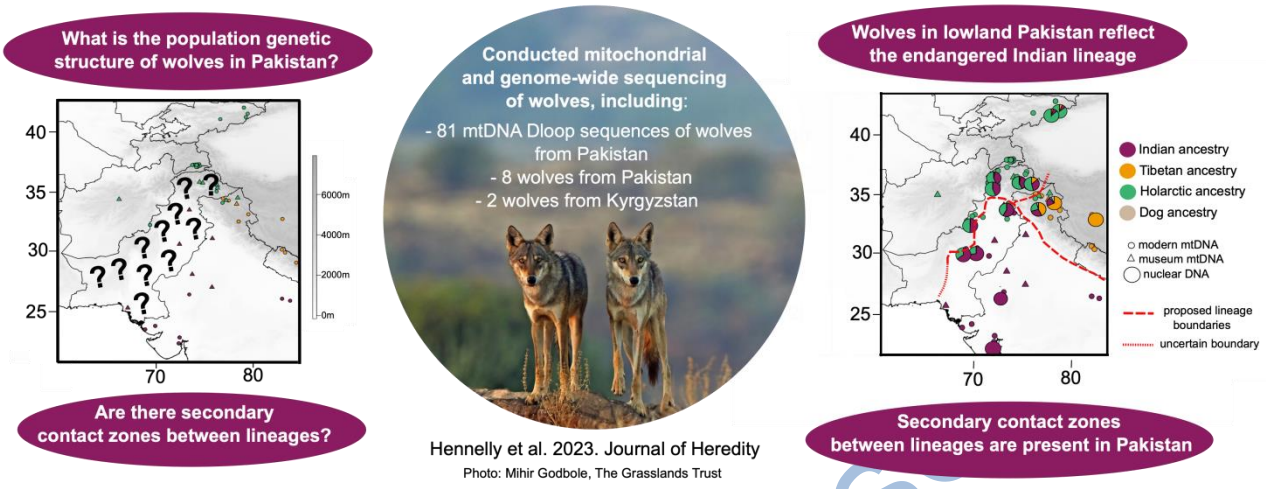
Graphical abstract



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Main Text

Introduction

Understanding the evolutionary history and distribution of genetically distinct lineages and populations is of key importance for conservation. Management decisions are often guided by and prioritized based on the taxonomic recognition of species and their populations. However, much of the world's biodiversity remains undescribed and data-deficient, which can lead to loss of taxa before they have even been identified (Ecksein et al. 2018; Borgelt et al. 2022). This problem is especially pronounced for many biodiverse countries near the equator, where climate change and increasing human-induced pressures have resulted in extensive loss of species and continue to threaten many others.

Although currently recognized as a single species, the gray wolf (*Canis lupus*) collectively includes three main divergent lineages, each of which represents an evolutionary significant unit and potentially a distinct species (Wang et al. 2020; Hennelly et al. 2021; Wang et al. 2022; Werhahn et al. 2022). Two of these lineages, Tibetan (also called Himalayan) and Indian, are restricted to relatively small regions of southern Asia, where all three lineages occur (Sharma et al. 2004; Wang et al. 2020; Hennelly et al. 2021; Wang et al. 2022). The Holarctic lineage is the most widespread and reflects massive Pleistocene expansions across much of Eurasia and into North America, as well as recurrent gene flow among its populations over the past 100,000 years (Loog et al. 2020; Bergström et al. 2022). First suggested based on mtDNA two decades ago, recent genomic data has confirmed the Indian and Tibetan lineages as evolutionarily distinct and are hundreds of thousands of years divergent from each other and the Holarctic wolves (Sharma et al. 2004; Wang et al. 2020; Hennelly et al. 2021; Krofel et al. 2022; Wang et al. 2022; Werhahn et al. 2022). Despite their distinctiveness, wolves of the Indian lineage are among the least studied and most endangered in the world. Generally, these wolves inhabit arid grasslands of the Indian subcontinent and are among the smallest bodied wolves (Jhala 2003). Wolves within the Tibetan lineage are larger-bodied, more densely furred, and

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possess multiple genes known or suspected to confer adaptation to high elevations (Zhang et al. 2014; Werhahn et al. 2018; Werhahn et al. 2020). The distribution of the Indian and Tibetan lineages remains largely undescribed and challenging to assess partly due to the phenotypic similarity of Holarctic wolf populations in southwest Asia to Indian wolves, and those of the high-elevation mountains of Central Asia to Tibetan wolves.

Recent studies suggest the ranges of these three lineages converge in the vicinity of Pakistan (Sharma et al. 2004; Hennelly et a. 2021). The Indian mitochondrial lineage has been documented in Pakistan using museum samples collected in the lower-elevation regions of Sindh and Punjab, where wolves are morphologically similar (small-bodied, short hair) to arid regions of low-elevation India and southwest Asia (Roberts 1977; Sharma et al. 2004; Sarwar et al. 2021). In contrast, Holarctic mitochondrial haplotypes have been documented from larger-bodied and thickly furred wolves inhabiting the mountains of the Sulieman and Karakoram ranges of Pakistan (Sharma et al. 2004; Hamid et al. 2019; Fig. S1). To the east of the Karakoram range, wolves sampled from the Ladakh region of northwest India exhibit Tibetan mitochondrial and nuclear genomic ancestry, suggesting a transition between Tibetan and Holarctic lineages in the Ladakh-Karakoram region spanning India and Pakistan (Sharma et al. 2004; Hennelly et al. 2021). Currently no genetic data exist for wolves in the southwest region of Pakistan. However, wolves in this region have been described as morphologically intermediate between Indian wolves of India and high elevation wolves of Northern Pakistan (Robert 1977). Thus, these observations suggest Pakistan may be situated where multiple divergent populations are in contact with each other, termed as contact zones. Contact zones in these regions of Pakistan also have been described for several other mammal species (Hoffman and Smith 2005; Statham et al. 2014; Jacobson et al. 2016).

Delineating the distributions of wolf lineages in Pakistan would provide essential information to inform their conservation in Pakistan, where wolves are among the last few remaining large

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carnivores (Sheikh and Molur 2003). Wolves are currently classified as an endangered species in Pakistan based on the IUCN Red List assessment of Pakistan's mammals conducted in 2003 (Sheikh and Molur 2003). Wolf populations are especially rare in Sindh and Punjab, where they have been described as declining and increasingly rare since the 1970's (Roberts 1977). While two subspecies of gray wolf are currently described in Pakistan – the Indian wolf (*Canis lupus pallipes*) and Tibetan wolf (*Canis lupus chanco*) – this classification is based primarily on morphological data (Roberts 1977). Genomic data are therefore needed to re-assess and revise if necessary the taxonomic classification of wolves in Pakistan.

In this study, we sequenced a portion of the mitochondrial D-loop region of 81 wolf samples from Pakistan and 1 golden jackal from Pakistan (*Canis aureus*). We also used a genotyping-by-sequencing (GBS) approach to obtain genome-wide sequence of 8 wolves and 1 dog from Pakistan as well as resequenced the whole genomes of 2 wolves from Kyrgyzstan. We then combined these data with 57 previously sequenced canid genomes to more precisely locate contact zones among the three wolf lineages in Pakistan and use these findings to identify taxonomically informed management units among Pakistan's wolf populations.

Methods

Samples and Sequencing procedures

For mitochondrial D-loop sequencing, we used 81 wolf samples and 1 golden jackal from Pakistan that were mostly non-invasively collected as scats (Table S1). These samples were collected from various regions of Pakistan, including Gilgit-Baltistan, Khyber Pakhtunkhwa (KPK), and Punjab. For genome wide sequencing, we used 12 new canid samples, including 9 wolves from Pakistan for which we conducted GBS and 2 wolves from the Tien Shan mountain range in Kyrgyzstan, which we sequenced entirely (Table S2). The nine new samples from Pakistan included Chitral Gol National

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Park in the Hindu Kush mountain range (n=2), Central Karakoram National Park in the Karakoram mountain range (n=2), the Sulieman mountain range near Kohlu in Baluchistan (n=1), South Waziristan (n=1), the Indus plains near Dera Ghazi Khan (n=1), the Pothwar Plateau near Kallar Syedan (n=1), and a free-ranging dog from Islamabad (n=1). We also included 392 previously published mitochondrial sequences and 56 genomic short read datasets for reference (Tables S2, S3).

We extracted DNA from blood and tissue samples using the DNeasy Blood and Tissue Kit (Qiagen, CA, USA) and from scat samples using QiaAmp Stool Kit (Qiagen, CA, USA), in both cases following the manufacturer's protocols. For sequencing the mitochondrial regions, we amplified a 402-bp region of the mitochondrial D-loop region using the primers HV1A-F: CCCTGACACCCCTACATTCA and HV1C-R: TTATGTGTGATCATGGGCTGA. We used 25 µl reactions, which involved 3 µl of DNA, 0.2 µM primers, 0.2mM dNTPs , 0.2 µl of 50X TITANIUM® Taq DNA Polymerase (Takura Bio, Inc. Kusatsu, Shiga, Japan), and 2.5 µl 10X TITANIUM® Taq PCR Buffer. We then conducted polymerase chain reaction (PCR), where the cycle conditions were 95ºC for 1 minute, followed by 36 cycles of 95ºC for 20 seconds, then 51ºC for 30 seconds and 72 ºC for 40 seconds, and lastly 5 min at 72 ºC (Sacks et al. 2021). We used Big Dye sequencing chemistry (Applied Biosystems, Inc.) to sequence the D-loop products and we used Sequencher 5.4 (Gene Codes Inc., Ann Arbor, Michigan) to inspect the electropherograms. Lastly, we identified a 12-bp region that we were not able to confidently align (positions 15518-15530) (Kim et al. 1998). Therefore, we removed this section from our newly sequenced and previously published D-loop sequences, resulting in our final trimmed sequence to be a length of 366bp.

For the nine new samples from Pakistan, we performed GBS to obtain genome-wide single nucleotide polymorphisms (SNPs) (Elshire et al. 2011). We digested 100 ng of DNA per

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sample at 37°C for 2 hours with a restriction enzyme (Nsil-HF, an equivalent to EcoT22I; New England BioLabs Inc., Ipswich, MA). Subsequently, we ligated a common and uniquely barcoded adapter to each DNA sample with one negative control per run. To minimize unevenness among individual libraries in the final sequencing pool, we pooled ligated samples into 8 separate sub-pools before purifying via QiaQuick PCR columns (Qiagen Inc.) prior to the PCR reaction. To increase the complexity of our final library, we then conducted 4 replicate PCR reactions for each of our 8 library sub-pools, for a total of 36 reactions. Each 50 µL reaction contained 10 µL of purified adapter ligated DNA, 25 µL of NEB 2X Master Mix, 25 pmol of both forward and reverse PCR Primer, and dH_2O. The PCR conditions were: 5 minutes at 72°C, 30 seconds at 98°C, followed by 18 cycles of 10 seconds at 98°C, 30 seconds at 65°C, and 30 seconds at 72°C, and lastly a final extension at 72°C for five minutes. We used QiaQuick PCR columns to purified PCR products and used Qubit fluorometer (Qiagen Inc.) to quantified library concentrations. All libraries were run on 1% agarose gels and a Bioanalyzer (Agilent Technologies, Santa Clara, CA) trace before pooling in equal masses for sequencing (96 libraries/lane) on an Illumina HiSeq4000 (SR100) at the University of California, Davis Genome Center DNA Technologies core. For the two wolf samples from Kyrgyzstan, tissue (DNA) was sent to BGI for DNA extraction, library preparation, and sequencing of the whole genome by sequencing prepared libraries on a BGISEQ-500 paired end at 100-bp.

Alignment and SNP dataset for nuclear data

Raw reads for all of our samples were trimmed using Trim Galore v0.6.5 (https://www.bioinformatics.babraham.ac.uk/proje cts/trim_galore/). We then mapped the reads to the domestic dog genome assembly (CanFam3.1) with an attached gray wolf Y chromosome from Smeds et al using BWA MEM v0.7.17. r1188 (Li and Durbin 2009; Smeds et al. 2019). For whole genome

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resequenced samples, we identified and removed PCR duplicates using MarkDuplicates tool v2.18.25 from the Picard suite (http://broadinstitute.github.io/picard). All BAM files originating from GBS and WGS were then sorted and filtered to retain only properly paired reads (-F 1024) and with a minimum mapping quality score of 10 (-q 10) using SAMtools (Li et al. 2009). Because our dataset included both GBS and WGS data, we only retained reads of each whole genome-sequenced sample that overlapped with read positions in the GBS data. To achieve this, we first merged all GBS bam files to obtain a list of all positions found in our GBS data and then subsetted each WGS bam files to keep reads that overlapped with those GBS positions (samtools view -L).

Phylogenetic relationships

To determine the mitochondrial lineages of our 81 wolf (and one golden jackal) D-loop haplotypes from Pakistan, we constructed a phylogenetic tree including these and 75 previously typed reference sequences (Table S3). We trimmed all sequences to 322 bp, including removal of a 12-bp region that could not be reliably aligned (Sacks et al. 2021). We used ModelFinder to select the HKY substitution model and used BEAST v1.10.4 to construct the phylogenetic tree using the Speciation: Birth-Death Process tree prior, relaxed lognormal clock (Drummond and Rambaut 2007; Kalyaanamoorthy et al. 2017). We ran the analysis for 50 million MCMC cycles, sampling every 5,000 and discarding the first 5 million states as burn-in. We used Tracer v1.7.1 (Rambaut et al. 2018) to assess the outputs and effective sample sizes (ESS), ensuring that ESS > 200. Finally, we used TreeAnnotator (v2.5.2) to infer the maximum credibility tree and FigTree v1.4.4 to display the trees.

Population Structure

To gain insight into the distribution of mitochondrial lineages across Asia, we assembled a comprehensive dataset of locations where previous studies have assigned mtDNA haplotypes to one of the three main wolf lineages (Table S3). We then combined this dataset with the lineage assignments of our newly sequenced samples to create a map of mitochondrial lineages of wolves in

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South and Central Asia. In addition, we recorded the elevation at which the samples originated to investigate the relationship between elevation and distribution of the mitochondrial lineages.

To assess nuclear genetic structure, we conducted a principal component analysis (PCA) using PCangsd (Meisner and Albrecthsen 2018) with our new genomic samples along with previously published reference sequences. We inferred the PCA using genotype likelihoods from ANGSD and filtered the dataset by including only properly paired reads (-only_proper_pairs 1) from autosomes, excluding reads with excessive mismatches (-C 50) or mapping quality lower than 20 (-minMapQ20) (Korneliussen et al. 2014). We also removed transitions (-noTrans 1) and bases with quality scores <20 (-minQ 20) and retained SNPs represented that had at least ~90% of individuals present (≥40 of the 45 total; -minInd 40). We obtained the covariance matrix from PCAngsd and used the R function *eigen* to infer the PCA.

To estimate the individual ancestry proportions, we inferred genotype likelihoods using ANGSD and ran NGSadmix (Skotte et al. 2013) assuming K = 2–7 genetic clusters. We also included additional canids by including 4 North American gray wolves, 12 dogs, a coyote (*Canis latrans*), African wolf (*Canis lupaster*), Golden Jackal from India (*Canis aureus*), and an Ethiopian wolf (*Canis simensis*) (Table S1). We used the same criteria for filtering in ANGSD as for PCAngsd, except we required a minimum of 58 individuals out of the total of 65 individuals used in this analysis to obtain sites that had at least ~90% of individuals present (-minInd 58). Lastly, to assess how nuclear ancestry relates to elevation, we recorded the elevation of the Pakistan wolf samples and samples from adjacent regions.

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Inferring shared ancestry from D-statistics

To investigate patterns of shared ancestry of wolves in Pakistan to ancestral Indian and Tibetan wolf lineages, we calculated the D statistic (Green et al. 2010). An excess of alleles derived in a lineage (P3) that are shared with one of two more recently diverged (i.e., sister) lineages (P1, P2) is suggestive of gene flow or shared phylogenetic relatedness between P3 and P1 or P2. To test for gene flow and/or phylogenetic relatedness between Eurasian wolf individuals (P1, P2) and the Indian and Tibetan lineages (P3), we estimated D statistics to test for shared ancestry between P2 and P3 in the topology structure (((P1, P2), P3), P4), where P4 indicates an outgroup as is necessary to infer the derived state of alleles in P3. With our wolf samples, we used the topologies (((Portugal wolf, X), Indian wolf), Andean Fox) and (((Portugal wolf, X), Tibetan wolf), Andean Fox), where X is an individual wolf from Eurasia. We ran the D-statistic test in ANGSD using genotype likelihoods (-doAbbababa 1) and a similar set of filtering criteria as in PCAngsd and NGSadmix except we included reads that had at least ~90% of individuals present (-minInd 57).

Results

Mitochondrial Population Structure

Using the 322-bp mitochondrial D-loop sequences of 81 wolf samples and 1 golden jackal from Pakistan, along with 75 previously published D-loop sequences, we constructed a Bayesian phylogenetic tree (Fig. S2). Most wolves were sampled from the mountainous regions of northern Pakistan and were dominated by the Holarctic lineage, which were distributed throughout the northern Sulieman range, Hindu Kush range, and Karakoram range in Pakistan, with most samples at elevations >1000 m (Fig. 1, Fig. S3, Table S4). Although comparably fewer, most of the samples collected from lowland Pakistan exhibited Indian wolf haplotypes, as did previously sampled wolves from this region and the adjacent peninsular subcontinent of India (Fig. 1, Fig S3, Table S3, Table S4). Conversely, no Indian haplotypes were found far from lowland Pakistan, resulting in a relatively sharp contact zone with the other two lineages. Similarly, despite previous detection of Tibetan

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mitochondrial haplotypes in the adjacent Ladakh region of the Himalayas in India, we detected no Tibetan mitochondrial haplotypes among our newly sequenced samples from the Karakoram Mountains of Gilgit Baltistan (n=48) (Fig. 1). As evidenced by the distribution of previously published sequences, Holarctic haplotypes were distributed throughout the Central Asian mountain ranges, many regions of Iran, and the lower elevation regions west, north, and east of the Tibetan Plateau (Fig. S4). Tibetan haplotypes were restricted to the Tibetan Plateau and the Himalayan Mountain range east of the Kashmir region (Fig. S4).

Population Genomic Structure

We successfully sequenced all eight wolves, one golden jackal from Pakistan, one dog from Pakistan and the two wolves from Kyrgyzstan (Fig. 2A). The average depth of our GBS samples was 39x (range: 9.75–78.41x) and that of our newly whole-genome-sequenced samples averaged 19.45x and 15.21x. Within our GBS loci, we obtained 61,510 autosomal SNPs for the PCA, 90,394 autosomal SNPs for the Admixture analyses, and 79,985 autosomal SNPs for the D-statistic analysis.

The PCA indicated that our newly sequenced wolves from Pakistan fell intermediate between the Indian and Holarctic wolf lineages, with one individual from the Karakoram Mountains also showing a small amount of Tibetan ancestry (Fig. 2B). Generally, wolves from Pakistan that were sampled from the low elevation regions fell closer to wolves of the Indian lineage on the PCA. The admixture analysis similarly showed higher levels of ancestry shared between wolves from lower elevations of Pakistan and Indian wolves from India (Fig 1C, Fig. S5). Wolves sampled from northern Pakistan at elevations <1,750 m tended to possess >50% Indian ancestry (Fig. S6A).

Despite being classified as the Tibetan wolf (*Canis lupus chanco*) in Pakistan, our wolves from mountainous regions of the Hindu Kush and Karakoram ranges of Pakistan had majority Holarctic

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ancestry and high amounts of Indian wolf ancestry with little to no Tibetan ancestry. Generally, we found proportions of Tibetan ancestry increased with elevation; wolves sampled from >2700 m tended to possess >50% Tibetan ancestry (Figure S6B). This relationship likely corresponds to the geography of this region where the deep valleys of the Karakoram Mountain range gradually increase in elevation until reaching the Tibetan Plateau. In general, wolves from the Karakoram Mountains of Pakistan and Ladakh region of India showed admixture from all three wolf lineages.

D statistics

We treated each wolf, including wolves from Pakistan, as sister (P2) to a representative Holarctic wolf from Portugal (P1) and assessed shared ancestry with Indian wolves using an Indian wolf as the third clade (P3) with the Andean fox set as the outgroup (P4). All of the wolves sampled in Pakistan, as well as two Tibetan wolves sampled in Ladakh, India, showed highly significant D statistics, consistent with high levels of Indian wolf introgression; alternatively, these wolves could have been ancestrally Indian wolves with Holarctic introgression (Fig. 3). Wolves from the lowland Indus plains of DG Khan, Pothwar Plateau near Kallar Syaden, and the Sulieman Mountains near Kolhu in Baluchistan showed the highest D-statistic values (Fig. 3). Otherwise, most wolves outside of Pakistan showed no statistically significant evidence of gene flow from Indian wolves (i.e., positive D statistics). Exceptions were one wolf from Syria and three from Iran that showed marginally significant levels of gene flow with Indian wolves. Except for the Ladakh wolves, which are geographically close to the Indian lineage, Tibetan wolves exhibited among the lowest D-statistic values, suggesting little shared ancestry deriving from phylogenetic relatedness or gene flow between Indian and Tibetan wolves.

Using a Tibetan wolf as P3 and treating each wolf as the sister (P2) to the wolf from Portugal (P1) resulted in one of the wolves from the Karakoram range of Pakistan showing a strongly significant D statistic, consistent with gene flow from the Tibetan lineage (Fig. 3). This was the same wolf for

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which the PCA and Admixture analyses suggested Tibetan admixture. Otherwise, wolves from Pakistan, including from high-elevation regions, such as Chitral and the other wolf from the Karakoram range, showed only marginally significant or non-significant D statistics. Outside of Pakistan and the Tibetan wolf range, evidence of gene flow (positive D statistic) with the Tibetan lineage was only significant in Central and East Asian wolves directly adjacent to the Tibetan Plateau. Generally, D-statistic evidence of gene flow declined rapidly with distance from the Tibetan Plateau and into the Karakoram Mountains of Pakistan (Fig. 3).

Discussion

We investigated the population structure and genome-wide patterns of ancestry of wolves from Pakistan to identify contact zones and work towards delineating boundaries among the three wolf lineages in Eurasia. Our genome-wide analyses using 10 newly sequenced wolves indicated wolves from Pakistan were universally admixed, with contributions primarily from Holarctic and Indian lineages, with relative proportions depending on geography. Both mitochondrial and nuclear findings suggest the presence of contact zones between the Indian and Holarctic wolves likely surrounding the Sulieman, Hindu Kush, and Himalayan mountain ranges and forming a three-way contact zone in the Kashmir region (Fig. 4). A contact zone between Tibetan and Holarctic wolf lineages appears to be present within the Ladakh region of India. We suggest these contact zones form the most western and northern boundary for the Indian lineage and the most western boundary for the Tibetan lineage. (Fig. 4). Further genomic work using additional wolf samples can provide greater understanding of the geographic extent of hybridization and resolution in defining lineage boundaries.

Secondary contact zones in Pakistan

Our mitochondrial and nuclear datasets were concordant in indicating the presence of multiple contact zones in Pakistan occurring among the Indian, Tibetan, and Holarctic wolf lineages. The clearest contact zone we detected was between the Holarctic and Tibetan lineages in the Ladakh-Karakoram region of India and Pakistan. All modern and museum-derived mitochondrial data from the

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Karakoram Mountains in Gilgit Baltistan fell within the Holarctic lineage (n=52), whereas Ladakh harbored a mix of Tibetan and Holarctic mitochondrial lineages ($n_{Tibetan}$= 5; $n_{Holarctic}$= 4). Consistent with the mitochondrial data, a sharp cline in Tibetan genome-wide ancestry crosses through the Ladakh-Karakoram region. Our discovery of this contact zone was unexpected because wolves on either side are phenotypically similar and have historically been described as a single subspecies. The narrowness of the contact zone suggests some form of reproductive barrier could be hindering gene flow. For example, selective forces associated with high elevation habitats could explain this subdivision, as has been hypothesized to explain the limited gene flow observed between Tibetan and Holarctic wolves in other regions of Asia (Werhahn et al. 2020). Alternatively, natal habitat-biased dispersal could result in partial reproductive barriers as has been suggested for other gray wolf populations (Pilot et al. 2006; Musiani et al. 2007; Munoz-Fuentes et al. 2009). Further work that incorporates more genomic data and movement data of wolves in this region would provide insights into mechanisms maintaining lineage boundaries in this highly mobile species.

We detected a second contact zone between the Indian and Holarctic lineage along the mountain ranges that surround the lowland Indus valley of Pakistan. Wolves inhabiting lower elevation and southern regions of Pakistan showed the highest proportion of genome-wide Indian ancestry and Indian mitochondrial haplotypes. These results are consistent with museum-derived mitochondrial and morphological evidence that these wolves largely reflect the Indian lineage (Roberts 1977; Sharma et al. 2004; Sarwar et al. 2021). Based on PCA, admixture analyses, and D statistics, wolves of Iran showed no strong cline in Indian ancestry, suggesting a major shift between Indian and Holarctic ancestry occurs somewhere between the Indus River of Pakistan and eastern Iran. However, no genetic data from modern wolf samples is available from wolf populations of Sindh and Southern Baluchistan of Pakistan. Thus, sequencing wolves from this region will be needed to investigate whether this contact zone extends along the southern mountain ranges of Pakistan and how narrow this contact zone may be. To the north, our data suggests this contact zone between the Indian and Holarctic lineage extends onto the Pothwar Plateau. We detected a mix of Indian and Holarctic

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haplotypes on this Plateau (n_{Indian}=2, $n_{Holarctic}$=2) and an individual with admixed Indian and Holarctic nuclear ancestry (Fig 2B, 2C). However, we found neither genomic nor mitochondrial Tibetan ancestry on the Pothwar Plateau, which taken together with all our results, suggests the contact zone between the Tibetan and Holarctic lineage is much more narrow than for the Indian-Holarctic contact zones. In agreement with past studies, our results suggest the presence of a three-way contact zone among all three lineages is likely located in the geographically small region of the Kashmir Valley (Sharma et al. 2004).

Multiple lines of evidence suggest these secondary contact zones in Pakistan have likely been in existence for thousands of years rather than reflecting recent anthropogenic range dynamics. There are multiple scenarios in which these contact zones could have arisen. One possible scenario is that as the Holarctic lineage expanded during the late Pleistocene, it came into contact with the Indian subcontinent and Tibetan Plateau, where the Indian and Tibetan lineages had been isolated in glacial refugia. In line with this scenario, modern and ancient genomes suggest the Holarctic lineage underwent multiple expansion events and its populations were highly connected during the late Pleistocene (Loog et al. 2020, Bergstrom et al. 2022). This includes wolves from Iran where their demographic history is similar to other Holarctic wolves (Wang et al. 2022). In contrast, the demographic history of Indian and Tibetan lineages suggests they were isolated within glacial refugia in the Indian subcontinent and the Tibetan Plateau, respectively (Wang et al. 2022, Wang et al. 2021). A second scenario is that the historical distribution of the Indian and Tibetan lineages encompassed a larger region, and the Holarctic lineage gradually replaced them until reaching these prominent contact zones in Pakistan. Our results show wolves in Southwest Asia have some Indian ancestry that could be remnants of the Indian wolf's past distribution. Ancient wolf genomes from southwest Asia and further analyses that time admixture events would be needed to clarify the process in which these contact zones formed.

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Conservation Implications

These results have implications for the taxonomy and conservation of wolves in Pakistan. Our genome-wide data indicate that the endangered Indian wolf lineage is present throughout lowland Pakistan. Wolves most fully representing the Indian lineage are likely present in the low elevation regions of Sindh and Southern Punjab, where wolves are increasingly rare and declining (Rais et al. 2011; Sheikh and Molur 2003). Due to the lack of sampling, it is unclear whether wolves in Baluchistan primarily reflect the Indian or Holarctic lineage. Wolves within the northwest mountain ranges and Pothwar Plateau have admixed genomic backgrounds with Indian and Holarctic ancestry. Conserving these admixed populations may contribute to preserving alleles and genetic variation that derives from the Indian lineage. The importance of such admixed populations has been recognized to be a reservoir of genetic variation, whether rare alleles from the Indian lineage or new Holarctic alleles, in which natural selection can act upon (Supple and Shapiro 2018). Our findings support previous work that a taxonomic revision is recommended to differentiate wolves of Southwest Asia and the Indian subcontinent, which collectively are considered as *Canis lupus pallipes* (Hennelly et al. 2021; Krofel et al. 2022). More broadly, the presence of seemingly stable contact zones between divergent lineages in such a highly mobile species supports recent calls for the Indian and Tibetan wolf to be recognized as distinct subspecies or species (Werhahn et al. 2022).

These findings highlight the need to conserve the remaining wolves that most fully represent the Indian lineage in Sindh and Punjab, where wolves are extremely rare and in many regions could be already extirpated (Sharma et al. 2004; Saad et al. 2015). Major threats to this wolf population include lack of information and human-wolf conflict. There are almost no published studies on wolf populations in Sindh and Punjab as almost all studies on wolves in Pakistan have been conducted in the northern mountain ranges. As a result, there are no population abundance estimates for wolves in these regions, thus hindering our understanding of the threat status of this population. Wolf-human conflict associated with livestock depredation by wolves is documented in all regions of Pakistan,

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resulting in severe persecution and negative attitudes of many local people towards wolves (Khan et al. 2019; Hamid et al. 2019; Khattak et al. 2022). For example, in villages in the Northwest region of Pakistan, ~65% of people interviewed were in favor of reducing or complete eradication of wolves, with 35% preferring complete eradication (Khattak et al. 2022). Lastly, wolves in Pakistan are viewed as less threatened by extinction by people in urban regions of Pakistan compared to other species listed as Endangered or Vulnerable in Pakistan, such as the common leopard (*Panthera pardus*), snow leopard (*Panthera uncia*), and Asiatic black bear (*Ursus thibetanus*) (Sheikh and Molur 2003; Ali et al. 2022).

With only an estimated ~3,000 Indian wolves in India and an unknown yet declining number in Pakistan, this endemic lineage is one of the most endangered canid populations in the world (Jhala et al. 2022). We recommend a more comprehensive genetic assessment of the Indian wolf in Pakistan is needed, especially in Sindh, Southern Punjab, and Baluchistan. These results would aid in identifying potentially isolated, inbred, or otherwise at-risk populations in greatest need of conservation efforts. Targeted conservation efforts need to include methods to reduce human-wolf conflict, which is a major driver of persecution and negative attitudes of local people (Khattak et al. 2022, Hamid et al. 2019, Ali et al. 2016, Rehman et al. 2021). Currently, we are not aware of any existing compensation scheme for livestock depredation by wolves in Pakistan. Establishing compensation schemes for livestock depredation by wolves may help alleviate the economic burden of wolves and improve their public perception by local people living in these landscapes. Other conservation efforts can include awareness programs highlighting the ecological importance of wolves and adopting tools for non-lethal mitigation of wolf depredation on livestock. Together, our results confirm the presence of the endangered and divergent Indian lineage in Pakistan and informs delineation of wolf populations to aid in identifying populations of conservation concern.

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Data Availability

The raw DNA sequence reads are publicly available in the NCBI SRA Bioproject PRJNA990466 with Biosample numbers SAMN36267796- SAMN36267806. All scripts are publicly available at https://github.com/hennelly/Genomic_analyisis_wolves_of_Pakistan

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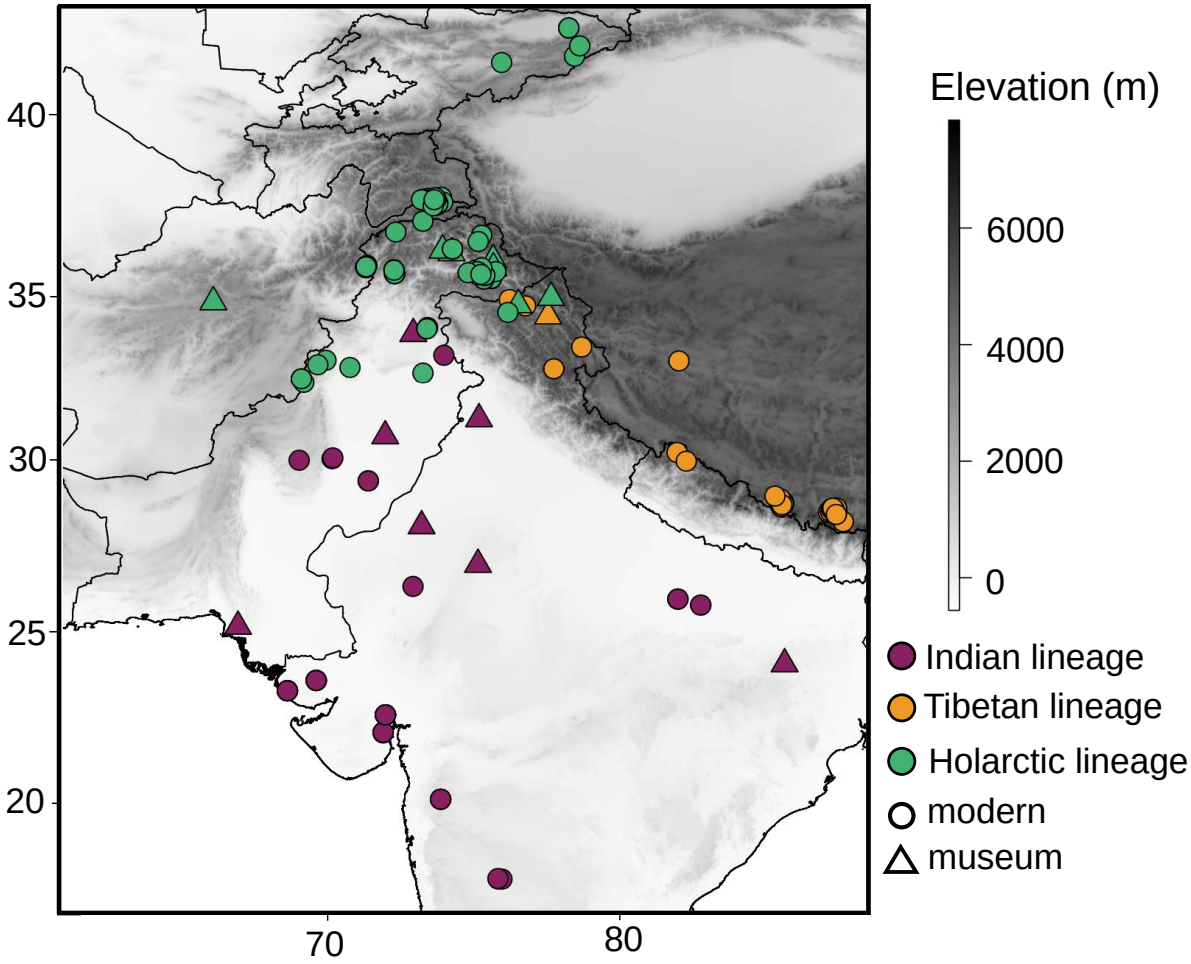

Figure 1. Distribution of the Indian, Tibetan, and Holarctic mitochondrial lineages in southern Asia using 261 wolf samples. Each point represents a single individual, with the color indicating its mitochondrial lineage. Circles indicate modern-day samples and triangles indicate samples from museum collections initially collected 1875–1964 (Sharma et al. 2004).

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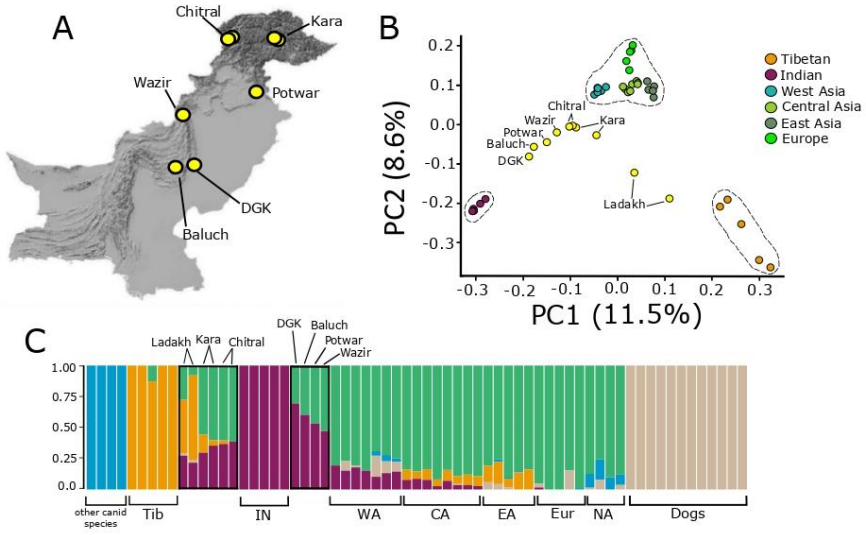

Figure 2. Gray wolf samples from Pakistan and population structure of Eurasian gray wolf populations. (A) Locations of gray wolf samples in Pakistan, which include wolves from Dera Ghazi Khan (DGK), Sulieman mountain range of Baluchistan (Baluch) and South Waziristan (Wazir), Kallar Syedan on the Pothwar Plateau (Pothwar), the Hindu Kush mountain range in Chitral-Gol National Park (Chitral), and the Karakoram Mountains (Kara). (B) Principle component analysis consisting of ~61,000 autosomal SNPs and 45 wolves showing the three lineages – Indian, Tibetan, and Holarctic – enclosed by a dashed polygon. Wolves that fell between these three lineages shown in yellow, which include our newly sequenced wolves from Pakistan and two wolves from Ladakh region of India. (C) Individual admixture proportions at K = 5 using ~90,000 autosomal SNPs across 49 gray wolves, 12 dogs, and four other canid species (Ethiopian wolf *Canis simensis*, Golden Jackal *Canis aureus*, African wolf *Canis lupaster*, Coyote *Canis latrans*). Each bar represents an individual and colors within each bar represents the estimated ancestry belonging to a specific population. Labels indicate the following populations: Tibetan (Tib), Indian (IN), West Asia (WA), Central Asia (CA), East Asia (EA), European (EUR), and North American (NA).

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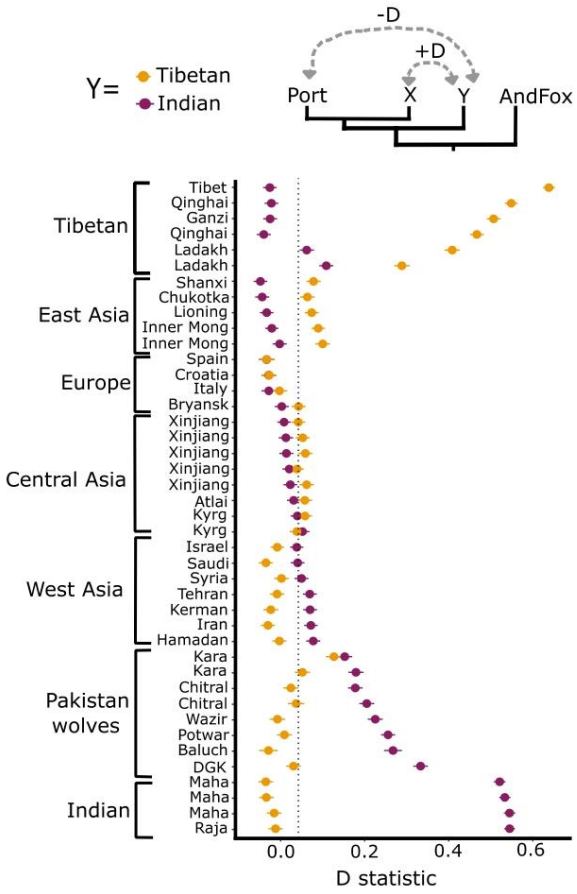

Figure 3. Genome-wide patterns of shared ancestry across Eurasian wolves using D-statistics. Positive D-statistic values indicate shared ancestry between individual X and an Indian wolf individual (purple points) and individual X and a Tibetan wolf individual (orange points). All analyses used an Andean fox (*Lycalopex culpaeus*) as an outgroup and an individual wolf from Portugal as a representative Holarctic wolf to compare shared ancestry of individual X. Dotted line depicts where gene flow/shared ancestry is significant (Z score > 3).

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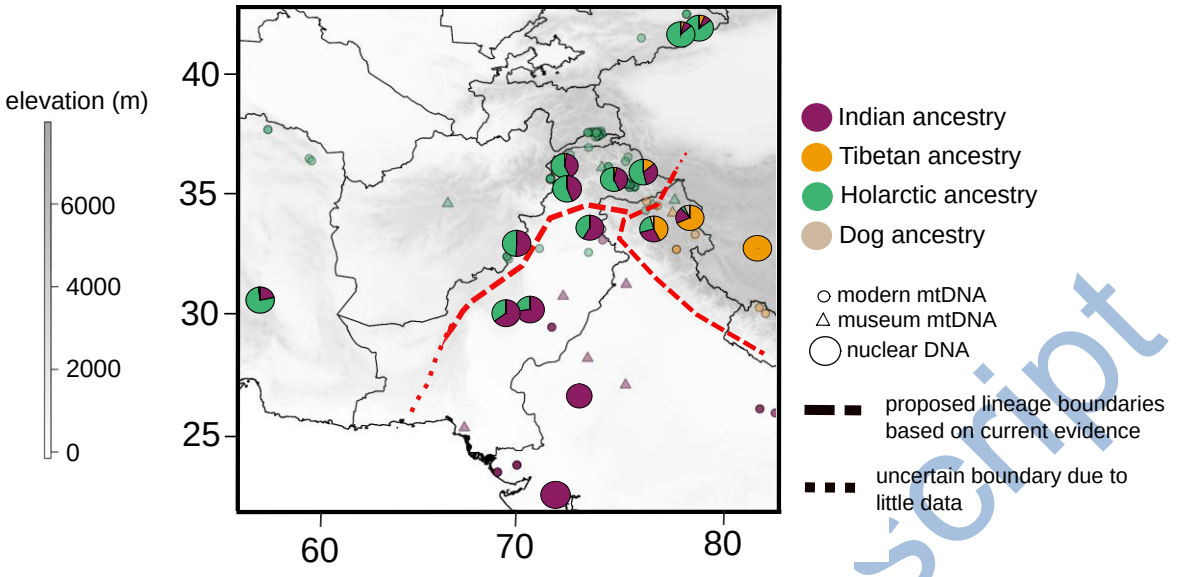

Figure 4. Proposed lineage boundaries of the three major wolf lineages based on currently available mitochondrial and genomic data. Each point represents an individual wolf sample with colors indicating which of the three ancestries each wolf assigns to based on K=5 in our NGSadmix analysis. The red thick dashed line indicates the proposed lineage boundaries for the Indian, Tibetan, and Holarctic lineages in this region of Southern Asia. We use a dashed rather than solid line to indicate uncertainty in the lineage boundaries. The thinner dashed line indicates regions where there is higher uncertainty for estimating the boundary between the Indian, Tibetan, and/or Holarctic lineages due to lack of sampling.

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