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RESEARCH ARTICLE

Genetic diversity, structure, and demographic histories of unique and ancient wolf lineages in India

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Abstract

Assessing genetic diversity, population connectivity, demographic patterns, and phylogeographic relationships is vital for understanding the evolutionary history of species and thus aid in conservation management decisions. Indian wolves (currently, *Canis lupus pallipes* and *Canis lupus chanco*) are considered ancient, unique and divergent lineages among grey wolves, yet their population genetics are poorly understood. To void this knowledge gap, we collected samples from Indian peninsular ($n = 77$) and Himalayan wolves ($n = 24$) and used a combination of maternal (mtDNA CR and Cyt *b*) and biparental (nuclear microsatellites) markers to estimate levels of genetic diversity, examine the patterns of genetic structuring between them and within their distribution range, and assess their demographic histories. Both the wolf populations showed moderate levels of genetic variability, comparable to other grey wolves. Low levels of genetic differentiation were observed within both the Indian peninsular and Himalayan wolves indicating high levels of gene flow within their populations. On the other hand, high levels of genetic differentiation were observed between the two wolves indicating absence of gene flow. Molecular analysis highlighted the uniqueness of both the Indian wolves which was further supported by the presence of unique haplotypes indicating no admixture between them. Demographic analysis using both mtDNA and microsatellites revealed decline in population sizes of both the wolf lineages and both have undergone bottlenecks. Estimates of past effective population size revealed recent population declines of both lineages of Indian wolves at around 25–50 generations corresponding to about 100–200 years ago. Our results further support the designation of both lineages of Indian wolves as two distinct species *Canis pallipes* and *Canis himalayensis* and suggest increasing conservation efforts to save the unique and ancient wolf species from extinction.

Keywords Grey wolf · Phylogeography · Phylogenetics · Population structure · Bottleneck

Introduction

Habitat modification and direct persecution by humans is considered the prime reason for the recent historic declines in most of the carnivores over the past centuries (Ripple et al. 2014). Species occurring in low densities are highly vulnerable to habitat loss and fragmentation, which operates through demographic and genetic stochasticity to enhance chances of local population extinctions (Lande 1993; Crooks 2002). Land use changes at landscape scales can severely affect the ability of animals to move between suitable habitat patches, thereby causing population fragmentation, reproductive isolation, and loss of fitness (Frankham 2006; Allendorf et al. 2013). Genetic diversity plays an important role in individual and population level fitness by endowing ability to adapt to environmental changes (Hughes et al. 2008). Genetic diversity and population sizes are negatively correlated and therefore small populations that have reduced genetic diversity are more prone to extinction events due to demographic stochasticity and inbreeding depression (Reed and Frankham 2003; Frankham 2006). Low genetic diversity is therefore concerning especially with species that exists as small populations and with limited gene flow between them (Roelke et al. 1993; Banks et al. 2013). Hence, assessing genetic diversity and connectivity for species populations is vital for formulating conservation policy and management.

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The grey wolf *Canis lupus,* is usually considered to be one of the most widely distributed large carnivore inhabiting major parts of the Northern hemisphere from the High Arctic to the deserts and dry shrub lands of the Middle East and southern North America (Mech 1974; Mech and Boitani 2010). Although considered abundant, global reduction in the wolf's historic range by 68% and total elimination from a few areas have been recorded in the last few hundred years due to persecution (Musiani et al. 2010; Ripple et al. 2014). Thirty-two subspecies of *Canis lupus* have been recognized across their global distribution range (Wilson and Reeders 2005). Numerous morphological, mitochondrial DNA (mtDNA), nuclear markers, and genomic analysis have enhanced our understanding of the evolutionary history of grey wolves. Whole mitogenome analysis revealed that some of the mitochondrial lineages observed in ancient wolves were absent in contemporary wolves, indicating extinction or replacement of these ancient lineages (Thalmann et al. 2013; Koblmüller et al. 2016). Similarly, genome wide analysis revealed widespread admixture among *Canis* species including grey wolves-dogs, golden jackals-grey wolves, coyotes-grey wolves, and undefined ghost lineage present day species (VonHoldt et al., 2011; Fan et al. 2016; VonHoldt et al. 2016; Gopalakrishnan et al. 2018; Pilot et al. 2019; Wang et al. 2020).

Despite grey wolf's wide distribution and complex evolutionary history, the majority of the molecular studies have been from North America and Europe with comparatively few studies from Asia. Based on phylogenetic analysis using mtDNA, wolf lineages: African wolf *Canis lupaster*, Himalayan wolf *Canis lupus himalayensis* and Indian wolf *Canis lupus pallipes* diverged as monophyletic sister clades and are proposed to be genetically distinct from contemporary Holarctic grey wolves (*C. lupus*) and merit distinct species status (Aggarwal et al. 2003, 2007; Sharma et al. 2003; Rueness et al. 2011; Gaubert et al. 2012; Werhahn et al. 2017, 2018; Hennelly et al. 2021; Wang et al. 2022). Divergence date estimates based on mitochondrial sequences splits Himalayan wolves and other wolves between 0.55 and 0.8 Mya (Million years ago) and Indian peninsular wolves and other wolves between 0.27 and 0.4 Mya (Sharma et al. 2003; Werhahn et al. 2018). While, Fan et al., (2016) estimated the split time between Eurasian and North American wolves to be 12,500 years using nuclear genomic data. Based on this analysis it seems likely that the split times between the Indian wolves and other wolves could be more recent compared to earlier estimates obtained from mtDNA sequence data.

IUCN lists both of these Indian wolf lineages as least concern (Boitani et al. 2020), since they are not formally considered as distinct species or sub-species. But regionally, both are considered endangered and protected under Schedule I of the Indian Wildlife (Protection) Act, 1972 and appendix 1 of Convention on International trade in Endangered Species of Wild Fauna and Flora (CITES). Most of the research carried out on Indian wolves is limited to ecology with few studies on molecular phylogenetics highlighting their unique ancestry. Srinivas and Jhala (2021) analysed cranial measurements of Indian wolf museum specimens and found that Himalayan wolves had largest skulls but could not reliably distinguish them from Indian peninsular wolves even after considering cranial measurements and structural morphology of the hair. The Indian peninsular wolf population is estimated at 3200 individuals (Jhala et al., 2022) and Himalayan wolves estimated at 400–1000 individuals (Fox and Chundawat 1995). Poisoning of wolves, poaching for pelts in the Himalayas, hybridizing and swamping of wild wolf gene pools with dogs, and diseases spread by dogs are considered major threats to the survival of Indian wolves (Jhala and Giles 1991; Jhala 2003; Vanak and Gompper 2009).

Population genetics and hybridization studies suggest that wolves disperse over long distances and can hybridize with other members of the genus *Canis*, a process that promotes gene flow, retains high level of genetic diversity and reduce levels of population diversification (Vilà and Wayne 1999; Pilot et al. 2010; Fan et al. 2016). However, due to increasing anthropogenic activities like modification of landscapes and infrastructure developments, dispersal can be compromised, thereby fragmenting wolf populations and causing decline in genetic diversity. Moreover, wolves from India were considered as unique and ancient, but still nothing was known about their population genetics. Therefore, in this study, we use nuclear microsatellite genotyping and mtDNA sequencing for both Indian peninsular and Himalayan wolves to estimate levels of genetic diversity, examine the patterns of genetic structuring across their distribution range, and assess their demographic parameters over historical times. The results obtained will contribute to a better understanding of their evolutionary history, current genetic diversity, and gene flow within and between extant wolves.

Materials and methods

Sampling and DNA extraction

Tissue, blood and hair samples (Indian wolves, $n = 77$; Himalayan wolves, $n = 24$) used in this study were collected between 1995 and 2020 from a radio-telemetry study on Indian wolves, collections from known origin wolves in zoological parks, and those provided by State Forest Departments (Fig. 1A). Capture and radio-collaring permissions were obtained from the Director of Wildlife Preservation of the Government of India and the Chief Wildlife Warden of the States as per Wildlife (Protection)

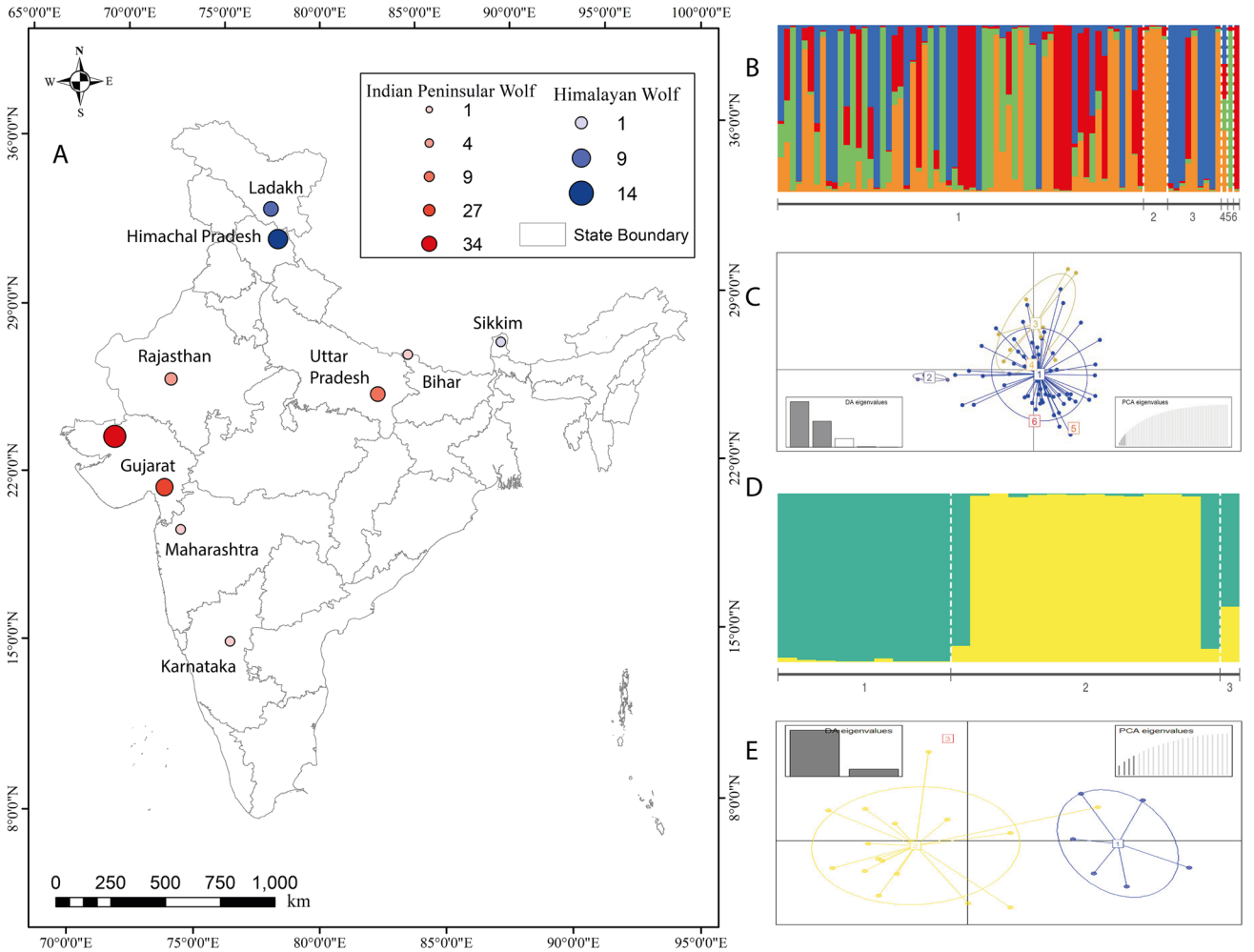

Fig. 1 Map showing the sampling locations of both the Indian peninsular and Himalayan wolves used in this study (**A**). Population genetic structure analysis results of Indian peninsular (**B, C**) and Himalayan wolves (**D, E**) based on 25 microsatellite loci. Summary bar plots indicating genetic structure of 77 individual Indian peninsular wolves using STRUCTURE runs at K = 4 (**B**). Each individual is represented by a vertical bar, and the coloured length of each bar indicates the probability of membership in each cluster. Scatterplot of DAPC (**C**) analysis with each Indian peninsular wolf population indicated by different colour codes and inertia ellipses shown on the first two axes. The populations 1, 2, 3, 4, 5 and 6 represents Gujarat, Rajasthan, Uttar Pradesh, Bihar, Maharashtra, and Karnataka respectively. Summary bar plots indicating genetic structure of 24 individual Himalayan wolves using STRUCTURE runs at $K = 2$ (**D**). Each individual is represented by a vertical bar, and the coloured length of each bar indicates the probability of membership in each cluster. Population differentiation of Himalayan wolves (**E**) as represented on the first axis of the discriminant analysis of principal components. The sampling localities 1, 2, and 3 represents Ladakh, Himachal Pradesh and North Sikkim respectively

Act, 1972. The research was approved by the Training, Research & Academic Council of the Wildlife Institute of India that also considers the ethical aspects of the research before approval. Samples were either stored in 90% ethanol or buffered solutions and later shifted to the laboratory for storage at − 20 °C until DNA extraction. Genomic DNA was extracted from DNeasy blood and tissue kit (Qiagen, Germany) following the manufacturer's conditions. All extractions were carried out in batches that included negative controls to monitor for potential contamination.

Mitochondrial sequencing

Two mitochondrial markers: a) ThrL15926 and DL-H16340 were used to amplify 440 bp fragment of control region (CR, Vilà et al. 1999) and b) Canid L1 (Paxinos et al. 1997) and H15149 (Kocher et al. 1989) to amplify 412 bp fragment of cytochrome b gene (Cyt *b*). Polymerase chain reactions (PCR) were carried out in a volume of 10 µl including 1X PCR buffer, 0.2 mM of each deoxynucleotide triphosphate, 0.5 µM of each primer, 0.5 units of Taq DNA polymerase (Thermo Fisher Scientific, USA) and 20–300 ng of template DNA, with thermocycling conditions as detailed in

earlier study on Indian canids (Sharma et al. 2003). A small aliquot of each amplified PCR product was visualized on a 2% agarose gel, to ensure amplification of Cyt *b* and CR fragments. The PCR products were then cleaned using ExoSAP (Thermo Fisher Scientific, USA) and sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following manufacturer's instructions on ABI 3500 sequencer (Applied Biosystems, USA). All PCR reactions had negative and positive controls to check for contamination and amplification success.

Microsatellite genotyping

A panel of 25 fluorescently labelled autosomal microsatellites which were used in earlier studies on wolves, jackals and dogs were amplified (Supplementary Table 1Ostrander et al. 1993; Fredholm and Winterø, 1995; Francisco et al. 1996; Neff et al. 1999; Breen et al. 2001; Ichikawa et al. 2002; Hellmann et al. 2006). The microsatellite markers were selected based on their polymorphism, effective number of alleles, and amplification success in other canid studies. All the PCR amplifications were carried out in four multiplex reactions (detailed in Supplementary Table 1) using the Qiagen Multiplex master mix Kit (Qiagen, Germany) following the manufacturer's protocol in a total volume of 10 µl with 5 µl PCR buffer, 2 pmol of each primer pair and 2 µl DNA for each reaction. The thermocycling conditions for all the four multiplex reactions includes: initial denaturation at 95 °C for 15 min, followed by 11 cycles at 94 °C for 30 s, 58 °C for 30 s with touchdown of 0.5 °C per cycle and 72 °C for 1 min, and 28 cycles at 94 °C for 30 s, 52 °C for 30 s with touchdown of 0.5 °C per cycle and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. Molecular size standard, GeneScanTM 500 LIZ (Applied Biosystems, USA) was added to the diluted PCR products to identify the size of amplified microsatellite loci and analysed using an ABI PRISM 3500 (Applied Biosystems, USA) automatic sequencer. Negative controls were included for all the microsatellite genotyping amplification reactions to monitor for contamination. Finally, all the alleles observed were scored manually for each microsatellite using Genemapper v4.0 (Applied Biosystems, USA).

Data analysis

Mitochondrial DNA

Mitochondrial diversity estimates including number of haplotypes N_H, total number of polymorphic sites N_S, nucleotide diversity π, and haplotype diversity H using CR and Cyt *b* alignments were estimated using the program Arlequin v. 3.5 (Excoffier and Lischer 2010). To test for demographic signatures from CR sequences, Tajima's D (Tajima 1989), Fu's *F*s (Fu 1997) statistics and associated significance values were inferred using Arlequin v. 3.5 (Excoffier and Lischer 2010) and DnaSP v. 6.12 (Rozas et al. 2017). Additionally, to look for changes in population abundance in the recent past, we used mismatch distribution tests and Harpending's raggedness index for both the Indian peninsular and Himalayan wolves using DnaSP v. 6.12 (Rozas et al. 2017).

Median joining haplotype networks were constructed with CR and Cyt *b* sequences to understand the phylogeographic structure within both the Indian wolves using the program PopArt v. 1.7 (Leigh and Bryant 2015). Samples used for mtDNA based phylogeographic network analyses were inclusive of samples previously reported in earlier studies on Indian wolves (Sharma et al. 2003; Joshi et al. 2020). The network calculations were carried out using default values for the epsilon parameter (epsilon = 0) by assigning equal weights to all the variable sites. Bayesian phylogenetic tree was constructed using control region ($n = 184$) and Cyt *b* ($n = 187$) haplotypes to infer the evolutionary relationship of Indian peninsular and Himalayan wolves in relation to other wolf and wolf like canids for which genetic data from the same markers was available. Akaike information criterion (Akaike 1974) criterion implemented in the jModelTest program (Posada 2008) was used to estimate most plausible and parsimonious nucleotide substitution for the data set. Phylogenetic analysis was carried out in MrBayes v. 3.2 (Ronquist et al. 2012) with chain length of 25 million generations of Markov chain Monte Carlo (MCMC) simulations, sampled every 1000 generations, with the first 5 million runs discarded as burn-ins. Finally, FigTree v. 1.4 (http://tree.bio.ed.ac.uk/software/ figtree/) was used to view and annotate the consensus phylogenetic tree. Dhole (*Cuon alpinus*), African wild dog (*Lycaon pictus*), golden jackals (*Canis aureus*), side striped jackals (*Canis adustus*), black backed jackals (*Canis mesomelas*), Ethiopian wolf (*Canis simensis*), and African golden wolf (*Canis lupaster*) sequences were used along with wolf sequences in the phylogenetic tree construction.

Microsatellites

The program CREATE (Coombs et al. 2008) was used to prepare input files for all the microsatellite based population genetic analyses. Presence of null alleles and typographic errors due to stuttering patterns and small allele dominance were tested using MICRO-CHECKER v 2.2 (Van Oosterhout et al. 2004). Microsatellite replicate data was quantified using PEDANT v1.0 (Johnson and Haydon 2007), a maximum likelihood approach, to estimate allele dropout (ADO) and false allele (FA) error rates. Probability of individual identity $P_{(ID)}$ and probability of identity of siblings $P_{(ID\text{-}sibs)}$

were calculated using identity analysis option implemented in the program CERVUS v 3.0 (Kalinowski et al. 2007). Microsatellite based genetic diversity estimates including number of alleles (*Na*), expected (*He*) and observed (*Ho*) heterozygosity, polymorphic information criterion (PIC) were estimated for both the Indian peninsular and Himalayan wolves using CERVUS v 3.0 (Kalinowski et al. 2007). Additionally, allelic richness (AR), mean number of alleles (MNA) and Inbreeding co-efficient (F_{IS}) values were estimated using FSTAT v 2.9 (Goudet 1995). Deviations from Hardy–Weinberg Equilibrium (HWE) and linkage disequilibrium among pairs of loci were tested using exact tests in GENEPOP v 1.2 (Raymond and Rousset 1995). Significance levels were adjusted with sequential Bonferroni corrections for all multiple comparisons (Rice 1989).

Multi-locus genotypes were analysed using Bayesian clustering and non-Bayesian multivariate approaches to detect population structuring between the predefined sites (Fig. 1A) within and between the Indian peninsular and Himalayan wolf populations. Non-spatially explicit Bayesian clustering approach implemented in STRUCTURE v 2.3 (Pritchard et al. 2000) was used to identify the optimal number of genetic clusters (K). Analysis was carried out by running 500,000 Markov chain Monte Carlo (MCMC) iterations with a burn-in runs of 100,000 without prior (LOCPRIOR = 0) location information, following admixture model and correlated allele frequencies within populations. Each K was allowed to vary between 1 and 10 with 20 independent runs for each value of K. The optimal value of K was identified using the web version of STRUCTURE HARVESTER v 0.6.94 (Earl and vonHoldt 2012) by computing i) mean posterior probability for each K value, LnP(D) and ii) rate of change in the likelihood of K values (delta K). A stringent criterion of $q > 0.8$ value was used for assigning individuals as residents of a population and value less than 0.8 were considered with admixed ancestry (Yumnam et al. 2014). The assignment probability plots were produced using the web version PopHelper (Francis 2017). A non-Bayesian multivariate analysis was carried out using the Discriminant Analysis of Principal Components (DAPC) using the package Adegenet (Jombart 2008) in R v 3.2 (R Core Team 2015). DAPC analysis is not constrained by the assumptions of HWE or LD which applies to STRUCTURE analysis in identifying the genetic clusters.

Genetic signatures of historic demographic contractions or bottleneck events were tested using two approaches. First, M-ratio test or Garza–Williamson index (G-W) (Garza and Williamson 2001) implemented in Arlequin v. 3.5 (Excoffier and Lischer 2010) was used by comparing number of alleles (k) with allelic size range (r). Second, Wilcoxon sign rank test (Cornuet and Luikart 1996) implemented in the program BOTTLENECK v 1.2 (Piry et al. 1999) was used to test for departure from mutation-drift equilibrium based on heterozygote deficiency or excess. Analyses were performed with 10,000 simulations for each of the following mutation models: single stepwise mutation model (SSM) and two phase model (TPM) with frequency of 0.12 and 0.95 as generic values and variance as recommended (Piry et al. 1999). The allelic distribution curve for both the Indian peninsular and Himalayan wolves were plotted using program BOTTLENECK v 1.2 (Piry et al. 1999), to check for L shape (indicative of population equilibrium) or mode shift (typically observed in bottlenecked populations) curves.

Estimating posterior distributions of demographic parameters and history using coalescence theory and MCMC sampling were considered more robust to certain violations of mutation model assumptions (Girod et al. 2011) and bottleneck duration (Peery et al. 2012). Therefore, algorithm and methods available in program VarEff v 1.2 (Chevalet and Nikolic 2010; Nikolic and Chevalet 2014) were used in the R package (https://forge-dga.jouy.inra.fr/projects/package-vareff-variation-of-effective-population-size/) to estimate historic contractions of effective population size using autosomal microsatellite markers. VarEff v 1.2 (Chevalet and Nikolic 2010) assumes a stepwise mutation model, making use of an approximate likelihood of data and an MCMC framework to simulate past demography by sampling step functions. Moreover, VarEff provides information on the occurrence and time of bottleneck events in the past by recovering the posterior distribution at the time to the most recent common ancestor (TMRCA) between alleles. The observed peaks in these distributions indicate times when coalescence events likely occurred, and intervals between peaks may indicate periods when bottlenecks occurred. Analysis was carried out using a Two-phase Mutation Model (TPM) with proportion for multi-step mutations of $c = 0.2$ and an average microsatellite mutation rate (μ) of 3×10^{-3} per generation (Francisco et al. 1996; Parra et al. 2010). Effective population size was estimated from present to the last 10,000 generations in the past, assuming the generation time (G) of 4.3–4.7 years for wolves (Mech et al. 2016).

Results

Mitochondrial sequencing analysis

Uniqueness of Indian wolves

The Bayesian phylogenetic trees constructed using both the CR and Cyt *b* sequences separately converged in showing the uniqueness of both the Indian peninsular and Himalayan wolves. The Bayesian phylogenetic tree constructed using CR clustered all the Himalayan wolves into a single basal clade to all other Holarctic wolves and dogs. The Indian wolves (*Canis lupus pallipes*) which includes Indian

peninsular and wolves from Bangladesh clustered together within a single clade but separated from the large clade consisting of Holarctic wolves and dogs. However, wolves from Pakistan and Iran clustered within the clade of Holarctic wolves (Fig. 2). On the other hand, the phylogenetic tree constructed using Cyt *b* gene fragments clustered the Himalayan wolves and Indian peninsular wolves basal and distinct from the clade of Holarctic grey wolves and dogs (Supplementary Fig. 1).

Indian peninsular wolf

Indian peninsular wolves ($n = 105$) had mt-DNA diversity values of $H = 0.704 \pm 0.029$, $\pi = 0.003 \pm 0.002$, with mean number of pairwise differences of 1.085 ± 0.719 and seven polymorphic sites within the 336 bp CR gene fragment. A total of six haplotypes were observed for the CR gene fragment including the four haplotypes reported earlier (Table 1 and Supplementary Table 2). Similarly, within Cyt *b* gene fragment of 329 bps, genetic diversity of $H = 0.694 \pm 0.038$, $\pi = 0.003 \pm 0.002$ ($n = 88$), mean number of pairwise differences of 1.089 ± 0.7223 and five polymorphic sites were observed. Six haplotypes were identified in the Cyt *b* gene fragment and all the haplotypes were reported for the first time for Indian peninsular wolves (Table 1 and Supplementary Table 3). The demographic statistics based on Tajima's D (-0.441, $p = 0.388$) and Fu's Fs (-0.072, $p = 0.556$) values were negative using CR for Indian peninsular wolves, but values were non-significant indicating population equilibrium. Observed values for mismatch distribution of pairwise differences among all the Indian peninsular wolves showed right-skewed unimodal peak. On the other hand, distribution of observed mismatch values showed a good fit with the expected distribution values further supported with the low and non-significant Harpending's raggedness index with value of $r = 0.095$ ($p = 0.53$) rejecting the null hypothesis of population expansion (Supplementary Fig. 2A).

The median joining haplotype network constructed for Indian peninsular wolves using CR sequences revealed weakly structured haplotype network with six haplotypes distributed in multiple alternative connections (Fig. 3A). All six haplotypes were separated from one another by one to four single base pair substitutions. Hap_1 was observed to be the most dominant distributed in Gujarat ($n = 38$), Uttar Pradesh ($n = 2$), Maharashtra ($n = 2$), Tamil Nadu ($n = 4$), and ancient samples ($n = 2$, collected from Rajasthan and West Bengal), followed by Hap_2 ($n = 21$) and Hap_4 ($n = 21$) haplotypes (Supplementary Table 2 and Fig. 3A). The median joining haplotype network constructed using Cyt *b* sequences also revealed a weakly structured haplotype network with six haplotypes distributed in multiple alternative connections (Fig. 3C). All six haplotypes were separated from one another by one to two single base pair substitutions. Hap_2 was observed to be the most dominant haplotype distributed in Gujarat ($n = 27$), Rajasthan ($n = 3$), Uttar Pradesh ($n = 5$), and Tamil Nadu ($n = 4$), followed by Hap_1, which is distributed in Gujarat ($n = 18$), Rajasthan ($n = 3$), and Maharashtra ($n = 1$) regions (Supplementary Table 3 and Fig. 3B).

Himalayan wolf

Himalayan wolves ($n = 49$) had mt-DNA diversity values of $H = 0.751 \pm 0.038$, $\pi = 0.136 \pm 0.066$ with mean number of pairwise differences of 5.275 ± 3.961 and 10 polymorphic sites observed within the 335 bp CR gene fragment. A total of seven haplotypes were observed in CR gene fragment including the six haplotypes reported earlier (Table 1 and Supplementary Table 4). Similarly, low genetic diversity was also observed for the 279 bps Cyt *b* gene fragment ($n = 42$; $H = 0.459 \pm 0.080$, $\pi = 0.001 \pm 0.001$) with mean number of pairwise differences of 0.501 ± 0.435 and two polymorphic sites observed. Three haplotypes were identified in the Cyt *b* gene fragment including the two haplotypes reported earlier (Table 1 and Supplementary Table 5). Tajima's D (-0.072, $p = 0.556$) and Fu's Fs (-3.067, $p = 0.718$) values for CR were negative and non-significant for Himalayan wolves, indicative of population equilibrium. Observed values for mismatch distribution of pairwise differences among all the Himalayan wolves showed multimodal peak. Distribution of observed mismatch values showed a good fit with the expected values which further supported by significant Harpending's raggedness index value of $r = 0.125$ ($p = 0.02$) indicative of population equilibrium (Supplementary Fig. 2B).

The median joining haplotype network constructed for Himalayan wolves using CR sequences revealed weakly structured haplotype network with seven haplotypes distributed in multiple alternative connections (Fig. 3B). All seven haplotypes were separated from one another by one to three single base pair substitutions. Hap_2 was observed to be the most dominant haplotype distributed in Himachal Pradesh ($n = 15$), Uttarakhand (1), Ladakh (5), and ancient samples ($n = 2$), followed by Hap_1 ($n = 18$) which was observed in the Himachal Pradesh ($n = 16$) and ancient samples ($n = 2$, collected from Nepal) regions (Supplementary Table 4 and Fig. 3B). The median joining haplotype network constructed using Cyt *b* sequences also revealed a weakly structured haplotype network with three haplotypes (Fig. 3D). All three haplotypes were separated from one another by a single base pair substitution. Hap_2 was observed to be the most dominant haplotype distributed in Himachal Pradesh ($n = 30$), followed by Hap_1, restricted to Himachal Pradesh ($n = 6$), and Hap_3 restricted to the Ladakh ($n = 6$) region (Supplementary Table 5 and Fig. 3D).

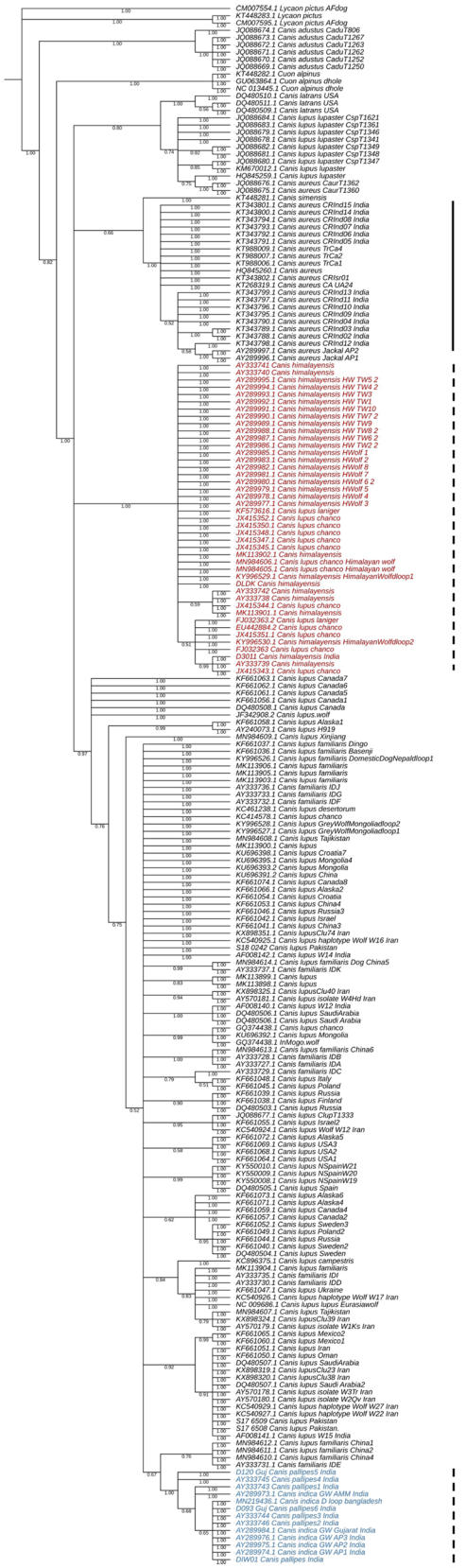

Fig. 2 Bayesian phylogenetic relationships of the Indian peninsular and Himalayan wolves to Holarctic wolves and other *Canis* species based on CR DNA sequences with GenBank accession numbers

Table 1 Measures of genetic diversity estimates (microsatellite and mtDNA) for Indian peninsular and Himalayan wolves

Diversity estimates	Indian Peninsular wolf	Himalayan wolf
mtDNA control region		
Sample size	105	49
No. of haplotypes	6	6
Haplotype diversity	0.704 (0.029)	0.751 (0.038)
Nucleotide diversity	0.003 (0.002)	0.136 (0.066)
Tajima's D (*p*-value)	(− 0.441 (0.388)	(− 0.072 (0.556)
Fu's Fs (*p*-value)	(− 0.072 (0.556)	(− 3.067 (0.718)
Microsatellite DNA		
Sample size	77	24
Mean alleles/locus	7 (1.658)	4.24 (0.831)
Allelic range	21.440 (8.973)	12.960 (5.660)
Expected heterozygosity	0.702 (0.065)	0.638 (0.057)
Observed heterozygosity	0.686 (0.079)	0.695 (0.097)
FIS	0.153	− 0.079

*SD values are denoted in brackets

Microsatellite genotyping analysis

Genetic structure between the Indian wolves

STRUCTURE v 2.3 analysis considering both the Indian peninsular and Himalayan wolves suggested the most probable number of genetic clusters to be two ($k=2$) based on ΔK values using no prior locations in the model (locprior = 0) following the criterion of Evanno. The bar plot clearly showed Indian peninsular and Himalayan wolves are clustered separately, indicating absence of admixture or gene flow between them (Fig. 4A). DAPC analysis also suggested the presence of two genetic clusters, with no overlap, further supporting the results of STRUCTURE analysis (Fig. 4B).

Indian peninsular wolf

Multilocus genotypes were obtained at all the 25 microsatellites in both the Indian peninsular and Himalayan wolves with all individuals typed for all 25 loci (100% success rate). The number of alleles in Indian peninsular wolves ranged between 4 (VBUFX) to 11 (F2140) with a mean number of alleles per locus at seven, the allelic size range was 21.40, the observed and expected heterozygosity values were 0.68

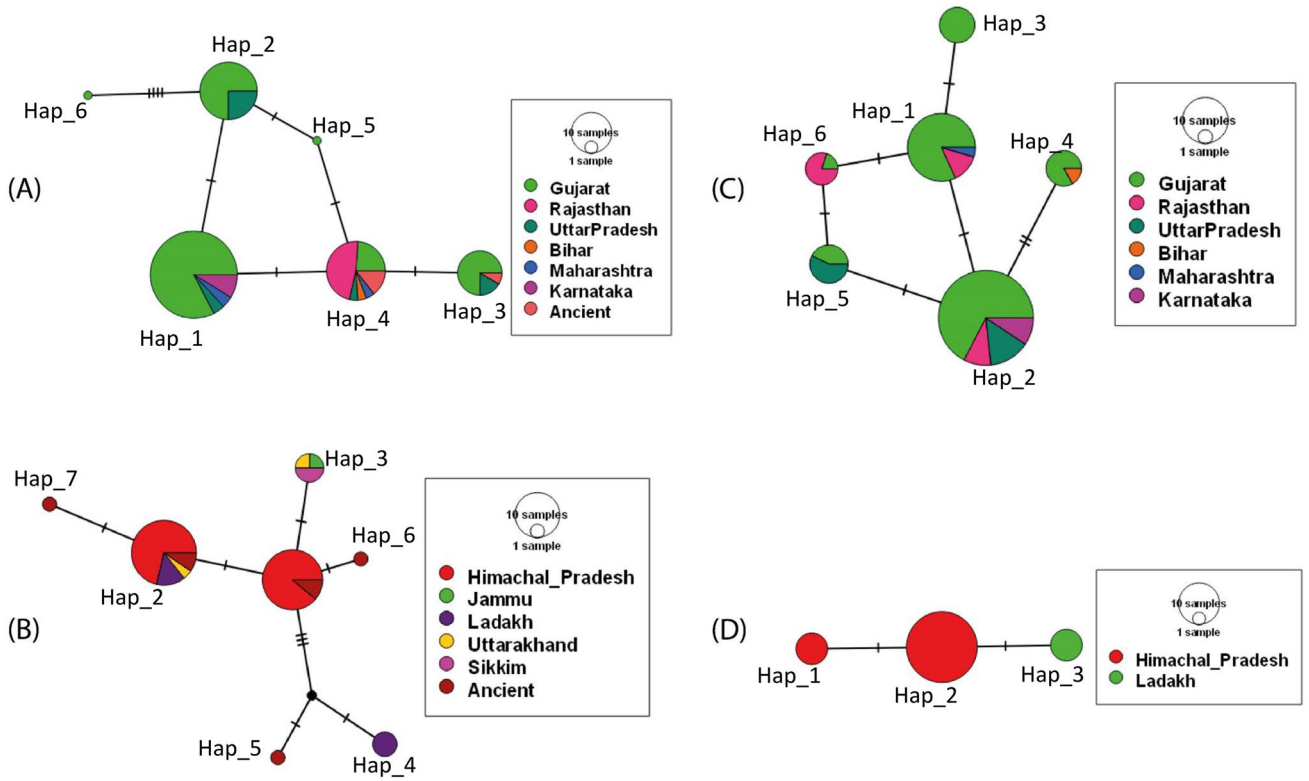

Fig. 3 Median joining haplotype network constructed using mtDNA CR (**A**) and Cyt *b* (**C**) in Indian peninsular wolves and mtDNA CR (**B**) and Cyt *b* (**D**) in Himalayan wolves. Haplotype circles are colour coded according to geographic locality, and circle size is proportional to haplotype frequency

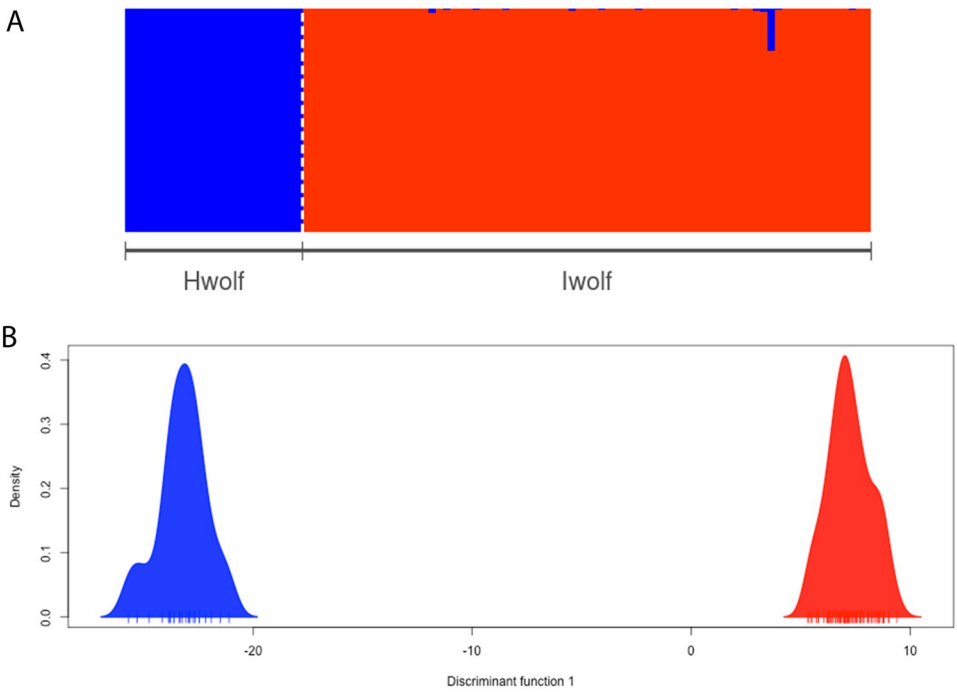

Fig. 4 Genetic structure of both Indian peninsular and Himalayan wolves based on 25 nuclear microsatellites. STRUCTURE results of Indian wolves ($N=77$) and Himalayan wolves ($N=24$) considering admixture model implemented in STRUCTU RE v 2.3 with no prior location information (**A**). Scatterplot of DAPC with each wolf indicated by different colour codes and inertia ellipses shown on first two axes (**B**). The red colour represents Indian peninsular wolves and blue represents Himalayan wolves for both figures

and 0.70 respectively, and PIC value was 0.76 (Table 1 and Supplementary Table 6). The panel of microsatellite markers used for individual identification had a cumulative probability (P_{ID}) value of 1.13×10^{-30} and probability of correctly identifying siblings ($P_{ID}-_{sibs}$) from each other was 1.33×10^{-11} indicating sufficient power of the microsatellites to discriminate amongst individuals and siblings. Genotyping error rates varied among microsatellite loci with mean maximum likelihood allelic drop out per genotype at 0.007 and false allele per genotype at 0.014. Presence of null alleles was detected at four loci (CXX436, CXX253, MCPH2, and CXX279) with no evidence of stuttering and small allele dominance. After Bonferroni corrections, significant deviation from HWE was observed for one locus (FH2010) and LD was detected between 8 pairs of loci. The mean inbreeding coefficient (F_{IS}) was positive (0.15) for Indian peninsular wolves (Table 1 and Supplementary Table 6).

Bayesian analysis in STRUCTURE v 2.3 without prior location data (locprior = 0) suggested the most probable number of genetic clusters based on delta K (ΔK, Supplementary Fig. 3A) and log likelihood values for K (Ln (K) = − 6310.62, Supplementary Fig. 3B) to be four ($K=4$). The STRUCTURE bar plots showed no clear clustering patterns across populations indicating high levels of admixture (Fig. 1B and Supplementary Fig. 3C). DAPC analysis suggested the presence of two genetic clusters with individuals from Rajasthan showing distinctiveness whereas the remaining populations showed admixture (Fig. 1C).

Bottleneck analysis using sign test for detecting heterozygosity excess showed evidence of genetic bottlenecks. Wilcoxon test for heterozygosity excess was also significant for two microsatellite mutation models i.e. TPM and SMM (Supplementary Table 8). The allelic frequency distribution curve was of shifted mode for Indian peninsular wolves, indicative of population decline and genetic bottleneck. On the other hand, M-ratio or G-W index values for Indian peninsular wolves was 0.33 which is less than 0.68, indicative of a genetic bottleneck (Supplementary Table 8). Estimates of past effective population based on MCMC approach implemented in Vareff indicated a mean effective population size (*Ne*) of ~ 3500 at ~ 5000 generations (quantile range of 5 and 95%: 51–15,219), showed a declining trend until ~ 1100 generations before present. The population later showed an incline with a massive increase in effective population size till ~ 800 generations and further maintained the effective population size till almost the last ~ 50 generations (Ne: 8421, quantile range: 1382–20,000) which corresponds to around 225 Ybp. The population then showed a drastic decline or a bottleneck event during the past 25–50 generations which corresponds to ~ 115–225 Ybp considering the generation time of wolves as 4.5 years (Fig. 5A).

Himalayan wolf

The number of alleles in Himalayan wolves ranged between 3 and 6 with mean number of alleles per locus at 4.24, the mean allelic size range was 12.96, the observed and expected heterozygosity values were 0.69 and 0.63

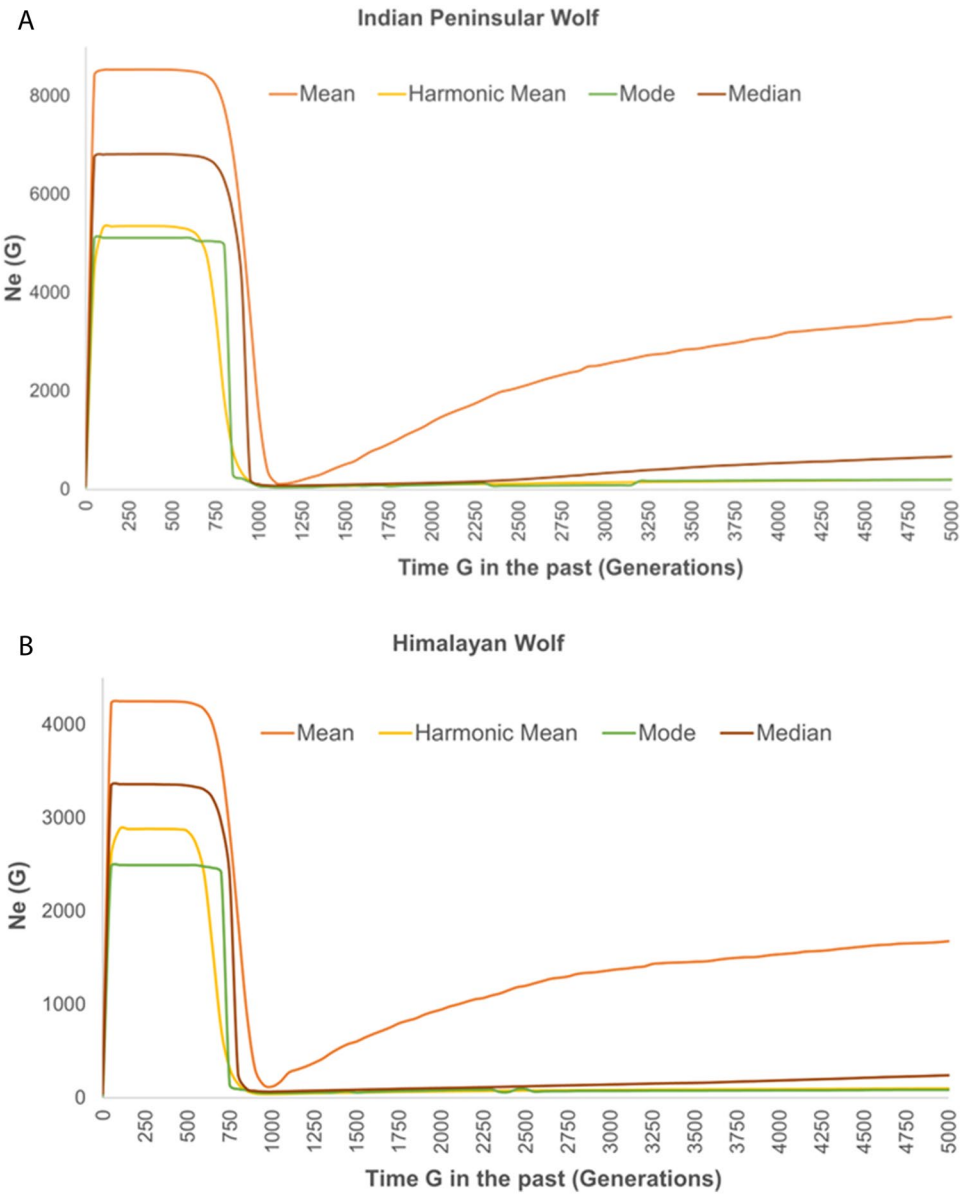

Fig. 5 Estimates of effective population size (Ne) of Indian peninsular wolves (**A**) and Himalayan wolves (**B**) based on 25 polymorphic microsatellite loci using approximate likelihood MCMC approach in VarEff v.1.2 package in R. The coloured lines indicate Arithmetic mean of Ne (red), Harmonic mean (orange), mode (blue) and median (black) respectively

respectively, and PIC value was 0.67 (Table 1 and Supplementary Table 7). The mean observed heterozygosity (Ho = 0.69) was slightly higher in comparison with expected heterozygosity (He = 0.63). The panel of microsatellite markers used for individual identification had cumulative probability of identity (P_{ID}) value of 1.38×10^{-23} and probability of identity siblings ($P_{ID\text{-}sibs}$) value of 3.66×10^{-10} indicating sufficient power of the microsatellites to discriminate between related individuals. Genotyping error rates varied among microsatellite loci with mean maximum likelihood allelic drop out per genotype was 0.001 and false allele per genotype was 0.006 (Table 1 and Supplementary Table 7). None of the loci showed presence of null alleles with no evidence of stuttering and small allele dominance. After Bonferroni corrections, none of the loci deviated from HWE and no LD was detected between any pair of loci. The mean inbreeding coefficient (F_{IS}) value was negative (− 0.07) for Himalayan wolves (Table 1).

Non-spatially explicit Bayesian analysis in STRUCTURE v 2.3 suggested the most probable number of genetic clusters based on delta K (ΔK) values to be two ($K = 2$) with no prior location model (locprior = 0) (Supplementary Fig. 4A). On the other hand, the log likelihood values for no prior (Ln (K) = − 1599.46) information reached an inflection point at $K = 3$ (Supplementary Fig. 4B) but with a flat curve with wide error margins and were therefore quite uninformative. The bar plots showed clear clustering patterns across populations where few samples from the Spiti region showed as a separate cluster whereas samples from the remaining areas of North Sikkim, Ladakh and few samples from Spiti

showed as a second cluster (Fig. 1D and Supplementary Fig. 4C). DAPC analysis also suggested the presence of two genetic clusters with patterns similar to the analysis of STRUCTURE (Fig. 1E).

Bottleneck analysis using sign test for detecting heterozygosity excess showed evidence of significant signatures of genetic bottleneck. Wilcoxon test for heterozygosity excess was also significant for the two microsatellite mutation models, i.e., TPM and SMM (Supplementary Table 8). The allelic frequency distribution curve was of shifted mode for Himalayan wolves, indicative of population declines and genetic bottleneck. M-ratio or G-W index value was 0.33, indicative of a genetic bottleneck (Supplementary Table 8). Estimates of past effective population (*Ne*) were 1676 wolves at ~ 5000 generations (quantile range of 5 and 95%: 28–6631), showed a declining trend until about ~ 1000 generations before present. The population increased substantially between ~ 1000 generations to ~ 600 generations and remained stable at this high abundance until the last ~ 50 generations (Ne: 4221, quantile range: 1476–9739) which corresponds to around 225 Ybp. The population then showed a drastic decline or a bottleneck event during the past 25–50 generations which corresponds to ~ 115–225 Ybp considering the generation time of wolves as 4.5 years (Fig. 5B).

Discussion

Indian peninsular wolf and Himalayan wolves are considered ancestral to and share a common ancestor with the contemporary Holarctic wolves and diverged as separate lineages (Sharma et al. 2003; Aggarwal et al. 2007; Werhahn et al. 2017; Hennelly et al. 2021). Interestingly, both the ancient lineages exist in India and are geographically separated with Himalayan wolves restricted to high altitudes whereas Indian peninsular wolves restricted to the lowland plains. Our findings, provide the first investigation of genetic diversity estimates, population genetic structure and demographic histories of both these Indian peninsular and Himalayan wolves.

Mitochondrial based genetic diversity estimates for Indian peninsular wolves and Himalayan wolves were moderate and almost similar. Mitochondrial DNA CR based diversity indices of Indian wolves were high when compared with other European wolf populations from Russia, Poland, Iberian Peninsula whose range of diversity indices were $H = 0.34–0.67$ and $\pi = 0.009–0.016$ for the same CR fragment (Czarnomska et al. 2013; Sastre et al. 2011). Nuclear microsatellite based genetic diversity in both Himalayan wolves ($Ho = 0.69$) and Indian peninsular wolves ($Ho = 0.68$) were similar (Table 1). The observed heterozygosity values (0.69) were slightly higher than the expected value (0.63) in Himalayan wolves (Table 1). Such scenarios were observed when previously homogenous and isolated populations were interbred allowing mixing of alleles (isolate-breaking effect). Such a scenario is not difficult to visualize amongst the Himalayan wolves, where often the high mountain ranges separating valley habitats would have become barriers to gene flow with the advent of cooler climate and perma-frost on these ranges, while allowing valley populations to mix during warmer climatic regimes. The observed heterozygosity levels in Indian wolves were comparable to wolves from European populations (Ho range: 0.65–0.75) from Poland, Bulgaria, Serbia, the Baltic States and Central Russia (Sastre et al. 2011; Czarnomska et al. 2013; Hindrikson et al. 2013; Moura et al. 2014; Szewczyk et al. 2019; Korablev et al. 2021). Heterozygosity levels in both the Indian wolves exceeded the range of heterozygosity reported in wolf populations from Italy, Finland, Spain, Slovakia, Estonia and Siberian territories (Ho:0.44-0.61) (Lucchini et al. 2002; Aspi et al. 2006; Plumer et al. 2016; Montana et al. 2017; Talala et al. 2020). Although, genetic diversity estimates across different wolf populations are strictly not comparable because of different mtDNA sequence lengths and different microsatellites used, but the estimates suggests that genetic variability observed in Indian wolves is comparable to other wolf populations. The negative F_{IS} value (− 0.07) observed in Himalayan wolves could be due to the presence of high observed heterozygosity values and a similar pattern was also observed in Siberian wolves (− 0.057; Talala et al. 2020). On the other hand, positive F_{IS} values observed in Indian peninsular wolves (0.15) denotes no inbreeding events and could be attributed to high dispersal ability of wolves thereby causing high gene flow. The mean inbreeding coefficient values found in Indian peninsular wolves were comparable with wolves from Iberia, Bulgaria, Serbia, and Slovakia (0.10–0.25) (Verardi et al. 2006; Sastre et al. 2011) but comparatively higher than wolves from Russia, Arkhangelsk, and Apennines, ($F_{IS} = 0.03–0.06$; Aspi et al. 2006; Korablev et al. 2021).

Using a combination of individual and population-based Bayesian clustering and multivariate analysis revealed low genetic differentiation within both the Indian peninsular and Himalayan wolf populations (Fig. 1B and D). Low genetic differentiation observed could be attributed to high dispersal ability of wolves (Mech and Boitani 2010) and lack of any barriers to the gene flow within and between the landscapes occupied by Indian peninsular and Himalayan wolves (Jhala and Giles 1991; Jhala 2003; Jhala et al., 2022). Moreover, the structuring observed in Himalayan wolves, especially the separation of the Himachal population, was likely due to an over representation of samples from a zoo population that was known to have originated from a single sibling pair of wild origin founders i.e. from Spiti valley, Himachal Pradesh (Fig. 1D). Himalayan wolves are critically endangered and obtaining samples was extremely difficult, hence, we choose to include these potentially related samples in the analysis.

Indian peninsular wolves also showed low genetic differentiation at geographic level, wherein individuals from all the sampled locations i.e. Gujarat, Rajasthan, Bihar, and Maharashtra, and Karnataka clustered together with high levels of admixture between them. However, wolves from Rajasthan segregated out as a distinct population cluster (Fig. 1B). We believe that the sampled wolves from Rajasthan possibly had introgression of dog DNA, since we have recorded several morphological wolf-dog hybrids from this region (Jhala unpublished data). Mitochondrial analysis also revealed no historical gene flow between Himalayan and peninsular Indian wolves, but individuals across different populations of wolves from the same lineage were observed to share haplotypes (Fig. 3A–D) suggestive of high dispersal and interbreeding within the wolf populations of the same lineages.

Microsatellite based population structuring showed total absence of any admixture between the Indian peninsular wolves and Himalayan wolves suggesting absence of gene flow between them. The result was surprising since there are no physical barriers to dispersal between the geographic ranges of these two wolves (Fig. 4A and B). Similar pattern was also observed between tundra/taiga and boreal coniferous forest wolves (Musiani et al. 2007) and Coastal British Columbia and Southeast Alaska Wolves (Weckworth et al. 2011). The Himalayan wolves are specialized to live in high altitude desert conditions (Werhahn et al. 2018; Wang et al. 2020), whereas the Indian peninsular wolves prefer semi-arid lowland open habitats (Jhala and Giles 1991; Jhala 2003; Jhala et al., 2022). The Himalayan wolves were not reported from the temperate and forest zone of the Himalayas, while the Indian peninsular wolves were not reported from the Himalayan foothills. The closest record of Indian wolves to the Himalayan Mountain range has been from the Shivalik and Shivalik foothills in Rajaji Tiger Reserve (https://www.newsclick.in/Photo-captures-Indian-grey-wolf-northern-Himalayas) and from the Churya hills of Valmiki Tiger Reserve (Maurya et al. 2021) in India. Thus, there seems to be a wide strip of the forested Himalayan ranges that are devoid of wolves separating the two wolf lineages of India. Himalayan wolves breed in the summer while the Indian peninsular wolves are the only wolves that breed in the winter (Jhala 2003), it is also possible that these wolf lineages have behavioural barriers to interbreeding.

The mitochondrial marker based Bayesian phylogenetic analysis (Fig. 2 and Supplementary Fig. 1) also supported earlier reports that suggested distinct lineages of both Indian peninsular and Himalayan wolves that were basal to the Holarctic grey wolves (Aggarwal et al. 2003, 2007; Sharma et al. 2003; Werhahn et al. 2017, 2018). The range of these two wolves and the Holarctic wolf-dog clade are likely to overlap or be in close proximity in Baluchistan and Waziristan provinces of Pakistan and further genetic sampling from this geographical region may provide better insights to gene flow between these wolves. One wolf sample from Pakistan had its CR sequence similar to that of the Holarctic wolf-dog clade (Fig. 2). Hennelly et al., (2021) using full genome sequences of Indian peninsular and Himalayan wolves also found that Indian peninsular and Himalayan wolves form evolutionary distinct and ancient lineages basal to other grey wolf populations. The same authors also highlighted that southern Asia has acted as refugia for both the Indian peninsular and Himalayan wolves during the glaciation period.

Signatures of either population decline, or equilibrium were observed in both the Indian wolves using mitochondrial and microsatellite markers. Non-significant negative values of Tajima's D, Fu's F s statistics, unimodal with non-significant raggedness index value (Indian peninsular wolf, Supplementary Fig. 2A) and multimodal peaks (Himalayan wolf, Supplementary Fig. 2B) in mismatch distribution tests are characteristics of demographic contraction. Similarly, bottleneck analysis with Wilcoxon's-Sign rank test for testing heterozygosity excess and shifted mode allele frequency distribution curve and M-ratio or G-W index values were supportive of population declines and genetic bottleneck events in the recent past (Supplementary Table 8). The more refined bottleneck analysis using MCMC approach on microsatellite data further corroborated a declining trend in effective population size until ~ 1100 generations ago for the Indian peninsular wolf (Fig. 5A), and ~ 800 generations for the Himalayan wolf (Fig. 5B). This decline was likely due to climate fluctuation that resulted in the changes in the vegetation patterns along with the wide spread of early humans. The decline also coincides with the Meghalayan event that caused a prolonged drought that lasted for over 200 years some 4500 Ybp affecting several civilizations worldwide (Góis-Marques et al. 2020). Later effective population size increased till ~ 600–800 generations and further showed drastic decline or a bottleneck event during the past 25–50 generations which corresponds to 100–250 Ybp (Fig. 4A and B). The latest decline in both the Indian wolf populations was possibly due to increased anthropogenic activities, intense agricultural practices, subsequent habitat modifications, and persecution during the last 100–150 years.

Conservation implications

Our data improve on the earlier mt-DNA based inferences by adding nuclear markers that clearly show that India has two distinct ancient wolves with no admixture between them. We further show that both wolves also had different demographic histories. Both wolves have experienced substantial decline in the population sizes in the recent past. Reasonable genetic diversity and evidence of gene flow across regional populations bode well for the conservation of these unique and ancient wolf lineages. However, the recent trend in

declining effective population size is a cause of concern and needs to be addressed through conservation policy, active management and protection. Some evidence of potential genetic introgression from dogs amongst Indian peninsular wolves from Rajasthan and Himalayan wolves was also a matter of concern (Wang et al. 2022, Jhala unpublished data). Feral dogs are a major conservation problem across India which is home to over 30 million dogs (Vanak and Gompper 2009). The problem is severe especially for wolves which can hybridize with dogs, contract their diseases and dogs compete for the same food as wolves (Jhala 2003). Future studies on both the Indian wolves with genome-wide datasets from across representative geographical regions will be useful to evaluate fine-scale population genetic structure and allow for investigating loci that are adaptive for specialized ecological traits and climatic regimes.

Recent genomic analysis (Hennelly et al. 2021; Wang et al. 2022) have shown that both Indian wolf lineages were distinct and basal to the Holarctic wolves and dogs. These wolves diverged from the Holarctic wolves between approximately 80 (Himalayan wolves) to 150 (Peninsular wolves) thousand years ago and show distinct historic demographic trajectories from other wolves (Hennelly et al. 2021; Wang et al. 2022). Besides, Wang et al (2020) suggest that an unknown canid ancestor contributed 39% to the genetic makeup of the Himalayan wolf which endowed the species with adaptive advantage for a life in the high altitudes. Indian peninsular wolves are the only species of wolves that breed in winter (Jhala 2003). Due to morphological differences that are suggestive of survival adaptations for a life at high altitudes, Hodgson (1847) had classified the Himalayan wolf as a distinct species (*Canis laniger*). Though our microsatellite panel did not detect any admixture between Himalayan wolves and Indian peninsular wolves, genome sequencing data has revealed some level of geneflow from Indian peninsular wolves into Persian wolves and Himalayan wolves (Hennelly et al. 2021; Wang et al. 2022). Given the wolf's dispersal ability, lack of any physical barriers to geneflow between the three wolf lineages, and the propensity of canids to interbreed, it is not surprising that this level of gene flow was detected. What is surprising, is that the Himalayan wolf and the Indian peninsular wolves have retained their uniqueness and distinctiveness from the Holarctic wolves, suggestive of behavioral or genetic isolating mechanisms. Currently, these wolves are classified as *C. l. chanco* (Himalayan wolf) and *C. l. pallipes* (Indian peninsular wolf) which clubs them with the Holarctic wolves of China and Western Asia, which the IUCN considers to be of least concern (Boitani, Phillips and Jhala 2018). Considering that these ancient wolf lineages likely survived in isolation within Pleistocene refugia of the Indian subcontinent and have retained their genetic distinctiveness, they warrant the status as distinct species. This recognition as separate species would enhance their conservation value and encourage the global community and range countries to develop policy and allocate resources for prioritizing their conservation.

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Author contributions YVJ and YS conceptualised the study and collected samples. YS performed the experiments, analysed the data and wrote the manuscript. YVJ supervised the study, reviewed the drafts and finalised the manuscript.

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Data availability The datasets presented in this study can be found in online repositories. Mitochondrial DNA sequence data can be found using accession numbers ON010580- ON010589 on GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Nuclear microsatellite genotypes data can be found on repository with https://doi.org/10.6084/m9.figshare.19385912

Declarations

Conflict of interest The authors declare no conflict of interest.

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