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ORIGINAL RESEARCH

Snow leopard phylogeography and population structure supports two global populations with single refugial origin

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Abstract

Snow leopards (*Panthera uncia*) inhabit the mountainous regions of High Asia, which experienced serial glacial contraction and expansion during climatic cycles of the Pleistocene. The corresponding impacts of glacial vicariance may have alternately promoted or constrained genetic differentiation to shape the distribution of genetic lineages and population structure. We studied snow leopard phylogeography across High Asia by examining range-wide historical and contemporary genetic structure with mitochondrial DNA and microsatellite markers. We genotyped 182 individuals from across snow leopard range and sequenced portions of the mitogenome in a spatially stratified subset of 80 individuals to infer historical biogeographic and contemporary patterns of genetic diversity. We observed a lack of phylogeographic structure, and analyses suggested a single refugial origin for all sampled populations. Molecular data provided tentative evidence of a hypothesized glacial refugia in the Tian Shan-Pamir-Hindu Kush-Karakoram mountain ranges, and detected mixed signatures of population expansion. Concordant assessments of microsatellite data indicated two global genetic populations, though we detected geographic differences between historical and contemporary population structure and connectivity inferred from mitochondrial and microsatellite data, respectively. Using the largest sample size and geographic coverage to date, we demonstrate novel information on the phylogeographic history of snow leopards, and corroborate existing interpretations of snow leopard connectivity and genetic structure. We recommend that conservation efforts incorporate genetic data to define and protect meaningful conservation units and their underlying genetic

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diversity, and to maintain the snow leopard's adaptive potential and continued resilience to environmental changes.

Keywords Conservation genetics · Glacial refugia · High Asia · *Panthera uncia* · Phylogeography · Snow leopard

Introduction

In the high elevation regions of the Tibetan Plateau and adjacent mountain ranges known as High Asia, Pleistocene glaciation cycles through the Last Glacial Maximum (LGM; 20,000 BP) significantly drove the biogeographic history of the region's ecosystems. Many taxa persisted through these dynamic events in climatically-stable refugia, where periods of refugial isolation operated on evolutionary timescales to form distinct phylogenetic patterns (Keppel et al. 2012; Řičánková et al. 2015). There are telltale genetic signatures of populations that persisted in glacial refugia versus those more recently established by post-glacial expansion from refugia. These genetic signals can include significant differentiation from other refugial populations, due to drift, and, if refugial populations were large, higher levels of genetic diversity owing to longer demographic histories (Hewitt 2004; Provan and Bennett 2008). Compared to populations within refugia, populations expanding from refugia into newly available habitats tend to exhibit genetic consequences of expansion including reduced genetic diversity, via founder events and population bottlenecks, as they comprise only a subset of genetic diversity from refugial source populations (Hewitt 2000). Phylogeographic studies can help identify the distribution of these evolutionarily distinct populations, and thereby inform scale-appropriate management and policy priorities for biologically relevant conservation units (Bermingham and Moritz 1998; Avise 2000). Conservation efforts that consider phylogenetic diversity are more likely to ensure that the intra-specific diversity, evolutionary history, and adaptive potential present in distinct lineages are maintained (Hickerson et al. 2010).

In High Asia, phylogeographic investigations have revealed genetic signals consistent with refugial populations in multiple taxa, including green toads (*Bufo viridis*; Zhang et al. 2008), Delphinium (*Delphinium naviculare*; Zhang and Zhang 2012), Tibetan wolves (*Canis lupus chanco*; Werhahn et al. 2020), and likely (*Panthera uncia*; Janečka et al. 2017). Snow leopards are specialists of the montane ecosystems of High Asia (hereafter High Asia is inclusive of snow leopard range; (McCarthy et al. 2023). They are currently IUCN Red Listed as Vulnerable across their 12-country distribution (IUCN 2017). Contemporary snow leopard range includes 1,776,000 to 3,300,000 km^2 of the naturally fragmented Altai, Himalayan, Hindu Kush, Karakoram, Khangai, Kunlun, Pamir, Qilian, Sayan, and Tian Shan mountain ranges (Li et al. 2020). They are adapted to extreme ruggedness, high elevation, low temperatures, and hypoxic conditions, which, coupled with their elusive nature, has made gathering natural history data difficult. Substantial knowledge gaps for snow leopards include a lack of sufficient understanding of their distribution, numbers, population structure, and connectivity, as well as fully comprehending the impacts of both historical and ongoing climate change (Snow Leopard Working Secretariat 2013; Weckworth 2021). This lack of information is noted as a primary conservation challenge (McCarthy et al. 2023) and limits effective policy and conservation interventions.

In the face of increasingly fragmented and rapidly changing environments in High Asia, large-scale snow leopard conservation efforts are better served by understanding the species' genetic diversity. Yet, despite recent efforts to describe the molecular ecology of snow leopards (Hacker et al. 2023a) characterizing population genetic parameters remains incomplete throughout much of snow leopard range. For example, genetic data has not yet been integrated into snow leopard landscape connectivity models and priority landscape delineation efforts (Li et al. 2020). Instead, such efforts have relied on habitat suitability modelling, expert opinion, and contemporary occurrence records to identify important landscapes for global snow leopard conservation and connectivity. A dedicated application of genetic data and analysis could fill many knowledge gaps, including characterizing range-wide phylogeography, validating putative climate refugia (Li et al. 2016) and subsequent regions of evolutionary significance, and identifying routes of functional connectivity. Such knowledge could help identify distinct genetic units that warrant conservation attention and patterns of low or declining genetic diversity that could threaten population persistence.

The results of a range-wide snow leopard habitat suitability modeling exercise based on contemporary occurrence records (Li et al. 2016) hypothesized three large climate refugia; the Altai mountains, the Tian Shan Pamir-Hindu Kush-Karakoram (TPHK) mountain ranges, and the Qilian mountains on the Tibetan Plateau. These three climate refugia were modeled to be climatically stable across past and future global climate events. Li et al. (2016) proposed that these refugia facilitated snow leopard persistence during Pleistocene glacial-interglacial cycles, and may provide stable habitat for long-term snow leopard survival into the future. Through phylogeographic approaches, we can test if patterns of snow leopard genetic diversity reflect isolation in Pleistocene refugia and if those patterns support the modeled refugia (Li et al. 2016).

Molecular methods are generally underutilized for snow leopards (Weckworth 2021). Past genetic studies focused on small spatial and temporal scales corresponding to local (Shrestha and Kindlmann 2020; Atzeni et al. 2021) and regional (Korablev et al. 2021; Hacker et al. 2023b) populations. So far, only Janečka et al. (2017) have published on range-wide snow leopard genetic diversity. They reported three genetic clusters based on 70 individuals and 33 microsatellite loci, but identified no mitochondrial (mtDNA) diversity (Janečka et al. 2017). They proposed the three genetic clusters were representative of snow leopard subspecies. The validity of this conclusion was debated with key points revolving around the utility vs. limitations of mtDNA, as well as challenges of sample size and distribution (Janečka et al. 2018; Senn et al. 2018). Resolving this debate requires more extensive genetic research on snow leopards, including improved sampling and additional marker data for phylogenetic evaluation.

We initiated a multi-national, trans-boundary collaborative effort to amass the largest sample size and geographic coverage to date to evaluate range-wide phylogeographic relationships and contemporary patterns of genetic diversity of snow leopards. Based on variation at mtDNA and microsatellite markers, we aimed to answer four lines of inquiry for snow leopards. (1) What are the range-wide phylogeographic dynamics of snow leopards, and how might intraspecific variation be structured? (2) Do patterns of genetic variability in snow leopard genetic lineages corroborate the hypothesized Pleistocene glacial refugia modeled by Li et al. (2016)? (3) Do phylogenetic results support the hypothesized subspecies proposed by Janečka et al. (2017)? Finally, (4) how do contemporary range-wide genetic patterns compare to phylogeographic dynamics, and are there signals of connectivity? From our results we draw inferences on past demographic processes, describe range-wide genetic connectivity, and provide insight into the uses of genetic data for large-scale snow leopard conservation efforts.

Materials and methods

Sample collection and laboratory procedures

Snow leopard samples were collected from ten of the twelve snow leopard range countries (Russia, Mongolia, Kyrgyzstan, Tajikistan, Uzbekistan, Afghanistan, Pakistan, Nepal, Bhutan, and China; see Appendix S1 in Supporting Information). Scat samples were collected opportunistically from 2008 to 2019 via extensive international collaborative efforts between researchers, academic institutions, NGOs, and local communities (Table 1). Scats were georeferenced and stored until DNA extraction in either silica gel beads or ethanol. Blood samples were collected for research activities outside this study, and were obtained using proper permits and transported to the United States according to CITES guidelines. DNA from a total of 507 samples (scat = 443, blood = 25, and DNA previously extracted from scats = 39) were analyzed, 336 were confirmed as snow leopards and suitable for analysis. Of the 336 individuals, 108 were previously genotyped for the same loci we used (Caragiulo et al. unpublished data, American Museum of Natural History, New York, NY, USA) and 99 were genotyped for other markers (Korablev et al. 2021) and re-genotyped using our loci (see below), and the remaining 129 were processed for the first time in this study. Genetic analysis, DNA extraction, species ID (as needed), and genotyping were conducted at the National Genomics Center for Wildlife and Fish Conservation (NGC), United States Forest Service, Rocky Mountain Research Station (Missoula, MT, USA). DNA was extracted from scats with QIAmp DNA Stool Mini Kits (Qiagen, Inc., Valencia, CA, USA). DNA from blood samples was extracted with DNeasy Tissue Kits (Qiagen, Inc., Valencia, CA, USA). Quality and quantity of template DNA were determined by 1.6% agarose gel electrophoresis.

To determine species in unknown samples, we amplified ~ 360 base pairs (bp) of mitochondrial DNA using 16 S rRNA universal primers, (modified from Hoelzel and Green 1992). Reaction volumes of 30 µl contained 50–100 ng DNA, 1× reaction buffer (Life Tech-

Table 1 Individual snow leopards organized by mountain range and sample size for mtDNA/nDNA analysis (*n*). Standard indices of genetic variability include number of different alleles (A_N), allelic richness (A_R), effective alleles (A_E), observed heterozygosity (H_O) expected heterozygosity (H_E), number of haplotypes (*k*), haplotype diversity (*h*), and nucleotide diversity (π)

Mountain range	*n*	A_N	A_R	A_E	H_O	H_E	k	h	π
Sayan	12/22	4.3	2.3	2.9	0.60	0.58	2	0.41	0.00067
Altai	15/25	4.5	2.3	2.8	0.58	0.58	3	0.53	0.00097
Gobi Desert	3/34	4.3	2.4	2.8	0.56	0.62	2	0.67	0.00110
Tian Shan	17/29	5.5	2.6	3.4	0.71	0.69	3	0.41	0.00073
Pamir	20/55	5.8	2.6	3.4	0.60	0.69	6	0.78	0.00288
Karakoram	8/9	5.9	3.0	4.0	0.62	0.73	3	0.61	0.00129
Himalayas	5/5	2.5	1.9	1.8	0.48	0.41	2	0.60	0.00099
Tibetan Plateau	0/3	2.6	2.2	2.1	0.54	0.47	-	-	-
Global	80/182	4.4	2.4	3.9	0.58	0.60	12	0.64	0.00169

nologies, NY, USA), 2.5 mM $MgCl_2$, 200 µM each dNTP, 1 µM each primer, 1 U AmpliTaq Gold polymerase (Life Technologies, NY, USA). The polymerase chain reaction (PCR) program was 94 ◦C/5 min, [94 ◦C/1 min, 50 ◦C/1 min, 72 ◦C/90s] × 34 cycles, 72 ◦C/5 min. PCR products were purified using ExoSap-IT (Affymetrix-USB Corporation, OH, USA) according to manufacturer’s instructions. Reactions were sequenced at Eurofins Genomics (Louisville, KY) using standard Sanger sequencing protocols. DNA sequence data were viewed and aligned with Sequencher v.5.4.6 (Gene Codes Corp. MI), and compared to reference sequences from known species.

We assessed genetic variation from nuclear DNA to characterize range-wide population structure to determine if patterns of microsatellite diversity differed from those observed in mtDNA. We amplified 12 microsatellites known to be variable in snow leopards: FCA032, FCA075, FCA096, FCA100, FCA124, FCA126, FCA132, FCA208, FCA212, FCA225, FCA229, FCA275 (Menotti-Raymond et al. 1999). Microsatellite loci were amplified in PCR multiplexes, where total volume (10 µl) contained 1.0 µl DNA, 1x reaction buffer (Life Technologies Grand Island, NY, USA), 2.0 mM $MgCl_2$, 200 mM of each dNTP, 1 mM reverse primer, 1 mM dye-labeled forward primer, 1.5 mg/ml BSA, and 1U Taq polymerase (Life Technologies). The PCR profile was 94 °C/5 min, ([94 °C/1 min, 55 °C/1 min, 72 °C/30 s] x 36 cycles). Resultant products were visualized on a LI-COR DNA analyzer (LI-COR Biotechnology).

To standardize the previous genotypes (Caragiulo et al. unpublished) with new genotypes, 15 individuals from the Caragiulo et al. dataset that encompassed the range of observed alleles were used as standards in scoring the new genotypes, thus ensuring consistency across scoring profiles and allowing us to confidently combine datasets. To screen for allelic dropout, we performed replicate genotyping for all new samples. Samples with amplification failure, or discrepancies between replicates, were again repeated in duplicate to minimize genotyping error. For statistical analyses, we only used unique individuals that successfully amplified at 8 or more (≥66%) of our loci, which provided 43 individuals from the 129 unknown samples, 87 of 108 individuals from Caragiulo et al. (unpublished), and 52 of 99 individuals from Korablev et al. (2021), for a microsatellite dataset of 182 individual snow leopards.

For phylogenetic analyses, we sequenced a spatially stratified subset of 80 individual snow leopard samples across 607 bp concatenated mtDNA sequences: 445 bp of cytochrome *b* using primers mcb398 and mcb869 (Verma and Singh 2003), and 162 bp of control region using primers PUN-CCR-F and PUN-CCR-R254 (Janečka et al. 2017). PCR profile and sequencing steps for both markers followed the same protocol used for species identification.

Fifty-eight individuals were represented in both the mitochondrial and the microsatellite datasets; the remaining individuals in the mitochondrial dataset ($n = 22$) and microsatellite dataset ($n = 124$) represented different individuals from different sampling locations (Table 1). No individuals from the Tibetan Plateau successfully amplified for the mitochondrial dataset.

Data analysis

Previous studies on snow leopard connectivity suggest mountain ranges are important linkages for global snow leopard populations (Riordan et al. 2016; Li et al. 2020). Therefore, we assigned individual samples to groups based on their geographic location in one of eight major mountain ranges (Altai, Gobi Desert, Himalaya, Karakoram, Pamir, Sayan, Tian Shan, Tibetan Plateau; see Table 1 for sample size by range). These mountain ranges served as a priori population units for subsequent analyses requiring sample groupings.

We described phylogeographic dynamics of snow leopards using two approaches with mtDNA data: (i) comparing phylogenies using Bayesian and maximum likelihood (ML) methods, and (ii) testing for signatures of population expansion. This phylogeographic framework simultaneously addressed our second question regarding genetic signatures of glacial refugia, as spatial structuring of distinct genetic lineages may indicate evidence of divergence in glacial refugia (Provan and Bennett 2008). We estimated number of polymorphic sites, number of haplotypes (k), haplotype diversity (h), and nucleotide diversity (π) for mtDNA sequences (Table 1) using DnaSP v6 (Rozas et al. 2017).

To infer phylogenetic relationships among mtDNA haplotypes, we used Bayesian and maximum likelihood criteria. We used the software jModelTest (Posada 2008) to identify the best substitution model for concatenated snow leopard sequences based on consensus between corrected Akaike Information Criterion (AICc) and Bayesian Information Criterion (BIC). A Bayesian maximum clade credibility tree was created using BEAST v1.10 (Suchard et al. 2018) using a strict clock model, the best substitution model, default optimization schedule, and Markov chain Monte Carlo (MCMC) chain-length of ten million. We sampled every 10,000 generations, discarded the first 10% of samples, and estimated Bayesian posterior probabilities (BPP) on the 50% majority rule consensus of remaining trees. We analyzed results in Tracer v1.7.2 (Rambaut et al. 2018). Phylogenetic trees were summarized in TreeAnnotator v1.10.4 (Drummond et al. 2007) and visualized and stylized in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). We also calculated a maximum likelihood phylogeny with software MEGA X (Kumar et al. 2018), and assessed resulting relationships with 1000 bootstrap replicates. All phylogenies were rooted with a concatenated sequence from a tiger mitogenome (Zhang et al. 2011- Genbank accession HM589215.1). To further examine the intraspecific genetic variation of phylogenetic relationships, we also conducted a haplotype network analysis on the mtDNA sequences with PopART software (https://popart.maths.otago.ac.nz/) using the median joining algorithm (Bandelt et al. 1999).

Prior work has identified common genetic signatures underlying population dynamics that occur through glaciation cycles (Stewart et al. 2010). Demographic events such as rapid population growth and expansion exhibit either high levels of haplotype diversity, with low levels of nucleotide diversity, or low levels of haplotype diversity over large areas (Avise 2000). We calculated Tajima's D (Tajima 1989) and Fu's F_S statistic (Fu 1997), using DnaSp (Rozas et al. 2017), for the global population and any distinctive clades denoted in the phylogenies. These two tests of neutrality are indicative of population expansion with significant negative departures from zero. To distinguish patterns of expansion from background selection, we calculated Fu and Li's (1993) D^* and F^* statistics. If F_S is significant but D^* and F^* are not, population growth or range expansion is supported (Fu 1997). We also calculated mismatch distributions of pairwise nucleotide differences in DnaSp, where unimodal distributions indicate population expansion, and multimodal distributions indicate stable populations.

Using our microsatellite data, we used two approaches to address our third question to characterize contemporary population genetic structure and connectivity: (i) determining range-wide population genetic structure with non-spatial Bayesian clustering approaches, and (ii) testing for concurrence amongst multiple exploratory multivariate analyses. We derived all standard estimates of genetic diversity for each mountain range in GenAlEx 6.503 (Peakall and Smouse 2012), and used the R package *genepop* (Rousset et al. 2008) to calculate allelic richness (Table 1). We tested loci for linkage disequilibrium (LD) and deviations from Hardy- Weinberg equilibrium (HWE) with significant P-values adjusted for multiple comparisons (Rice 1989). For populations experiencing spatially limited dispersal, genetic differentiation among individuals increases as geographic distance increases, resulting in spatially-autocorrelated mating patterns (isolation by distance; Wright 1943). We tested for isolation by distance (IBD) for all cats via a simple Mantel test, which quantifies the correlation between individual pairwise genetic and Euclidian distances (calculated in GenAlEx using default genetic distances; statistical significance assessed with 10,000 permutations), and evaluated patterns of spatial autocorrelation for all 182 individuals to detect departures from random mating within 100 km distance categories reflecting snow leopard dispersal (Riordan et al. 2016).

We used the non-spatial, Bayesian clustering program STRUCTURE (Pritchard et al. 2000) to determine the optimal number of populations (K) based on genotype data. This approach minimizes Hardy-Weinberg proportions and linkage disequilibrium within each cluster. However, simulation studies have shown STRUCTURE to be unreliable at identifying population structure when sampling is unbalanced, including merging populations comprising small sample sizes (Puechmaille 2016; Wang 2017). Wang (2017) showed this reduced performance is primarily caused by using the default ancestry prior, which assumes all populations contribute equally, and the default alpha value ($\alpha = 1$), which inhibits the mixing of the MCMC sampler and falsely attributes individual ancestry to come from a single population. Instead of using these default parameter settings, a combination of the alternative ancestry prior, which assumes populations contribute variably to the sampled individuals, the uncorrelated allele frequency model, and adopting a smaller alpha value improved STRUCTURE's ability to obtain accurate individual assignments (Wang 2017). To address the likely influence of uneven sampling on population clustering, we explored multiple parameter combinations in STRUCTURE to optimize our inferences on snow leopard population genetic structure. We tested the following parameter combinations: for all models we used the alternative ancestry prior, an initial alpha value $\alpha = 1/K$, where K is the assumed number of clusters 1–5, and either a correlated or uncorrelated allele frequency model. For all models, we also applied the prior model parameter LOCPRIOR, which can be informative when there are weak population signals due to small sampling sizes or low levels of genetic differentiation between populations (Hubisz et al. 2009). This resulted in ten separate STRUCTURE runs for the microsatellite dataset. For each model, we performed ten independent runs of $K = 1$–5 with 1,000,000 Markov Chain Monte Carlo (MCMC) steps and 300,000 burn-in steps under admixture, uncorrelated allele frequency model. Optimal K among tested values was determined by visual examination of likelihood scores in STRUCTURE HARVESTER (Earl and vonHoldt 2012) using both the standard procedure and ΔK statistic (Evanno et al. 2005). When genetic differentiation is strong, the ΔK statistic performs well, but under moderate to low genetic differentiation the standard procedure performs better (Waples and Gaggiotti 2006). We applied both approaches to identify the uppermost level of population structure in each model, and evaluated pairwise F_{ST} values for the most highly supported K in Arlequin 3.5.2 (Excoffier and Lischer 2010). Individual snow leopards were assigned to a putative population based on their highest ancestry coefficient (q), which represents the estimated probability an individual belongs to a given cluster. We created STRUCTURE bar plots in STRUCTURE PLOT (Ramasamy et al. 2014). For populations with weak genetic differentiation, congruence amongst multiple methods may be especially useful in determining the true *K* while minimizing various criticisms associated with each method (Kanno et al. 2011; Latch et al. 2006), thus we used principal component analysis (PCA) as implemented in R package *adegenet* (Jombart 2008). PCA complements Bayesian analysis by not requiring data to meet the assumptions of LD and HWE. For PCA, we retained the first three axes, and performed k-means clustering to evaluate the optimal number of clusters. We assessed recent population bottlenecks in each mountain range by testing for heterozygote excess using a two-phase model of mutation (TPM) using the program BOTTLENECK (Piry, Luikart, and Cornuet; 1999). We constrained models by defining multistep mutations to account for 5%, 10%, 20%, and 25% of mutations for cats in each mountain range. For all four scenarios, the Wilcoxon signed rank test was used to determine which mountain ranges exhibited a heterozygous excess (Luikart et al. 1998).

Results

mtDNA diversity

Across High Asia, we identified 12 distinct haplotypes in 80 individuals. There were 12 polymorphic sites with haplotype and nucleotide diversity at 0.74 and 0.00193, respectively. Of these 12 distinct haplotypes, eight were represented by multiple individuals and four by only a single individual each. The dominant haplotype (Hap2) was found in 47 individuals with a geographic coverage across seven of eight mountain ranges (excluding the Tibetan Plateau, which was not represented in the mtDNA dataset). Haplotypes represented by single individuals (Table 1) were found in the Pamir (3) and Altai (1).

For the Bayesian maximum clade credibility tree, we identified HKY + G as the best substitution model for concatenated snow leopard sequences. All effective sample sizes (ESS) were > 3,200, indicating MCMC chain length was sufficient (Drummond et al. 2007). The rooted maximum clade credibility tree showed relatively shallow divergence among snow leopards across High Asia (Fig. 1). The tree topology indicated strong support (BPP = 100%) for divergence of a single haplotype (Hap11), which was found in four individuals from the Pamir mountains. For the remaining nodes, which consisted of individuals from throughout snow leopard range (including the Pamir mountains), there was no clear geographic pattern of the distribution of mtDNA diversity, indicating either current gene flow or incomplete lineage sorting among maternal lineages. Because within-group branch lengths for these topologies were short, and branch nodes had low support values, geographic admixture among these maternal lineages must have been relatively recent. The maximum likelihood tree (Appendix S2) showed no pattern in the geographic distribution of haplotypes. The internal topologies within this arrangement were similarly not well-supported, with short branch lengths. Haplotype network analysis revealed a star-like pattern, reinforcing a lack of geographic subdivision of mtDNA diversity, and a pattern consistent with population expansion (Fig. 2). The network showed that haplotype Hap11 differs from the most common haplotype (Hap2) by two base pairs.

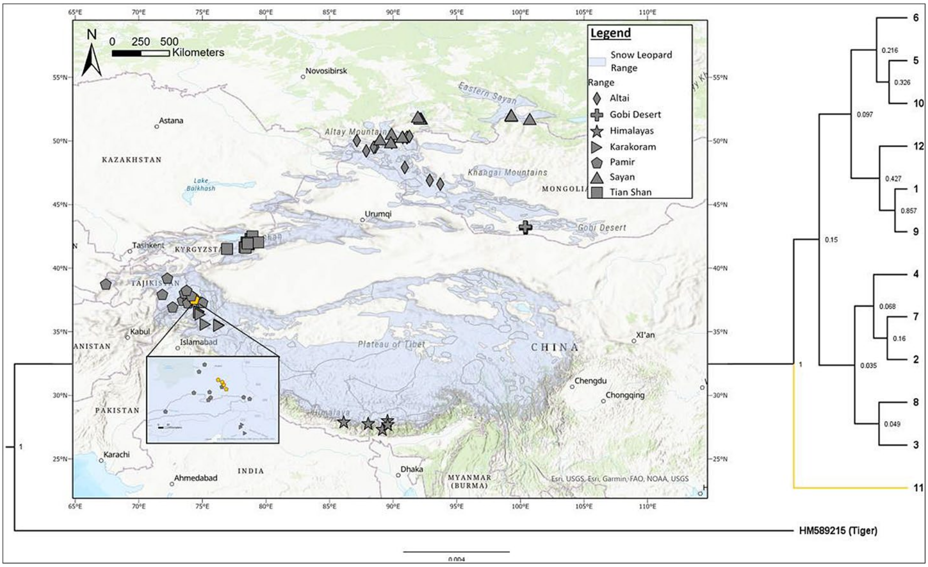

Fig. 1 Bayesian maximum clade credibility tree showing 12 haplotypes representing 607 base pairs of the mitochondrial genome, including cytochrome *b* and control region, from 80 snow leopards. Rooted with a tiger from China. Inset shows sample locations of individuals comprising a geographically restricted haplotype in the Pamir mountains (yellow). Mountain ranges are represented by unique polygons

Fu's F_S was significantly negative for the global dataset (Fu's F_S = -6.77, $P=0.003$), providing support for expansion. D* and F* ($P>0.10$) was non-significant for all groups. In contrast, Tajima's D was not significant (Tajima's D = -1.13, $P>0.10$), however, Fu's simulations suggest F_S is a more sensitive indicator of population expansion than Tajima's D (Fu 1997). Mismatch distributions showed unimodal shapes for the global dataset, further supporting the genetic signal expected in an expanding population (Appendix S3).

Microsatellite diversity

Measures of genetic diversity for 182 snow leopards varied slightly by mountain range (Table 1). When we tested for deviations from HWE by mountain range, loci from the Gobi Desert (FCA212, FCA225, FCA229), Tian Shan (FCA225), and Pamir (FCA132, FCA225, FCA229) were out of HWE. Following Bonferroni correction for multiple tests in the global dataset, no loci exhibited heterozygote deficiency ($\alpha=0.05$). Seven locus-pairs exhibited LD (FCA032 and FCA100; FCA126 and FCA212; FCA132 and FCA229; FCA100 and FCA275; FCA124 and FCA275; FCA229 and FCA275; FCA124 and FCA229); taken together, these patterns might be explained by population admixture, or recent population reduction (Slatkin 2008). We observed a moderate amount of pairwise individual genetic variation explained by distance (IBD; $r=0.325$, $P=0.01$). We detected positive spatial autocorrelation among individuals up to 1,200 km apart ($r=0.035$, $P=0.001$).

For each STRUCTURE analysis of microsatellite data, clustering patterns were consistent and all supported two groups. The first cluster consisted entirely of individuals from the Sayan, Altai, and Gobi Desert ranges (hereafter, North population), and a second cluster

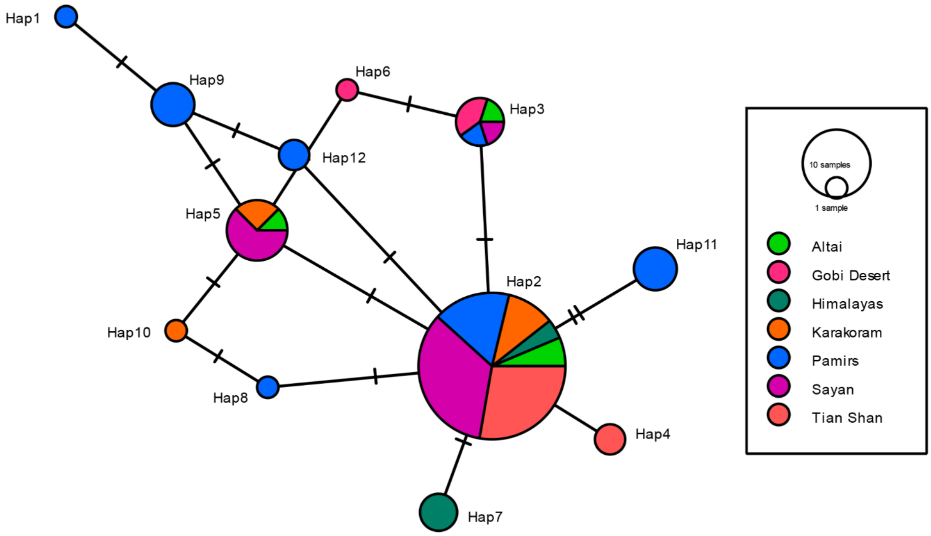

Fig. 2 Median joining network showing 12 mitochondrial genome haplotypes representing 607 base pairs of the mitochondrial genome, including cytochrome *b* and control region, from 80 snow leopards. Haplotypes are represented by circles whose sizes are proportional to the number of individuals. Dashed lines represent the number of base pairs differentiating haplotypes. Different colors represent each mountain range

comprised mostly from the remaining ranges (hereafter, Core population). Across all ten models testing $K=1$–5, individual assignments to populations were consistent, and individual proportion of membership (q) for values of K were similar across all runs, suggesting accuracy of both individual ancestry assignment and an estimate of $K=2$. As a result, we report results for an uncorrelated allele frequency model using the alternative prior and alpha set to $\alpha=0.5$, based on $1/K$ when $K=2$. To evaluate for additional plausible spatial substructuring not captured by $K=2$, we visually inspected q-values for $K=2$–4. We observed increased spatial clustering for mountain ranges outside of Russia and Mongolia, but ΔK method indicated values beyond $K=2$ are above optimum (Fig. 3; see Appendix S4 for STRUCTURE HARVESTER outputs and Appendix S5 for STRUCTUREq-plots at $K=2$–4 and). Pairwise F_{ST} (calculated in Arlequin) between the two putative clusters from STRUCTURE was significant (0.096; $P<0.001$).

Genetic structure from the PCA showed support for the same two groups as STRUCTURE (Fig. 3); Sayan, Altai, and Gobi Desert into a North cluster, and the Tian Shan, Pamir, Karakoram, Himalaya, and Tibetan Plateau into the Core cluster. Allele frequency distribution overlapped considerably, indicating our markers weakly distinguished populations. K-means clustering to the PCA space validated our choice to use mountain range as a grouping metric, as there were 7 optimal clusters across 8 mountain ranges, with individual assignments in the Tibetan Plateau ($n=3$) absorbed into the Himalayas and Tian Shan mountains, likely reflecting low sample size for this region.

Significance of the one-tailed Wilcoxon tests for heterozygote excess varied slightly across all four TPM scenarios. Significant heterozygote excess was detected in the Gobi Desert with a TPM of 10%, 20%, and 25% ($P=0.046$, 0.001, 0.0008, respectively). The Tian Shan population exhibited heterozygote excess with a TPM of 10%, 20%, and 25%

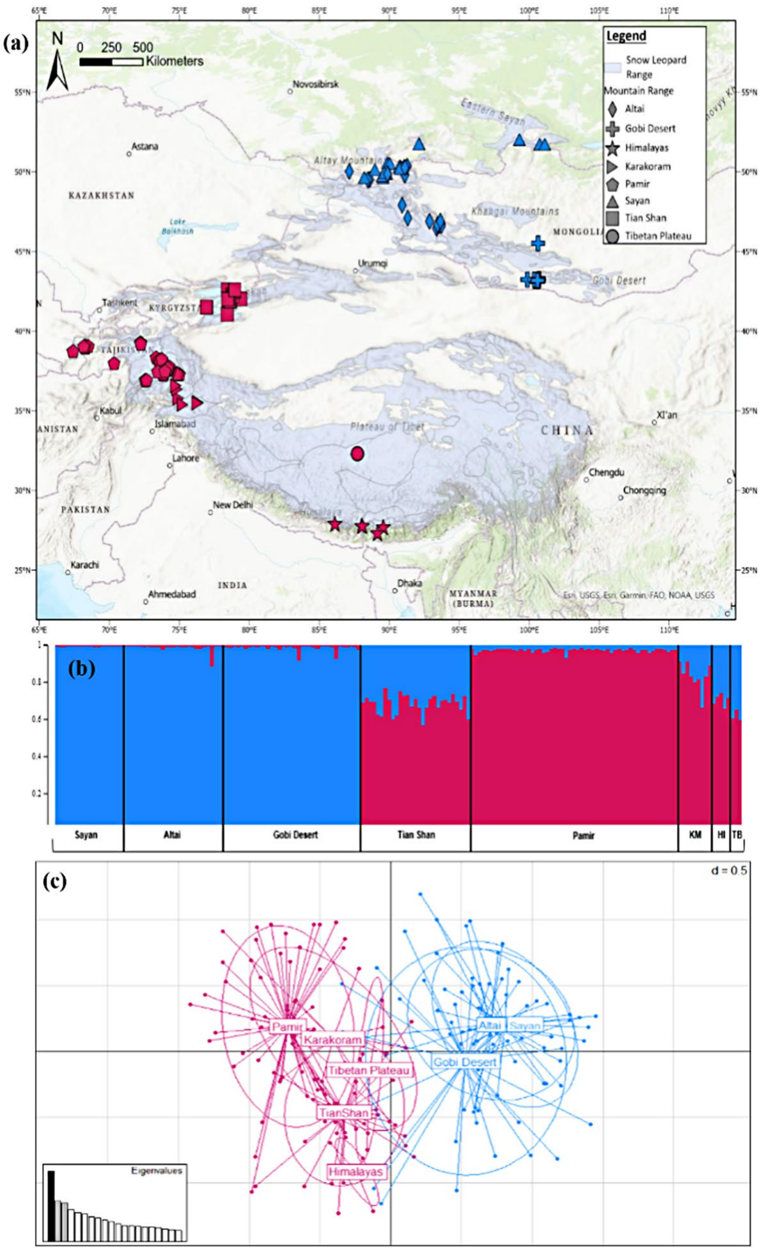

Fig. 3 (**a**) Map of global snow leopard population structure based on Bayesian clustering analysis of genotypes from 12 autosomal microsatellites. Individual snow leopards ($n=182$) were assigned to one of two populations based on q-values from STRUCTURE, where $K=2$ received the most support. Population is denoted by color (blue=North population, red=Core population), and sample location is indicated by polygon shape. (**b**) STRUCTURE plot of microsatellite results for $K=2$, which received the most support. The mountain range of samples are listed at the bottom, with colors corresponding to the map. (**c**) PCA plot indicating genetic distance between mountain ranges containing samples from 182 snow leopards. PCA is based on pairwise F_{ST} values using microsatellite data. Individuals are denoted by colored polygons and organized by mountain range

($P = 0.021$, 0.005, 0.005, respectively). The Sayan exhibited heterozygote excess at 10%, 20% and 25% TPM ($P = 0.051$, 0.040 and 0.010, respectively), and the Altai was significant at 20% and 25% ($P = 0.046$ for both). Under these scenarios, the hypothesis of mutation-drift equilibrium was rejected, indicating a recent genetic bottleneck may have occurred for snow leopards in the Sayan, Altai, Gobi Desert, and Tian Shan mountain ranges.

Discussion

Snow leopard phylogeography

Based on analyses of mtDNA variation, snow leopards in High Asia showed a lack of phylogeographic structure. The overlapping geographic distributions of haplotypes, combined with the widespread presence of the most common haplotype, is consistent with expectations of expansion from a single refugial origin for all sampled populations (Slatkin and Hudson 1991; Avise 2000). Mixed support from the expansion tests suggests a pattern of contracted habitat restricted by glacial ice followed by population growth and geographic expansion during inter-glacial periods. While we observed high BPP support in the Bayesian tree for divergence of one haplotype in the Pamir mountains, this was not supported in the maximum likelihood tree. Notably, mtDNA diversity was highest in the Pamir ranges, and haplotype network analysis showed that the Pamir mountains had several private haplotypes, including the most divergent. This suggests that this region overlapped with a climate refugium. Areas of high diversity, as compared to lower diversity elsewhere, can indicate refugial cores; this pattern of spatially restricted and isolated glacial refugia is apparent in other montane species (Provan and Bennett 2008; Holderegger and Thiel-Egenter 2009; Zigouris et al. 2013). While species' genetic diversity tends to be greatest in regions of origin, high mtDNA diversity can also be indicative of increased time of isolation in refugia, or lineage admixture (Provan and Bennett 2008; Weckworth et al. 2012). However, cold-adapted species like snow leopards are generally expected to have spent less time in refugia compared to temperate species (Stewart et al. 2010). Other big cat species, such as African lions (*Panthera leo*), exhibited regional lineages that were lost during recent glacial-interglacial cycles, a likely result of expansion and contraction events from/to regional refugia (Bertola et al. 2016). If snow leopards shared a similar evolutionary history of expansion and contraction in regional refugia, the overlapping distribution of haplotypes, combined with genetic patterns in the Pamir mountains, might provide tentative genetic evidence for Li et al.'s (2016) hypothesis that the TPHK system served as a climate refugium for snow leopards. However, without mtDNA data from the Tibetan Plateau, which comprises nearly 60% of known snow leopard range, we cannot rule out the presence of other refugia, and their effects on contemporary structure of mtDNA, across snow leopard distribution.

In contrast to mtDNA analysis, concordance between STRUCTURE and PCA analyses indicated two range-wide populations. More data are needed to determine if our results reflect expansion from a single, genetically diverse refugial population, or expansion and mixing of groups from multiple refugia. One explanation for the discordance in our results might be a lack of statistical power to distinguish refugial diversity. It is also possible demographic events resulted in lineage extinctions and thus we are sampling only a subset of original diversity, as suggested for the Andean cat (*Leopardus jacobita)*, another high-altitude specialist felid (Cossíos et al. 2012). While complete mitogenomes have revealed phylogeographic relationships not previously detected with shorter sequences (Keis et al. 2013), *Panthera* species have reduced intraspecific genetic diversity relative to other carnivores owing to more recent divergence times (Farhadinia et al. 2020). Similarly, common leopards (*Panthera pardus*) have shown low haplotype diversity in parts of West Asia (Rozhnov et al. 2011; Farhadinia et al. 2015; Asad et al. 2019), and Andean cats have exhibited lower genetic diversity compared to lower elevation neotropical cats (Ruiz-García et al. 2022).

Range-wide genetic diversity

Contemporary geographic structure of snow leopard genetic diversity appears to be a function of isolation by distance as well as signatures of spatial autocorrelation, which we detected up to 1,200 km. This distance is beyond the estimated 250–500 km dispersal limit of snow leopards (Janečka et al. 2017). Because the sampling scale of this study exceeded the asymptote of spatial autocorrelation, it is unlikely fine-scale spatial autocorrelation is inflating optimal K (Schwartz and McKelvey 2008). While our analyses consistently identified $K=2$ as the most probable population structure, our plots for $K=3$ (Appendix S5) corresponded to the geographic population structure identified in Janečka et al. (2017). As a result, we cannot discount potential homogenization caused by gene flow. For example, STRUCTURE output when $K=3$ may represent a patch or clinal population rather than a third, discrete population, although cryptic subpopulations have been shown to blend together and resulted in low population assignment in Eurasian lynx (*Lynx lynx*; Rueness et al. 2014). Despite our efforts to address the statistical difficulties in inferring K by STRUCTURE (Wang 2017), increased sampling within and between groups is needed to further characterize genetic variation and structure in these regions.

Many alpine species have low dispersal rates and exhibit sex-biased dispersal, and thus retain phylogeographic patterns in present-day genetic variation (Sim et al. 2016), yet, we show evidence of temporal and geographic differences between phylogeographic and contemporary population structure. Li et al. (2020) proposed that linkages between the Altai and Tian Shan-Pamir-Hindu Kush-Karakoram mountain ranges play a critical role in maintaining global snow leopard connectivity. Results from microsatellite data corroborates high structural connectivity of contemporary snow leopard habitat within the west Himalayan-Karakoram-Pamir region (Riordan et al. 2016). However, we found evidence of genetic bottlenecks in the Altai, Gobi Desert, Sayan, and Tian Shan ranges, which flank narrow regions of connectivity in habitat linkages identified by Li et al. (2020). This could indicate recent challenges to dispersal for snow leopards between the North and Core populations, between mountain ranges within each population, or recent population decline, as indicated by heterozygote excess. However, statistical power in BOTTLENECK is highest when sampling at least 30 individuals per population (Piry, Luikart, and Cornuet; 1999), so it is possible we were unable to detect signatures in some mountain ranges due to low sample size. Taken together, snow leopard populations may be spatially unstructured in some portions of their range, but varying degrees of connectivity, isolation by distance, and movement resistance may facilitate spatially-explicit gradient or stepping stone processes (Balkenhol et al. 2015).

Sub-species status and conservation implications

Our results corroborate a post-Pleistocene differentiation between northern and western High Asia groups seen in Janečka et al. (2017), but little distinctiveness of the Himalayas and Tibetan Plateau, and no deeper phylogenetic divergence that would support subspecies status for any snow leopard population (Janečka et al. 2017). Yet, IUCN Red List assessments do not require subspecies status to warrant assessment of population units below the species level (IUCN Standards and Petitions Committee 2022). For example, our results suggest the North population of snow leopards may continue to be partially isolated from the Core population; in the future, the North population may have need for special protection warranting an IUCN Red List assessment and management as a distinct conservation unit below the species level. Efforts to maintain connectivity between these portions of snow leopard range should be prioritized, including filling data gaps for snow leopards in Kazakhstan, India, and northern China. Further, more research is needed to understand the evolutionary significance of snow leopards on the Tibetan Plateau, including defining conservation units at appropriate geographic scales and their political contexts for management range-wide. To that end, three large-scale, collaborative assessments have tried to prioritize snow leopard conservation using a variety of analytical frameworks, but none included genetic data, and the conservation priorities that resulted from each effort varied (Li et al. 2020). For snow leopards, a lack of habitat connectivity in some portions of their range, combined with small populations typical of low-density species, can have demographic consequences related to genetic drift and gene flow, including inbreeding depression and loss of adaptive potential, among others (Weckworth 2021). In addition to the evidence that the Pamir mountain range may overlap with a climate refugium for snow leopards, the high genetic diversity of snow leopards in this region also indicates an area important for global snow leopard connectivity, and should be a consideration for transboundary conservation efforts.

Implementing genetic research into landscape planning efforts can provide crucial information on spatio-temporal changes in the distribution of snow leopard genetic diversity, which can in turn provide context by which certain regions or groups are evaluated. We recommend ongoing snow leopard conservation and management programs incorporate genetic research into their strategies, and implement interventions that ensure the preservation of the species' range-wide genetic diversity.

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Author contributions IAC, BW, TMC, and KPM conceived and designed the study; IAC, BW, AC, KPM, TMC, XB, EB, LH, SJ, OJJ, SK, AK, MK, ZK, RK, XL, PL, TKM, BM, SM, MAN, SO, RP, JP, AP, TR, TR, VVR, BNS, and TW provided and/or donated samples; IAC and KLP conducted laboratory work at the National Genomics Center with MKS providing laboratory space for the project; AC, AA, GA, IDF, MG, LH, SJ, AK, MP, JP, SR, MK, AP, VVR, BNS, and TW conducted previous laboratory work on donated samples; IAC performed the mtDNA and nDNA analyses and evaluated the results; IAC led the writing with assistance from BW and KPM. All authors contributed to manuscript writing and approved it for publication.

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Data availability Mitochondrial sequence data have been deposited on GenBank: D-loop sequences (accession numbers OQ863595- OQ863598), and cytochrome-b sequences (accession numbers OQ863585-OQ863594). Data released upon publication.

Declarations

Competing interests The authors declare no competing interests.

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