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Exceedingly low genetic diversity in snow leopards due to persistently small population size

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Snow leopards (*Panthera uncia*) serve as an umbrella species whose conservation benefits their high-elevation Asian habitat. Their numbers are believed to be in decline due to numerous anthropogenic threats; however, their conservation is hindered by numerous knowledge gaps. In particular, the dearth of genetic data, unique among all big cat species, hinders a full understanding of their population structure, historical population size, and current levels of genetic diversity. Here, we use whole-genome sequencing data for 41 snow leopards (37 newly sequenced) to offer insights into these unresolved aspects of snow leopard biology. Among our samples, we find evidence of a primary genetic divide between the northern and southern part of the range around the Dzungarian Basin—as previously identified using landscape models and fecal microsatellite markers—and a secondary divide south of Kyrgyzstan around the Taklamakan Desert. Most noteworthy, we find that snow leopards have the lowest genetic diversity of any big cat species, likely due to a persistently small population size throughout their evolutionary history rather than recent inbreeding. We also find that snow leopards have significantly less highly deleterious homozygous load compared to numerous *Panthera* species, suggesting effective purging during their evolutionary history at small population sizes. Without a large population size or ample standing genetic variation to help buffer them from any forthcoming anthropogenic challenges, snow leopard persistence may be more tenuous than currently appreciated.

snow leopard | *Panthera uncia* | heterozygosity | structure

Significance

Through an international effort, we have generated whole-genome sequencing data for 37 snow leopards, increasing the number of snow leopards sequenced by an order of magnitude. We have used these data to show that snow leopards have the lowest genetic diversity of any big cat species, even the cheetah. We do not see evidence of high levels of inbreeding or genetic load in snow leopards compared to other *Panthera* species; however, their dearth of genetic diversity and small population size should be kept in mind when assessing their risk of extinction in the Anthropocene.

Residing in some of the most extreme and remote areas of the world, snow leopards (*Panthera uncia*) are rarely encountered and are challenging to study, making them one of the most enigmatic of the large charismatic mammals. They are among the largest carnivores in the high-elevation habitat in which they reside and their persistence relies on healthy mountain ungulate populations (1) sometimes supplemented by livestock (2–9). Snow leopard habitat consists of mountainous areas of Asia, spanning 12 countries (Fig. 1*A*), habitat that offers immense ecosystem services–acting as an important source of carbon storage (10) and providing water to almost two billion people. Snow leopards serve as an umbrella species whose conservation benefits this globally crucial Asian mountain ecosystem. In spite of its apparent benefits, snow leopard conservation is impeded by the many knowledge gaps regarding this elusive species (11).

Snow leopards were listed as Endangered by the International Union for Conservation of Nature (IUCN) for 45 y but were downlisted to Vulnerable in 2017 as they did not meet specific criteria for population size (fewer than 2,500 mature individuals) and percent population decline (more than 20% over two generations) for Endangered status. This change of status has been controversial (17, 18) as snow leopard numbers are presumed to be in decline due to habitat loss, decreased availability of primary prey (high-elevation, mountain-dwelling ungulates), retaliatory killings for livestock predation (19), and poaching for their skins (20). As climate change in high mountain Asia is occurring at an even more rapid rate than elsewhere in the Northern hemisphere, excluding the Arctic (21), it is also likely to become an increasing threat to snow leopards (22).

Currently, the global snow leopard population size is estimated to be anywhere from 4,700 to 7,500 individuals and little is known about their historical population size and range (23) or their current population trends (12). While many other big cat species experienced historical declines due to range contractions during the Last Glacial Maximum (24, 25) and are facing contemporary human-driven declines (26, 27), it remains unclear

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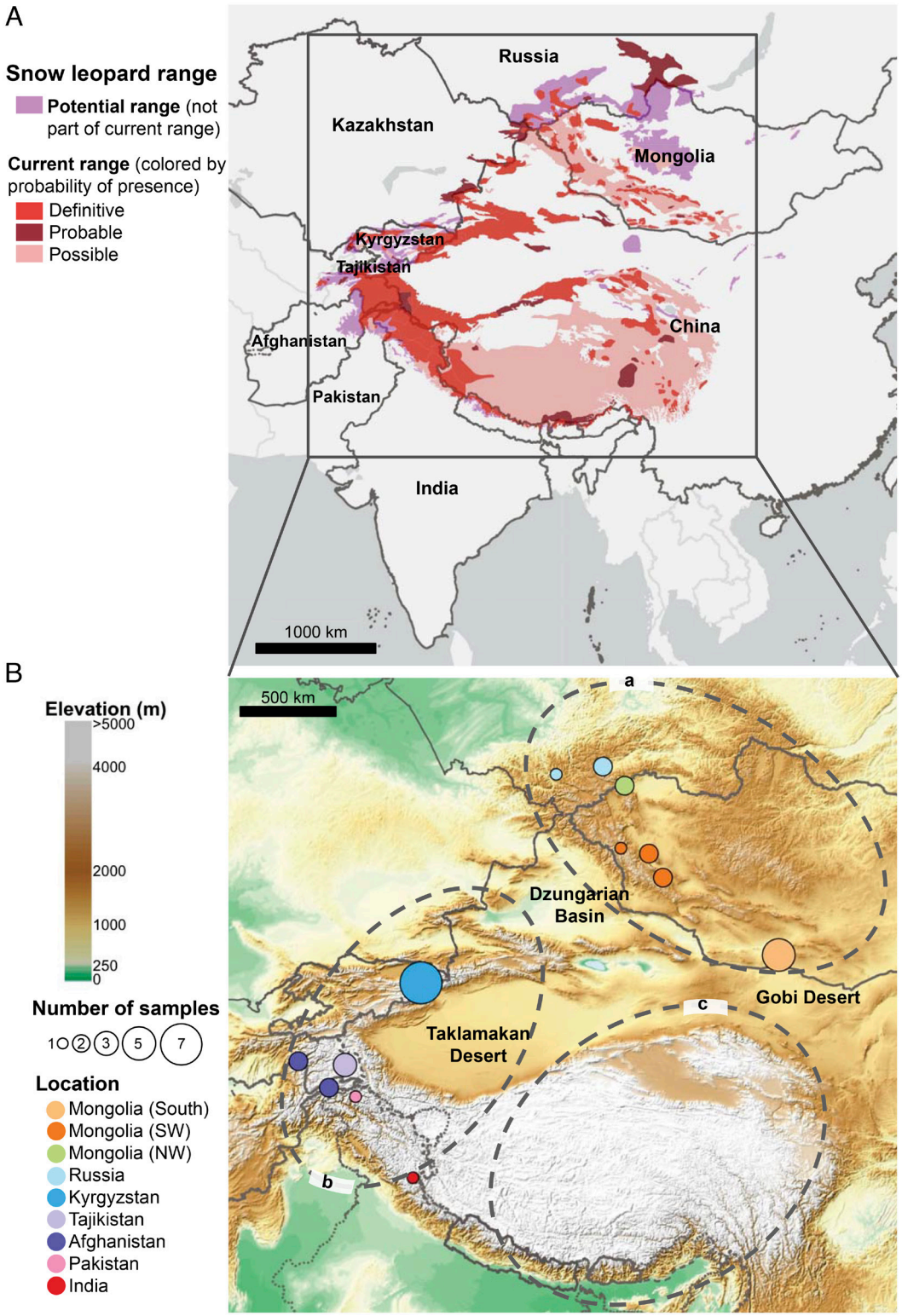

Fig. 1. Snow leopard distribution and sample maps. (*A*) The IUCN snow leopard distribution (12), taken from (13), is shown and the largest snow leopard range countries are labeled. The total potential snow leopard range (all shades of red and pink) is 3,256,841 km^2, of which 2,778,309 km^2 is considered to be the current range. Within the current range, snow leopards are only definitively present in 32% of the area [889,059 km^2, definitive observation of snow leopards within the year leading up to the assessment (2007–2008)], probable in 8% (222,265 km^2, likely present based on habitat, prey, and connectivity and there is recent nondefinitive information or definitive information of snow leopard presence within the last 5 y), and possible in 60% (1,666,985 km^2, possibility present due to habitat and connectivity to known populations, but no specific information about snow leopards in this area within the last 5 y) (13). (*B*) Sample locations are indicated with different sized circles indicating the number of samples from each location. The basemap (14) indicates elevation and landscape features discussed in the text are labeled. Gray dashed ovals indicate the geographic distribution of the three subspecies suggested by Janecka et al. (15)– a) *P. u. irbis*, b) *P. u. uncia*, c) *P. u. uncioides*. In both maps, country boundaries available from the Snow Leopard Trust (16) are shown in dark gray. Not all country boundaries are in agreement, so a dotted line is used for India and a dashed line is used for China to make overlapping boundaries visible. Maps were created using ArcGIS software by Environmental Systems Research Institute, Inc.

if the snow leopard was previously more abundant than is currently estimated. One study using fecal microsatellite data suggests that snow leopards may have undergone a bottleneck ~8,000 y ago (15). Estimates of genetic diversity and levels of inbreeding are also limited due to a dearth of genomic data for the species. They are the least studied genetically of all the big cat species (28) with whole-genome sequencing (WGS) data available for only two wild snow leopards (29) and two captive individuals (30) prior to this study. Genetic diversity assessed using fecal microsatellite data (15, 31–34) as well as genomic data from one of the previously sequenced wild snow leopards suggests low diversity (29), but genomic data from additional samples are required to determine whether this is a characteristic of the species across its range.

Additionally, there is still more to learn about snow leopard population structure and connectivity (13). Numerous studies have used microsatellite markers from fecal samples to assess genetic structure and connectivity at varying geographic scales. These studies have found connectivity at the local scale to be location dependent, with evidence of continuous habitat connectivity across at least 75 km in Pakistan (34) and >1,000 km in Mongolia (32), weak genetic differentiation among snow leopards samples across 400 km of mountainous terrain in Northern China (33), and signs of genetic structure between sampling areas about 500 km apart in Russia (31). At a larger geographic scale, fecal microsatellite studies (15, 31, 32) and other lines of evidence (35, 36) suggest a geographic divide between Mongolia/Russia and the southern part of the range due to the Dzungarian Basin and Gobi Desert. However, snow leopards are known to cross long distances between mountain ranges (37, 38). In addition to this prominent north–south divide, the largest scale fecal microsatellite study also identified a second divide within the southern group separating the east and west of the Himalayas-Tibetan Plateau complex (15). Janecka et al. (15) argue that each of these three distinct groups constitute unique subspecies; however, this subspecies designation and the level of connectivity across the landscape remains controversial (39, 40). Population structure and connectivity can be assessed with greater resolution using whole-genome data (41); however, until now, this has not been possible.

In addition to the wild snow leopard population, the international community has worked for decades to establish a sustainable zoo population. As of 2008 there were 445 snow leopards across 205 institutions globally, not including China, representing the genetic diversity of 56 wild founders (42), most of which came from the wild in the 1960s–1990s, often from unknown locations. As it is the goal of zoos to maintain a genetically diverse population (43) and to act as reserves of genetic diversity for endangered species, it is important to know what portion of the global genomic diversity of snow leopards this population represents.

Here, we generate WGS data for 33 wild snow leopards from multiple locations across their range in addition to four captive snow leopards from the North American zoo population. We combine this data with existing data for four individuals to achieve three main objectives: 1) characterize snow leopard population structure and connectivity to see how estimates from WGS data compare to fecal microsatellite data; 2) assess the current level of genomic diversity in snow leopards, how this compares to other big cat species, and how this relates to historical population size, inbreeding levels, and genetic load; and 3) assess the ancestry of the current zoo snow leopard population. Among our samples, which do not include most of the central/southern part of the range, we find evidence of the previously identified genetic divide between the north and south, as well as a divide between the Kyrgyz population and populations farther south. However, low levels of genetic differentiation among groups suggests some level of connectivity. We also find the current North American zoo population to be dominated by Kyrgyzstan-region ancestry. Most notably, we find snow leopards to be the least genetically diverse contemporary big cat species, likely due to a persistently small population throughout their evolutionary history rather than to recent inbreeding. Additionally, we find snow leopards to have significantly lower large-effect homozygous genetic load compared to many other *Panthera* species suggesting purging of highly deleterious recessive mutations throughout their evolutionary history. We believe these results have significant implications for snow leopard conservation.

Results

After filtering samples based on sequence quality and breadth of coverage we were left with a final dataset consisting of 37 snow leopard samples. This final dataset included 34 samples from our newly generated data with an average individual depth of coverage of 7.3 × (minimum of 3 × and maximum of 16.8 ×) and three previously published samples with an average depth of coverage of 23 × (minimum of 12 × and maximum of 28.9 ×). These samples consisted of 32 wild born snow leopards representing seven countries (Mongolia, Russia, Kyrgyzstan, Tajikistan, Afghanistan, Pakistan, and India) (Fig. 1*B*) and five captive samples (*SI Appendix*, Table S1).

We mapped these data to the snow leopard reference genome (NCBI accession PRJNA602938) (30) and called single nucleotide polymorphisms (SNPs) as described in the methods, resulting in a final SNP set of 1,591,978. Within this final dataset, we identified one pair of first-degree relatives and three pairs of second-degree relatives (all within the wild samples). When conducting analyses that could be affected by the presence of related individuals, we removed one representative from each of these four related pairs (the sample with lower sequencing coverage), leaving a total of 28 wild and five captive snow leopards. Details of which samples were included in each analysis and why are shown in *SI Appendix*, Table S5.

Population Structure and Dispersal Barriers. We assessed population structure among our samples using principal component analysis (PCA). PCA results indicated that the one Indian sample (12 × coverage), and to a lesser extent, one of the Tajikistan samples (U13, 6.7 × coverage) were genetically distinct from all of the other samples (Fig. 2*A*). In order to more clearly visualize the groupings among the other samples, we also visualized the PCA with the Indian and U13 sample removed. In this PCA, three distinct groups are apparent–Mongolia and Russia; Kyrgyzstan and captive; and Tajikistan, Afghanistan, and Pakistan (Fig. 2*B*).

We also investigated population structure using Admixture (44) to test models with one to ten ancestral populations (K = 1 to 10) over ten independent runs. Due to issues that can arise with having a small sample size for a genetically distinct group (45), we first ran Admixture without the Indian sample (Fig. 2*C*). At K = 2, Mongolia and Russia separated clearly from all other samples with the same ancestry assignments supported in all ten iterations. At K = 3, nine of the ten iterations supported a clear separation of the samples into three groups–Mongolia and Russia; Tajikistan, Afghanistan, and Pakistan; captives and Kyrgyzstan (Fig. 2*C* and *SI Appendix*, Fig. S1*B*)–recapitulating what was observed in the PCA. Admixture and PCA results were robust to the removal of captive samples from the analyses (*SI Appendix*, Fig. S2).

When including the Indian sample, Admixture had difficulty assigning it at K = 2 with five runs grouping it with the southern samples and five runs showing ancestry split between the north

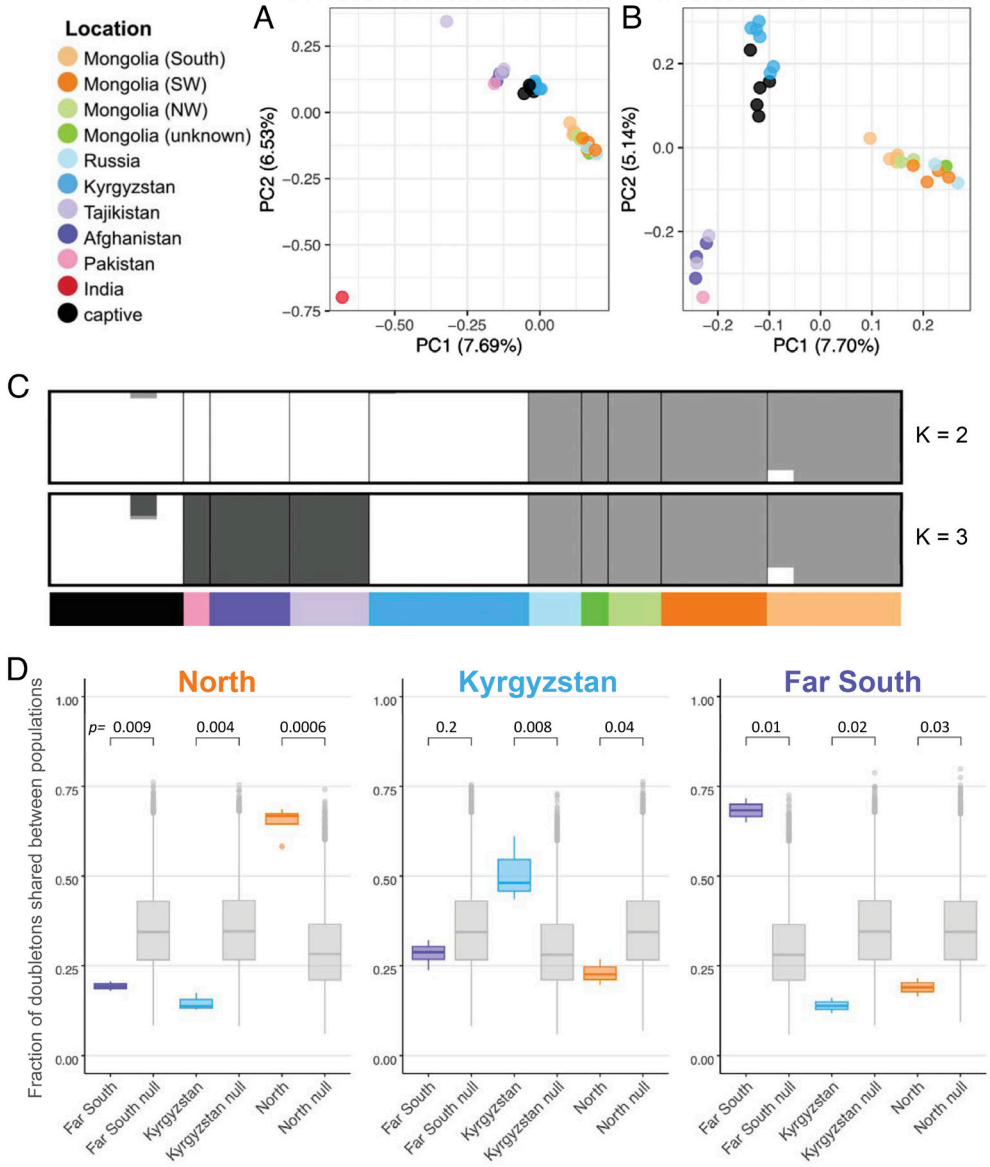

Fig. 2. Principal components analysis (PCA), Admixture, and rare allele sharing. (*A*) PCA of genetic variation of 37 unrelated snow leopards using 1,448,657 SNPs. (*B*) PCA after removing two outlier samples—India and sample U13 from Tajikistan. PCA axis labels include the percent variation explained by PC1 and PC2. (*C*) Admixture results for ten independent runs for K = 2 and K = 3 distinct ancestry groups. The ancestry assignments shown for K = 2 were supported by all ten iterations and the ancestry assignments shown for K = 3 were supported by nine of the ten iterations (the alternate ancestry assignments supported by one run is shown in *SI Appendix*, Fig. S1*B*). (*D*) Doubleton sharing between populations compared to null distributions under panmixia. We identified all doubletons where each minor allele occurred in a different individual. For each group, identified above each graph, we then calculated the fraction of doubletons occurring in an individual of that group that were shared with individuals of each other group. Observed values are shown in color and null distributions are shown in gray. We made null distributions by randomly shuffling population assignment among the samples and recalculating doubleton sharing 10,000 times. *P*-values for comparisons between observed data and the null distribution using the Wilcoxon Rank Sum Test are shown. The lower and upper edges of the boxes correspond to the first and third quartiles and the whiskers extend to the lowest/highest value that is no further than 1.5*IQR (interquartile range) from the box. Points falling further than 1.5*IQR from the box are plotted individually. In all analyses, we have removed one member of each related pair and in the case of doubleton sharing, we have downsampled populations to n = 6 for each group.

and the south (*SI Appendix*, Fig. S1*A*). Maximum likelihood phylogeny construction also showed the Indian sample to be genetically distinct (*SI Appendix*, Fig. S3). Thus, PCA, Admixture, and phylogenetic results all corroborate that the one Indian sample included in this study is genetically distinct from all of the other samples.

Among the other samples (excluding India), Admixture and PCA results suggest three genetically distinct groups (Fig. 2 *B* and *C*). We see a primary genetic divide between the north (Mongolia and Russia) and the south (all other samples) with a secondary divide within the southern group between Kyrgyzstan and populations farther south (Afghanistan, Tajikistan, and Pakistan). These results also show the five captive samples, whose lineages encompass more than half of all of the founders of the current captive population (33 of 56 founders are represented, *SI Appendix*, Fig. S4), group most closely with the Kyrgyzstan samples.

Assessment of Gene Flow. Based on Admixture and PCA results, we quantified population structure between the two groups identified in Admixture at K = 2 and among the three groups identified at K = 3 (Fig. 2*C*) by assessing shared versus private SNPs, F_{ST}, and rare allele sharing. We excluded captive samples and the Indian sample from these analyses. We also excluded one sample from each related pair (U01, U08, KGZ_F4, and AF_06)

from F_{ST} and rare allele sharing analyses, but not shared versus private SNP assessments.

At K = 2, we compared groups that we will refer to as "North" (consisting of Russia and Mongolia) and "South" (consisting of Kyrgyzstan, Tajikistan, Afghanistan, and Pakistan). Each group (downsampled to n = 15) had more shared SNPs (598,449) than private SNPs (379,861 private to the North and 364,010 private to the South) (*SI Appendix*, Fig. S5*A*). At K = 3, we compared groups that we will refer to as "North" (consisting of Russia and Mongolia), "Kyrgyzstan", and "Far South" (consisting of Tajikistan, Afghanistan, and Pakistan). We downsampled the North and Far South groups to seven individuals such that all three groups had the same sample size. Here, and in all analyses requiring downsampling, first, samples that were geographically close were thinned, then samples were chosen for removal based on coverage, such that the most geographically unique and highest coverage samples were retained (*SI Appendix*, Table S5). We found that the Far South and North group had more than twice as many private SNPs (222,569 and 219,175, respectively) as Kyrgyzstan (111,392) (*SI Appendix*, Fig. S5*B*) and that Kyrgyzstan shared a similar number of SNPs with both the North and the Far South (394,020 shared among all three groups, 65,181 shared only between Kyrgyzstan and North, 64,871 shared only between Kyrgyzstan and Far South). It is worth noting that the Kyrgyz group represents the smallest geographic area and this limitation in sampling could contribute to our observation of fewer private SNPs in this group.

We calculated Weir and Cockerham's weighted pairwise F_{ST} after removing one individual for each first and second degree related pair and downsampling groups to equal size. At K = 2, F_{ST} between the North and South was 0.091. At K = 3, the pairwise F_{ST} between the North and Far South was 0.123, between North and Kyrgyzstan was 0.115, and between Kyrgyzstan and the Far South was 0.093. Although F_{ST} can not be directly compared between species, for rough context, these values fall below F_{ST} observed between different tiger subspecies, which range from 0.164 to 0.318, and are on par with the lower bound F_{ST} observed between Bengal tiger subpopulations, which range from 0.094 to 0.3 (46). Observed pairwise F_{ST} at K = 3 was compared to a null distribution representing panmixia created by randomly shuffling population assignments 10,000 times and recalculating F_{ST} between populations. This analysis showed observed F_{ST}values, although somewhat low, to be highly significant ($P < 0.0001$) (*SI Appendix*, Fig. S6).

We identified doubletons (SNPs with a minor allele count of two) where each of the two minor alleles occurs in separate individuals. We then assessed doubleton sharing (47, 48) among the three groups–North, Kyrgyzstan, and Far South–after downsampling each group to six individuals. Rare variants, such as doubletons, are typically the most recent variants. In human populations, doubletons are found most commonly within the same population, and doubleton sharing between populations is interpreted to reflect recent connectivity between those populations (49). Here, in addition to calculating doubleton sharing among snow leopard groups, we also compared the observed doubleton sharing to a null distribution representing panmixia in the same way as pairwise F_{ST}–sample population assignments were randomly shuffled and observed doubleton sharing between populations was recalculated 10,000 times. Doubleton sharing corroborated the preidentified groups with individuals sharing a significantly higher fraction of doubletons (0.40 to 0.72) with individuals within the same group (*P*-value North - 0.0006, Kyrgyzstan - 0.008, Far South - 0.01) (Fig. 2*D*). However, the fraction of doubletons that each individual shared with each of the other groups was 0.11 to 0.32. The Kyrgyzstan group showed the highest fraction of doubleton sharing with outside groups– sharing enough with the Far South to not be significantly different from panmixia ($P = 0.2$). Both Kyrgyzstan and Far South also share enough rare alleles with the North to only be marginally significantly different from panmixia ($P = 0.04$ and $P = 0.03$, respectively). These results support the presence of genetic divergence among these three groups, but also indicate some amount of gene flow among the groups. Note that our assessments of gene flow are limited by our small sample size and will benefit from additional samples in future studies.

Heterozygosity and Historical Population Size. We used publicly available data to call SNPs in all big cat species using the Genome Analysis Toolkit (GATK) (50) and calculated heterozygosity using VCFtools (51). Here, we defined big cat species as species with an average adult body weight of 40 kg or more, which includes all *Panthera* species (lion (*P. leo*), tiger (*P. tigris*), leopard (*P. pardus*), jaguar (*P. onca*), and snow leopard) as well as cheetah (*Acinonyx jubatus*) and puma (*Puma concolor*) (52). We found snow leopards to have the lowest heterozygosity of any big cat species, with heterozygosity for every snow leopard sample included in this study falling lower than that observed in any other big cat (Fig. 3*A*). Notably, snow leopard heterozygosity was lower than that of cheetahs, which have long been considered the archetype of low heterozygosity in big cats (26, 53). The relative values of observed heterozygosity among all the other species for which we calculated heterozygosity were consistent with previous work (27, 54–56).

We used pairwise sequentially Markovian coalescent (PSMC) (58) to reconstruct historical effective population size using the highest coverage individual from each genetically distinct group (North-29 ×, Kyrgyzstan/captive-28 ×, Far South-9 ×, India-12 ×). Reconstructions showed consistent results across all populations sampled and across all bootstrap replicates (Fig. 3*B*). All reconstructions show a consistently small effective population size over the last ~900,000 y with snow leopard effective population size never exceeding 28,000 individuals. Reconstructions suggested that snow leopards had an effective population size of 13,000 to 17,000 individuals from ~300,000 to 75,000 y ago and underwent a slow decline between ~75,000 to 30,000 y ago to an effective population size of ~6,000 to 8,000 individuals. This decline is coincident with the maximum extent of glaciation in mountainous areas of Asia during the Last Glacial Maximum (~40,000 to 100,000 y ago) which was well before the global Last Glacial Maximum (~20,000 y ago) (57). Effective population size is generally smaller than census size (59) and PSMC historical effective population size estimates can be impacted by numerous species-specific parameters (e.g., population structure, inbreeding, mutation rate, generation time) (60, 61) which have not been thoroughly characterized in snow leopards, and thus exact effective population size estimates should be interpreted cautiously.

Inbreeding and Runs of Homozygosity (ROH). Using the same dataset that we used to calculate genome-wide heterozygosity across big cat species, we also calculated the inbreeding coefficient (*F*) across big cats. These results showed the inbreeding coefficient of snow leopards was not significantly higher than other big cats and was even significantly lower than Asian leopard and puma indicating that the lower genetic diversity observed in snow leopards is not explained by higher inbreeding (Fig. 4*A*).

We also used the software GARLIC (62) to assess ROH in snow leopard samples with 8 × sequencing coverage or more. ROH of different sizes are likely the results of different processes– long ROH reflects recent inbreeding while shorter ROH can reflect

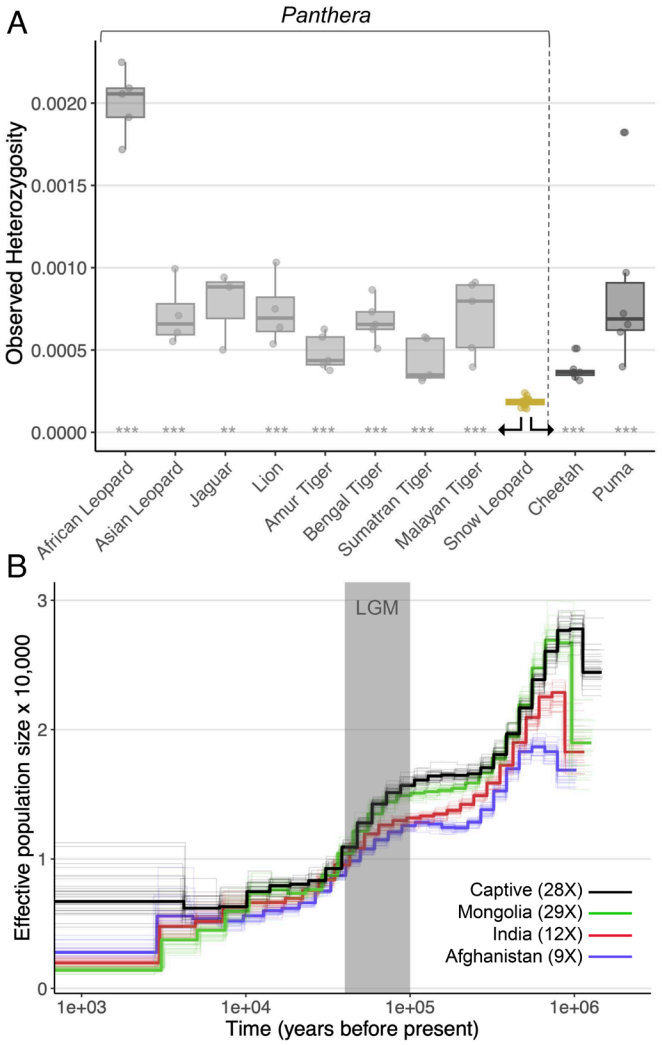

Fig. 3. Genome-wide heterozygosity across all big cats and demographic history of snow leopards. (*A*) Comparison of genome-wide heterozygosity across all big cat species. We used publicly available data to call SNPs for every big cat species using GATK and calculated observed heterozygosity using VCFtools. In the case of leopard and tiger, we called SNPs separately for genetically distinct groups. We calculated snow leopard heterozygosity from SNPs called using the same pipeline that was used for SNP calling in all the other big cat species. Only snow leopard samples with a depth of 8 × or higher are included (n = 15). The lower and upper edges of the boxes correspond to the first and third quartiles and the whiskers extend to the lowest/highest value that is no further than 1.5*IQR (interquartile range) from the box. Points falling further than 1.5*IQR from the box are plotted individually. Stars below each boxplot indicate the *P*-value when comparing each species/subspecies to snow leopards using the Wilcoxon rank-sum test ($P < 0.01$** and $P < 0.001$***). (*B*) Reconstruction of effective population sizes using PSMC with a mutation rate of 3.62×10^{-9} per site per generation and a generation time of 5 y (29). Thirty bootstraps are shown for each sample in thinner, fainter lines of the same color. The timing of the Last Glacial Maximum (LGM) in the mountains of Asia (~40,000 to 100,000 y ago) (57) and the average depth of coverage of each sample used is indicated.

shared population history or background levels of relatedness due to small population size. GARLIC identified 130 bp to be the ideal window size for our dataset. GARLIC uses a sliding window along the chromosome and makes a logarithm of the odds (LOD) calculation of autozygosity for each window which is an estimate of how likely homozygous regions are to be identical by descent. We divided ROH into four size bins – 0.1 to 1, 1 to 5, 5 to 10 Mb, and >10 Mb – and the proportion of the genome in each size bin for each individual was calculated by dividing the total sequence length in each size bin by the total mappable length of the genome (1,818,166,894 bp). These proportions were compared to those observed in tigers using GARLIC output provided by Armstrong et al. (56).

We found the general trends in the proportion of each ROH size class to be different between snow leopards and tigers, which are known to have undergone recent inbreeding due to small population sizes (63). Across all samples, snow leopards had a larger proportion of the genome (average of 16%) in short ROH (0.1 to 1 Mb) compared to tigers (average of 3%). On average, snow leopards also had a much lower proportion of their genome in ROH longer than 10 Mb (<1% on average) compared to tigers (9% on average). The average proportion of the genome in intermediate sized ROH (1 to 10 Mb) was similar in snow leopards (12%) and tigers (11%) (Fig. 4*B*).

Genetic Load. We used our SNP calls from across the five *Panthera* species to assess how genetic load in snow leopards compares to the other *Panthera* species. Only snow leopard samples with 8 × coverage or higher and one representative from each related pair were used for this analysis. We used the software SnpEff (64) to annotate SNPs and the protocol outlined in (65) to identify derived SNPs in each species. We used this information to filter our dataset to only derived SNPs in protein coding transcripts and counted the total number of SNPs, highly deleterious SNPs, and moderately deleterious SNPs in the homozygous and heterozygous state in each sample. We used these counts to calculate what proportion of each individuals' total homozygous and heterozygous SNPs were highly deleterious and what proportion were moderately deleterious. Looking at the proportion of deleterious mutations out of the total number of protein-coding mutations in this way allows us to assess the relative load across *Panthera* while accounting for variability among the species, such as evolutionary rates, and is ideal for comparisons between species (66).

We investigated homozygous and heterozygous load separately because highly deleterious mutations are more likely to be recessive (67), resulting in purging of highly deleterious mutations only in the homozygous state. Additionally, we expect to see a different trend when looking at moderately deleterious mutations compared to highly deleterious mutations—while small populations are better at removing highly deleterious mutations through purifying selection, this is not the case with mildly deleterious mutations where drift will dominate in small populations (68).

We found that snow leopards had significantly less derived highly deleterious genetic load than numerous *Panthera* species/subspecies– African leopard, jaguar, lion, and Sumatran tiger– in the homozygous state (Fig. 4*C*), and no significant differences in the heterozygous state (Fig. 4*D*). Conversely, snow leopards had slightly more derived moderately deleterious genetic load than lions and Bengal tigers in the homozygous state (*SI Appendix*, Fig. S9*A*) and no significant differences in the heterozygous state (*SI Appendix*, Fig. S9*B*).

Discussion

Population Structure and Dispersal Barriers. We used WGS from 37 snow leopards to investigate population structure of the species. Our results corroborate results from previous studies. Among our samples, which do not include the southeast part of the range, we identify three genetically distinct groups. Admixture and PCA results identify the most pronounced divide among our samples to occur between the northern and southern part of the range around the Dzungarian Basin (Fig. 2 *B* and *C*), consistent with previous microsatellite analyses (15, 31, 32) and models (35, 36). Admixture and PCA results also identify a secondary divide occurring south

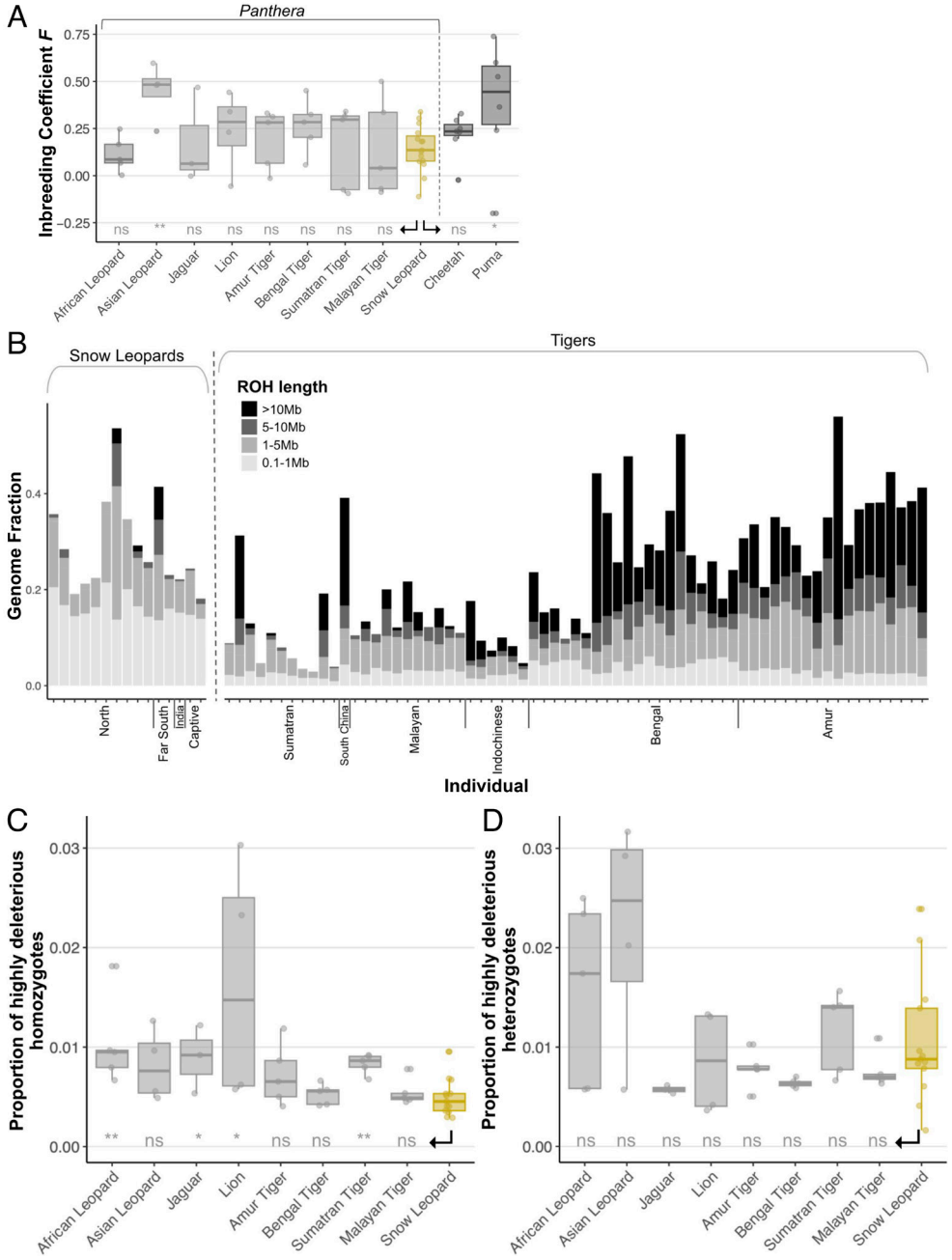

Fig. 4. Inbreeding estimates across all big cats, ROH estimates for snow leopards and tigers, and highly deleterious load across *Panthera*. (*A*) Comparison of inbreeding coefficient *F* measured by method of moments across all big cat species. We used publicly available data to call SNPs for every big cat species using GATK and calculated the inbreeding coefficient *F* using VCFtools. Only snow leopard samples with 8 × coverage or greater are included (n = 15). (*B*) The fraction of the genome in ROH of each size bin (0.1 to 1, 1 to 5, 5 to 10 Mb, and >10 Mb) for snow leopards and tigers. All ROH estimates were calculated using GARLIC. We pulled tiger ROH data from Armstrong et al. (56). Each bar is one individual. Snow leopard samples are ordered by group as indicated along the *x*-axis and tigers are ordered by subspecies. All ROH estimates, for snow leopard and tiger, only include samples with greater than 8 × coverage. (*C*) Comparison of the proportion of homozygous derived protein-coding SNPs that are highly deleterious across *Panthera*. (*D*) Comparison of the proportion of heterozygous derived protein-coding SNPs that are highly deleterious across *Panthera*. In all boxplots, each point represents one individual. The lower and upper edges of the boxes correspond to the first and third quartiles and the whiskers extend to the lowest/highest value that is no further than 1.5*IRQ (interquartile range) from the box. Points falling further than 1.5*IRQ from the box are plotted individually. The *P*-value when comparing each species/subspecies to snow leopards using the Wilcoxon rank-sum test is indicated below each boxplot (ns = nonsignificant, $*P < 0.05$, $**P < 0.01$).

of Kyrgyzstan (Fig. 2 *B* and *C*), around the Taklamakan Desert, consistent with previous microsatellite analyses (31). Both of these genetic divides are also supported by highly significant F_{ST} values between groups (*SI Appendix*, Fig. S6).

These results, in combination with significant F_{ST} among these regions, suggest that the Dzungarian Basin and Taklamakan Desert present barriers to dispersal for snow leopards. However, the level of genetic differentiation among these regions was modest as measured through shared versus private alleles (*SI Appendix*, Fig. S5), and shared rare alleles (Fig. 2*D*) suggesting that there is some level of connectivity among these groups. This is consistent with previous fecal microsatellite work which has also found evidence of weak connectivity between Mongolia and China (32), as well as between Kyrgyzstan and Mongolia/Russia and between Kyrgyzstan and Tajikistan (31). Additional samples will be necessary to confidently estimate gene flow in future analyses.

Population structure analyses suggest that India is genetically distinct from all other samples (Fig. 2*A* and *SI Appendix*, Fig. S3). The uniqueness of this sample could suggest that this individual is our one representative of the southeastern phylogenetic group (*P. u. uncioides*, Fig. 1*B*) suggested by Janečka et al. (15), but more samples from this area will be necessary to resolve how genetically distinct Indian snow leopards may be.

Heterozygosity, Demographic History, ROH, and Genetic Load. Our results show that snow leopards have the lowest genetic diversity of any big cat species (Fig. 3*A*), lower than previously appreciated (27, 69). Demographic history and ROH assessments indicate the exceptionally low heterozygosity in snow leopards is likely due to a persistently small population size over the last 900,000 y (Fig. 3*B*) rather than recent inbreeding events. Our demographic history assessment (Fig. 3*B*) did not pick up the more recent population bottleneck ~8,000 y ago suggested by Janecka et al. (15) from microsatellite data likely because PSMC analyses loose power at more recent time scales.

With a persistently small population size and low genetic diversity throughout their evolutionary history, snow leopards serve as yet another example that high genetic diversity is not a requirement for the long-term persistence of a species (70–75). Such long term persistence has been suggested to be facilitated by purging of deleterious mutations over long time scales in relatively small populations (70–72, 75). Our assessment of genetic load across *Panthera* supports this hypothesis, showing that snow leopards have a significantly smaller proportion of highly deleterious homozygous SNPs than many other *Panthera* species (Fig. 4*C*). We also find that snow leopards have slightly more moderately deleterious homozygous SNPs than some other *Panthera* species (*SI Appendix*, Fig. S9), which is also consistent with a long-term small population size resulting in less effective purging of mildly deleterious mutations.

Consistent with demographic history assessments, ROH analyses show snow leopards have a greater proportion of their genome in shorter ROH (Fig. 4*B*), which likely reflects shared population history and small historic population size (76). Conversely, many tiger subspecies, which are known to have high levels of recent inbreeding due to small isolated populations (63), show the opposite trend, with the highest proportion of their genome in longer ROH (56) (Fig. 4*B*). Taken together, all of our results–heterozygosity, demographic history, ROH, and load–are all consistent with snow leopards having a persistently small population size.

Note that during the review process of this manuscript, a different study (77), using partially overlapping samples with ours, published results consistent with what we have presented here—very low heterozygosity in snow leopards, purging of highly deleterious variants, and lengths of ROH indicating historic inbreeding due to long-term small population size rather than recent inbreeding.

Origin of Captive Samples. Studbook-based pedigrees for the five captive individuals sequenced in this study show that the origin of their wild ancestors is mostly unknown. The few ancestors of known origin are documented as originating from the USSR, Kyrgyzstan, Kazakhstan, and the western Himalayas (*SI Appendix*, Fig. S4). Admixture and PCA results both show a strong signal of genetic similarity between the five captive individuals and the current Kyrgyzstan population (Fig. 2 *A*–*C*). The current Kyrgyzstan population is likely genetically similar to that in Kazakhstan and northeastern China, for which we have no genomic data. Thus, these results suggest that the ancestors of the captive individuals largely came from what is currently Kyrgyzstan, or the surrounding area, and fewer ancestors have come from other portions of the range. Given that it is a goal of zoos to maintain a genetically diverse population and potentially be a reserve of genetic diversity, the fact that the current North American zoo population only represents a small subset of the current wild diversity indicates that this captive population should not be perceived as a reserve of rangewide wild genetic diversity.

Conservation Implications. Snow leopards live in arid, cold, low-productivity, high-elevation habitats where few species can persist—an environment that has evidently only ever been able to support a limited number of snow leopards (Fig. 3*B*). As a result, our data show that they have likely always had an effective population size much lower than other big cats [as suggested by Pečnerová et al. (27) and Cho et al. (29)], and harbor less genetic diversity than even the cheetah (Fig. 3*A*).

Thanks to their extreme environment, snow leopards have not yet been exposed to the same level of acute anthropogenic pressures as have big cats living in habitats more easily accessible to humans. Yet, even having been spared the most intense human impacts, our data indicate that they already have extremely low genomic diversity and population sizes compared to other big cats. Although these characteristics have not hindered their long-term persistence thus far, and have likely resulted in the purging of highly deleterious mutations from the population (Fig. 4*C*), this means that snow leopards cannot rely on a large population size or standing genetic variation to help them survive any forthcoming anthropogenic challenges, as other big cats have done (78, 79). Additionally, snow leopards carry similar amounts of highly deleterious heterozygous load as other *Panthera* (Fig. 4*D*), load that would become unmasked with future inbreeding, suggesting that snow leopards are just as vulnerable to future inbreeding depression as other *Panthera* species. Unfortunately, the most intense anthropogenic pressures may lie ahead for snow leopards. Anthropogenic climate change threatens to shrink snow leopard range through habitat change (22) and more intense interspecific competition [e.g. (80), (81)]; shifting grazing practices risk facilitating spillover of novel pathogens from domestic animals into snow leopards and their prey (82); and accelerating mining, energy, and infrastructure development threaten to fragment and degrade previously remote snow leopard habitats (83, 84). Protection of snow leopards and their habitat, to the greatest degree possible, will be pivotal as this low-density carnivore appears genetically and demographically ill-equipped to bounce back from anthropogenic perturbations (85).

Materials and Methods

Sample Collection and Sequencing. Through an international collaborative effort, we collected a total of 37 snow leopard blood or tissue samples, composed of 32 wild caught samples from five countries (Afghanistan, Kyrgyzstan, Tajikistan, Mongolia, and Russia), one currently captive but wild-born individual from Pakistan (included in the wild group throughout the manuscript), and four captive samples from mixed/unknown ancestry. Sample details, including DNA extraction and library preparation methods used, can be found in *SI Appendix*, Table S1. We sequenced all samples on an Illumina sequencing platform using paired end 150 bp reads with an aim of sequencing each sample to ~5 to 8 × coverage.

All samples from captive individuals were collected as part of routine animal care in the respective Association of Zoos and Aquariums zoos and all wild samples were collected as part of ongoing monitoring of snow leopards by local conservation groups with all necessary collection permits. Samples were processed in labs in the United States, France, and Russia and were transported to respective locations with appropriate transport permits.

Additionally, we collected all currently published WGS data for snow leopards, which included data for two captive individuals (NCBI accessions SRR16227515 (30) and SRR12437590), one wild individual from Mongolia [NCBI accession SRR836372 (29)], and one wild individual from India (NCBI BioProject PRJNA1051290). In total, we gathered WGS data for 41 snow leopards.

Note that there are WGS data for an additional sample identified as a snow leopard on Genbank (biosample SAMN17432540) that we did not use in this study because we concluded that these data were from an Asian leopard (*P. pardus*) (Supplementary Methods and *SI Appendix*, Fig. S7).

We were unable to include any samples from a large portion of the snow leopard range in the southeast, but are hopeful that data from this area will be available in future analyses.

Variant Calling. Reads were mapped to the snow leopard reference genome [NCBI accession PRJNA602938 (30). Mapping, using BWA-MEM (86), and SNP calling, using GATK (87), were performed by Gencove Inc., a service provider. We calculated the depth and breadth of coverage for each sample from BAM files using SAMtools (88) (*SI Appendix*, Table S1). Variants were filtered for quality as described in detail in the Supplementary Methods resulting in a final set of 1,591,978 SNPs.

Relatedness Assessment. We estimated relatedness among samples using SNPrelate (89) in R (90) as described in detail in the Supplementary Methods. We identified one sample pair with a kinship coefficient greater than that expected from first-order relatives–U01 and U09 (kinship coefficient of 0.335), and we identified three sample pairs with kinship coefficients consistent with second-order related pairs–SL_KGZ_F1 and SL_KGZ_F4 (0.148), AF_SL_07 and AF_SL_06 (0.135), and U14 and U08 (0.124). We removed the sample with lower sequencing coverage from each of these pairs (U01, SL_KGZ_F4, AF_SL_06, and U08) from indicated analyses. All sample pairs with nonzero kinship coefficients are listed in *SI Appendix*, Table S2.

Pedigrees for Captive-Bred Samples. Using snow leopard studbooks (42, 91), we compiled information on all of the ancestors of the five captive-born individuals included in this study and drew pedigrees with the kinship2 package (92) in R (*SI Appendix*, Fig. S4). The wild origin of their ancestors is mostly unknown; however, each sample has a few ancestors for which there is a wild origin location listed (*SI Appendix*, Fig. S4), these origin locations include the USSR (between 1964–1974), Kazakhstan (in 1972), Kyrgyzstan (between 1974–1980), Przewalsk (between 1974–1975, which we believe to refer to Karakol, Kyrgyzstan which was previously named Przewalsk), and Aksai (a contested region between China and India in the Western Himalayas, in 1979).

Population Structure Assessments. All population structure assessments were conducted on the dataset after filtering for first and second degree relatives (N = 33, 1,448,657 SNPs). We conducted PCA using PLINK2 (93), admixture analyses using Admixture (44), and constructed a phylogenetic tree using IQtree (94). PCA and admixture analyses were also conducted without the India samples as well as without the captive samples. Details can be found in the *SI Appendix, Supplementary Methods*.

Quantifying Population Structure. We further characterized population divides identified in Admixture and PCA by calculating the number of shared versus private SNPs among groups using BCFtools (95), pairwise F_{ST} using VCFtools, and the rate of rare variant sharing among groups using VCFtools and PLINK. We excluded captive samples from these analyses as well as the sample from India as the PCA indicates that this sample is genetically distinct from all groups. Details of these analyses can be found in the *SI Appendix, Supplementary Methods*.

PSMC. We used PSMC (58) to estimate snow leopard effective population size back in time using the highest coverage sample for each population cluster. Starting with BAM files filtered to include only putative autosomes, we used SAMtools *mpileup* and BCFtools *call* to generate a VCF. We then used *vcfutils.pl vcf2fq* to generate diploid FASTQ files using the "-D" flag to set the maximum read depth to twice the average depth for each sample and the "-d" flag to set the minimum depth to a third of the average depth for each sample, as recommended by PSMC for generating PSMC input. We then ran PSMC with the default settings. We also performed 30 rounds of bootstrapping using random sampling with replacement for each sample. For plotting, we used a mutation rate of 3.62×10^{-9} per site per generation as calculated by Armstrong et al. 2025 (96) and a generation time of 5 y as suggested for snow leopards by Cho et al. (29).

Heterozygosity in Other Big Cats. We calculated heterozygosity in all big cat species using publicly available data in order to see how snow leopard heterozygosity levels compared to other big cat species. We included all other species in the genus *Panthera* (leopard, lion, tiger, jaguar) as well as cheetah and puma. Accession numbers of publicly available WGS data and reference genomes used in this analysis are listed in *SI Appendix*, Tables S3 and S4, respectively. We mapped all FASTQ data to the corresponding reference genome using BWA-MEM (86), called SNPs using GATK (87), and filtered the resultant VCF as described in detail in the *SI Appendix, Supplementary Methods*.

We calculated the mappable length of each genome using mappability BED files to calculate the number of nucleotides with a mappability score of one. Using the filtered VCF for each species or group, we calculated observed homozygosity for each sample using VCFtools (51) with the flag "--het." In R, we calculated the number of heterozygous sites by subtracting the number of observed homozygous sites column ((O)HOM) from the total number of sites column (NSITES). We then calculated heterozygosity by dividing the number of heterozygous sites by the length of the genome consisting of putative autosomes with a mappability score of one. We used ggplot2 in R to create boxplots of heterozygosity results.

Heterozygosity in Snow Leopards. Although SNPs had already been called by Gencove Inc. (as described above), we recalled SNPs from the snow leopard dataset using the same pipeline used to call SNPs in all of the other big cat species to ensure comparability of heterozygosity calculations among species. We calculated observed heterozygosity in snow leopards using both SNP datasets (Gencove's and ours) in the same way as described above. We calculated heterozygosity for all individuals, regardless of relatedness; however, because heterozygous SNPs are most accurately called with higher coverage data (97), we only used samples with 8 × coverage or more.

We compared snow leopard heterozygosity calculated from SNPs called using our pipeline to heterozygosity calculated from SNPs called by Gencove using Pearson correlation coefficient calculated using the ggpubr (98) package in R (*SI Appendix*, Fig. S8). We found heterozygosity calculated from the two different SNP calling pipelines to be extremely correlated ($R = 0.97$, $P = 5.1E-9$); however, all heterozygosity estimates calculated from our SNP calls were slightly higher (average of ~0.000056) than that estimated from Gencove SNP calls.

ROH. We converted VCF files to transposed PLINK files using PLINK1.9 with the flags "--allow-extra-chr –const-fid 0 --recode transpose." We then inferred ROH in each individual using the software GARLIC (62) with an error rate of 0.001 and the centromere location of each chromosome set to 0, 0; since centromere location is unknown. In addition, we used "--auto-win-size, --auto-overlap-frac, –winsize 100" to allow GARLIC to determine the ideal window size to use.

We divided ROH into four size bins–0.1 to 1, 1 to 5, 5 to 10 Mb, and >10 Mb, and then calculated the proportion of the genome in each size bin by dividing the total sequence length in each size bin by the total sequence length of putative autosomes with a mappability score greater than one. The proportion of the genome in each size bin was visualized in boxplots using ggplot2 in R.

We pulled ROH values for tigers, calculated using GARLIC, from Armstrong et al. (56) and calculated the proportion of the genome in each size bin of ROH for each individual in the same way as described above, but using the total mappable length across all autosomes in the tiger reference genome.

As with heterozygosity assessments in snow leopards, in order to limit any biases caused by allelic drop out in lower coverage samples, we only used samples with 8 × coverage or higher in ROH assessments in both snow leopards and tigers.

Inbreeding Across Big Cats. We calculated the inbreeding coefficient *F* in all big cat species using the same SNP datasets used to calculate heterozygosity. As with heterozygosity assessments, we only calculated inbreeding for snow leopards samples with 8 × coverage or higher. We calculated the inbreeding coefficient *F* using the method of moments [(observed homozygous count - expected count)/(total observations - expected count)] using VCFtools with the flag "-het."

Genetic Load Across *Panthera*. We assessed genetic load across *Panthera* using the same SNP datasets used to calculate heterozygosity. We first built a database for each *Panthera* species in SnpEff (64) using an annotated genome for each species. We then used SnpEff to characterize the effect of each SNP in each species. In the case of tiger and lion, the reference genome used to call SNPs was not

annotated, so we first used LiftOverVCF (99) to project our SNP calls onto the annotated tiger and lion genome, respectively. We used the pipeline from (65) to identify derived SNPs by comparing across the *Panthera* clade. We used this information to limit our SNP dataset to only derived SNPs in protein coding transcripts and counted the number of total SNPs, high impact SNPs, and moderate impact SNPs in the heterozygous and homozygous state in each individual. Highly deleterious homozygous load in each individual was calculated as the number of high impact homozygous SNPs divided by the total number of homozygous SNPs in that individual. Highly deleterious heterozygous load was calculated for each individual by dividing the number of high impact heterozygous SNPs by the total number of heterozygous SNPs. Moderately deleterious homozygous and heterozygous load was calculated in the same way. Additional details can be found in the *SI Appendix, Supplementary Methods*.

Samples Included in Each Analysis. We provide a detailed list of which samples were included in each analysis in *SI Appendix*, Table S5.

Data, Materials, and Software Availability. Data associated with this study has been deposited into bioproject PRJNA1048427 (100) and will be released upon publication. The code used for analyses in this project is available on the project's github: https://github.com/ksolari/SL_WGS (101).

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