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Next-Generation Sequencing of Fecal DNA: A Novel Insight Into the Mitogenome Phylogeography of the Snow Leopard (*Panthera Uncia*)

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ABSTRACT

Fecal samples are commonly used in conservation genetics for endangered and elusive species such as the snow leopard (*Panthera uncia*). However, the limited quantity and low quality of endogenous DNA in these samples present a challenge for acquiring genetic and genomic data. Previous studies of snow leopard mitochondrial DNA (mtDNA) phylogeography have produced inconsistent results, likely due to the limited sequencing length of PCR-based methods. To address this limitation, we performed Next-Generation Sequencing (NGS) on 19 fecal samples obtained from the eastern Qinghai-Tibet Plateau, resulting in 6.51–12.72 Gb of raw data per sample. We successfully assembled 17 complete mitogenome sequences (~16,720 bp) and identified 67 SNPs. Phylogeographic analysis revealed two divergent mtDNA lineages with a patristic distance of 0.31%, comparable to the divergence observed between major lineages of lions (0.38%–0.82%) and tigers (0.24%–0.56%). One lineage was predominantly found in the Qilian Mountains, while the other was more broadly distributed across the Sanjiangyuan Region and the Hengduan Mountains. These results reveal a considerable level of mtDNA diversity at a local scale, which was missed in a previous study focusing on short mtDNA segments. Our study demonstrates the promising applicability of mitogenome assembly via NGS of fecal DNA, and we anticipate that it may advance global snow leopard conservation genetics by alleviating technical hurdles and enhancing data sharing.

Chen Cheng and Huaiqing Chen have contributed equally to this study.

Summary

Studying snow leopards is challenging due to their elusive nature, but their feces offer a practical way to explore their genetics. To extract the maximum genetic information from these fecal samples, which typically contain very little high-quality DNA, we employed next-generation sequencing (NGS) technology to analyze their mitogenomes. From samples collected in the eastern Qinghai-Tibet Plateau, we successfully reconstructed and examined the complete mitogenomes of 17 snow leopards. Our analysis uncovered two distinct genetic lineages, with levels of difference comparable to those found between major lineages of lions and tigers. One lineage was predominantly found in the Qilian Mountains, while the other was more widely distributed across the Sanjiangyuan Region and the Hengduan Mountains. This study highlights significant local-scale genetic diversity that previous studies may have overlooked. The workflow established in this study may bolster global snow leopard conservation genetics by reducing technical obstacles and promoting data sharing.

- **Practitioner Points**
  - This study developed a robust and cost-efficient workflow for assembling snow leopard mitogenomes using NGS of fecal DNA.
  - Our results show two divergent mtDNA lineages in the eastern Qinghai-Tibet Plateau, suggesting substantial local-scale genetic diversity.

1 | Introduction

The snow leopard (*Panthera uncia*) is an apex predator predominantly found in the alpine mountainous regions of the Qinghai-Tibet Plateau and Central Asia. Its distribution spans 12 countries, with China harboring approximately 60% of the species' total habitat range (McCarthy et al. 2016). Globally, snow leopards are classified as Vulnerable by the International Union for Conservation of Nature (IUCN) and are mainly threatened by habitat degradation, poaching, retaliatory killing, and prey depletion (Li et al. 2016b, 2020, 2019; McCarthy et al. 2017).

Conservation genetic research is crucial for comprehending the genetic diversity of the snow leopard and identifying priority populations and habitats (Weckworth 2021). Fecal samples are commonly used in conservation genetic studies of endangered and elusive species due to their noninvasive nature and convenience of field collection (Rodgers and Janečka 2013). However, such samples typically contain low quantities and poor quality of endogenous DNA, which presents challenges for acquiring genetic and genomic data (Andrews et al. 2018).

Previous studies on the mitochondrial DNA (mtDNA) phylogeography of snow leopards have yielded conflicting results. One study detected virtually no mitochondrial DNA (mtDNA) variation across 70 fecal samples collected from the species' entire range (Janecka et al. 2017). However, this result was likely compromised by limited sequencing length, as the study employed PCR-based methods to analyze only 683 bp mtDNA segments. In contrast, mitogenome sequences assembled from captive individuals and confiscated tissue samples have revealed substantial genetic diversity (Janecka et al. 2020; Wang et al. 2025). These findings underscore the need to obtain complete mitogenome sequences from range-wide populations for a more accurate understanding of snow leopard phylogeography. However, traditional approaches for mitogenome assembly in *Panthera* species have relied almost exclusively on tissue samples, which are impractical for population-scale studies in snow leopards (Li et al. 2016a; Wei et al. 2011; Buddhakosai et al. 2016; Liu et al. 2018; Bertola et al. 2016).

Recent advances in next-generation sequencing (NGS) provide a powerful solution for analyzing noninvasive samples (Andrews et al. 2018). Mitogenome assembly via NGS of fecal DNA has been successfully implemented in studies of various species, including primates (Perry et al. 2010; Ang et al. 2020; Srivathsan et al. 2016; Van Der Valk et al. 2017; Wanner et al. 2021), rabbits (Baeza et al. 2023), and elephants (De Flamingh et al. 2023). The core procedure involves the construction of a metagenomic library, followed by the filtering and assembly of endogenous mtDNA reads using bioinformatic pipelines (Baeza et al. 2023; De Flamingh et al. 2023).

To overcome the limitations of mtDNA phylogeographic research on snow leopards, our study has two primary objectives: 1) to establish a workflow for mitogenome assembly using NGS of fecal DNA, and 2) to reconstruct the mitogenome phylogeography of wild snow leopard populations. Here, we present preliminary results based on fecal samples collected from the eastern Qinghai-Tibet Plateau. Spanning approximately 490,000 km^2 of snow leopard habitat, this region—located in Qinghai and Sichuan provinces—probably represents the world's largest contiguous habitat for the species (Li 2012; Xiao et al. 2019). Our results demonstrate the potential of this approach to advance snow leopard conservation genetics.

2 | Methods

2.1 | Study Area and Fecal Sample Collection

Our study area comprises Qinghai and Sichuan provinces, subdivided into three major geographic units: the Qilian Mountains in northern Qinghai, the Sanjiangyuan Region in southern Qinghai, and the Hengduan Mountains in northwestern Sichuan (Figure 1). Fecal samples from wild carnivores were collected during field transect surveys conducted between 2014 and 2019 (Lu et al. 2023). Each sample was individually preserved in 95% ethanol in 50 mL centrifuge tubes, maintained at cool room temperature during transport, and subsequently stored at −20°C upon arrival at the laboratory at Peking University (Beijing, China).

2.2 | Fecal DNA Extraction and Carnivore Species Identification

DNA was extracted from 100 to 200 mg of each fecal sample using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany), with extraction blanks included to detect potential contamination. Final DNA pellets for each extraction were resuspended in 160 μL

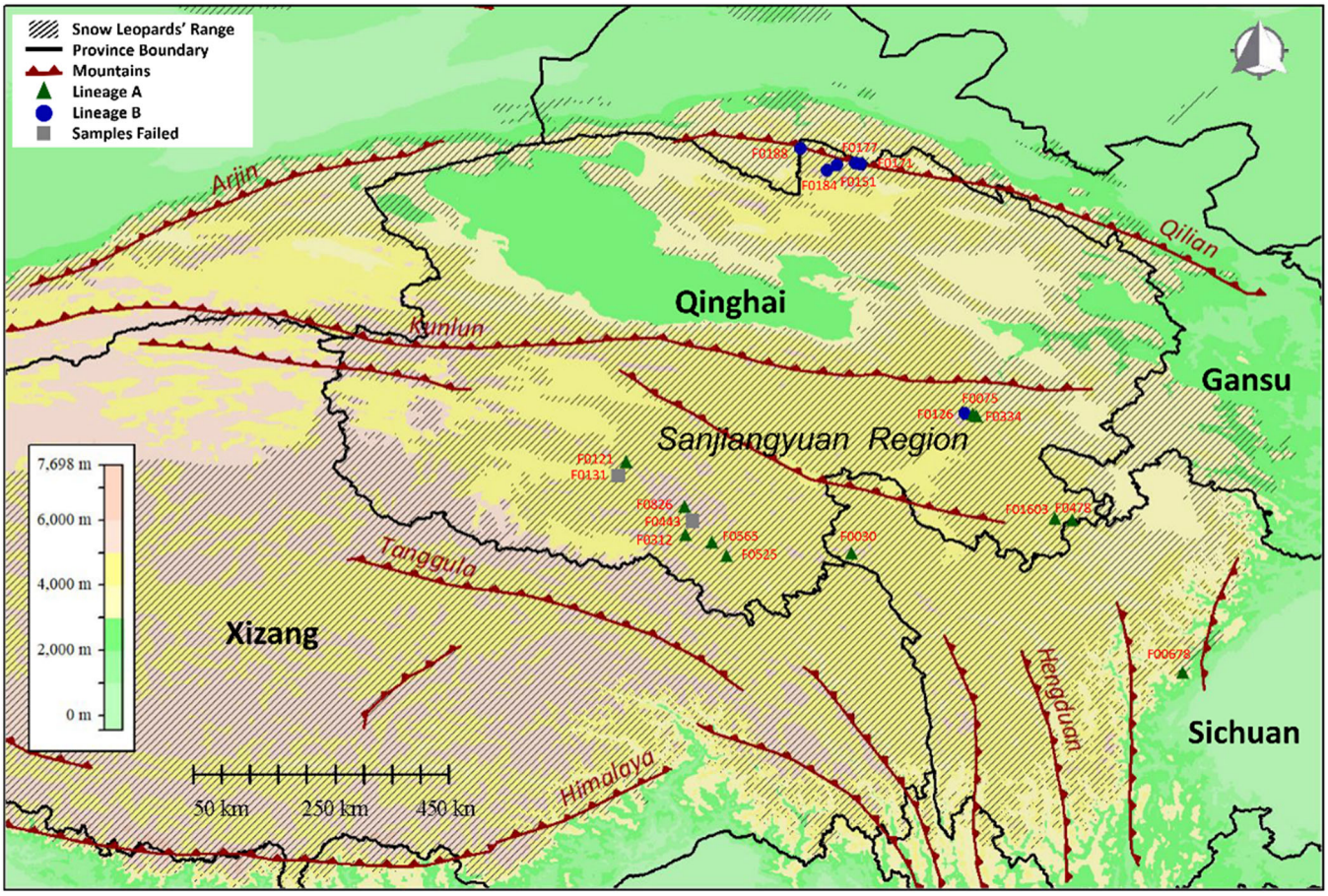

FIGURE 1 | Study area and sample localities. The eastern Qinghai-Tibet Plateau, shown here, supports one of the world's largest continuous snow leopard habitats. The region is subdivided into three major geographic units: the Qilian Mountains (northern Qinghai), the Sanjiangyuan Region (southern Qinghai), and the Hengduan Mountains (northwestern Sichuan). Green triangles denote samples assigned to mitochondrial Lineage A; blue squares indicate Lineage B. The snow leopard distribution range is adapted from the IUCN Red List (McCarthy et al. 2017).

double-distilled water. To identify the host carnivore species, a ~ 400 bp fragment of the mitochondrial *cytochrome b* gene was amplified using a combination of mammalian universal primers H15149 (Kocher et al. 1989) and CanidL1 (Paxinos et al. 1997), and then sequenced bidirectionally using a 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA).

Each obtained sequence was blasted against the GenBank nucleotide database (Madden 2013), and the host species was identified based on the reference sequence with the highest similarity score (98%–100%) (Lu et al. 2023). Nineteen samples identified as snow leopard were selected for subsequent NGS analysis to maximize the geographic coverage of sample localities (Figure 1 and Table 1).

2.3 | High-Throughput Sequencing and Mapping

DNA solutions were submitted for quality testing, library construction, and sequencing using the Illumina NovaSeq. 6000 system at Berry Genomics (Biotechnical Company, Beijing, China). Standard NGS protocols were adopted, except that ultrasonic fragmentation was skipped to avoid over-fragmentation of fecal DNA. Each sample yielded approximately 6 Gb of raw data in 150 bp pair-end reads. Reads with > 3% "N" bases or > 50% bases below Q30 were filtered out.

MtDNA reads were extracted from the total data by mapping them to a 16,717 bp reference mitogenome sequence from a snow leopard of undisclosed geographic origin (NCBI ID: KP202269). Mapping was conducted using *Low Sensitivity/Fastest* settings with Fine Tuning (None) in Geneious Prime v.2020.0.3. The mapped reads were processed using BBDuk Trimmer v.1.0 to remove Illumina adapters at the right end and low-quality bases at both ends. Subsequently, duplicate reads were removed. Finally, the cleaned mtDNA reads were re-mapped to the reference to assemble a circular contig, and consensus mitogenome sequences were generated for bases with at least 2× coverage using the *Highest Quality* setting and annotated using the *Transfer Annotations* function. Mitogenome sequences were aligned using ClustalW algorithm in MEGA-X to identify sequence variation (Kumar et al. 2018). The two short mtDNA segments sequenced by Janecka et al. 2017 (NCBI ID: KY967523, KY967522) were compared with the resulting alignment.

2.4 | Phylogeny Reconstruction

Mitogenome sequences for one snow leopard from Mongolia and other closely related species—lion (*Panthera leo*), leopard (*P. pardas*), jaguar (*P. once*) tiger (*P. tigris*), and clouded leopard (*Neofelis nebulosa*) as the outgroup—were either downloaded

TABLE 1 | Information on snow leopard fecal samples and next-generation sequencing results.

Region	Locality	Sample ID	Total DNA (μg)	Raw data (Gb)	Mean insert size (bp)	Mean (min, max) depth (X)	Output ratio (X/Gb)	Mitogenome size (bp)
Sanjiangyuan region	Zaduo, Qinghai	F0522	0.17	9.45	329	76.8 (43, 114)	8.13	16718
	Zaduo, Qinghai	F0565	10.03	8.68	263	55.8 (20, 196)	6.42	16718
	Zaduo, Qinghai	F0312	0.18	8.08	270	3500.2 (1616, 15544)	432.95	16716
	Zaduo, Qinghai	F0826	0.24	6.51	301	389.8 (176, 497)	59.91	16716
	Jiuzhi, Qinghai	F0478	0.22	9.22	343	27.2 (10, 50)	2.95	16720
	Jiuzhi, Qinghai	F1603	0.17	9.28	312	56.4 (17, 86)	6.08	16716
	Maqin, Qinghai	F0075	0.24	12.72	262	1053.3 (457, 1492)	82.78	16716
	Maqin, Qinghai	F0126	1.78	7.18	264	85.6 (41, 230)	11.92	16724
	Maqin, Qinghai	F0334	0.57	7.59	252	551.1 (215, 798)	72.60	16720
	Zhiduo, Qinghai	F0121	1.33	9.91	318	147.1 (65, 204)	14.84	16717
	Zhiduo, Qinghai	F0131	1.96	9.00	300	7.2 (0, 17)	0.80	*Failed due to low coverage*
	Zaduo, Qinghai	F0443	0.32	9.69	308	12.3 (2, 42)	1.27	*Failed due to Numt*
Hengduan mountains	Shiqu, Sichuan	F0030	0.24	7.14	302	254.8 (92, 366)	35.69	16720
	Wenchuan, Sichuan	F0678	0.33	7.99	348	98.2 (28, 148)	12.30	16720
Qilian mountains	Tianjun, Qinghai	F0151	1.5	7.12	270	1308.5 (719, 2285)	183.76	16720
	Tianjun, Qinghai	F0171	0.5	11.26	244	16.3 (2, 38)	1.45	16720
	Tianjun, Qinghai	F0177	0.27	9.46	276	296 (144, 399)	31.30	16716
	Tianjun, Qinghai	F0184	0.08	8.21	286	890.9 (469, 1463)	108.51	16717
	Tianjun, Qinghai	F0188	0.52	9.84	306	1766.5 (1014, 2455)	179.51	16716

from GenBank or assembled with SRA reads (see Figure 2 for NCBI ID) (Cho et al. 2013; Buddhakosai et al. 2016; Liu et al. 2018; Bertola et al. 2016). These sequences were aligned with our own using MAFFT v7.450 (Katoh and Standley 2013). The data set was trimmed to a 15,419 bp contig of coding regions, with the D-loop region excluded. Phylogenetic reconstruction was conducted using Bayesian inference in MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), under the GTR + I + G substitution model as determined by Modeltest (Posada and Crandall 1998). The analysis ran for 1,100,000 iterations, with the initial 100,000 iterations discarded as burn-in.

3 | Results

3.1 | Sequencing and Variations

The total DNA content of the 19 fecal samples ranged from 0.08 to 10.03 μg (Table 1). Library building and sequencing

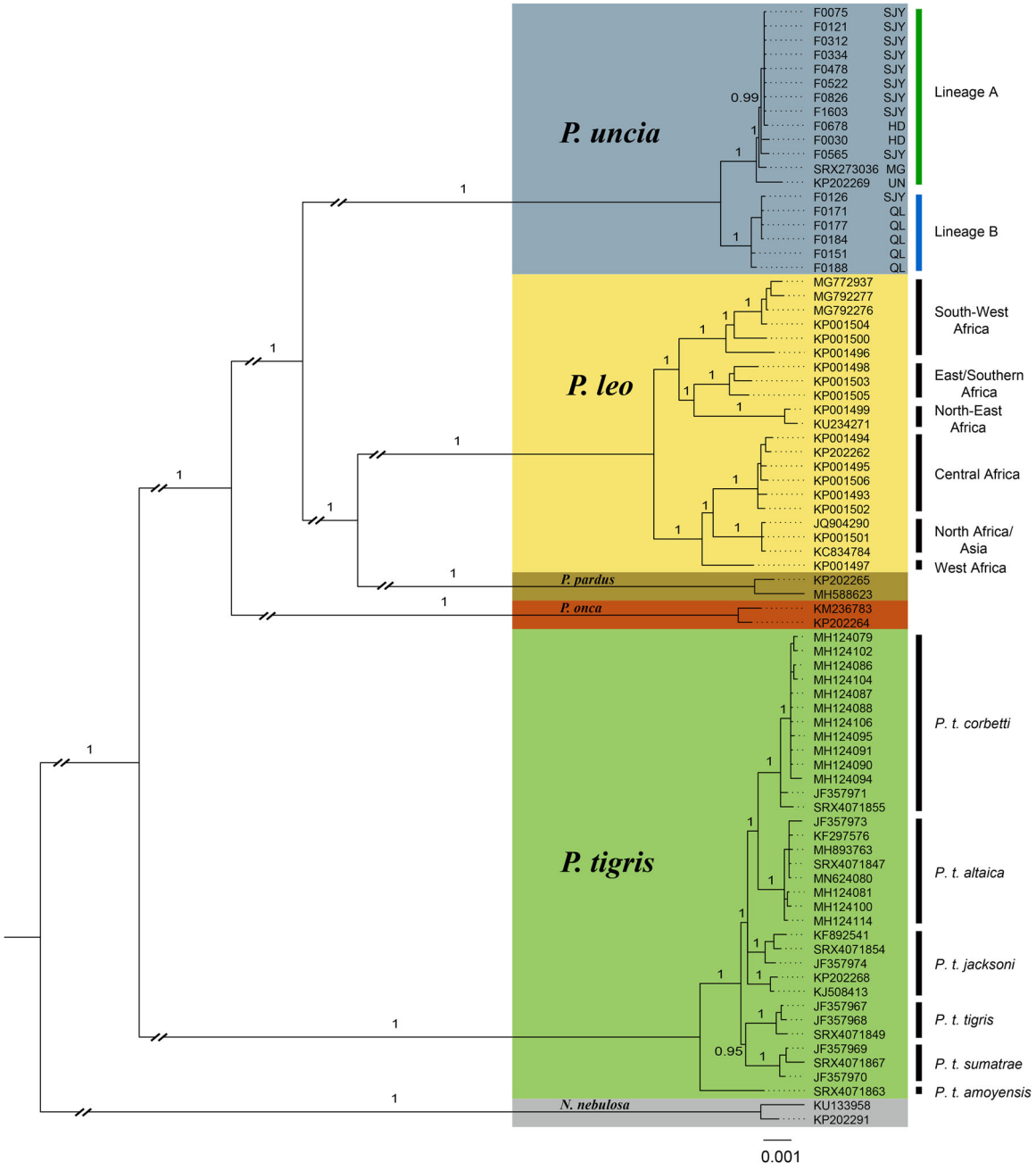

FIGURE 2 | Mitogenome phylogeny of the snow leopard (*Panthera uncia*) and other big cats, including the lion (*P. leo*), leopard (*P. pardas*), jaguar (*P. once*), and tiger (*P. tigris*), with the clouded leopard (*Neofelis nebulosa*) as the outgroup. The analysis is based on a 15,419-bp mitochondrial DNA coding region data set. Bayesian posterior probability values are shown at the branches. Reference sequences (NCBI IDs) and sample IDs from this study are labeled at the tips. Snow leopard sample localities are abbreviated as follows: SJY (Sanjiangyuan Region), HD (Hengduan Mountains), QL (Qilian Mountains), MG (Mongolia), and UN (unknown). Subspecies/lineages are denoted by right-hand bars. Species-level branches have been truncated for clarity.

were successfully completed for all samples, including those with minimal DNA content. For each sample, 6.51 to 12.72 Gb of raw data (or 5.98–11.04 Gb of clean data) were generated. Mitogenome contigs were constructed with mean read coverage ranging from 7.2× to 3500.2×. The mean insert size varied from 244 to 349 bp. Complete mitogenome sequences were ultimately obtained for 17 samples. Two samples, F0131 and F0443, were excluded due to low coverage and an enormous number of heterozygous sites, presumably caused by nuclear mitochondrial pseudogenes (*Numt*s) (Kim et al. 2006).

The mitogenome sequences ranged from 16,716 bp to 16,724 bp in length. A total of 67 single-nucleotide polymorphisms (SNPs) and three indels were identified in the alignment (Table 2). Variation was more frequent at the junction of *ATP6* and *COX3* (8622–8717 bp), *ND4* (10,945-11,127 bp), and hypervariable region I (15,756–15,890 bp), where four SNPs occur within 200 bp. The anterior portion of hypervariable region II (16,332–16,395 bp) exhibited the highest level of variability, with two indels and four SNPs detected.

3.2 | Phylogeography Reveals Two Divergent Lineages

Our mitogenome phylogeny presents the same species-level topology as previous studies of the genus *Panthera* (Figure 2) (Johnson et al. 2006; Li et al. 2016a). The tiger forms the basal lineage, while the snow leopard is placed in a sister group to the common ancestor of the lion and leopard. This pattern is inconsistent with the nuclear phylogeny, in which the snow leopard and tiger form sister groups. The observed mito-nuclear discordance has been interpreted as evidence of post-speciation hybridization between the ancestors of snow leopards and those of lions and leopards (Figueiró et al. 2017).

Within snow leopards, two divergent lineages were found. Lineage A is broadly distributed across the Sanjiangyuan Region and the Hengduan Mountains, whereas Lineage B is concentrated in the Qilian Mountains. Notably, both lineages were detected in Maqin, Qinghai, on the northwestern edge of the Sanjiangyuan Region (Figure 1). These findings suggest a phylogeographic pattern consistent with a north–south split in the eastern Qinghai-Tibet Plateau. Unexpectedly, a geographically distant sample from Mongolia clustered within Lineage A at its basal location.

Representative sequences from lions and tigers were included in the phylogenetic analysis to characterize intraspecific diversity and provide a comparative framework. Six major lineages were observed in lions, corresponding to the proposed northern and southern subspecies (Bertola et al. 2016). Seven major lineages were identified in tigers, aligning with the six extant subspecies (Liu et al. 2018). The average patristic distances among major lineages were 0.38%–0.82% in lions and 0.24%–0.56% in tigers. The average patristic distance between the two snow leopard lineages was 0.31%, indicating a comparable level of intraspecific divergence.

4 | Discussion

4.1 | Implications for Methodology

Mitogenome sequences represent a superior genetic marker that overcomes the limitations of partial mtDNA segments, and their use has become increasingly common in conservation genetics (Smith 2016; Gouda et al. 2020). This study provides a case where mitogenome sequencing identified considerable mtDNA diversity, whereas the analysis of short segments missed almost all variation. According to our results, the two segments analyzed in the previous study (Janecka et al. 2017) showed minimal genetic variation: the 323-bp central conserved region (15,902–16,225 bp) exhibited no variation, and the 244-bp reverse segment of hypervariable region II (16,468–16,712 bp) contained only two SNPs. Given that PCR-based sequencing remains a prevalent technique, particularly when dealing with substantial sample sizes, we advocate for the prior assembly of mitogenome sequences to inform the design of PCR target segments. This strategy can help prevent the misidentification of genetic variants and ensure more comprehensive detection. Based on our mitogenome data, we identify three highly variable short regions—the junction of *ATP6* and *COX3*, *ND4*, and hypervariable region I—as suitable target segments for PCR-based sequencing (Table 2). However, as our sample localities are confined to the eastern Qinghai-Tibet plateau, a broader sampling range for mitogenome sequencing is needed to guide global PCR-based study designs.

Our study developed a robust and cost-efficient workflow for mitogenome assembly via NGS of fecal DNA. The significant DNA fragmentation observed in fecal samples, typically around 300 bp in length (as detailed in Table 1), conveniently aligns with the shotgun approach employed in NGS. Consequently, the only modification required to standard NGS protocols is the omission of ultrasonic or enzymatic fragmentation steps. Additionally, the high copy number of mtDNA per cell, ranging from 75 to 2500 times that of nuclear DNA (Picard 2021), renders it practical to derive mitogenome sequences from fecal DNA using low-coverage NGS, consistent with the principle of "metagenome skimming" (Andrews et al. 2018). The output ratio of mtDNA depth varied from 0.80 to 432.95× (median 14.84×) per 1 Gb of raw data, indicating large variation in endogenous mtDNA content across fecal samples. We suggest that generating 4 Gb of raw data per sample is a cost-effective strategy in the absence of prior information, as 79% of our samples (15 out of 19) yielded mtDNA coverage exceeding 20× with this volume of sequencing data. For the remaining samples (including the two unsuccessful ones in this study), their mitogenome sequences may still be recoverable through increased sequencing depth and refined bioinformatic processing.

4.2 | Insights for Snow Leopard Conservation Genetics

This study provides novel insights into the phylogeography of snow leopards and underscores the need for more extensive genetic research. Our results indicate two divergent mtDNA

TABLE 2 | Variable sites in mitogenome sequences. An asterisk indicates variable sites previously identified in Janecka et al. 2017. Green-shaded cells highlight three highly variable short regions, each containing at least 4 SNPs within a 200 bp window.

Lineage	Variable sites / Sample ID	1	1	2	2	2	2	3	3	4	4	4	4	4	4	5	6	6	6	7	7	7	8	8	8	8	8	8	8	8	9	9	1	1	1
		7	7	2	6	8	9	2	6	0	1	2	5	6	7	7	0	1	6	2	4	6	0	1	4	5	6	6	6	7	9	6	9	3	8	9
		1	9	7	2	0	8	2	8	4	2	9	3	7	9	1	5	3	3	9	3	4	6	8	9	2	2	5	7	1	9	6	9	1	2	4
		9	5	5	7	3	0	6	4	1	5	9	9	5	4	9	6	6	7	7	2	2	1	8	9	0	2	8	5	7	0	9	2	9	3	5
Lineage A	F0030	A	G	T	T	T	C	T	C	C	A	A	T	G	C	A	C	T	C	T	T	C	C	T	C	C	C	A	T	G	A	T	C	C	C	T
	F0075	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	F0121	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	F0312	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	F0334	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	F0478	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	F0522	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	F0565	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.
	F0678	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	F0826	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	F1603	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lineage B	F0126	T	A	C	C	C	T	C	T	T	G	G	C	A	.	G	T	.	T	C	C	T	T	C	T	T	T	G	C	A	G	C	.	T	.	C
	F0151	T	A	C	C	C	T	C	T	T	G	G	C	A	.	G	T	.	T	C	C	T	.	C	T	T	T	G	C	A	.	.	.	T	.	C
	F0171	T	A	C	C	C	T	C	T	T	G	G	C	A	.	G	T	.	T	C	C	T	T	C	T	T	T	G	C	A	G	C	.	T	.	C
	F0177	T	A	C	C	C	T	C	T	T	G	G	C	A	.	G	T	.	T	C	C	T	T	C	T	T	T	G	C	A	G	C	.	T	.	C
	F0184	T	A	C	C	C	T	C	T	T	G	G	C	A	.	G	T	.	T	C	C	T	T	C	T	T	T	G	C	A	G	C	.	T	.	C
	F0188	T	A	C	C	C	T	C	T	T	G	G	C	A	.	G	T	.	T	C	C	T	.	C	T	T	T	G	C	A	.	.	.	T	.	C
Lineage	Variable sites / Sample ID	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1*	1*	1
		0	1	1	1	2	2	2	2	2	2	2	3	3	3	3	3	3	4	4	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6
		9	0	1	2	1	1	2	3	7	8	9	1	2	4	6	7	8	6	8	2	5	7	8	8	8	2	3	3	3	3	3	3	7	7	7
		8	3	2	2	4	6	8	8	5	9	4	7	1	9	3	2	1	4	7	5	4	5	1	3	9	3	3	4	5	9	9	9	0	0	3
		6	5	7	1	6	0	7	9	2	9	7	3	7	9	0	9	0	3	9	7	3	6	0	3	0	4	2	8	6	3	4	5	3	8	0
Lineage A	F0030	C	A	C	G	T	T	A	A	C	T	C	C	T	T	C	C	T	T	T	C	G	G	G	T	C	G	C	C	?	G	T	A	T	C	—
	F0075	.	.	T	.	.	.	.	.	.	C	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	?	T	.	—	—	.	.	C
	F0121	.	.	T	.	.	.	.	.	.	C	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	Y	T	.	—	—	.	.	—

(Continues)

TABLE 2 | (Continued)

	F0312	.	.	T	.	.	.	.	.	.	C	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	Y	T	.	—	—	.	.	G
	F0334	.	.	T	.	.	.	.	.	.	C	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	Y	T	.	.	.	.	.	C
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	F0522	.	.	T	.	.	.	.	.	.	C	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	?	.	—	—	.	.	T
	F0565	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	A	.	T	T	.	.	.	.	.	T
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	F0826	.	.	T	.	.	.	.	.	.	C	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	Y	T	.	—	—	.	.	C
	F1603	.	.	T	.	.	.	.	.	.	C	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	?	T	.	—	—	.	.	—
Lineage B	F0126	T	G	.	A	.	.	G	G	T	C	T	T	C	C	T	T	C	C	C	.	A	A	A	C	T	.	.	.	?	A	C	R	C	T	—
	F0151	T	G	.	A	C	.	G	G	.	C	T	T	C	C	.	T	C	C	.	.	A	A	A	C	T	A	T	T	T	.	.	.	C	.	C
	F0171	T	G	.	A	.	.	G	G	.	C	T	T	C	C	T	T	C	C	C	.	A	A	A	C	T	.	.	Y	?	A	C	R	C	T	C
	F0177	T	G	.	A	.	.	G	G	.	C	T	T	C	C	T	T	C	C	C	.	A	A	A	C	T	.	.	Y	?	A	—	—	C	T	C
	F0184	T	G	.	A	.	.	G	G	.	C	T	T	C	C	T	T	C	C	C	.	A	A	A	C	T	.	.	Y	?	A	—	—	C	T	—
	F0188	T	G	.	A	.	C	G	G	.	C	T	T	C	C	.	T	C	C	.	A	A	A	A	C	T	A	.	.	T	.	—	—	C	.	G

lineages, exhibiting a north–south split in the eastern Qinghai-Tibet Plateau (Figures 1 and 2). These two lineages were also reported in a recent study using confiscated samples from the Qinghai-Tibet Plateau, although that study lacked associated geographic metadata (Wang et al. 2025). While we did not directly estimate divergence times, the level of divergence appears comparable to that of tiger subspecies, with an estimated divergence time of approximately 50,000 to 80,000 years (Liu et al. 2018; Sun et al. 2023). The inclusion of the Mongolian sample within Lineage A suggests that this mitochondrial lineage may have a wide distribution range. A recent population genomics study also revealed two major genetic lineages in global snow leopards: a southern lineage (Qinghai-Tibet Plateau) and a northern lineage (Altai Mountains, Tianshan, Pamir, and western Himalayas), which diverged approximately 25,000 years ago (Yang et al. 2025). Notably, nuclear genetic differentiation was significantly lower than the divergence observed between the two mtDNA lineages among populations in the eastern Qinghai-Tibet Plateau. Mito-nuclear discordance is common in felids and may result from sex-biased dispersal, historical hybridization, or incomplete lineage sorting (Figueiró et al. 2017; Li et al. 2016a). We hypothesize that the observed discordance reflects an ancient divergence of two distinct mtDNA lineages, followed by recent male-biased dispersal and admixture that have shaped current nuclear genetic patterns. While snow leopard dispersal behavior remains poorly characterized, male-biased dispersal has been well documented in other felids such as jaguars and lynxes (*Lynx* spp.) (Janečka et al. 2007; Kantek et al. 2021; Herrero et al. 2021), suggesting a potentially similar pattern in snow leopards. However, limitations in sample size and geographic scope render our current findings preliminary. Broader sampling is necessary to uncover a comprehensive phylogeographic structure of the species, which is crucial for refining intraspecific taxonomy and identifying conservation priority units.

We anticipate that our research will contribute significantly to global snow leopard conservation genetics by lowering technical hurdles and enhancing data sharing. We provide a practical protocol for mitogenome assembly from fecal DNA using conventional NGS methods. This approach enables the efficient generation of extensive genetic data with minimal technical complexity and is easily implemented using established commercial sequencing services. It not only decreases the barrier of data collection but also fosters the sharing and comparative analysis of data across various studies, holding the promise to ignite and enhance international research collaborations. Looking ahead, we envision that coordinated global efforts involving widespread fecal sampling and NGS-based mitogenome analysis will accelerate progress toward a more profound and comprehensive understanding of the global genetic architecture of snow leopards. This enhanced knowledge will aid in the refinement of their taxonomic classification and support the identification of conservation priority units.

5 | Conclusion

In summary, this study established a robust and cost-effective workflow for mitogenome assembly via NGS using fecal DNA, specifically for snow leopard research. Phylogeographic analysis

revealed two divergent mtDNA lineages exhibiting a north–south split pattern in the eastern Qinghai-Tibet Plateau. We anticipate that our research will advance global conservation genetics of snow leopards by alleviating technical barriers and facilitating data sharing.

Author Contributions

Chen Cheng: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, writing – original draft, writing – review and editing. **Huaiqing Chen:** conceptualization, data curation, formal analysis, methodology, software, visualization, writing – original draft, writing – review and editing. **Xueyang Li:** data curation, formal analysis, investigation, methodology, writing – original draft, writing – review and editing. **Moyan Chu:** data curation, methodology, validation, visualization, writing – original draft, writing – review and editing. **Xiang Zhao:** formal analysis, funding acquisition, project administration, resources. **Lingyun Xiao:** funding acquisition, investigation, project administration, resources, writing – review and editing. **Cunxin Ma:** funding acquisition, resources. **Zhi Lu:** conceptualization, funding acquisition, project administration, supervision, writing – review and editing.

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Ethics Statement

The authors declare that this study was conducted ethically, following all applicable guidelines for animal care and human consent. The data are original, accurate, and have not been previously published or manipulated. All authors have significantly contributed to the work and disclosed any conflicts of interest. We adhere to scientific integrity and will correct any errors and cooperate with the publication process.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Mitogenome sequences generated in this study have been submitted to NCBI with accession numbers MT423707-MT423723. Original sequencing data and additional information related to this article are available upon reasonable request from the authors.

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