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Genetic insights and conservation strategies for Amur tigers in Southwest Primorye Russia

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Southwest Primorye hosts approximately 9% of the remaining wild Amur tiger population and represents hope for the revival of tigers in Northeast China and the Korean peninsula. Decades of conservation efforts have led to a significant increase in population size, from less than 10 individuals surviving in the region in 1996 to multiple folds today. However, while the population size has recovered since the mid-1900s, the effects of genetic depletion on evolutionary potential are not easily reversed. In this study, a non-invasive genetic analysis of the Amur tiger subpopulation in Southwest Primorye was conducted using microsatellite loci and mitochondrial genes to estimate genetic diversity, relatedness, and determine the impact of historical demographic dynamics. A total of 32 individuals (16 males, 15 females, and 1 unidentified sex) were identified, and signs of bottlenecks were detected, reflecting past demographic events. Low genetic variation observed in mitochondrial DNA also revealed genetic depletion within the population. Most individuals were found to be closely related to each other, raising concerns about inbreeding given the small population size and somewhat isolated environment from the main population in Sikhote-Alin. These findings emphasize the urgent need to establish ecological corridors to neighboring areas to restore genetic diversity and ensure the conservation of the Amur tiger population in Southwest Primorye.

Keywords Amur tiger, Southwest Primorye, Non-invasive survey, Genetic bottleneck, Isolate-breaking Effect, Ecological corridor

The effects of human activities such as habitat destruction, pollution, overhunting, and climate change have significantly impacted apex predators and their habitats, leading to declining populations and increased risk of extinction, thereby disrupting the delicate balance of ecosystems they inhabit[1–5]. With the low population density and insufficient prey resource, apex predators may expand their home ranges, become more solitary, and exhibit decreased reproductive rates due to reduced prey availability[6,7]. This adaptation to resource scarcity can lead to heightened aggression and vulnerability to environmental threats, posing significant challenges to their survival. Therefore, conservation efforts aimed at restoring population densities and protecting habitat are crucial for maintaining apex predator populations and preserving ecosystem balance[8].

The Amur tiger (*Panthera tigris altaica*) is a big cat subspecies representing Northeast Asia that reigns as an apex predator in its local ecosystem. It was once widely distributed from Northeast China and Far Eastern Russia to the Korean Peninsula. However, the relentless pressures of indiscriminate hunting, capture for economic gain, and habitat destruction have led to the virtual extinction of Amur tigers across much of their natural range. The

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species is now declared extinct in South Korea, with the last recorded capture dating back to 1921[9]. In Northeast China, camera trap surveys recorded a total of 55 wild Amur tigers between 2013 and 2018[10]. Russia is home to the majority of the remaining wild Amur tigers, with the current estimated census size standing above 750 individuals, representing a remarkable recovery from the less than 40 tigers that survived by the 1940s[11,12].

The tiger habitat in Russia has largely remained preserved and connected. However, the anthropogenic development corridor between Vladivostok and Khanka Lake has divided the tiger range into two sections: the main Sikhote-Alin landscape, home to 90% of Russian tigers, and Southwest Primorye, a narrow strip of land in the southernmost area of Primorsky province spanning at least 5400 km^2[13,14]. This small land also supports the last remaining Amur leopards[15]. Microsatellite analyses confirm the genetic distinctness of the Southwest Primorye tiger population[16,17]. Despite supporting a small isolated tiger population, Southwest Primorye holds great significance for the survival and expansion of big cats in China and the Korean peninsula, as it is geographically adjacent to the Laoyeling landscape, which constitutes the major tiger range in China[18].

The tiger population of Southwest Primorye was estimated to be less than 10 individuals by 1996[19,20], and there were estimated 11–13 tiger (including 2–4 cubs) by 2005[21]. Since then, the population has been gradually increasing, with increased sightings of tigers on the Chinese side, attributed to conservation measures and government policies[22–24]. In the Southwest Primorye region, camera-trap monitoring recorded 39 adult tigers during the winter of 2013–2016[25], with this figure rising to 58 tigers (including cubs) according to the 2021–2022 census[14].

Genetic diversity is directly linked to a species' long-term survival, as it provides evolutionary potential and accounts for the ability to adapt to both internal and external fluctuations[26]. The genetic potential of a population depends on the strength of the founding genes and how many individuals are in the population. This is often a problem in small, isolated populations that have gone through significant declines[15,27]. Therefore, these populations require regular genetic monitoring and management to ensure their viability. Microsatellites, which are co-dominant, polymorphic, and neutral, are commonly employed in genetic studies[28]. They are particularly useful for endangered species when working with non-invasive samples such as feces, urine, hair, saliva, and so on. Microsatellites serve as valuable tools for studying genetic diversity, population structure, historical dynamics, and animal movement patterns within human-dominated and fragmented landscapes[29–33].

In this study, we investigated the quantitative and qualitative status of the Amur tiger sub-population in Southwest Primorye using DNA extracted from fecal samples. We also genotyped the population using microsatellite markers developed from a genome-wide survey of big cats[34], which can be reliably applied to multiple feline species, and sequenced mitochondrial DNA using newly designed markers. Based on the genetic data obtained, we aimed to estimate the number of individuals and the genetic diversity, and furthermore, to uncover the demographic history, providing background for better understanding this endangered population and formulating appropriate conservation strategies.

Results

Sample collection and species & sex identification

A total of 342 feces were collected from 2014 to 2019 (Fig. 1). With an exception of the first year of the survey, more than 50 samples were collected annually in this period (18 in 2014, 51 in 2015, 63 in 2016, 89 in 2017, 51 in 2018, 65 in 2019, and 5 samples with unknown collection dates). Following species identification, 247 out of a total of 342 were identified as tiger, 89 as leopard and 6 remained unidentified. The number of collected tiger samples ranged between 30 and 50 per year, except for 2014 and 2017 (13 in 2014, 31 in 2015, 49 in 2016, 74 in 2017, 33 in 2018, 43 in 2019, with 4 samples having unknown collection dates). Out of the 247 tiger samples, sex was successfully identified in 172 samples ($n=120$, male; $n=52$, female), showing a success rate of 69.6% and male biased sample collection.

Pre-screening processes and individual identification

Sample screening

Out of the 247 tiger samples, 142 were selected in the initial screening step based on a genotyping success rate more than 50% for four markers (Pan6A1, Pan7C2, Pan2A1, and Pan1A1). In the secondary screening step, 78 out of these 142 samples, having a genotyping success rate of more than 75% for 32 markers were chosen to use for marker evaluation.

Marker screening

Of the 32 microsatellite markers, 14 were monomorphic and 18 were polymorphic. Among 18 polymorphic markers, less informative markers were excluded from individual identification markers for following reasons: relatively high error rates (Pan1C1, Pan9C2, Pan14C2, and Pan6A1); low polymorphism (Pan8C2, Pan1C1, and Pan3A2); ambiguous peak calls (Pan3A2, and Pan2D2). Finally, 10 markers were selected and utilized for individual identification (Supplementary Table 3).

Individual identification

Out of the 142 samples, 137 were utilized for individual identification, with the breakdown by year as follows: 6 in 2014, 22 in 2015, 19 in 2016, 39 in 2017, 22 in 2018, 28 in 2019, and 1 with an unknown collection date. These samples had missing data for 2 loci or fewer out of the total 10 loci. With a threshold of allowing 3 mismatches, 32 Amur tigers were successfully identified. Among them, 16 were identified as male (M1 ~ M16), 15 as female (F1 ~ F15), and one (U1) with unidentified sex. Twenty individuals were detected multiple times within the 137 samples, with three individuals notably collected in high numbers (> 10) (Supplementary Table 4). The product of P_{ID} and P_{IDsib} were 1.41×10^{-5} and 4.51×10^{-3}, respectively (Fig. 2).

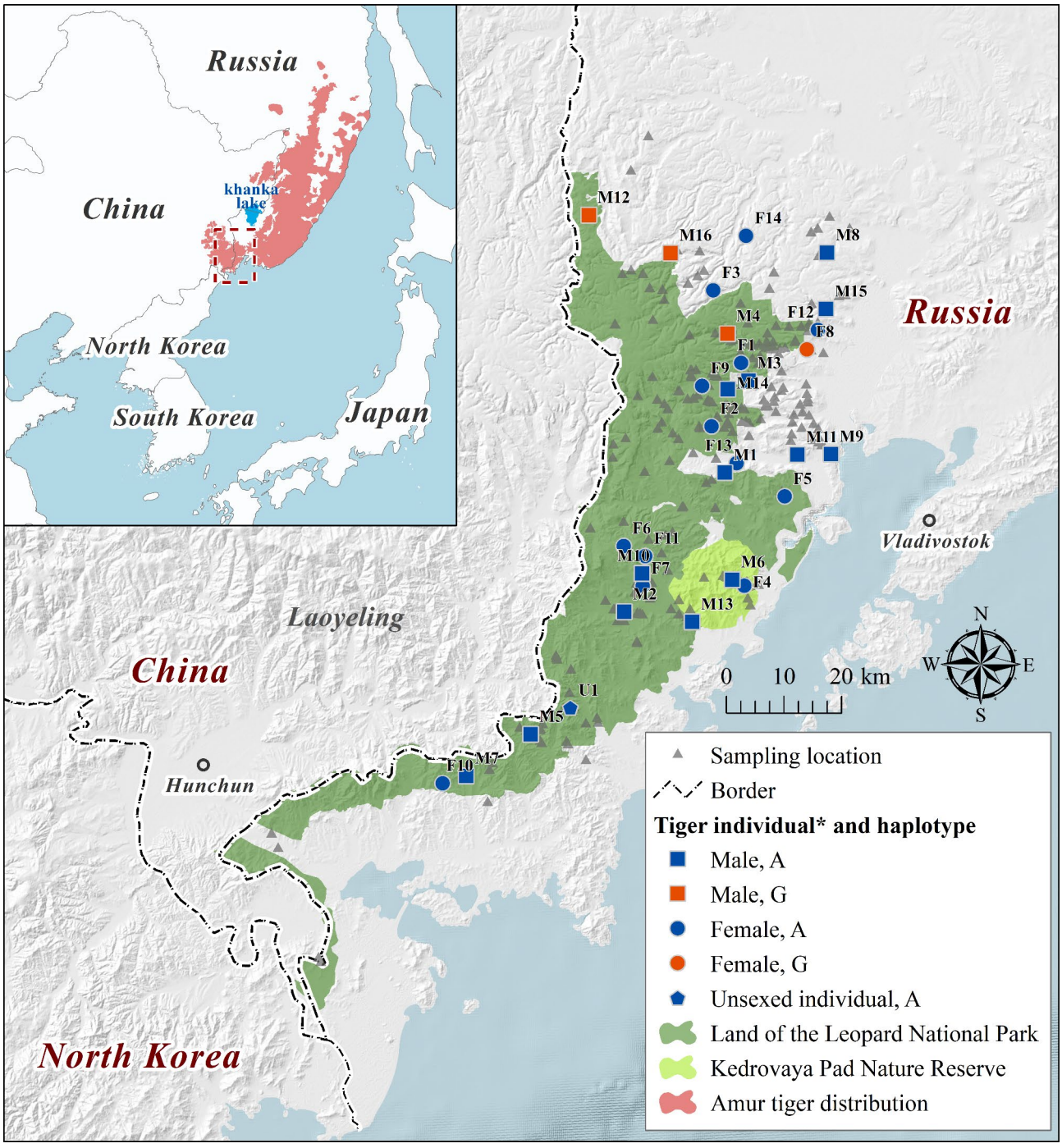

Fig. 1. Study area and capture location of Amur tiger individual samples (any one of the multiple recaptures, if applicable) identified by genetic analysis. Note: All layers have been processed using ArcMap 10.8 (desktop. arcgis.com, accessed on 9 July 2024; ESRI, Redlands, USA); Individual F15 not marked due to missing GPS coordinates.

Genetic diversity and historical demography dynamics

No significant linkage disequilibrium, null alleles, or HWE deviations were detected in the 18 polymorphic loci, all of which were available for following analysis. The number of alleles (N_A) ranged from two to four, with an average of 2.556, while the effective number of alleles (N_E) averaged 1.911, ranging from 1.041 to 2.981. The average observed heterozygosity (H_O) was 0.468 (ranging from 0.040 to 0.667), slightly higher than the average expected heterozygosity (H_E) of 0.435 (ranging from 0.039 to 0.665). Additionally, we recalculated all genetic diversity indices with data excluding three loci (Pan8C2, Pan1C1, Pan9C2) that showed markedly low polymorphism (Table 1).

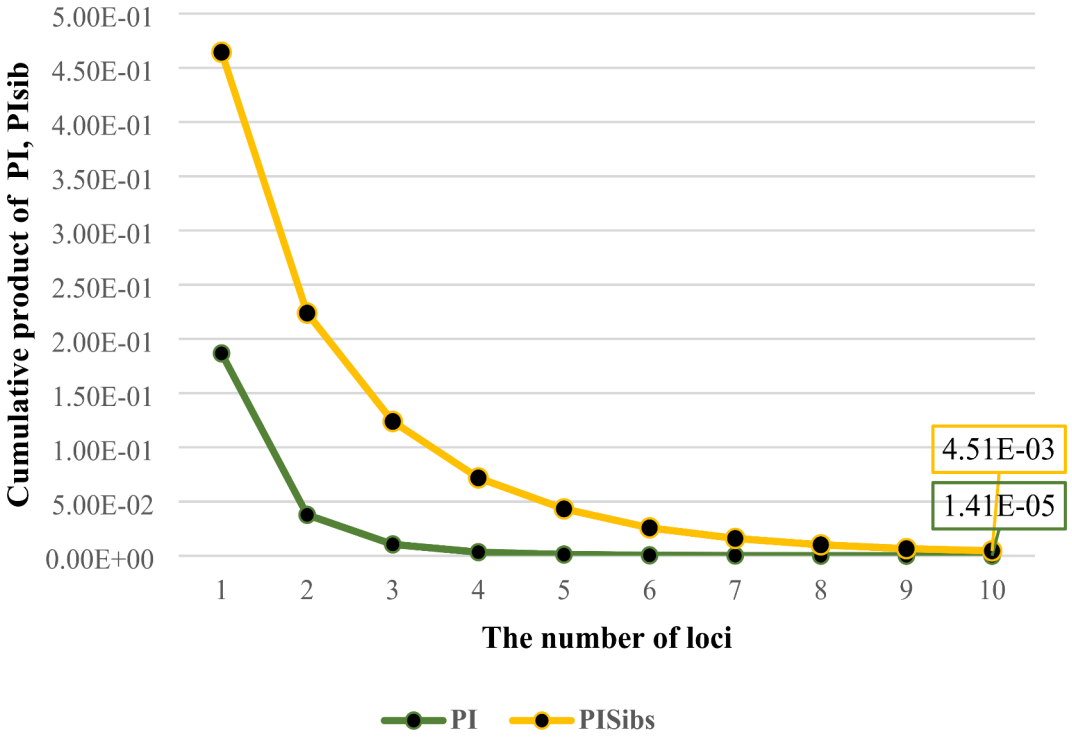

Fig. 2. Cumulative product plot of probability of identity for unrelated individuals (PI) and sibling (PIsib) for individual identification markers and their products.

Recent reduction in abundance was detected by the bottleneck test with significant p-values in all mutation models (infinite allele model, stepwise mutation model, two phase model) and statistical tests (sign test, standardized differences test, Wilcoxon test), along with a distortion in allele frequency class distribution (Fig. 3; Table 2). The Garza-Williamson index (mean = 0.38878, S.D. = 0.17755) was less than 0.68, indicating the possibility of a historical bottleneck in the population.

Population structure and relatedness

Population structure

Microsatellite markers detected no evidence of population structure for Amur tigers in LLNP. The plot of delta K showed local peaks at K = 2, 6, and 9, but those were not supported by visualized bar plots representing the proportion of inferred clusters for each individual (Supplementary Fig. 2). Additionally, no optimal number of clusters was obtained in the BIC plot during de novo clustering implemented in DAPC (Supplementary Fig. 3). Furthermore, clustering pattern were not evident in PCoA. (Supplementary Fig. 4).

Relatedness

The average relatedness calculated by each estimator was as follows: Wang = 0.04793 ± 0.31644, LynchLi = 0.04408 ± 0.31952, LynchRd = -0.0243 ± 0.257, Ritland = -0.0237 ± 0.20602, QuallerGt = -0.0245 ± 0.29721, TrioML = 0.12487 ± 0.18612, DyadML = 0.1422 ± 0.20362 (Supplementary Table 5). Simulation results showed that TrioML had the lowest MSE (data not shown) and was used to build network. When a network was drawn connecting pairs with a relatedness greater than 0.25, all individuals were connected to each other in a single network (Fig. 4), meaning that all relationships were more than half-sib, avuncular, grandparent-grandchild or double first cousin.

Analysis of mitochondrial DNA sequence

In total of 1,888 bp was sequenced from 6 partial regions of Cyt b, COI, COII, CR. No variation was found in the entire region among the 32 individual samples, except for a single point mutation (adenine to guanine transition) detected in Cyt b fragment of 306 bp in size (ptcb-16671 & ptcb-17020). This mutation divided the Amur tiger population into two haplotypes, designed as type A ($n = 28$) and type G ($n = 4$) (Table 3). Nucleotide diversity (π) was estimated to be 0.00014, indicating an extremely low level of genetic diversity in mitochondrial DNA. The same haplotypes were observed in multiple samples of the same individual, revalidating the outcome of individual identification based on microsatellite markers. Interestingly, individuals with type G haplotype were found to be confined to the northern part of sampling area (Fig. 1), prompting several hypotheses for discussion (which will be covered in the discussion part).

Locus	*N*	Size range	N_A	N_E	H_O	H_E	PIC	P_{ID}	P_{IDsib}
Pan6A1	29	157–161	3.000	2.346	0.552	0.574	0.496	2.59E-01	5.28E-01
Pan7C2*	32	200–209	4.000	2.756	0.625	0.637	0.567	2.02E-01	4.82E-01
Pan2A1*	32	223–229	2.000	1.560	0.406	0.359	0.294	4.75E-01	6.89E-01
Pan1A1*	32	233–235	2.000	1.992	0.563	0.498	0.374	3.76E-01	5.95E-01
Pan4A1*	32	180–188	3.000	2.981	0.656	0.665	0.590	1.87E-01	4.64E-01
Pan7A1*	32	166–182	3.000	1.992	0.563	0.498	0.436	3.14E-01	5.80E-01
Pan8C2	28	129–135	2.000	1.153	0.143	0.133	0.124	7.61E-01	8.74E-01
Pan3D2*	31	168–174	3.000	2.138	0.548	0.532	0.473	2.78E-01	5.53E-01
Pan6A2*	32	124–126	2.000	1.717	0.531	0.417	0.330	4.26E-01	6.48E-01
Pan1C1	25	155–161	2.000	1.083	0.080	0.077	0.074	8.55E-01	9.25E-01
Pan3D1*	32	230–238	3.000	1.874	0.500	0.466	0.408	3.43E-01	6.03E-01
Pan3A2	24	210–214	2.000	1.986	0.500	0.497	0.373	3.77E-01	5.96E-01
Pan9C2	25	220–226	2.000	1.041	0.040	0.039	0.038	9.24E-01	9.61E-01
Pan14C2	25	195–199	3.000	2.094	0.440	0.522	0.465	2.86E-01	5.60E-01
Pan15C2	24	189–201	3.000	1.917	0.667	0.478	0.409	3.41E-01	5.96E-01
Pan3A1*	31	189–191	2.000	1.845	0.516	0.458	0.353	3.99E-01	6.21E-01
Pan4D1*	32	167–175	2.000	1.789	0.469	0.441	0.344	4.10E-01	6.32E-01
Pan2D2	24	123–145	3.000	2.133	0.625	0.531	0.468	2.83E-01	5.55E-01
Average	29		2.556	1.911	0.468	0.435	0.368	1.41E-05†	4.51E-03‡
Average¶	29.6		2.667	2.075	0.544	0.505	0.425		

Table 1. Genetic variation of 18 polymorphic microsatellite loci based on 32 individuals. N: samples size, N_A: the number of alleles, N_E: the effective number of alleles, H_O: observed heterozygosity, H_E: expected heterozygosity, PIC: polymorphism information content, P_{ID}: probability of identity for unrelated individuals, P_{IDsib}: probability of identity for siblings. * 10 markers used for individual identification. † Product of probability of identity for unrelated individuals of 10 markers. ‡ Product of probability of identity for siblings of 10 markers. ¶ Averages calculated without three less polymorphic loci (Pan8C2, Pan1C1, Pan9C2).

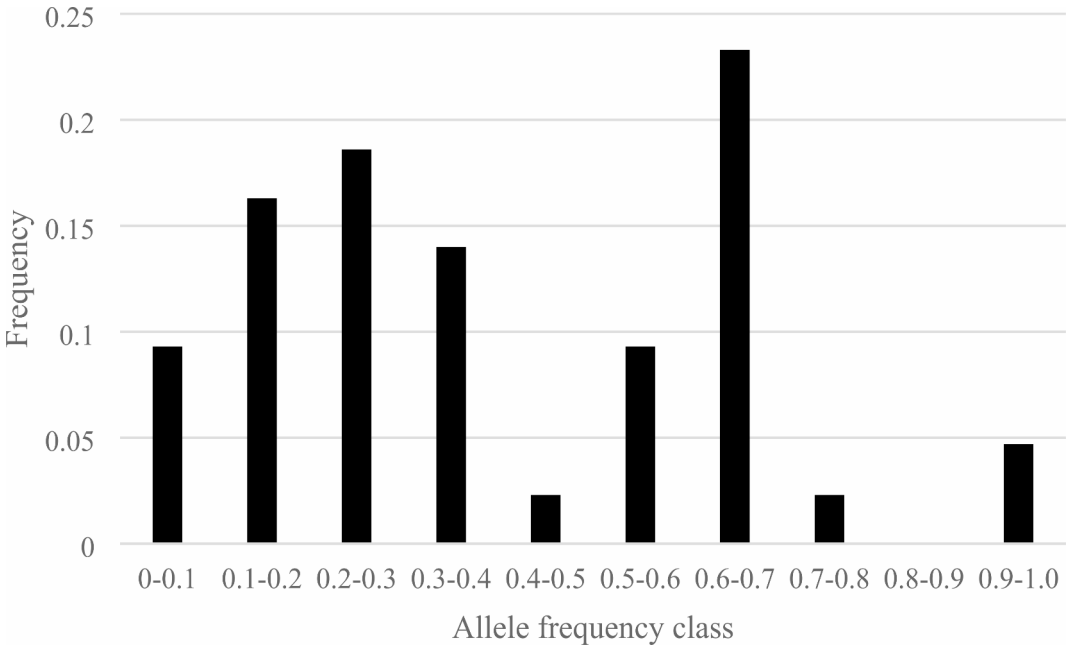

Fig. 3. Distribution of allele frequency class. Graph shows the frequencies (Y-axis) of allele frequency classes (X-axis). Population that have experienced demographic fluctuation in the past may have distorted distribution that are skewed away from an L-shaped distribution.

	Statistical test	Sign test	Standardized differences test	Wilcoxon signed-rank test
Mutation model	I.A.M	0.00083	0.00004	0.00016
	T.P.M	0.00019	0.00087	0.00192
	S.M.M	0.00268	0.01037	0.00278

Table 2. P-values of statistical tests for detecting bottleneck sign.

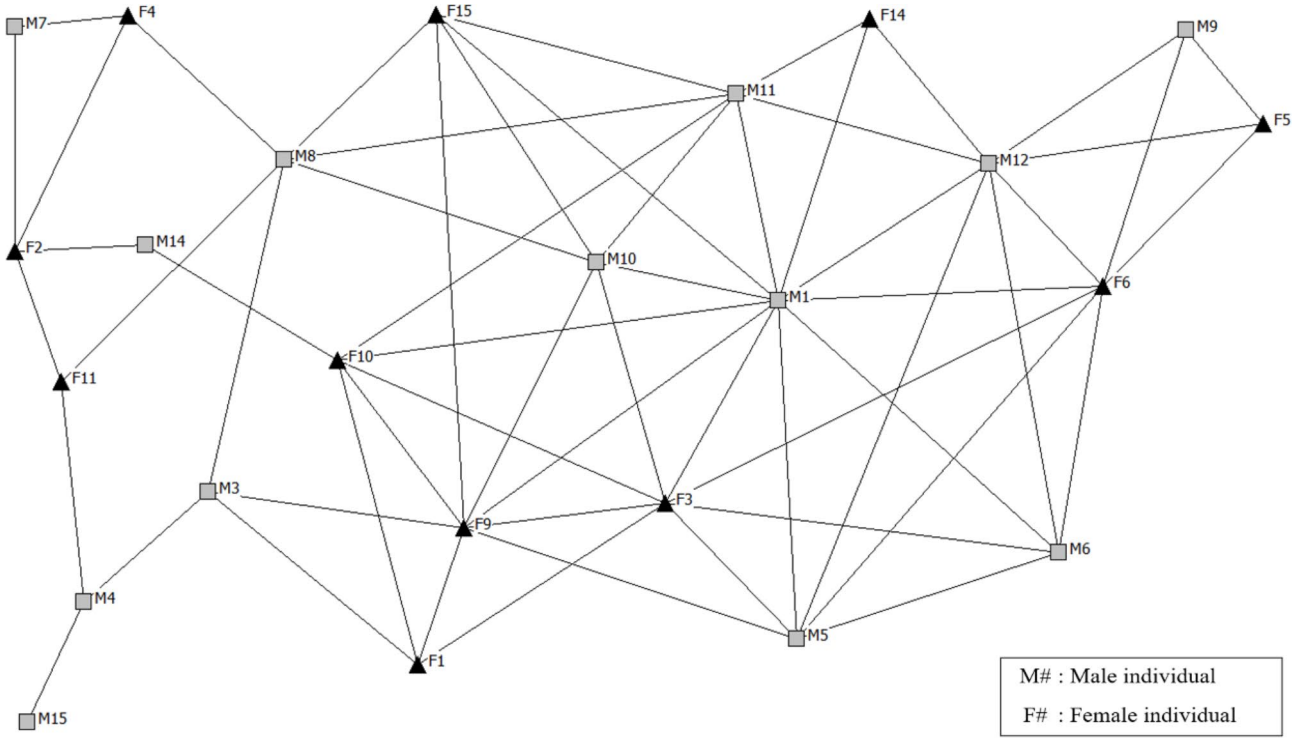

Fig. 4. Relatedness network connecting pairs (TrioML > 0.25) constructed by UCINET. Males were symbolized by gray square and females by black triangle.

Discussion

Southwest Primorye (SW) is integral to the Russian Far East – China Global Priority Tiger Conservation Landscape (TCL) and serves as a core breeding site for Amur tigers at the southwestern edge of their distribution range[36]. SW tiger population is small and forms a continuous link to the tiger population in northeast China's Jilin and Heilongjiang Provinces[13,37]. Populations located at the edges of their range (like SW) are vulnerable to the effects of demographic stochasticity from external disturbances[38]. Noninvasive genetic monitoring serves as an effective strategy to safeguard such populations. In addition to providing a conservative estimate of the population, this methodology allows for the assessment of genetic diversity, mating strategies, and animal movement and dispersal within the landscape.

Long-term extensive surveys were conducted throughout the Land of the Leopard (LL) to collect fecal samples. Molecular sexing revealed that the majority of these samples originated from male tigers. In intuitive perspective, the large number of male feces might be attributed to relatively larger number of males in the sampling area. However, individual identification in this study has observed that there was no significant difference in the number of male and female tigers in SW, suggesting that another factor contributed to this sampling pattern. Tigers, like other felids, are known to construct their own paths by leaving marks along the route, using them to demarcate their territories and communicate with other individuals[39–41]. Cheek rubbing, urine spraying, glandular secretions, and feces are used to deposit scent mainly by males to "proclaim" their presence to other competing individuals or potential mates[42,43]. As the field surveys were conducted along the conspicuous paths fitting for exhibition, it is reasonable that males' instinctive behavior to show off themselves has contributed to the male-biased sample collection.

Based on the genotypic profile constructed by 10 microsatellite markers, 32 Amur tigers were identified, including 16 males, 15 females and 1 of unidentified sex. In earlier genetic monitoring studies using noninvasive samples, fewer individuals were identified from the SW ($n = 12$ in Sugimoto et al.[44], $n = 19$ in Sorokin et al.[17]). The growing number of individuals has been identified according to the chronological order of studies, indicating a gradual increase in the census population size. Given the similar sample size, sampling period and survey

	Gene	COI		COII	Cyt b		CR
	Position*	31–228	1139–1436	1849–2132	8847–9244	9638–9943	10,241–10,644
	Polymorphic site	none	none	none	none	9911	none
Individual	M1	-	-	-	-	A	-
	M2	-	-	-	-	A	-
	M3	-	-	-	-	A	-
	M4	-	-	-	-	G	-
	M5	-	-	-	-	A	-
	M6	-	-	-	-	A	-
	M7	-	-	-	-	A	-
	M8	-	-	-	-	A	-
	M9	-	-	-	-	A	-
	M10	-	-	-	-	A	-
	M11	-	-	-	-	A	-
	M12	-	-	-	-	G	-
	M13	-	-	-	-	A	-
	M14	-	-	-	-	A	-
	M15	-	-	-	-	A	-
	M16	-	-	-	-	G	-
	F1	-	-	-	-	A	-
	F2	-	-	-	-	A	-
	F3	-	-	-	-	A	-
	F4	-	-	-	-	A	-
	F5	-	-	-	-	A	-
	F6	-	-	-	-	A	-
	F7	-	-	-	-	A	-
	F8	-	-	-	-	G	-
	F9	-	-	-	-	A	-
	F10	-	-	-	-	A	-
	F11	-	-	-	-	A	-
	F12	-	-	-	-	A	-
	F13	-	-	-	-	A	-
	F14	-	-	-	-	A	-
	F15	-	-	-	-	A	-
	U1	-	-	-	-	A	-

Table 3. Nucleotide polymorphism in mitochondrial DNA. * nucleotide position numbers correspond to the complete reference mitochondrial genome sequence of *Panthera tigris altaica* provided by Choi et al.[35] (accession number: HM185182.2).

area between studies, it can be reliably interpreted as a reflection of the population growth, which has also been detected in field survey and camera trap monitoring[19,25]. Likewise, the increasing registrations of Amur tigers in Northeast China have been observed along the border of Primorsky province in recent years, implying spatial expansion attributed to increased population density in SW[10,45–48].

Using 18 polymorphic microsatellite markers, we estimated H_O and H_E, parameters widely used for characterizing genetic diversity, as 0.468 and 0.435, respectively, which are numerically moderate compared to previous studies. By analyzing 12 microsatellite loci of 14 individuals, Henry et al.[16] obtained 0.25 for median H_E (H_O was not described). Sugimoto et al.[44] reported H_O as 0.59 and H_E as 0.58 using 12 individuals and 10 loci, while Sorokin et al.[17] estimated H_O as 0.61 and H_E as 0.62 from 19 individuals using 9 loci. Each study used a different marker subset developed from the genomic DNA of domestic cat, snow leopard, Sumatran tiger or Bengal tiger[49–52]. Therefore, direct comparisons between parameters estimated by different markers seems unreasonable, as they vary depending on the utilized loci. This emphasizes the necessity to use the same markers that can be applied to multiple species.

SW population is small, isolated, and genetically distinct from the larger Sikhote-Alin tiger population[16,17]. The mean relatedness value of 0.12487 found in this study is close to first cousin relationship, and is very similar to the results of Ning et al.[53], who analyzed relatedness between 30 Amur tigers sampled near SW (mean DyadML = 0.124). Relatedness network results reveal that Amur tigers in SW are related to each other through close relationship. Except for a few individuals on the fringes of the network, every individual is connected to at least three other individuals, suggesting that inbreeding between closely related individuals is very likely to occur in the SW population in the near future. Interestingly, a tendency of heterozygote excess was observed in the

microsatellite data ($H_O = 0.54$, $H_E = 0.51$; Table 1). There are several hypothesizes for this. First, gene pool might have been partially augmented by migrants from outside (isolate-breaking effect), allowing two homozygous individuals, one from the SW and one from elsewhere, to mate and produce a heterozygous offspring. Second, theoretically, difference in allele frequencies between male and female could lead to this phenomenon, particularly pronounced in small populations[54]. Third, during the individual identification, a sample possessing a heterozygote genotype rather than homozygote genotype was selected as representative individual among the samples with mismatches under the assumption that fecal DNA is vulnerable to allelic dropout. We found a low average difference in allele frequencies between males and females (mean = 0.066, S.D = 0.05), which implies a weak effect and nullifies the second hypothesis. The hypothesis of heterozygote-biased selection in the process of individual identification artificially contributing to an overestimation of the observed heterozygotes is less plausible but still possible, as similar results have been observed in previous non-invasive genetic research on SW tigers[44]. Previous genetic studies on SW tiger population support first hypothesis where they reported migrants sub-structuring and admixture[16–18]. However, in our study, we did not detect any sub-structuring, clustering, or migrants (Supplementary Figs. 2–5). It is possible that we failed to capture migrants, or the analysis of only a single population dataset resulted in poor resolution or polarity.

Amur tigers have suffered from intensive hunting, live capture for zoos, and habitat loss associated with human population growth, leading to significant demographic contraction from the late 18th century to the early 20th century[55,56]. There had been attempts to identify these events through genetic data. Henry et al.[16] found historical bottleneck signs in Sikhote-Alin population ($n = 81$) and Southwest Primory ($n = 14$) but failed to detect recent reduction in both populations. In contrast, Alasaad et al.[57] detected only recent bottlenecks in Sikhote-Alin population ($n = 15$), but not historical bottlenecks inconsistent with Henry et al.[16]. The discrepancy between the two studies, which may have been influenced by the high proportion of missing data (53–63%) in Henry et al.[16] and the small number of sporadically distributed samples in Alasaad et al.[57], have confounded a clear explanation of bottleneck in the Amur tiger population. Here, we found reliable evidence of demographic fluctuations by exploring the remnants of bottlenecks remaining in Amur tigers in Southwest Primorye based on a sufficient amount of evenly distributed samples and well-constructed genotypic profile with less missing data. Significant heterozygosity excess at mutation-drift equilibrium and the distorted allele frequency distribution indicated that severe recent reduction in abundance took place in this population[58–60], consistent with the demographic report from the 1940s[12]. Also, historical bottlenecks were detected by the Garza-Williamson index, revealing demographic events in late Pleistocene and subsequent period that caused severe population bottlenecks over a long time[61,62].

Extremely low diversity in the mitochondrial DNA was reported: two haplotypes (type A, type G) embedding single nucleotide variation. It is known that the Amur tiger has the lowest level of mitochondrial diversity among all tiger subspecies[63,64]. This exceptionally low mitochondrial diversity is probably attributable to chaotic demographic history[56,61,62,64]. The genetic diversity of modern tigers has been greatly reduced by multiple population oscillations, including the late Pleistocene bottleneck and post-bottleneck radiation during the interglacial period that shaped the current tiger subspecies[61,62]. Among the subspecies, the Amur tiger is the subspecies that migrated farthest northeast from the ancient tiger population in southwestern China, and due to the harsh climatic conditions of its habitat, it has likely maintained a small effective population size for a long period of time and has been strongly affected by selection pressures, resulting in lower genetic diversity compared to other subspecies. Along with the ancient demographic history, recent reductions have imposed adverse effects on the genetic diversity of Amur tigers. For SW population in particular, development corridors continues to disrupt genetic exchange with the Sikhote-Alin population, raising concerns about severe genetic deterioration[65,66]. One notable aspect in the mitochondrial DNA analysis is the geographical distribution of type G individuals, which is confined to northern area of SW (Fig. 1). One hypothesis for this phenomenon is that type G could be a new mutation in recent period, based on the fact that the South China and Indochinese tigers, a subspecies closely related to the Amur tiger, are type A (data not shown). However, type G was found in several captive amur tiger individuals whose matrilineal ancestors were captured in the Russian Far East in 1955 and 1975 by comparing our data to NCBI references and published data through alignment of overlapping sequences (Supplementary Table 6). This suggests that type G has been existed since at least the 1950s or earlier, giving it sufficient time to spread over a wider range. Another hypothesis is that it may represent recent migrants from either the Sikhote-Alin population or from across the border in Northeast China. Indeed, an Amur tiger captured in Mishan city, China[67] and individuals from Khabarovsk (not included in main context due to small sample size) were identified as type G (Supplementary Table 6). However, no genetic distinctiveness for type G individuals was observed in the clustering analysis based on microsatellites. It is still probable that type G has an external genetic origin, while its introduction to SW seems to have occurred long time ago. The confined haplotype distribution is likely due to maternal inheritance of mitochondrial DNA and strong fidelity to the territory of female tigers that restrict the maternal gene flow[64].

Conclusion

In this study, we investigated Amur tigers in the Southwest Primorye using non-invasive molecular approaches, which provide essential insights into the current status and genetic properties of this endangered apex carnivore. The number of individuals and their relatedness were estimated and past demographic event was detected from fecal samples, highlighting the advantage of non-invasive genetic monitoring. This method proves its efficiency for studying species rarely observed in the wild which enables the exploration of intrinsic properties.

Despite the quantitative increase in the SW tiger population, they are genetically closely related to each other and genetic depletion from past demographic events, described by low mitochondrial diversity, still persists. Therefore, delicate conservation strategies and continuous monitoring are imperative. Connectivity with the northern tiger population in Shikhote Alin is crucial for genetic recovery. The development corridors still

impede the movement between the two populations, and ecological corridors need to be established to connect them. Additionally, there is need of joint population monitoring efforts to manage SW-Laoyeling landscape tiger population. Furthermore, as the increase of Amur tiger population in the Russian Far East is accompanied by habitat expansion, connectivity between suitable habitats can reduce human conflicts and increase survival of tiger. Corridors along the China-North Korea border would extend the species' range into its vast former ranges, devoid of apex predators, providing optimal environments and abundant prey resources[37,68].

Lastly, the versatility of the microsatellite markers used in this study, along with established protocol, allows their application to other big cats. The microsatellite markers adopted to study tigers have already been used to monitor the critically endangered Amur leopard and other wild cats[15,34]. The wide adoption of these markers enables direct comparison between studies and the development of comprehensive, range-wide genetic information for big cats. This information is useful in transboundary population monitoring, species restoration, and forensic investigations.

Methods

Sample collection and DNA extraction

The scat samples were collected from the Land of the Leopard (LL) located in southern tip of Primorsky province in the Russian Far East bordering Hunchun, China on the west, which consists of two specially protected area – the Kedrovaya Pad Nature Reserve and the Land of the Leopard National Park (LLNP). Sample collection had been carried out along the forest roads and animal trail for six years from 2014 to 2019 by field researchers (Fig. 1). For each fecal sample, collection date, GPS coordinate, inferred freshness and presumed host were recorded. They were packed into plastic bags separately and kept frozen at -20 ? until DNA extraction.

Genomic DNA was extracted from all collected scat samples. To extract host's DNA, we first scratched surface of each scat samples and put it in 2 mL tubes. Thereafter, following steps of DNA extraction were performed using Fast stool DNA extraction kit (QIAGEN, Germany) based on the manufacturer's instructions except for the final step of DNA elution replaced by eluting twice with half volume of the elution buffer to maximize the yield of extracts.

Species and sex identification

Fecal samples lack reliable morphological distinct features to distinguish species and sex, the basic parameters necessary to monitor a wild population, and hence require additional efforts for species and sex identification using molecular methods. We followed the methodology devised by Sugimoto et al.[69] as the samples were collected from a wildlife sanctuary inhabited by both Amur tigers and Amur leopards.

For species identification, Pta-CbF/Pta-CbR and Ppo-CbF/Ppo-CbR primers were used to amplify cytochrome b gene fragment of Amur tiger and Amur leopard, respectively. Multiplex PCR was performed in a total volume of 15 μ L containing 1.5 μ L of PCR buffer (10 ×), 1.2 μ L of 2 mM dNTPs, 1.5 μ L of 1 μ g/ μ L BSA, 0.75 μ L of each 10 μ M primer, 1 μ L of i-Star *Taq* DNA polymerase (5U) and 1.5 μ L of DNA extract. PCR condition was set as follows: 1 cycle of 94 ? for 5 min, followed by 45 cycles of 94 ? for 30 s, 60 ? for 30 s, 72 ? for 45 s, and 1 cycle of 72 ? for 10 min. PCR products were visualized by gel electrophoresis and species was identified based on the amplicon size (271 bp for tiger & 156 bp for leopard). PCR assay was performed twice for each sample, followed by 2–3 additional assays in case of no consensus in initial assay. DNA sequencing was performed for 10 randomly selected samples for result re-validation using universal primers L14841 and H15149 amplifying 308 bp of the cytochrome *b*[70], followed by sequence alignment through the Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/).

Similarly, sex was identified using multiplex PCR targeting X and Y chromosome regions[69]. ZFX-PF/ZFX-PR and DBY7-PF/DBY7-PR primers were used to amplify partial ZFX (Zinc-finger, X chromosome) and DBY (DEAD box, Y chromosome), respectively. Sex was determined by the difference in fragment size on the agarose gel (205 bp for ZFX, 156 bp for DBY7). Multiplex PCR was performed in a total volume of 10 μ L containing 4 μ L of Qiagen Multiplex PCR kit master mix, 0.5 μ L of 1 μ g/ μ L BSA, 0.5 μ L of each 10 μ M primer and 2 μ L of DNA extract. PCR condition was set as follows: 1 cycle of 95 ? for 15 min, followed by 45 cycles of 95 ? for 40 s, 56 ? for 40 s, 72 ? for 40 s, and 1 cycle of 72 ? for 5 min. Initially, triplicate PCR assay was performed for each sample and 2–3 additional assays were carried out in case of no consensus in initial triplicate.

Genotypic profile construction

Genotyping was conducted using the panel of microsatellite markers designed for big cat species[34]. A total of 32 microsatellite loci were amplified using the multiplex PCR approach following the Hyun et al.[34]. PCR products were visualized on 2% agarose gel and amplified samples were genotyped using the ABI 3730 XL DNA Analyzer (Applied Biosystems, USA) with Genescan 500 LIZ internal size standard. Genotype calling was performed using the Geneious Prime 2022.1.1 (https://www.geneious.com). To construct genotypic profile, multiple-tube approach was carried out for each sample: initial triplicate PCR assays followed by additional 2–3 PCR assays for samples failed to make consensus genotype in initial step. Consensus genotype was constructed under following rules: genotype was confirmed when triplicate have the same genotype (for homozygote) or at least two replicates have the same genotype (for heterozygote).

Sample and marker screening for individual identification

For a cost-effective and time-efficient study, pre-screening steps were performed to select samples and markers for individual identification, with the following objectives for each step: (i) to remove samples of bad quality, (ii) selecting samples for marker evaluation, and (iii) selecting markers for individual identification. In the first step, amplification was carried out for 4 out of 32 loci to indirectly assess the quality of samples. Samples with

poor amplification (successfully amplified at less than 2 loci) were subsequently removed. Secondary screening was then performed using all 32 loci to select samples that successfully achieved consensus genotypes at most loci. And then, we calculated several indicators for each marker based on all replicate amplification data. In the final step, the minimum markers with accuracy and discriminatory power for individual identification were determined considering the error rates (allelic dropout (ADO) rate, false allele (FA) rate and type 1–5 error rates[71]), probability of identity (P_{ID}), probability of identity among siblings (P_{IDsib}), number of alleles (N_A), effective number of alleles (N_E), polymorphism information content (PIC), and the readability of microsatellite peaks.

The amplification success rate was simply calculated as the ratio of number of successful amplifications to the total number of PCR attempts. Genetic diversity parameters (N_A, N_E, H_O, H_E) were estimated using the GenAlEx 6.51b2[72,73], and PIC was calculated using Cervus v3.0.7[74]. Software Gimlet v1.3.3 was used to calculate P_{ID}, P_{IDsib}, ADO, FA and five types of errors (Type I-V)[75]. The genotyping results underwent thorough screening for the probable errors of the stuttering and large allele dropout using Micro-Checker v2.2.3[76].

Individual identification was then performed using 'multilocus matches' module in GenAlEx 6.51b2[72,73]. Among the first screened samples, only those with missing data at two or less loci were amplified by markers selected for individual identification. Recognizing the inevitable genotyping errors associated with fecal DNA, a certain number of mismatches were allowed to prevent the overestimation of population size. Individual identification was repeated several times with different number of allowed mismatches. As the number of allowed mismatches (N_{AM}) increased, the number of identified individuals gradually decreased, with significant drop occurring at $N_{AM} = 4$, indicating that the most conservative population size determination was achieved at $N_{AM} = 3$ (Supplementary Fig. 1). Following individual identification, the remaining polymorphic loci were genotyped for individuals with missing data, completing the genotypic profile for subsequent analysis.

Estimation of genetic diversity and detection of bottleneck

Based on individualized samples and polymorphic loci, analyses were conducted to estimate the genetic diversity of the Amur tiger population. Linkage disequilibrium was first examined to identify independent markers for analysis. This was achieved by confirming the significance of association in all pair of loci through Markov chain analysis with parameter set at 10,000 dememorization, 200 batches, and 5,000 iterations per batch using Genepop v4.7.5[77]. Subsequently, the Benjamini-Hochberg test was applied to control the false discovery rate and calculate adjusted p-values for multiple test[78]. Micro-Checker v2.2.3 was used to check the existence of null alleles and their frequencies[76]. Hardy-Weinberg equilibrium (HWE) was examined based on Fisher's exact test with parameter set at 10,000 dememorization, 200 batches and 5,000 iterations per batch using Genepop v4.7.5[77]. Genetic diversity indices, including the number of allele (N_A), effective number of allele (N_E), observed heterozygosity (H_O), expected heterozygosity (H_E), and fixation index (FIS) were estimated using GenAlEx 6.51b2[72,73].

BOTTLENECK v1.2.02 was employed to detect recent bottleneck under the assumption of the following three mutation model: infinite alleles model (IAM), stepwise mutation mode (SMM), and two-phase model (TPM), with the proportion of SMM set to 70% and variance set to 30[79]. Additionally, the Garza-Williamson index (M-ratio) was calculated using Arlequin v3.5.2.2 to detect evidence of bottleneck footprint[80,81].

Population structure and relatedness

Additional analyses were conducted to find out the existence of population structure and the relatedness between individuals. For these analyses, 24 individuals with no missing data were used to avoid distortion and increase reliability. The program STRUCTURE 2.3.4 was used to visualize population structure under 10 iterations of 400,000 MCMC generations, with 100,000 burn-in period[82]. The most likely number of cluster was determined based on Evanno method[83], implemented in STRUCTURER HARVESTER web 0.6.94[84]. Clustering pattern was also examined based on the genetic distance matrix through Principal Coordinate Analysis (PCoA) implemented in GenAlEx 6.51b2[72,73]. Additionally, Discriminant Analysis of Principal Components (DAPC) was employed to infer the optimal number of clusters using the R package 'adegenet' through Rstudio[85–88].

Both moment estimator and likelihood estimator methods were used to estimate relatedness between individuals. Five moment estimators[89–94] and two likelihood estimator[95,96] were calculated using COANCESTRY[97]. We simulated 1000 dyads for each of the four common relationships (parent-offspring, full-sib, half-sib and unrelated) based on the observed allele frequencies, and then compared the simulated relatedness to the true relatedness of each simulated relationship to calculate the mean squared error (MSE) of all estimators[53]. The estimator with the lowest MSE was then selected as the most reliable estimators and used to draw a network connecting individuals with high relatedness using UCINET v6.716[98].

Identification of sequence variation on mitochondrial DNA

Six novel primer pairs were developed to amplify partial regions of cytochrome b (Cyt b), cytochrome c oxidase I (COI), cytochrome c oxidase II (COII), and the control region (CR). These primers were designed using Primer 3[99] embedded in Geneious Prime 2022.1.1 (https://www.geneious.com). Target sites of the primers were determined to include polymorphic sites, referencing a previous study[63] and NCBI database (Supplementary Table 1). Partial mitochondrial DNA was amplified and sequenced in at least one sample per individual. Specifically, DNA was sequenced from two or more samples to verify the accuracy of individual identification by checking whether different haplotypes were found in the same individual sample for individuals identified in multiple feces. Primer pairs ptcb-15,878 & ptcb-16,317 (398 bp) and ptcb-16,671 & ptcb-17,020 (306 bp) were designed for the amplification of Cyt b; primer pairs ptCOI-7008 & ptCOI-7247 (198 bp) and ptCOI-8119 & ptCOI-8454 (298 bp) for COI; ptCOII-8836 & ptCOII-9117 (284 bp) for COII; and ptdl-241 & ptdl-969 (404 bp) for CR (Supplementary Table 2). PCR was performed in a total volume of 30 μL containing 3.0 μL of PCR

buffer (10 ×), 2.4 μL of 2 mM dNTPs, 1.5 μL of 1 μg/μL BSA, 1.0 μL of each 10 μM primer, 0.3 μL of *Ex Taq* DNA polymerase (1.5 U) and 2 μL of DNA extract. PCR conditions were set as follows: 1 cycle of 94 ? for 5 min, followed by 20 touchdown cycles of 94 ? for 30 s, 60 − 50 ? (decreasing by 0.5 ? for each cycle) for 30 s, 72 ? for 1 min, and 1 cycle of 72 ? for 7 min. The PCR products were subsequently purified and sequenced using an ABI 3730 XL DNA Analyzer (Applied Biosystems, USA). Haplotype diversity and nucleotide diversity were calculated using DnaSP 6.12.03[100].

Data availability

The data that support the findings of this study are included within the article and its supplementary information file. The raw datasets used for analysis are available from the corresponding author upon reasonable request.

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Author contributions

H.L. and P.P. conceptualized the study. P.P., Yo.L., and V.B. designed the research framework. T.M., D.M., Y.D. and V.B. contributed to sample collection. D.J., J.Y.H., T.M., S.C., J.L., D.Y.K., Yi.L. and P.P. participated in experimentation. H.L., M.S.M, Yo.L., and V.B. provided the resources. D.J. and P.P. performed the data analysis and wrote the first draft of the manuscript. Yi.L. created map figures. D.J., T.M., D.M., Y.D., V.B., P.P., Yo.L., and H.L. reviewed and edited the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Ethical declaration

This study was conducted based on the memorandum of understanding between the Tiger and leopard Conservation Fund in Korea (KTLCF), Republic of Korea and the Land of the Leopard National Park (LLNP), Russia. Samples were legally collected without direct disturbance or intimidation to the subject animals. DNA extraction was carried out in Russia, and the extracted DNA was subsequently exported to South Korea. The entire experimental process was conducted in accordance with the ethical guidelines on animal experiments provided by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University. In accordance with the import and export regulations of internationally endangered species, CITES permit was approved by the Han River Basin Environmental Office under the Ministry of Environment in Korea (ES2019-03989).

Additional information

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