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Source: Mammal Study, 51(2) : 113-127

Published By: Mammal Society of Japan

URL: <https://doi.org/10.3106/ms2025-0029>

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Local genetic diversity and structure of Asian black bear, *Ursus thibetanus*, populations in Nagano, Japan

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Published online 25 March, 2026; Print publication 30 April, 2026

Abstract. Human–bear conflict is a significant issue in Japan. Understanding genetic diversity, population structure, and local population size of the Asian black bear is essential for improving ecological knowledge and developing conservation and management measures of this species. We examined sex, mitochondrial (mt) DNA variations, and 12 nuclear microsatellite genotypes from hair-derived DNA collected in the Kiso region, Nagano Prefecture. After verifying microsatellite genotypes, we identified 30 distinct individuals (six males, 18 females) within an approximately 48 km² area. MtDNA analysis revealed that all males shared haplotype E01, while females possessed three haplotypes, including E07, detected in Nagano for the first time but previously reported in Tohoku region and Toyama Prefecture. STRUCTURE analysis did not reveal any genetic cluster in nuclear DNA, but significant spatial genetic structures were identified in both mtDNA and nuclear DNA. No evidence of a recent bottleneck was detected. This study is the first to examine the local genetic structure of the black bear in Nagano. Although no genetic clusters were detected, the estimated effective population size was not large, underscoring the importance of continued genetic monitoring. These findings provide insights into anticipating potential conflict zones and guiding conservation and management measures in the Kiso region.

Key words: genetic structure, hair traps, non-invasive genetic sampling, wildlife management.

Abstract in Japanese (要旨). 長野県に生息するツキノワグマ *Ursus thibetanus* 集団の地域的な遺伝的多様性と遺伝構造。人間とツキノワグマとの軋轢は、日本社会の大きな問題となっている。ツキノワグマの遺伝的多様性、集団構造、地域集団サイズの理解は、本種の生態学的知見の向上や保護管理策の指標を示すうえで不可欠である。本研究では、長野県木曽地域にて採取された体毛由来の DNA を用いて、性判別を行うとともに、ミトコンドリア DNA 多型および核 DNA のマイクロサテライト 12 遺伝子座の遺伝解析を行った。核マイクロサテライトの遺伝子型から、約 48 km² の調査範囲内において 30 個体（雄 6、雌 18）を識別した。ミトコンドリア DNA の解析の結果、雄の全個体はハプロタイプ E01 のみを示したが、雌は E07 を含む 3 つのハプロタイプを保有しており、E07 はこれまで東北地方や富山県では報告されていたが、長野県では初検出であった。STRUCTURE 解析は明瞭な核 DNA の遺伝的クラスターを検出しなかったが、ミトコンドリア・核 DNA の両方で有意な空間遺伝構造が検出された。また、近年のボトルネックの証拠は認められなかった。本研究は、長野県におけるツキノワグマの地域的遺伝構造を初めて明らかにしたものである。遺伝的クラスターは検出されなかったが、推定された有効集団サイズは大きくなかったことから、今後の継続的な遺伝モニタリングの必要性が示唆された。これら本研究成果は、木曽地域において、人間とツキノワグマとの軋轢が起こりえる地域の予想やツキノワグマの保護管理指標の指針づくりに新たな知見を提供する。

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Understanding species' population genetic structure, dispersal, and gene flow patterns are crucial, not only for improving ecological knowledge, but also for informing conservation and management measures (Manel et al. 2003; Quaglietta et al. 2013; Noble et al. 2016). Many studies have evaluated how the landscape affects dispersal and gene flow, using a genetic approach (Manel et al. 2003). However, as biogeographic structure, including genetic structure, is highly species- and scale-dependent (Cushman and Landguth 2010; Mullen et al. 2010), it is important to evaluate genetic structures at multiple scales, including wider scales (covering most of the target species' range), local scales (e.g., over tens to several hundreds of kilometers), and finer scales (e.g., several hundreds to thousands of meters) at a within-population level (Tsuda et al. 2010; Bodare et al. 2017). Knowing the local genetic structure has practical implications for species-specific conservation and management strategies at the local scale.

The Asian black bear, *Ursus thibetanus* (G. Cuvier, 1823, hereafter, black bear), is a widely distributed mammal in Japan. Its current distribution spans two of the main islands, Honshu and Shikoku, while it is thought to be extinct on Kyushu (Ohnishi and Yasukochi 2010; Ministry of the Environment 2012). As a key component of forest ecosystems, the black bear contributes to important ecological processes, such as seed dispersal (Takahashi et al. 2008). However, the species is under pressure from culling because of its potential for human–wildlife conflict (HWC). Because of its omnivorous diet (Ali et al. 2017; Koike 2025), the black bear can cause injury or mortality to humans and consume human-related foods such as crops, beehives, and livestock. These behaviors pose a significant challenge for the conservation of this species (Dawood et al. 2025).

Genetic monitoring offers a powerful research tool to assess population genetic structure, dispersal dynamics, and temporal changes in genetic diversity in the black bear (Zemanova 2021). However, obtaining blood and muscle samples, which provide high-quality and high-quantity DNA samples from mammals, is often challenging. Such sampling methods require physical capture, which can be expensive, logistically challenging, and potentially dangerous for both animals and handlers (Gompper et al. 2006; Mumma et al. 2015). In contrast, non-invasive genetic sampling (Waits and Paetkau 2005) provides a safe and cost-effective alternative to physical capture. A huge benefit of this method is that it has minimal or no impact on the animals' welfare, as it often does not require direct contact (Zemanova 2020). Non-invasive samples used for

DNA extraction commonly include saliva, feces, and hair (Taberlet et al. 1995; Ohnishi et al. 2011), but may also include urine, sperm, or other environmental materials such as footprint-derived eDNA (Carroll et al. 2018; Kinoshita et al. 2019; Ozga et al. 2021; Von Duyke et al. 2023). Systematic hair-trapping is an acknowledged method used by bear specialists (Miura and Oka 2003; Berezowska-Cnota et al. 2017) and is increasingly used in many countries.

In Japan, a Specified Wildlife Conservation and Management Plan (SWCMP) has been developed in 22 prefectures (Ministry of the Environment 2022). Nagano Prefecture, a highly mountainous region with extensive forests, supports one of the largest black bear populations in Japan, where HWC has become a significant and pressing social issue. In recent years, a consistent number of black bear-related incidents have occurred, with about ten cases reported annually (Nagano Prefecture 2022). Furthermore, agricultural and forestry sectors have suffered considerable damage, with estimated financial losses amounting to approximately 130 million yen per year (Nagano Prefecture 2022). These conflicts highlight the urgent need to mitigate human–wildlife interactions and ensure both human safety and wildlife conservation. To support such efforts in Nagano, monitoring the black bear populations and evaluating their spatial genetic structure at local scales is crucial for understanding not only their genetic diversity but also their distribution, gene flow, and dispersal dynamics. Such genetic information can also contribute to making practical management decisions, including the selection of appropriate release sites for individuals, translocated from urban to mountainous areas. Furthermore, understanding population connectivity can be helpful to consider potential movement routes into human settlements, supporting more strategic and informed conflict management planning. While previous studies have identified broad-scale genetic structures of the black bear populations across multiple prefectures (Ohnishi et al. 2009; Uno et al. 2015), local-scale patterns within Nagano Prefecture have not been well examined.

This study focused on the Kiso region of Nagano Prefecture, which has a long history of human–bear conflict (Huygens and Hayashi 2001). To gain further insight into the black bear's population genetic structure, we used two types of genetic data: mitochondrial (mt)DNA and nuclear DNA. As mtDNA is maternally inherited and nuclear DNA is bi-parentally inherited, by using both with the sex profile, population demography can be evaluated in more detail. The aim of this study was to assess the

genetic structure, diversity, and sex-biased dispersal patterns of the black bear in the Kiso region. Additionally, we aimed to evaluate the utility of hair samples as a non-invasive approach to investigating the genetic structure and gene flow of this species.

Materials and methods

Study site and sample collection

The study site was located within the national forest in the Kiso region of Nagano Prefecture (Fig. 1). Thirty-five barbed-wire hair traps (T1–35) were set across an area of about 48 km² in August 2020 (Fig. 1c). The study site is characterized by an undulating terrain at an elevation of 500–1300 m, intersected by multiple streams, with vegetation predominantly consisting of artificial coniferous forests, including larch (*Larix kaempferi*) and cypress (*Chamaecyparis obtusa*), especially in the southern area. Asian black bears have a polygamous mating system, as both sexes consort and potentially mate with multiple partners during a given mating season (Naganuma et al. 2021). They display male-biased dispersal and female philopatry patterns, which are commonly reported in mammals (Ishibashi and Saito 2004; Lawson Handley and Perrin 2007; Ohnishi and Osawa 2014; Oliveira et al. 2021; Takayama et al. 2023), and the home range size has been estimated to vary from 5 km² to 100 km², with an average home range size of 30–60 km² in Japan (Koike and Hazumi 2008; Izumiyama et al. 2009; Yamamoto et al. 2012). Compared to the species' average home range size, our study focused on a finer scale; therefore, we set

a high frequency of hair-trapping stations at regular intervals of approximately 1 km. In order to attract bears and maximize sampling success, the trap containers were filled with a nest of bees and honey as bait and were set at a height of 2 m above the ground (see Supplementary Fig. S1). Hair samples were collected regularly (about every 10–14 days) from August to November 2020. All the samples were stored at –25°C until DNA extraction.

DNA extraction, mtDNA sequencing, and microsatellite genotyping

Total genomic DNA was extracted from the hair samples using a DNA Extractor FM-Kit (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) and DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. From each sample, five or six hair roots were used. The concentration of individual DNA samples ranged from 2 to 20 ng/μL.

For nuclear DNA, 16 microsatellite loci were amplified using the microsatellite markers G1A, G10B, G1D, G10J, G10L, G10M, G10O, G10P, G10X, MSUT-1, MSUT-2, MSUT-6, MSUT-7, UarMU05, UarMU23, and UarMU50 (Ohnishi et al. 2019). The reactions were carried out as described by Ohnishi et al. (2019). Fragment sizes were determined using an ABI PRISM 3130 Genetic Analyzer and Gene Marker software (SoftGenetics, State College, PA, USA). The reproducibility of genotypes was confirmed by conducting polymerase chain reaction (PCR) and genotyping at least three times for each sample. In some cases, genotyping was repeated up to six times when

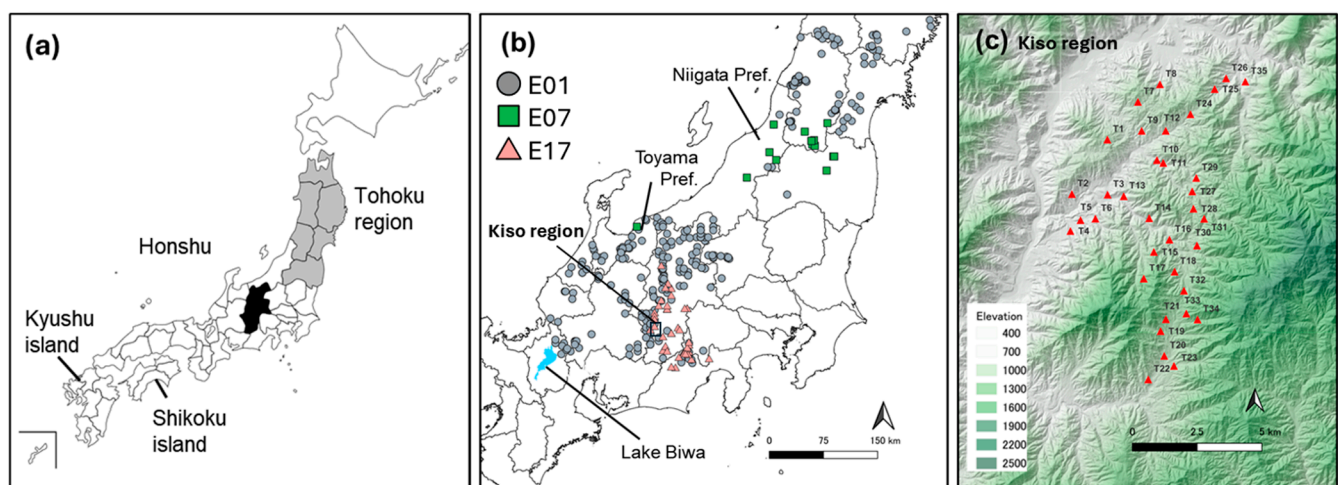


Fig. 1. (a) A map of Japan, with the location of Nagano Prefecture shown in black. (b) A map of central Honshu, with the location of the Kiso region indicated, and the distribution of three haplotypes (E01, E07, E17) as identified in Ohnishi et al. (2009); the study area is outlined in black. (c) The distribution of the 35 hair-trapping stations.

the electropherograms showed ambiguous or inconsistent allele peaks.

In the mtDNA analysis, we examined the control region (702–706 bp) of 47 individuals that have been successfully genotyped at nuclear microsatellite loci (see the Results), following the protocol of Ohnishi et al. (2009). The PCR reactions were carried out using *TaKaRa Ex Taq* (TaKaRa Bio Inc., Shiga, Japan). Each reaction contained 1.00 μ L genomic DNA, 0.80 μ L 10 $\times$ *Ex Taq* buffer, 0.80 μ L 25 mM $MgCl_2$, 0.64 μ L 2.5 mM dNTP mixture, 0.80 μ L pre-mixed 0.5 μ M forward and reverse primers (UT-5 and UT-7; Ohnishi et al. 2009), 0.04 μ L *Ex-Taq*, and 3.92 μ L RNase-free water in a final volume of 8.00 μ L. The amplification process consisted of an initial denaturation at 95°C for 10 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. Two microliters of each PCR product were loaded on a 2% agarose gel to confirm PCR success. After verification, each PCR product was purified using ExoSAP-IT Express PCR Product Cleanup (Applied Biosystems, Santa Clara, CA, USA). According to Ohnishi et al. (2009), two forward primers (UT-1; Ishibashi and Saitoh 2004; and UT-6; Ohnishi et al. 2009) were used for sequencing in the same direction: UT-6 as the main forward primer, and UT-1 as an internal primer to improve sequence data quality. The sequencing was performed by Eurofins Genomics Co., Ltd (Tokyo, Japan), and the mtDNA sequences obtained were edited using BioEdit software (Hall 1999). Individual haplotypes were identified using GenAlEx version 6.5 (hereafter, GenAlEx; Peakall and Smouse 2012). We reconstructed a Neighbor-net (Bryant and Moulton 2004) to evaluate the network of the detected mtDNA haplotypes using Split-tree5 (Huson and Bryant 2006), based on 52 published haplotypes for the black bear (Ohnishi et al. 2009). The published sequence data were obtained from the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/>). We visualized the distribution of haplotypes by creating a geographical map using QGIS3.8.3 (QGIS Development Team 2019).

Individual identification and sex determination

For the nuclear DNA microsatellites, 12 of the 16 loci that showed clear fragment patterns were used for further data analysis (see the Results). Prior to individual identification, allelic dropout rates were estimated by genotyping a subset of samples at each locus. The program Microsatellite Toolkit (Park 2001) was used to identify

individuals based on the multi-locus genotype, which permitted zero to three non-matching alleles. To check for the presence of the null alleles in the datasets, the genotype data were analyzed using Microchecker and FreeNA (van Oosterhout et al. 2004; Chapuis and Estoup 2007). Linkage disequilibrium was tested for locus pairs within a population by randomization, and the *P* values (= 0.05) obtained were adjusted by applying a sequential Bonferroni correction (Rice 1989) to avoid false positives, using FSTAT (Goudet 2001). The probability of identity (P_{ID}), the probability of identity of siblings ($P_{ID\ sib}$), and the probability of exclusion (P_E) were calculated based on the 12 loci using GenAlEx (Peakall and Smouse 2012). While individual identification is generally based on multilocus genotypes and probability-based approaches, we additionally calculated pairwise relatedness (Queller and Goodnight 1989) among the identified individuals using GenAlEx, as a supplementary check to confirm the consistency of individual identifications.

To identify the sex of the examined samples, we employed three sex-specific genes and their determination markers for ursids. Firstly, we examined the amelogenin (AMELX/Y) gene located on the X and Y chromosomes and employed a primer pair of SE47 and SE48 (Yamamoto et al. 2002). Males exhibited two bands corresponding to both AMEL-X and AMEL-Y loci, whereas females showed only a single AMEL-X band. The PCR reactions were carried out using *TaKaRa LA Taq* (TaKaRa Bio Inc., Shiga, Japan). Each reaction contained 2–10 ng genomic DNA (1.00 μ L), 0.80 μ L 10 $\times$ LA PCR buffer II, 0.80 μ L 25 mM $MgCl_2$, 1.28 μ L 2.5 mM dNTP mixture, 0.80 μ L pre-mixed 0.5 μ M forward and reverse primers, 0.08 μ L LA-Taq, and 3.24 μ L RNase-free water in a final volume of 8.00 μ L. The amplification process consisted of an initial denaturation at 94°C for 1 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 60 s, and extension at 72°C for 2 min; and a final extension at 72°C for 3 min. Secondly, we employed the sex-determining region of the Y chromosome (SRY) and genes encoding for the zinc finger proteins (ZF), using the primer pair MP-SRY-Ursus and MP-SRY-1R-Ursus for SRY, and MP-ZF-F and MP-ZF-R for ZFX/Y (Pagès et al. 2009). Following Pagès et al. (2009), males displayed two bands (ZF and SRY), whereas females showed a single ZF band, as the SRY gene is absent in females. The PCR reactions were carried out using *TaKaRa Ex Taq* (TaKaRa Bio Inc., Shiga, Japan). Each reaction contained 2–10 ng genomic DNA (1.00 μ L), 0.80 μ L 10 $\times$ LA PCR buffer II, 0.80 μ L 25 mM $MgCl_2$, 0.64 μ L 2.5 mM dNTP mixture, 0.80 μ L

pre-mixed 0.5 μ M forward and reverse primer, 0.04 μ L Ex-Taq, and 3.92 μ L RNase-free water in a final volume of 8.00 μ L. The PCR consisted of an initial denaturation at 94°C for 1 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s; and a final extension at 72°C for 3 min. The PCR products were electrophoresed in 2% agarose gel, and their sex-specific band patterns were evaluated. Each sample was tested at least twice, and up to four times depending on amplification quality, to ensure reproducibility. Only samples that had a clear band pattern for all three markers (AMELX/Y, SRY, and ZF) were used for sex determination.

Genetic diversity

We also grouped all identified individuals based on sex and mtDNA haplotype to compare genetic diversity among groups. Intra-population genetic diversity was assessed by calculating Nei's gene diversity index (Nei 1987), allelic richness (a measure of allelic diversity corrected for sample size differences among populations; El Mousadik and Petit 1996), and the fixation index (F_{IS} ; Wright 1951). Deviations from the Hardy-Weinberg equilibrium (HWE), indicated by F_{IS} values significantly different from zero, were tested by 1000 randomizations in FSTAT, applying a sequential Bonferroni correction (Rice 1989) as conducted in the linkage disequilibrium evaluation. In addition, to verify whether the observed deviation of F_{IS} in females is a signature of genetic sampling (e.g., inbreeding and outbreeding) or null alleles, heterozygosity excess in females was tested by using the empiric null distribution of F_{IS} under panmixia based on 1 000 000 permutations of alleles among the genotypes, using INEST 2.3 (Chybicki and Burczyk 2009). Chybicki and Burczyk (2009) have modified and improved procedures for mutation models and the likelihood of the mutation rate (θ). Their approach uses a method to detect a recent bottleneck, originally developed by Cornuet and Luikart (1996) and implemented in the software Bottleneck (Piry et al. 1999). The original method has been suggested to overestimate the reference equilibrium heterozygosity (Chybicki and Burczyk 2009). We examined the modified approach to evaluate recent bottlenecks using 30 individuals (see the Results). We employed the Wilcoxon signed-rank test to evaluate whether a recent bottleneck had occurred under an assumption of a two-phase mutation model (TPM; Di Rienzo et al. 1994); the P -value was determined based on the assumption of normality and 1 000 000 permutations to approximate the

exact value (normality was not assumed).

To estimate the effective population size (N_e) in the study region, we applied the linkage disequilibrium (LD) method (Waples 2006; Waples and Do 2008) as implemented in NeEstimator v2.1 (Do et al. 2014). As rare alleles can upwardly bias N_e estimates (Waples and Do 2010), we excluded rare alleles for which frequencies were less than 0.05, using the P_{crit} (a criterion for excluding rare alleles) function. Confidence intervals (CI) were determined using the Jackknife method, which has been shown to perform better than parametric methods (Waples and Do 2008).

Genealogical relationships, genetic structure, and spatial patterns

The pairwise genealogical relationship was evaluated using the program ML-Relate (Kalinowski et al. 2006). We calculated maximum likelihood estimates of relatedness (r ; Milligan 2003; Wagner et al. 2006) with a null allele correction (Wagner et al. 2006) and then classified pairs into one of four common pedigree relationships: unrelated (UL), siblings (S), half siblings (HS), and parent/offspring (PO). We determined the relationship between individuals when only one relationship from UL, S, HS, and PO remained after consideration of the 95% CI based on 1000 bootstraps.

To evaluate the genetic structure of identified individuals, a Bayesian clustering analysis at an individual level was carried out using STRUCTURE v. 2.3.4 (Pritchard et al. 2000; Falush et al. 2003), employing a combination of an admixture model and the F -model (Falush et al. 2003). The number of clusters (K) varying from 1 to 10 was evaluated under a correlated allele frequencies model using runs of 80 000 replicates, and Markov Chain Monte Carlo (MCMC) sampling after a burn-in of 20 000 with 20 runs for each cluster. STRUCTURE Harvester (Earl and vonHoldt 2012) was used to summarize the probability of the data (LnP(D)) for each K (Pritchard et al. 2000). Multimodality among runs and major clustering patterns at each K value were evaluated using the CLUMPAK server (Kopelman et al. 2015).

Genetic differentiation among the categorized genetic groups based on mtDNA haplotypes was evaluated by calculating F_{ST} values (Weir and Cockerham 1984) and the standardized value F'_{ST} , which always ranges from 0 to 1 (Meirmans and Hedrick 2011), using GenAlEx. The F_{ST} values corrected for null alleles were calculated with the software FreeNA (Chapuis and Estoup 2007).

We conducted individual-based spatial autocorrelation

analyses for the datasets using mitochondrial haplotype and 12 nuclear microsatellite genotype data, taking into consideration the different base numbers between haplotypes. For this analysis, multi-locus genotypic distances between individuals were calculated and then spatial autocorrelation coefficients, r (Smouse and Peakall 1999), were calculated for each distance class in 2 km increments, using GenAlEx (Peakall and Smouse 2012). For individuals detected at multiple hair-trap sites, one location was randomly selected to represent the individual in order to avoid pseudo-replication and reduce spatial bias. The upper and lower confidence limits or r values for the 95% CI, based on the null hypothesis of no spatial structure, and the autocorrelation of r , were tested for statistical significance based on 999 permutations. The spatial genetic structure based on nuclear DNA was assessed for all 30 identified individuals, whereas analyses for mtDNA were conducted on the 25 individuals for which haplotypes were determined. To analyze the heterogeneity of the spatial genetic structure (SGS) among distance classes, we used the method described by Smouse et al. (2008). The significance of the criterion (ω) was determined on the basis of 999 permutations. This heterogeneity test is considered significant when $P < 0.01$, taking into account type I errors as suggested by Banks and Peakall (2012).

We also conducted Mantel tests to examine the relationship between genetic distance (GD) and geographic distance (GGD) for each dataset (mtDNA and nuclear DNA), in order to test for isolation by distance (IBD; Wright 1943).

Results

Individual identification and sex information

A total of 210 hair samples were collected from 27 barbed-wired hair traps. Of these, 71 hair-derived DNA samples were successfully amplified and genotyped. From 16 microsatellite loci, we excluded four (G1A, G10M, G10O, and UarMU50) that showed an unclear fragment pattern and used the other 12 for further analyses to avoid genotyping errors. Multi-locus analyses showed a high probability of exclusion ($P_E = 0.9980$; Supplementary Fig. S2), a low probability of identity ($P_{ID} = 1.2E-09$), and a low probability of identity of siblings ($P_{ID\ sib} = 1.6E-04$) (Supplementary Fig. S3), suggesting a high resolution for identifying individuals. We confirmed the reproducibility of genotyping and obtained 64 alleles at 12 loci from the 71 samples. Allelic dropout was detected only at one locus (UT-6), with a dropout rate of 0.004. Forty-seven

individuals were identified from the 71 samples, considering genotypes of identified individuals that were 100% matched, and not assuming genotyping errors. When we allowed one to three non-matching alleles between genotypes, the estimated number of individuals varied accordingly: 34, 32, and 32 individuals, respectively. Among the 32-individual dataset (based on the two- and three-allele mismatch threshold), we rechecked fragment data and excluded two individuals for which we could not confidently distinguish unique genotypes, leading to a final dataset of 30 individuals. In all retained cases, matched samples exhibited consistent sex determination, mitochondrial haplotypes, and high relatedness values (close to 1, data not shown). Given the common occurrence of genotyping errors in hair-derived DNA (Gagneux et al. 1997) and the potential overestimation of individual counts in our initial dataset of 47 genotypes, we chose to adopt the conservative dataset of 30 individuals, defined using the three-allele mismatch threshold, as the primary dataset for this study. Moreover, although 20 of 66 locus pairs showed significant linkage disequilibrium in the 47 identified individuals ($P < 0.05$), no locus pairs showed significant linkage disequilibrium in the 30 identified individuals (Supplementary Table S1). Thus, we retained the 30-individual dataset for most of the subsequent analyses. Relatedness values based on the 30 individuals ranged from -0.567 to 0.814 (Supplementary Table S2), with a mean of -0.035 .

Among the 30, we confirmed the reproducibility of sex information and successfully detected six males and 18 females; the sex of six individuals remained undetermined, as they either did not show the X–Y homologous gene fragment or exhibited inconsistencies in sex information across multiple PCR trials, probably because of the low quality of the examined DNA. Representative electropherograms for male and female individuals are provided in Supplementary Fig. S4.

Based on the 12 loci, 61 alleles were detected among the 30 individuals. Although Microchecker detected null alleles at one locus (MU05), the estimated frequencies calculated using the Expectation Maximization (EM) algorithm in FreeNa were all below 0.12 (Supplementary Table S3). Notably, excluding the locus with null alleles (MU05) did not alter the number of identified individuals, which remained at 30. Considering the relatively low frequencies of null alleles and the finding by Huang et al. (2016) that such frequencies have minimal effects on population genetic inferences, we used the original genotype dataset for subsequent analyses. There were four cases of

hair samples collected from the same individuals among different traps. The four individuals consisted of one male and three females. Three of the four individuals' hair samples were collected at traps that were adjacent to each other and only about 1 km apart. The hair sampling period for a female individual identified at two hair traps, located 3 km apart, spanned 15 days.

MtDNA variation and genetic structure

We successfully obtained mtDNA sequences from 25 of the 30 individuals, representing three distinct haplotypes, UtCR-E01, UtCR-E07, and UtCR-E17, which have all been reported previously (Figs. 1b, 2a, and Supplementary Fig. S5a). E07 has a single nucleotide substitution with the haplotype E01, and is genetically closer to E01 than E17, which has three nucleotide substitutions (Fig. 2b). Most individuals were regarded as E01 and E17 haplotypes, with E01 tending to be distributed in the southern part of the study area and E17 in the northern part (Fig. 2a). While E01 has been widely distributed across Japan according to previous studies, E17 has shown a more localized distribution around the southern part of central Japan (Fig. 1b). Considering the spatial distribution and number of males and females, females were sampled more frequently and across a wider area compared to males (five males: approximately 15 km²; 15 females: approximately 37 km²; Supplementary Fig. S6).

All males were found to possess only the E01 haplotype, whereas females exhibited all three haplotypes, including the locally restricted haplotypes. Notably, only two individuals were assigned to the E07 haplotype, and both were females.

Nuclear DNA genetic diversity, relatedness, and structure

The overall gene diversity for the 30 individuals was calculated as 0.66. When we compared the genetic diversity of females ($n = 18$) and males ($n = 6$), gene diversity and allelic richness were slightly lower in females than in males (Supplementary Table S4), despite the larger sample size for females. The F_{IS} value showed a significant positive deviation from zero in the females ($F_{IS} = 0.146$, $P < 0.002$; Supplementary Table S4), whereas no significant deviation was observed in the males ($F_{IS} = 0.015$). However, when considering the presence of null alleles, the corrected F_{IS} value in females was within the 95% CI (Fig. 3). No evidence of a recent bottleneck was detected; a simulation analysis using the TPM model to assess heterozygosity excess did not yield significant values, indicating no detectable bottleneck (data not shown).

In addition to sex-based comparisons, we also assessed whether mitochondrial lineage (i.e., haplotypes E01 and E17) corresponded to genetic differentiation in nuclear markers. Among the 30 individuals identified, 23 could be assigned to either the E01 or E17 haplotype group.

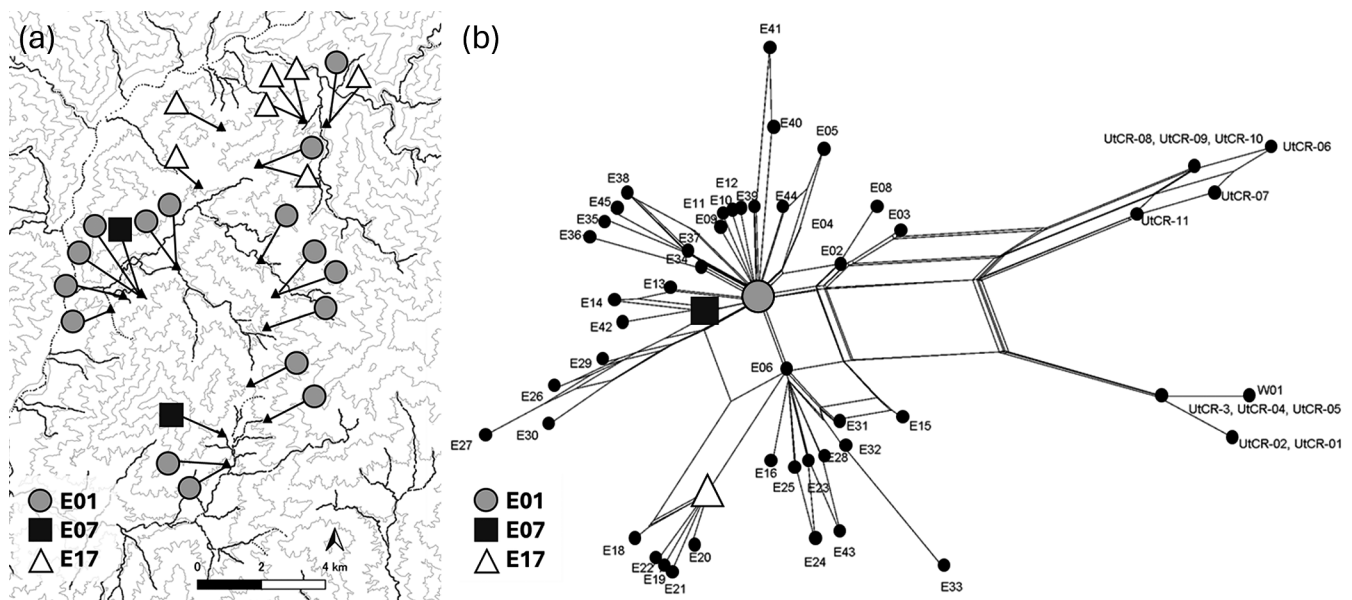


Fig. 2. The geographical distribution of the genetic structure of *Ursus thibetanus* in the study area based on mitochondrial (mt) DNA (a) and the phylogenetic relationship between mtDNA haplotypes as revealed using Neighbor-net (b). Individual points (a) show the geographical distribution of the hair traps where the DNA samples were collected, and the symbols show the haplotypes for each individual. The phylogenetic relationship was drawn using 52 published haplotypes; the sequence data were obtained from the National Center for Biotechnology Information (NCBI).

Gene diversity and allelic richness were slightly higher in the E01 group compared to the E17 group. The F_{IS} values did not reveal significant deviations from Hardy–

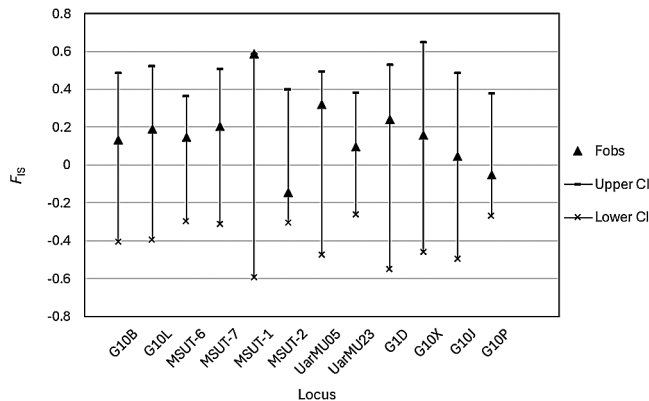


Fig. 3. The results of the permutation test for heterozygosity excess across loci in females ($n = 18$). The inbreeding coefficient F and its 95% confidence interval were calculated.

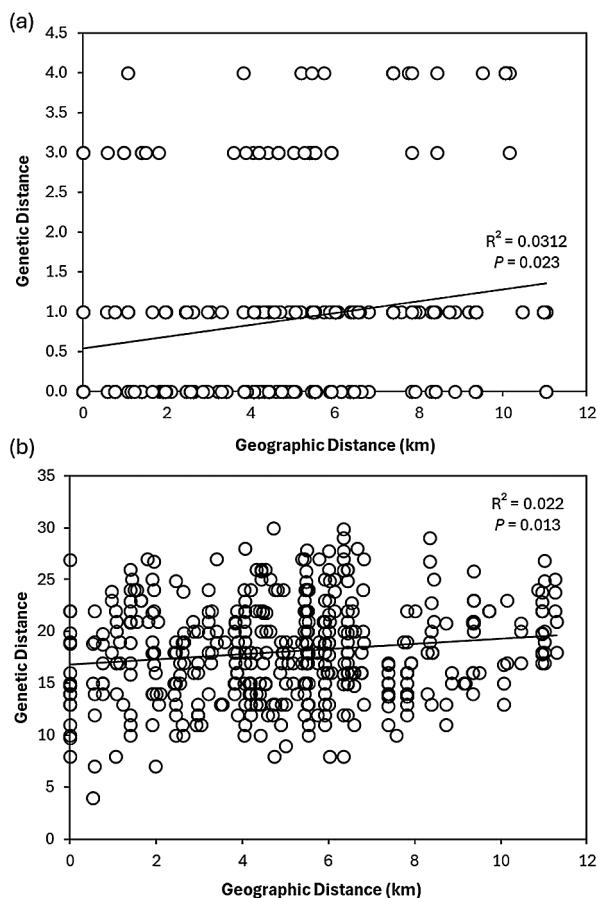


Fig. 4. Mantel scatterplots illustrating the relationship between genetic distance and geographic distance based on (a) mitochondrial DNA and (b) nuclear DNA. Each point represents a pairwise comparison between individuals, with the trend line indicating the overall correlation trend.

Weinberg equilibrium in either group (Supplementary Table S5). Furthermore, genetic differentiation between the two haplotype-based groups was not significant, with F_{ST} and F'_{ST} values of 0.015 ($P > 0.05$) and 0.044, respectively, and a null allele-corrected F_{ST} of 0.018 ($P > 0.05$).

The estimated N_e value based on all identified individuals was 44.8, with a Jackknife 95% CI ranging from 18.4 to “infinity”. Regarding the genealogical relationship analysis carried out with ML-Relate, among a total of 435 individual pairs, 141 could be assigned to a single most likely relationship category, at a 0.05 significance level. Among these, two pairs were estimated to be full siblings, one pair was identified as half siblings, and 138 pairs were estimated to be unrelated.

The STRUCTURE analysis based on 30 individuals showed the highest $\ln P(D)$ at $K = 1$, and no clear genetic clustering was detected (Supplementary Figs. S5b and S7).

Spatial autocorrelation analysis showed different patterns between mtDNA and nuclear DNA. For mtDNA, a significantly positive spatial autocorrelation was only detected within the first 0–4 km distance class ($P < 0.05$; Supplementary Fig. S8a). For nuclear DNA, which reflects dispersal by both sexes, no significant spatial autocorrelation was detected across all distance classes ($P > 0.05$; Supplementary Fig. S8b). While the ω criterion was significant in all five mtDNA correlograms, it was not significant in the nuclear DNA correlogram. In contrast, both mitochondrial and nuclear GD showed significant correlations with GGD ($P < 0.05$; Fig. 4), showing the existence of isolation by distance within the study area.

Discussion

Individual identification

While non-invasive sampling techniques are valuable for sampling large terrestrial mammal populations (Paetkau 2003), they are also prone to limitations, such as DNA degradation due to environmental exposure (Schultz et al. 2018; Bourgeois et al. 2019). Consequently, previous studies have often evaluated multi-locus genotypes using one to six independent PCR replicates to ensure accuracy (Ohnishi et al. 2011; Scarpulla et al. 2021; Buono et al. 2022). In this study, we used three to six independent PCR replicates per sample to obtain reliable multi-locus genotypes.

In addition, ensuring the accuracy of genotyping data from degraded samples requires close examination for potential issues such as genotyping errors and the presence of null alleles. Therefore, it is crucial that the degree

of linkage disequilibrium (LD) between loci is determined, to validate the biological relevance and reliability of the results. In this study, we checked the genotype of 16 microsatellite loci and selected 12, excluding those with unclear fragment patterns. By considering LD, we identified 30 individuals, with a P_{ID} of 1.20×10^{-9} . The value was comparable to previous studies conducted with 8–18 loci and across larger areas (Kadariya et al. 2018; López-Bao et al. 2020), supporting the reliability of our identification method.

Null allele frequencies across loci ranged from 0 to 0.117. According to Huang et al. (2016), frequency below 0.1 can be used directly without adjustment, while those above 0.5 can severely affect estimation accuracy and should be excluded. Considering these findings and our results, the influence of null alleles in our dataset is considered negligible. Thus, we estimated the minimum population size to be 30 individuals within a 48 km² study area.

MtDNA variation and its distribution

We clarified the local-scale genetic structure of the black bear population, considering sex, mtDNA, and nuclear DNA genetic variations. We found three mtDNA haplotypes, the distribution of which seemed to be divided between a valley and a small river (Fig. 2a). In a previous study, UtCR-E01 appeared to be widely distributed from Lake Biwa to northeastern Honshu, while UtCR-E17 was concentrated in the south of central Honshu (Ohnishi et al. 2009; Uno et al. 2015; Fig. 1b). Thus, detecting both mtDNA haplotypes in the southern part of central Honshu was expected. In contrast, UtCR-E07, previously found mainly in the southern Tohoku region and Toyama Prefecture (Ohnishi et al. 2009), was identified in Nagano for the first time in this study. As our sampling area was geographically limited, the origin of the E07 haplotype detected in Nagano remains unclear. However, two scenarios could be hypothesized. First, E07-carrying individuals may have dispersed from Toyama to Nagano across the Hida mountain range, which reaches elevations of 1500–3000 m (Supplementary Fig. S9). Notably, both individuals representing the E07 haplotype in Nagano were females, potentially suggesting long-distance dispersal by females. Although males generally exhibit larger home ranges than females in this species (Takayama et al. 2023), Takii et al. (2020) reported a case of long-distance dispersal by a subadult female during the summer. The individual moved a cumulative distance of 323 km over 91 days, with a home range size of 457.95 km² based on

the 95% minimum convex polygon (MCP) method. Second, E07 may have originally been present in Nagano, and the individual detected in Toyama by Ohnishi et al. (2009) could represent a disperser from Nagano. This remains a plausible explanation, particularly since the Toyama sample was from a single individual of unknown sex, which could have been a dispersing male. Furthermore, Ohnishi et al. (2009) defined E01 as the core haplotype, which had expanded extensively from the north of Lake Biwa to the Tohoku region before the last glacial maximum, rather than being a product of recent expansion. Based on this, it is possible that its close relative, E07, may have shared a similar ancient distribution but declined in many regions due to genetic drift. Given the limitations of our dataset in resolving the directionality of gene flow, comprehensive and fine-scale sampling across central Honshu is warranted to elucidate the current distribution and historical biogeographic dynamics of the E07 haplotype.

Nuclear DNA genetic diversity and structure

Analysis of nuclear microsatellite data from 30 individuals revealed a single genetic cluster within the approximately 48 km² study area, with gene diversity estimated at 0.66. They can be treated as a random mating population, as supported by the HWE tests. In comparison, Kadariya et al. (2018) reported no genetic structure among 60 black bears over a much larger area (approximately 525 km²) in the Annapurna Conservation Area, Nepal, with gene diversity estimated at 0.76, suggesting unrestricted movement across the landscape. Uno et al. (2015), however, detected four STRUCTURE clusters among 233 individuals across a vast area (30 790 km²) in northern Honshu, Japan, with a gene diversity value of 0.68. Although direct comparisons are limited due to the differences in loci and markers, the gene diversity observed in this study is similar to that of Uno et al. (2015). One possible explanation is that the study area may be situated in a region where multiple subpopulations interact, as suggested by mtDNA variation, facilitating occasional gene flow and maintaining genetic diversity. The lack of genetic clusters by the STRUCTURE analysis could be due to the smaller survey area than other studies because genetic structure is generally geographical scale dependent, as well as landscape heterogeneity. Indeed, as significant genetic differentiation was not detected between the mtDNA haplotype-based group in nuclear microsatellite markers, gene flow might have already occurred between maternal lineages in this region.

The effective population size (N_e) of the black bear population in this study was 44.8 (Jackknife 95% CI : 18.4 to infinite). Murphy et al. (2018) examined seven fragmented populations of the American black bear (*U. americanus*), and the estimated N_e ranged from 3 to 49 depending on the population, without “infinite” 95% CI s. Turnock et al. (2025) reported N_e values of 5.6–15.4 in fragmented populations of grizzly bear (*U. arctos*), and they frequently detected “infinite” 95% CI s in several populations or grouped individuals. However, the N_e value exceeded 200 with a fixed 95% CI in well-connected populations of European brown bear (*U. arctos*) (Skrbinšek et al. 2012). In general, the minimum threshold of N_e should be higher than 50 or even 100 to prevent inbreeding depression (Jamieson and Allendorf 2012; Frankham et al. 2014). These thresholds are derived from general population genetic theory and have not been specifically established for bear species. Although we need to be cautious of the estimated “infinite” 95% CI s, because of sampling errors (Do et al. 2014) and the unknown minimum threshold of N_e for the black bear (Murphy et al. 2018), our estimate represents the first LD-based N_e reported for the black bear in Japan and provides a valuable baseline. As no other N_e estimates have been reported from regions within Nagano Prefecture to date, this result provides a useful reference point for future comparisons. Local N_e estimates will facilitate the identification of genetically vulnerable subpopulations and support spatially explicit, adaptive conservation and management across the broader landscape.

These results underscore the importance of integrating spatial, genetic, and sex-specific data when assessing the local-scale structure and viability of mammalian populations, even within relatively continuous habitats. Local-scale genetic assessments, as demonstrated here, provide valuable insights into gene flow and demographic processes that may not be evident through broader scale studies alone. Continued long-term monitoring within the region will help detect temporal changes in genetic diversity and effective population size, while broader scale sampling will facilitate comparisons across different regions and spatial scales. Such efforts will deepen our understanding of population connectivity and structural variation and ultimately support the development of adaptive conservation strategies for the black bear.

Spatial genetic structure and female philopatry

Our results suggest that the observed genetic patterns may be influenced by sex-biased dispersal, particularly

male-mediated gene flow, a common characteristic in mammals. Although nuclear DNA microsatellite markers revealed no clear genetic structure, spatial autocorrelation was significant for mtDNA within 0–4 km distance class, but not for nuclear DNA. Furthermore, no significant nuclear genetic differentiation was detected between mtDNA haplotype-based groups. These contrasting patterns between maternally and biparentally inherited markers, together with slightly higher nuclear genetic diversity than females, despite a smaller sample size, may be attributable to female philopatry and male-biased dispersal, as reported in previous studies on bear populations (Ohnishi et al. 2011; Ohnishi and Osawa 2014; Fedorca et al. 2019; Draheim et al. 2021). Although the detection of a rare haplotype in females suggests the possibility of occasional long-distance dispersal, the overall pattern supports female philopatry within the population. This pattern reflects the tendency of mtDNA to maintain localized genetic structure under female philopatry, whereas nuclear DNA becomes homogenized through male-mediated gene flow.

Following the sampling process, females were sampled more frequently and over a broader area than males (six males: approximately 15 km²; 18 females: approximately 37 km²; Supplementary Fig. S4). In Ursidae species, sexually selected infanticide (SSI), i.e., where males kill dependent offspring but not their own progeny, has been widely reported (Bellemain et al. 2006; Naganuma et al. 2021; Tamatani et al. 2021). To mitigate the risk of SSI, female bears have been reported to alter their habitat use or avoid males during the summer (e.g., Pearson 1975; Rode et al. 2006; Steyaert et al. 2013). In our study, the northern part of the study area was characterized by complex and rich vegetation, including a variety of *Quercus* species (Fig. 5a), whereas the southern part primarily consists of coniferous forests dominated by *L. kaempferi* (Fig. 5b) and is located in a low-altitude valley. These environmental differences may partially explain the observed sex-based differences in detection rates. One possible interpretation is that adult males prefer higher-elevation areas with richer vegetation, while females may utilize lower larch-dominated areas, potentially to avoid males during certain seasons. As a result, hair traps in the southern area might have captured more female hair samples. However, this interpretation remains speculative, and further studies are needed to test these patterns.

Although the scale of the study area differs from previous studies (e.g., Takayama et al. 2023, which analyzed 14 nuclear microsatellites from 550 bears covering approximately 15 000 km²), this pattern is consistent with

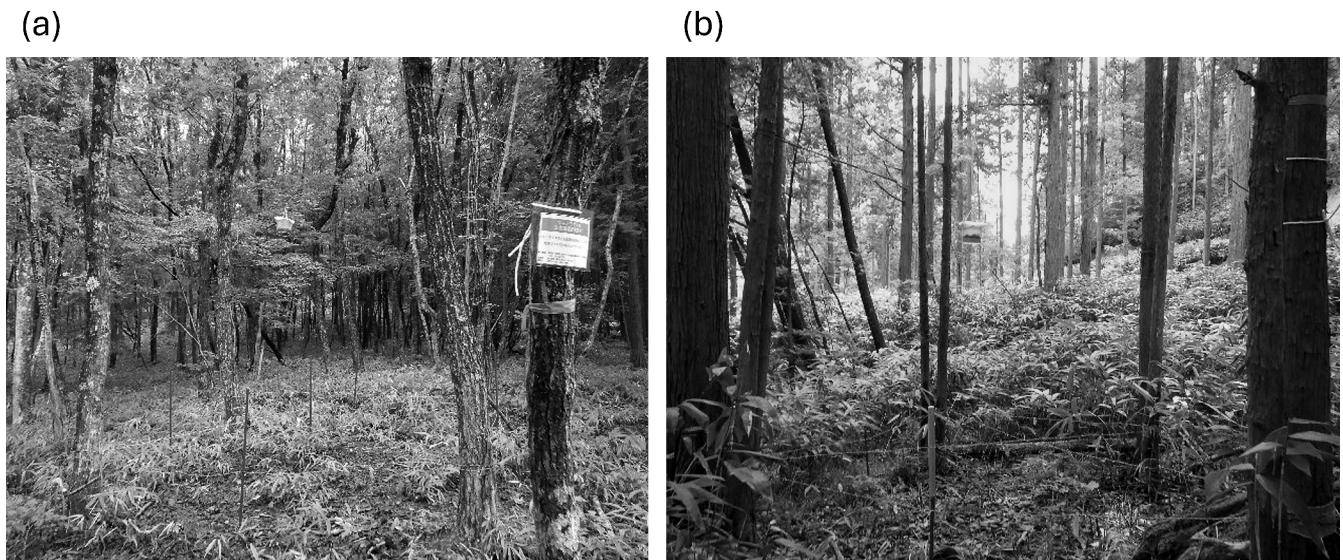


Fig. 5. (a) Vegetation and topography around the T35 trap site in the northern part of the study area, photographed on September 8, 2020. The northern area is typically dominated by deciduous broad-leaved forests, including *Quercus crispula* and other species. (b) Vegetation and topography around the T19 trap site in the southern part of the study area, photographed on October 1, 2020. The southern area is typically dominated by coniferous forests, including *Cryptomeria japonica* and *Larix kaempferi*.

established behavioral characteristics of bears, where males typically disperse farther than females (Shirane et al. 2019; Takayama et al. 2023).

Effectiveness and limitations of non-invasive hair traps and their application for conservation and management strategies

This study represents the first individual identification survey of the black bear in Nagano Prefecture using non-invasive genetic sampling, identifying at least 30 individuals within 48 km². The dense placement of hair traps enabled local-scale genetic analysis, revealing IBD patterns in both mtDNA and nuclear markers, even in the absence of distinct nuclear clusters. These findings underscore the importance of evaluating genetic structure at a local scale, particularly in heterogeneous mountain landscapes, and demonstrate the value of integrating mtDNA, sex, and microsatellite data to better understand patterns of gene flow and dispersal. While the dataset included some allele mismatches likely due to genotyping errors inherent in non-invasive samples, future studies would benefit from genome-wide analyses to minimize such errors and further improve accuracy.

Although we did not detect clear genetic structure within the study area, the estimated effective population size (N_e) was not particularly large. This underscores the importance of continued genetic monitoring to track temporal changes in genetic diversity and population size. In

addition, understanding local-scale genetic structure can predict potential conflict zones and design targeted management interventions (e.g., appropriate translocation or deterrent strategies). Thus, continued non-invasive genetic monitoring, combined with collaboration with local hunting associations, will help link ecological understanding with practical HWC mitigation strategies at both the prefectural and national levels.

Supplementary materials

Supplementary materials are available at *Mammal Study* online.

Supplementary Fig. S1. Photograph of a hair-trap station used in this study. The trap consists of barbed wire wrapped around trees, with a bait container (containing a nest of bees and honey) suspended approximately 2 meters above the ground.

Supplementary Fig. S2. The probability of exclusion (P_E) (a) when both parents known, (b) when only one parent is known, and (c) excluding both parents, based on 12 microsatellite (SSR) loci.

Supplementary Fig. S3. The probability of identity (P_{ID}) and the probability of sibling identity ($P_{ID\ sib}$) based on 12 microsatellite (SSR) loci.

Supplementary Fig. S4. Representative electrophoresis results for sex determination. (a) Amplification pattern using amelogenin primers. Males show two distinct bands

(X and Y alleles), while females show a single band (X allele only). (b) Amplification pattern using SRY and ZF primers. The SRY band (male-specific) is present only in males, while the ZF band (positive control) appears in both sexes.

Supplementary Fig. S5. The genetic structure analysis based on (a) mitochondrial (mt) DNA (white bars represent the lack of mtDNA haplotypes) and (b) nuclear microsatellite loci ($K = 2$) from 30 individuals. The individuals are shown as single vertical bar plots, with sex information, arranged from left to right in order from north to south.

Supplementary Fig. S6. The haplotype distribution for (a) males and (b) females.

Supplementary Fig. S7. Mean posterior probability (LnP(D)) values (a) and the delta K value (b) from $K = 1$ to $K = 10$ when $n = 30$.

Supplementary Fig. S8. Correlogram plots for the genetic correlation coefficients (r) for all individuals with increasing distance-class size, using (a) mitochondrial (mt)DNA, and (b) nuclear DNA. Closed symbols indicate a significantly positive r value ($P < 0.05$). The upper (U) and lower (L) confidence limits, which bound the 95% confidence interval, are shown as dashed lines. Upper (Ur error) and lower (Lr error) error bars bounding the 95% confidence interval are shown as vertical bars.

Supplementary Fig. S9. The location of the Hida mountain range in central Japan.

Supplementary Table S1. P -values for linkage disequilibrium between each locus pair, based on 30 individuals and 1320 permutations.

Supplementary Table S2. Pairwise relatedness values among 30 individuals using the Queller and Goodnight (1989) estimator.

Supplementary Table S3. Summary of the locus-by-locus polymorphism, allelic ranges, polymorphism information content (PIC), observed proportion of heterozygotes (H_{obs}), expected proportion of heterozygotes (H_{exp}), inbreeding coefficient (F_{IS}), and null allele frequency among 12 loci.

Supplementary Table S4. Genetic diversity within male and female groups based on 12 microsatellite loci.

Supplementary Table S5. Genetic diversity within two mitochondrial groups based on 12 microsatellite loci.

Acknowledgments: This study examined samples collected through a project conducted by Nagano Prefecture to investigate the population density of Asian black bears. We sincerely thank the Hokushinetsu Branch Office, Environmental Assessment Center, Nagano Prefecture,

for organizing the fieldwork and collecting hair trap samples. We also acknowledge support from the JSPS KAKENHI Grant-in-Aid for Scientific Research B (22H 02370, 25K02037), Grant-in-Aid for JSPS Research Fellow (23KJ0253), the Sumitomo Foundation, the Environment Research and Technology Development Fund (JPMEERF20254002) of the Environmental Restoration and Conservation Agency provided by the Ministry of the Environment of Japan, and the Mountain Science Center, University of Tsukuba. We also thank the reviewers for their appropriate comments to improve the manuscript. The mtDNA haplotypes and nuclear DNA genotype data used in this study were deposited at DRYAD entry <https://doi.org/10.5061/dryad.76hdr7t7s>.

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Received 4 June 2025. Accepted 22 August 2025.
Editor was Jun J Sato.