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Noninvasive genetic assessment of sloth bear diversity and population structure in Nepal

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Abstract: The genetic diversity of wildlife populations is crucial in maintaining their fitness and resilience to environmental changes and disease. Despite their rarity, ecological importance, and being an endangered species of public concern, information on sloth bears (*Melursus ursinus*) from Nepal is limited, and they are yet to receive conservation priority. To address this gap, we conducted Nepal's first noninvasive genetic surveys of sloth bear populations from 3 different habitat patches across their distribution range along the Churia-Terai landscape in Nepal between 2019 and 2021. Our study involved genotyping 127 samples using 12 microsatellite loci to determine the levels of genetic diversity and population genetic structure and using the control region of the mitochondrial genome for the haplotype analysis. We found 37 individuals in an area of approximately 1,000 km^2 comprising forest and grassland habitats. Our results indicate that the sloth bears in Nepal are characterized by low genetic diversity ($H_E = 0.48$) compared with other bear populations across its range. We had a limited number of individuals from different sampling areas, but their genotypes were consistent with there being no genetic structure between sampling areas. The study provides the crucial baseline information on the conservation genetics of sloth bears from Nepal and highlights the prospects for using noninvasive DNA sampling for research, monitoring, and conservation of bears.

Key words: genetic diversity, *Melursus ursinus*, Nepal, noninvasive DNA sampling, population genetics, population structure, sloth bear, wildlife conservation

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The sloth bear (*Melursus ursinus*, Shaw and Nodder 1791) is a carnivore in the Ursidae family with specialized adaptations for myrmecophagy (ant and termite feeding). Commonly referred to as *Kathe Bhalu* in Nepali, this species is endemic to the Indian subcontinent and Sri Lanka and has been listed as "Vulnerable" on the International Union for Conservation of Nature (IUCN) Red List. The IUCN recognizes genetic diversity as a key component of biodiversity that needs to be prioritized for conservation. Genetic diversity is crucial for maintaining population fitness, enabling them to adapt to changing environmental conditions and resist diseases (Frankham et al. 2002, Spielman et al. 2004). However, despite their rarity and ecological importance, ecological and genetic information of sloth bears is limited. This has contributed to the inadequate policy attention and bureaucratic inertia to conserve and manage sloth bear populations in Nepal.

Historically, sloth bears were abundant across the forest and grassland habitats along the Terai and Siwalik–Churia regions' alluvial plains and rugged hills. Their current distribution is limited to a few protected areas and forest patches (Dharaiya et al. 2020, Paudel et al. 2022, Subedi et al. 2021a). Trijuga forest (TJF) in the east, Bardiya National Park (BNP) in the west, and Chitwan National Park (CNP) in central Nepal are considered the major habitats with sloth bear presence, and only a few sporadic reports outside of

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these habitats have been reported (Sharma et al. 2023, Sadadev et al. 2024). Populations in small and isolated habitats can rapidly lose genetic diversity as a result of random genetic drift and inbreeding (Frankham 2003, 2010; Schlaepfer et al. 2018). Large mammals like sloth bears need large areas for successful dispersal and breeding, which makes them highly vulnerable to such genetic consequences (Thatte et al. 2020). A study investigating genetic variation and connectivity of sloth bears in India highlights the importance of well-connected habitats for gene flow and maintenance of genetic diversity (Dutta et al. 2015). Natural or anthropogenic barriers in a landscape can disrupt gene flow between populations and alter the population's genetic structure (Dixon et al. 2007, Ohnishi et al. 2007, Straka et al. 2012, Thatte et al. 2020). Populations with distinct genetic structures may require different management interventions as evolutionarily significant units. A demographically unstable population that has undergone a steep decline and has an inadequate genetic exchange with adjoining populations may be unable to maintain genetic diversity and population structure (Jansson et al. 2012). The situation becomes of particular concern for sloth bears because their population and geographic range have declined sharply and the remaining habitats are fragmented (Dharaiya et al. 2020). Sloth bears face the greatest impacts of human footprints among apex predators worldwide (Wolf and Ripple 2018, Quintana et al. 2022). Achieving human–bear co-existence in a human-dominated landscape requires judicious management of sloth bear populations and human communities' tolerance and behavior toward bears. Too many or too few sloth bears beyond the ecological and social carrying capacity can disrupt a delicate balance between development and wildlife conservation. Mixing of individuals from isolated patches and increasing numbers of individuals in subpopulations through translocations are recommended for the long-term conservation of sloth bears in Nepal (Jnawali et al. 2011), but little is known about genetic variation and population structure.

Without understanding the existing patterns of heterozygosity, the risk of gene homogenization increases, thereby reducing the species' resilience and adaptability for long-term survival (Bertola et al. 2022). Feces and hair samples collected without capturing and handling individuals have been widely used to obtain valuable ecological and genetic information on wildlife species (Dutta et al. 2015, Kadariya et al. 2018, Thapa et al. 2018). DNA obtained from noninvasive samples is usually degraded compared with blood or tissue samples; however, careful collection, storage, and transportation of fresh noninvasive samples can provide comparable results (Piggott and Taylor 2003, Andrews et al. 2021). Noninvasive DNA sampling offers a promising approach for studying bears given that use of other methods, such as camera-traps, is not feasible for individual identification. This method is suitable when the species under study is endangered, challenging to capture, and when the study has limited resources. However, no prior studies have assessed the genetic diversity and population structure of sloth bears in Nepal (Fig. 1), undermining their long-term conservation. In this first-of-its-kind study on sloth bears from Nepal, we aim to provide valuable baseline genetic information on sloth bears from Nepal that will support evidence-based conservation planning, enabling efficient allocation of limited resources to protect and manage sloth bear populations in Nepal.

Material and methods

Study area

The study was carried out along the Churia–Terai region of the outer Himalayan landscape in Nepal (Fig. 2). It consists of a geologically fragile mountain range along the foothills of the Himalayas known as 'Siwalik' or 'Churia' and alluvial flood plains formed by tributaries of the Ganges River and the associated valleys. Field sampling for genetic data was concentrated in the Chitwan National Park (CNP), Bardiya National Park (BNP), and Trijuga forest (TJF). Chitwan National Park covers 953 km^2 and is in the south-central part of Nepal along the floodplains of the Rapti, Reu, and Narayani rivers. Bardiya National Park covers 986 km^2 and is situated in the southwestern part of Nepal along the flood plains of the Karnali and Babai rivers. Trijuga forest covers 430 km^2 and is located in the southeastern part of Nepal along the bank of the Triyuga and Koshi rivers. These study sites are geographically $\geq$200 km apart from each other. The altitudinal range lies between 60 and 1,500 m above sea level, and the study area experiences a subtropical monsoonal climate. Currently, around 50% of the landscape is under agriculture and settlement, and another 50% comprises forests, shrublands, grasslands, and riverbeds (Ram et al. 2021). The major forest cover consists of the deciduous sal (*Shorea robusta*) forest. Tiger (*Panthera tigris*) is a dominant carnivore of the landscape that co-occurs with other mega herbivores like

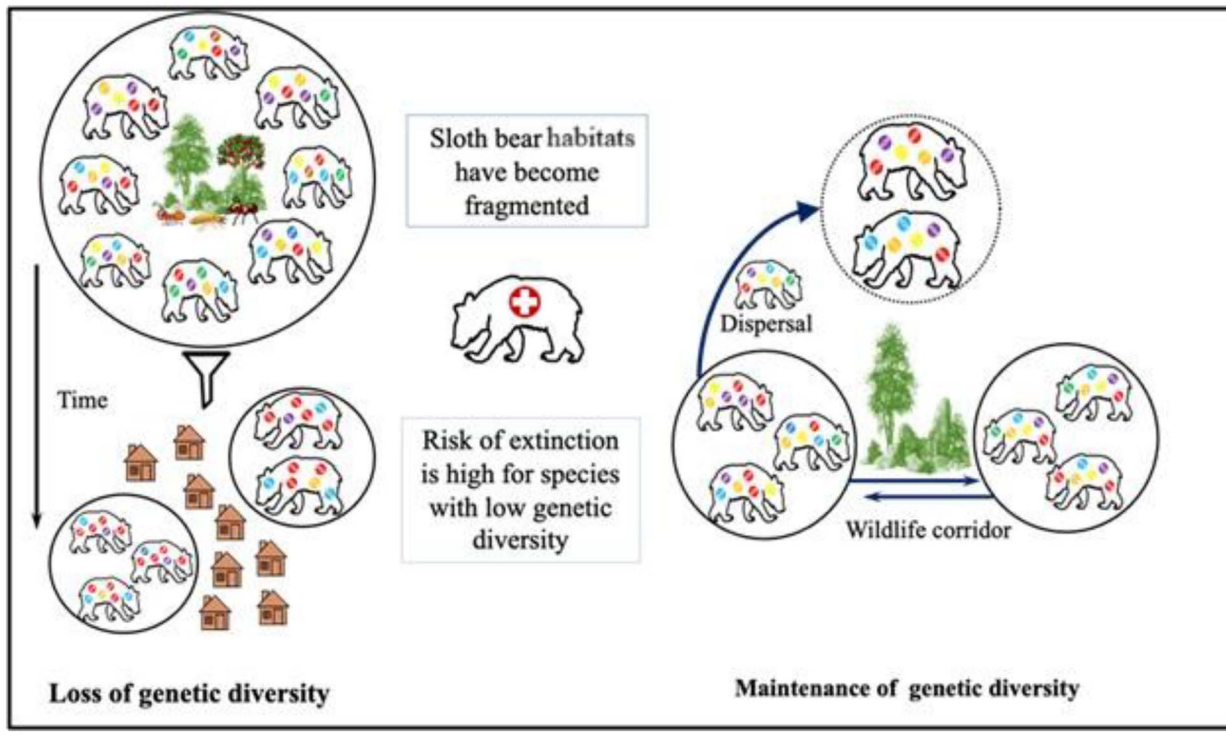

Fig. 1. Sloth bear (*Melursus ursinus*) habitats have become fragmented.

greater one-horned rhinoceros (*Rhinoceros unicornis*) and Asian elephants (*Elephas maximus*). Pangolins (*Manis pentadactyla* and *M. crassicaudata*) are other myrmecophagous species present in the landscape. The biological resources of this landscape are also of great importance to the livelihood of local people who depend intensely on forest resources for farming and livestock (Stræde and Treue 2006). Some fruit species, such as *Ficus* spp., *Syzygium* spp., *Zizyphus* spp., *Aegle marmelos*, *Cassia fistula*, *Phoenix* spp., *Mangifera indica*, *Bridelia retusa*, and *Bombax ceiba*, are used both by local people for their livelihood and sloth bears as an essential component of their diet (Shah et al. 2018). The collection of forest resources is regulated by the government and local communities under different management regimes.

Sampling design and data collection

We conducted an intensive search for sloth bear feces in the study area along forest trails, rivers, and animal tracks between 2019 and 2021. Survey effort (length of transect walked) across the sites CNP, BNP, and TJF was approximately 180 km, 80 km, and 40 km, respectively, based on the information available about habitat use by sloth bear and accessibility of the sites during the survey. We opportunistically collected hair samples when available. Sloth bear feces were distinguished mainly based on feces contents, such as the presence of termites, ants, and fruit remains. Presence of pugmarks, and/or the freshness of diggings, scrapes, and termite mound feedings in the nearby surroundings, aided in distinguishing fresh sloth bear samples. Experienced wildlife technicians involved in the survey determined freshness of sloth bear signs based on the visual patterns of the exterior surface and experience. When a fresh putative bear fecal sample was encountered, we rubbed the outer surface multiple times with a sterile flock swab (Puritan Medical Products Co., LLC, Guildford, Maine, USA) to ensure the mucus layer was properly scraped and adhered to the swab. The swab was stored in a 2.5-mL vial containing buffer solution (Inhibitex Buffer; Qiagen Inc., Tokyo, Japan). We collected hair samples using sterilized forceps and stored them in paper envelopes. Disposable latex gloves were replaced after each sample was collected, and forceps were immediately rinsed with 75% ethanol

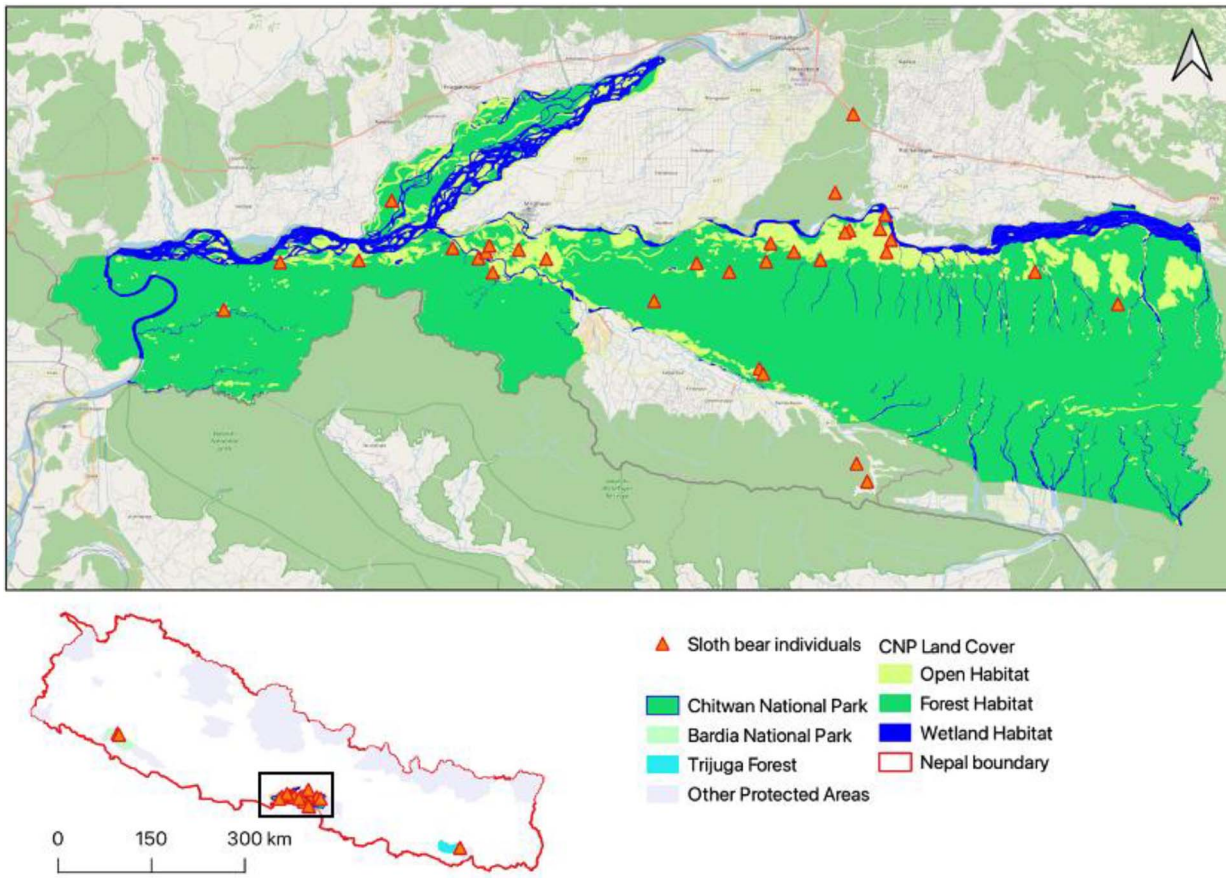

Fig. 2. Study area map showing sampling locations used in genetic surveys of sloth bears (*Melursus ursinus*) between 2019 and 2021; and distribution of individual genotypes and land cover types in Chitwan National Park, Nepal.

and flame sterilized to avoid contamination. Ethanol does not fully eliminate DNA carryover, so bleach treatment or flame sterilization is considered important to minimize cross-contamination risks, particularly for low-quantity DNA sources. We stored samples at −20°C until analysis. We recorded the global positioning system (GPS) location (Garmin; Garmin, Olathe, Texas, USA) and environmental characteristics of the sample location.

Genetic methods

We extracted the genomic DNA from the fecal swab and hair samples using the Qiagen QIAamp DNA Stool Mini Kit (Qiagen Inc.) for fecal samples and the Isohair easy (Nippon Gene, Inc., Tokyo, Japan) for hair samples following the manufacturer's instructions. Estimates of heterozygosity based on a few loci may not allow the differentiation of individuals, while a high number of loci can also have a negative impact on individual identification (Buono et al. 2022; i.e., increase the chances of genetic error and create false individuals). To determine the genotypes, we initially selected 7 polymorphic nuclear microsatellite loci optimized for the sloth bear study (Sharma et al. 2013). Poor-quality DNA from fecal samples could lead to the misidentification of individuals and biased estimates (Taberlet et al. 1999), so we discarded samples that were not successful in the first round of amplification. We first multiplexed samples with the primer set of 2 loci (MU26 and G10L) and then discarded if they did not produce scorable results at any locus, even after multiple rounds of polymerase chain reaction (PCR). PCR conditions for analysis are described in Text S1 (*Supplemental material*). Samples amplified at both loci were further amplified using primers for 5 additional loci (G1A, G10B, G10J, CXX203, and UMAR2) and sex primers.

In the initial set of 7 microsatellites, the MU26 locus was monomorphic, and the number of alleles was lower than that of Sharma et al. (2013). We included 8 additional microsatellite loci (G10H, CXX20, G10C, G1D, MU05, MU09, MU59, and G10M) previously used for studies in bears (Ostrander et al. 1993; Paetkau et al. 1995, 1998; Taberlet et al. 1997; Bellemain and Taberlet 2004; Cronin et al. 2009; Poissant and Davis 2011; Sharma et al. 2013) to check whether increasing microsatellite loci improved the results. We excluded monomorphic loci (MU26, G10C, and G1D), and finally considered 12 polymorphic loci for further genetic analysis. We used 2 Y-specific fragments (SMCY and 318.2) and 1 X-specific fragment (ZFX) for the molecular sexing of individual sloth bears following Bidon et al. (2013). We amplified all identified individuals' left variable region of the mitochondrial control region (CR)/D-loop (approx. 675 base-pair [bp]) for haplotype identification and phylogenetic analysis. Primers used for analysis are listed in Tables S1 and S2 (*Supplemental material*).

We did not carry out additional reactions if a sample produced a consensus genotype without ambiguous amplifications in both rounds. Otherwise, we conducted 3–4 amplifications for each sample to confirm any allele that was inconsistently scored. We constructed a consensus genotype if ≥2 replicates were matched in 8 loci for each sample; we excluded from our data set samples missing any locus. In some cases, we performed an additional singleplex PCR for the final confirmation of the allele.

Data analysis

We grouped identical consensus genotypes to identify the number of individuals using GIMLET software version 1.3.3 (Valière 2002). Each genotype's GPS coordinates were mapped using QGIS version 3.16. Genetic diversity (mean no. of alleles per locus [N_A], effective no. of alleles [N_E], observed heterozygosity [H_O], expected heterozygosity [H_E], Unbiased expected heterozygosity [uH_E], and Wright's inbreeding coefficient [F_{IS}] variables were calculated with GenALEx version 6.5 [Peakall and Smouse 2006]). We tested the Hardy–Weinberg equilibrium of loci following exact test and linkage disequilibrium between all pairs of loci using the web-based program GENEPOP version 4.2 (Rousset 2008). We applied Bonferroni corrections for multiple comparisons. We calculated the probability of identity (P_{ID}), the probability of identity of siblings (P_{IDSibs}), mean polymorphic information content (PIC), and the null allele frequency (F_{null}) of each locus using CERVUS version 3.0.7 (Kalinowski et al. 2007). We verified genotyping errors such as stutter bands, null alleles, and large allele dropouts using MICRO-CHECKER version 2.2.3. To determine the patterns of population genetic structure of the sloth bears population, we used a Bayesian clustering analysis in STRUCTURE version 2.3.4 (Pritchard et al. 2000). We assumed admixture and correlated allele frequencies using prior location information in STRUCTURE analysis. The admixture model was run with burn-in periods of 50,000 and 500,000 Markov Chain Monte Carlo iterations. The range of possible clusters (K) ranged from 1 to 6, and 5 independent runs were performed with and without prior information of sampling locations. We assigned each bear to a cluster if its membership coefficient (q) was >0.7 or classified as admixed if q was <0.7. To determine the most probable value of K, we used the mean LnProb values as in Pritchard et al. (2000), implemented in STRUCTURE HARVESTER (Earl and VonHoldt 2012). Mitochondrial sequences were visually inspected for errors, multiple peaks, and heteroplasmy using FinchTV version 1.4.0 (Geospiza Inc.) and aligned with Clustal W (Thompson et al. 1994). We deposited all sequences in the National Center for Biotechnology Information (NCBI) GenBank database (accession no. OQ200477–OQ200480) for the CR haplotypes. We obtained the reference sequence for the control region from the mitogenome sequence of sloth bears deposited in the NCBI GenBank database (Yu et al. 2007). We used the Himalayan black bear (*Ursus thibetanus laniger*; accession no. NC009331) as an outgroup for the phylogenetic analysis of sloth bears. We included base substitutions and the T-repeat variation in calculating haplotypes. Sequence alignment, haplotype identification, and phylogenetic tree construction was done using MEGA-X software (Kumar et al. 2018). We inferred the evolutionary history using the maximum likelihood method and the Kimura 2-parameter model. We inferred the bootstrap consensus tree from 1,000 replicates. We collapsed branches corresponding to partitions reproduced in <50% of bootstrap replicates. We obtained initial trees for the heuristic search automatically by applying neighbor-join, and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with a superior log likelihood value. We eliminated all positions containing gaps and missing data.

Table 1. Details of sloth bear (*Melursus ursinus*) samples collected between 2019 and 2021 in Nepal,[a] genotyping success, and sample origin. Total number of the unique individuals identified from forest and grassland habitats are included.

Location[a]	No. of samples	Sampling location		Genotyping			No. of unique sloth bears
		Forest	Grassland	Incomplete	Complete	% Success	
CNP	107	48	59	59	48	45	32
BNP	12	12	0	5	7	58	2
TJF	8	8	0	3	5	63	3
Total	127	68	59	67	60	47	37

[a]CNP is Chitwan National Park; BNP is Bardiya National Park; TJF is Trijuga forest.

Results

Noninvasive sampling and genotyping

We collected 127 samples (116 feces and 11 hairs) from approximately 1,000 km^2 of the area surveyed at 3 locations (Table 1). Sixty samples produced complete and reliable genotypes. The remaining samples were ambiguous for multiple loci or did not yield complete genotypes. We obtained an overall genotyping success of 47% (47.4% and 45.4% for fecal and hair samples, respectively). We identified 37 unique individuals from these 60 genotypes. Seven were females, and 18 were males. The sex of 12 individuals could not be determined because of poor amplification. Most of the individuals were recorded from the central habitat (CNP, $n = 32$), and very few individuals were recorded from the east (TJF, $n = 3$) and west (BNP, $n = 2$). Most samples were obtained in spring ($n = 90$) and winter ($n = 31$), and a few during monsoon ($n = 2$) and autumn season ($n = 4$). Despite the difference in land cover, almost an equal percentage of samples were obtained from the forest (53.5%) and grassland habitats (46.5%; Table 1).

Genetic diversity and population structure

The average allelic richness across 12 polymorphic loci was 3.58 (SE = 0.42), and the number of effective alleles was 2.15 (SE = 0.24). Three loci (MU26, G10C, and G1D) were monomorphic and excluded from the analysis. Other loci were polymorphic with 2 (MU59, MU09, and G10B), 3 (G10L, UMAR2, and G10M), 4 (MU05, G10H, CXX203, and CXX20), or 5 (G1A) or more (G10J) alleles per locus (Table 2). The observed heterozygosity (0.44, SE = 0.05) was lower than the expected heterozygosity (0.48, SE = 0.05). No significant deviation from Hardy Weinberg Equilibrium ($P > 0.05$) was detected between the microsatellite loci. No significant linkage disequilibrium was observed between microsatellite loci except for CXX203 and CXX20, which persisted even after Bonferroni correction. The mean polymorphic information content (PIC) was 0.42, ranging from 0.12 to 0.71. The cumulative P_{ID} and P_{ID}Sibs were 1.02×10^{-6} and 1.72×10^{-3} (Table 2). The fixation index F_{ST} was 0.07 (SE = 0.02). The Weir and Cockerham (1984) measure of the inbreeding coefficient (F_{IS}) was 0.08 and positive for most loci. Visualization of results from the STRUCTURE using Structure Harvester showed the highest mean LnProb value for $K = 1$ (Fig. S1 and Table S3 [*Supplemental material*]). The membership coefficient (q) did not show absolute values (0 or 1), and no individuals were assigned with high posterior probability ($q \geq 0.70$) to any of the clusters at $K = 2$ (Fig. 3 and Fig. S2 [*Supplemental material*]).

Haplotype distribution and phylogenetic relationship

We obtained a consensus sequence using the forward and reverse primers for the control region of mitochondrial DNA. The base substitutions at 2 variable positions and the repeat number variation at the thymine (T) and cytosine (C) repeat sites defined 4 unique haplotypes (Table 3). The base substitution detected in this analysis was a single position transition of Adenine (A)–Guanine (G) and C–T. No insertion or deletion was observed except for the T and C repeat number variation. Multiple substitutions were not observed at any variable positions. The substitutions were observed only in the samples from the eastern habitat (TJF). This eastern haplotype (MUNEP-E1, $n = 3$; accession no. OQ200480) is distributed approximately 200 km east of the central population in CNP. Variation in the T-repeat site was observed in the samples from the BNP. This western haplotype (MUNEP-B1, $n = 2$; accession no. OQ200479) is distributed approximately 300 km west of the central population in CNP. All other individuals belonged to the (MUNEP-C1, $n = 15$, accession no. OQ200477; and MUNEP-C2, $n = 17$, accession no. OQ200478) haplotype. Maximum likelihood phylogenetic

Table 2. Genetic diversity parameters[a] for the 12 polymorphic microsatellite loci used to evaluate the population of the sloth bears (*Melursus ursinus*) from Nepal.

Locus	Multiplex	N_A	N_E	H_O	H_E	$_UH_E$	PIC	P_{ID}	P_{ID}Sibs	F_{IS}	*P*	F_{null}	ADO
G10L	MP1	3	1.69	0.35	0.41	0.41	0.36	0.40	0.65	0.15	0.29	0.10	0.00
G1A	MP2	5	2.10	0.51	0.52	0.53	0.47	0.28	0.56	0.03	0.28	0.04	0.00
G10B	MP2	2	1.14	0.14	0.13	0.13	0.12	0.77	0.88	−0.06	1.00	−0.03	0.00
G10J	MP2	7	4.03	0.68	0.75	0.76	0.71	0.10	0.40	0.12	0.36	0.05	0.00
CXX203	MP3	4	2.13	0.46	0.53	0.54	0.47	0.28	0.56	0.15	0.15	0.10	0.00
UMAR2	MP3	3	1.24	0.16	0.20	0.20	0.18	0.66	0.82	0.18	0.36	0.09	0.00
G10H	MP4	4	3.56	0.62	0.72	0.73	0.67	0.13	0.42	0.15	0.07	0.07	0.00
CXX20	MP4	4	2.05	0.46	0.51	0.52	0.45	0.30	0.57	0.11	0.43	0.07	0.00
MU05	MP4	4	2.18	0.60	0.54	0.55	0.44	0.31	0.56	−0.08	0.75	−0.05	0.00
MU09	MP5	2	1.95	0.46	0.49	0.49	0.37	0.38	0.60	0.07	0.74	0.03	0.00
MU59	MP5	2	2.00	0.46	0.50	0.51	0.38	0.38	0.59	0.09	0.74	0.04	2.99
G10M	MP5	3	1.77	0.42	0.43	0.44	0.39	0.37	0.63	0.05	0.39	0.02	0.00
Mean/Cumulative		3.58	2.15	0.44	0.48	0.48	0.42	1.02×10^{-6}	1.72×10^{-3}				
SE		0.42	0.24	0.05	0.05	0.05							

[a]N_A, observed number of allele; N_E, effective number of allele; H_O, observed heterozygosity; H_E, expected heterozygosity; $_UH_E$, unbiased expected heterozygosity; PIC, polymorphic information content; P_{ID}, probability of identity (locus); P_{ID}Sibs, probability of siblings identity(locus); F_{IS}, Wright's inbreeding coefficient; *P*, *P*-values for exact tests of Hardy–Weinberg equilibrium; F_{null}, predicted frequency of null alleles; ADO, allele dropout %; SE, standard error.

analysis revealed that the haplotypes from Nepal formed a distinct clade compared with the reference sequence of sloth bear mitochondrial genome available in the gene bank (Fig. 4).

Discussion

Genotyping success

We obtained valuable information for the first time from the present study on the conservation genetics of sloth bears from Nepal. We had <50% success obtaining complete genotypes from our fecal samples and the success rate for hair samples was lower than the fecal samples. Noninvasive fecal and hair samples are vulnerable to rapid degradation in hot and humid conditions and thus are characterized by low DNA quality (Stetz et al. 2015, Andrews et al. 2021). Such noninvasively collected genetic samples are prone to high rates of incomplete genotyping, allelic dropouts, and false alleles (Taberlet et al. 1997, Kunde et al. 2020). The low success rate for hair compared with feces can be attributed to low-quality hair samples that were opportunistically obtained from the termite mounds and trees near the feeding sites. Systematic collection of hair samples from rub trees and hair traps can produce better DNA quality and, thus, relatively greater genotyping success (Shimozuru et al. 2019, 2022). However, the rubbing behavior has not been well-established in sloth bears, although it is widely documented for other bears species such as the American black bears (*Ursus americanus*) and the brown bear (*U. arctos*). Genotyping success rate could also be maximized using blood or tissue DNA samples, but it required capturing and handling wild sloth bears, which was logistically out of scope for this study. Noninvasive sampling techniques were better suited for our research and provided a more cost-effective option to obtain large samples within a short time frame in remote and challenging habitats. Further, repeated genotyping using a multitube approach reduced genotyping errors and increased the usability of the noninvasive samples (Bourgeois et al. 2019, Shimozuru et al. 2019). The Micro-checker program did not detect any errors due to stutter bands, large allele dropouts, or deficits of heterozygotes.

Genetic diversity

Evidence from the microsatellite markers indicates that the genetic diversity of sloth bears from Nepal is relatively lower than existing information on their genetics across their distribution range. The number of alleles was fewer in Nepal ($N_A = 3.57$) than those observed for the Indian sloth bear population ($N_A = 8.86$) if we focus on 7 microsatellite loci commonly used for both studies (Table 4a). The average number of alleles per locus and the associated heterozygosity can depend on the sample size. However, sloth bears in protected areas of central India, viz. Kanha, Pench, Satpura, and Melghat exhibited higher allele numbers and heterozygosity despite small samples from each study area relative to our study area (Table 4b). Moderate to high heterozygosity has been reported for other bear species, such as sun bears (*Helarctos malayanus*; $H_E =$

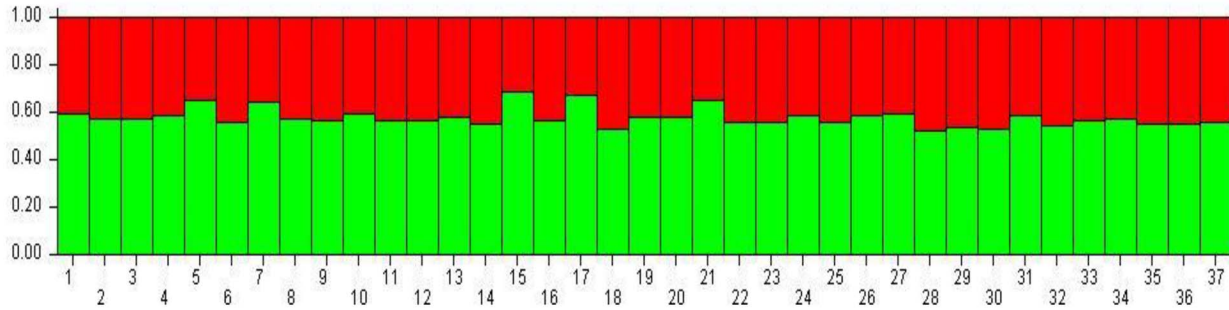

Fig. 3. Population structure of sloth bear (*Melursus ursinus*) derived from genetic surveys of sloth bears conducted between 2019 and 2021 in Nepal. Vertical bar represents individual bears, and the green color represents the membership coefficient (*q*) of each individual with value <0.70 when *K* = 2. Numbers 1–37 represent the individuals identified from microsatellite analysis. Numbers 5, 7, and 21 were individuals from Trijuga forest, numbers 15 and 17 were from Bardiya National Park, and others were from Chitwan National Park.

0.58; Kunde et al. 2020), Andean (spectacled) bears (*Tremarctos ornatus*; H_E = 0.40–0.57; Ruiz-García et al. 2005), Kermodian black bears (*U. americanus kermodei*; ${}_UH_E$ = 0.62–0.79; Marshall and Ritland 2002), Carpathian brown bears (*U. arctos arctos*; H_E = 0.65–0.80; Straka et al. 2012) and polar bears (*U. maritimus*; H_E = 0.64; Cronin et al. 2006). A high level of genetic diversity was observed in Himalayan black bears (H_E = 0.76) in the mountainous landscape of central Nepal (Kadariya et al. 2018). A moderate level of genetic diversity (H_E = 0.61) was reported in tigers from a similar landscape to our study in Nepal (Thapa et al. 2018). Parameters of genetic diversity across different species and the same species in different landscapes may show considerable variation because they evolve(d) under different demographic and environmental conditions.

The core-periphery hypothesis (CPH) or abundant-center hypothesis predicts that populations located at the periphery of a species' range should have lower levels of genetic variation than those at the center of the range. Peripheral ranges are usually characterized by suboptimal habitats, smaller populations, and reduced gene flow between populations. Our study's heterozygosity estimate at 7 microsatellite loci was 0.33, significantly lower than the 0.53 observed in central India, only a few hundred kilometers to the south (Dutta et al. 2015). The heterozygosity parameters marginally increased when the number of microsatellite loci increased from 7 to 12, but the average number of alleles did not significantly change (Table 4b). Historically, sloth bears' distribution range in the Indian subcontinent was contiguous across the lowlands, so it can be expected that both populations would have had higher levels of historical genetic diversity. Despite severe habitat loss in the central Indian landscape, sloth bears maintained moderate genetic diversity largely due to dispersal facilitated by corridors (Dutta et al. 2015, Thatte et al. 2020). Genetic diversity results of sloth bears from northernmost distribution range of the species in our study hint at predictions based on CPH. However, rigorous assessment may be required to move beyond assumption to provide more realistic and empirical evidences of this geographic patterns of abundant-center hypothesis (Sagarin et al. 2006).

Within these habitats, sloth bear activity is concentrated in a few suitable habitat patches with abundant food resources (Paudel et al. 2022). The home range for sloth bears in Nepal is relatively smaller, with extensive overlap within and between sexes (Joshi et al. 1995, Yoganand 2005, Ratnayeke et al. 2007). Small but positive inbreeding coefficient values (F_{IS}) in this study suggest that sloth bears in the study area are inbreeding. While inbreeding can greatly reduce the average individual fitness, and loss of genetic variability from random genetic drift can diminish future adaptability to a changing environment, other demographic factors can overshadow genetics (Lande 1988). Proportion of suitable habitat, vital rates of individuals, fluctuating environment, and other demographic factors are likely to be of more immediate importance than genetics in determining population viability of threatened wild mammals existing in a human-dominated landscapes.

Hunting can also alter a population's genetic characteristics through its influence on individual fitness, rate of gene flow, and changes in the effective population

Table 3. Variable positions and observed frequencies of the left domain of the control region for 4 haplotypes of sloth bears (*Melursus ursinus*) from genetic samples collected between 2019 and 2021 in Nepal. The dot indicates identity with the nucleotides of MUNEP-B1. Dashes indicate variation in the number of Ts and Cs.

		Position number						
Haplotype	Sequence length	07	59	60	190	Individuals	Location	GeneBank accession number
MUNEP-B1	466	G	T	C	C	2	BNP	OQ200479
MUNEP-E1	466	A	—	•	T	3	TJF	OQ200480
MUNEP-C1	466	•	—	—	•	15	CNP	OQ200477
MUNEP-C2	466	•	—	•	•	17	CNP	OQ200478

size (Harris et al. 2002). Sloth bears were killed in large numbers during royal hunting before the establishment of parks in the 1970s (Garshelis et al. 1999). Hunting is not practiced currently, but sloth bears are killed in poaching, collisions with vehicles, electrocution, and retaliation during incidents of human–bear conflicts (Acharya et al. 2016, Paudel et al. 2020). Although the genetic consequence of hunting, poaching, and retaliatory killing of sloth bears cannot be ascertained because their extent relative to the population size remains unclear, human-caused demographic changes including mortality related to habitat encroachment and conflicts may represent a far bigger threat than the long-term effects of the loss of genetic diversity.

Population differentiation

Although we have limited numbers of individuals in these sampling areas, their genotypes are consistent with there being no genetic structure between sampling areas. The variance of Ln likelihood and standard deviation for other values of K compared with $K = 1$ and the small difference in magnitude of ΔK and membership coefficients <0.7 for $K = 2$ suggested that $K = 1$ was most meaningful for our data. Our results are different from those reported for the population of sloth bears in central India (Dutta et al. 2015). Sloth bears in the central Indian landscape were interconnected by corridors that facilitated genetic exchange and prevented further genetic substructuring of the population.

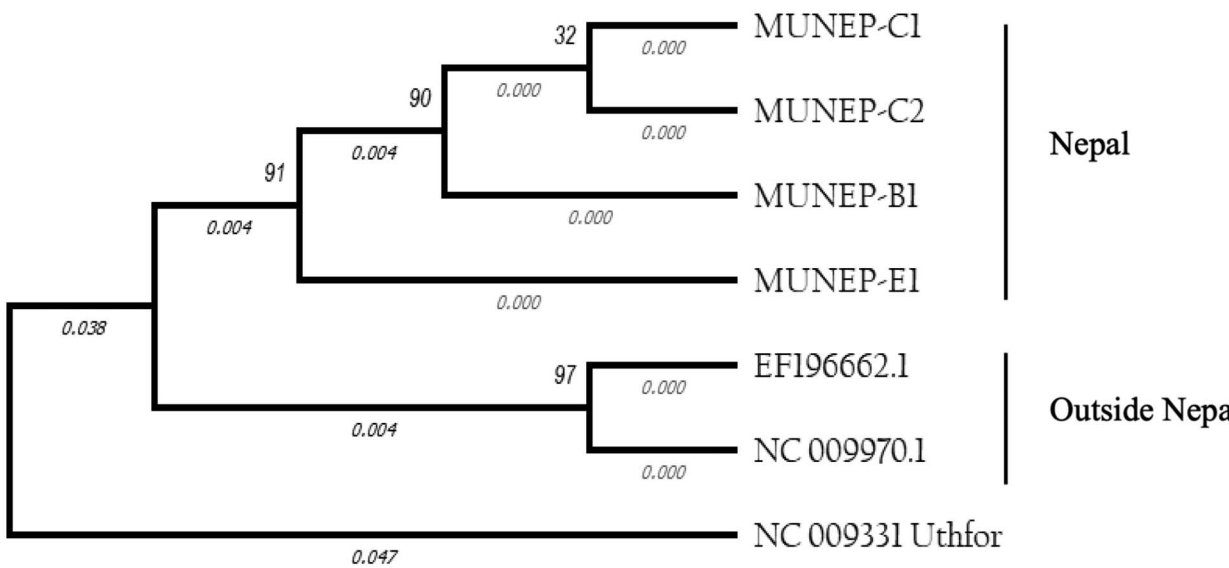

Fig. 4. Phylogenetic relationship between sloth bears (*Melursus ursinus*) from noninvasive genetic surveys conducted between 2019 and 2021 in Nepal. EF196662.1 and NC009970.1 are reference sloth bear samples obtained from the gene bank, and NC009331 is a Himalayan black bear (*Ursus thibetanus laniger*) sample used as an outgroup for analysis.

Table 4. Comparison of genetic diversity parameters[a] of sloth bears (*Melursus ursinus*) in our study with similar studies in Nepal and India. NP is National Park.

(a) Comparison of genetic diversity parameters across common microsatellite loci

	This study (Nepal)			Dutta et al. 2015 (Central India)		
Locus	**N_A**	**H_O**	**H_E**	**N_A**	**H_O**	**H_E**
MU26	1	0.00	0.00	7	0.38	0.69
G10L	3	0.35	0.41	7	0.89	0.68
G1A	5	0.51	0.52	11	0.38	0.72
G10B	2	0.14	0.13	4	0.27	0.47
G10J	7	0.68	0.75	12	0.58	0.88
CXX203	4	0.46	0.53	12	0.65	0.86
UMAR2	3	0.16	0.20	9	0.58	0.71
Mean	3.57	0.33	0.36	8.86	0.53	0.72

(b) Comparison of genetic diversity parameters across similar landscapes and species

Study	**Location (area)**	**No. of individual**	**N_A**	**H_O**	**H_E**
This study (sloth bears, Nepal)	Chitwan NP (953 km^2)	32	3.50	0.45	0.47
	Bardiya NP (986 km^2)	2	1.58	0.29	0.24
	Trijuga forest (430 km^2)	3	2.16	0.44	0.35
	Average (across 12 loci)	37	3.58	0.44	0.48
Dutta et al. 2015 (sloth bears, India)	Kanha (940 km^2)	9	5.71	0.62	0.75
	Pench (293 km^2)	8	5.29	0.52	0.61
	Satpura (646 km^2)	16	6.29	0.55	0.64
	Melghat (1,677 km^2)	22	6.29	0.49	0.65
	Average (across 7 loci)	55	8.86	0.53	0.72
Thatte et al. (2020) (sloth bear and tiger, India)	Central India (>5,000 km^2) sloth bears (across 11 loci)	104		0.39	0.51
	Central India (>5,000 km^2) tigers (across 12 loci)	117		0.52	0.72
Thapa et al. (2018) (tigers, Nepal)	Chitwan NP (953 km^2)	37	4	0.58	0.57
	Bardiya NP (986 km^2)	25	4	0.57	0.55
	Shuklaphanta NP (305 km^2)	16	3	0.46	0.52
	Average in Nepal (across 8 loci)	78	3.51	0.54	0.61
Kadariya et al. (2018) (black bears, Nepal)	Annapurna Conservation Area, Nepal (~525 km^2 surveyed across 8 loci)	60	7.63	0.79	0.76

[a]N_A, observed number of alleles; N_E, effective number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

We expected the structure profiles of TJF and BNP to show more variation from the CNP bears because of the fairly large geographic distance and the presence of human settlements, rivers, and escarpments between the studied habitats. When we included location information during the structural analysis, individuals' fractional membership coefficient (q), particularly TJF and BNP, increased slightly but was <0.7 to be assigned to a different cluster. Sloth bears have been reported to move frequently between the undulated outer Himalayas also known as "Siwalik" or "Churia" and Terai. This landscape was intact and provided uninterrupted movement of wildlife until the past few decades (<100 yr) when much of the land use change occurred. Currently, Trijuga forest (TJF) in the east, Bardiya National Park (BNP) in the west, and Chitwan National Park (CNP) in central Nepal are considered the major habitats with sloth bear presence, and only a few sporadic reports outside of these habitats have been reported (Sharma et al. 2023, Sadadev et al. 2024). On an evolutionary timescale, current fragmentation may not be long enough for genetic drift to occur and create a detectable genetic population signature.

Different populations may contribute variably to the ancestry of sampled individuals and uneven sampling can influence the number of populations and individual ancestry. A minimal sample from the eastern and western habitats, compared with the central habitat in our study, could have introduced some level of uncertainty in the population structuring. Further, subsampling,

new analysis, and parameter adjustments are proposed to improve the robustness of the structure algorithm in predicting the correct number of population clusters. The use of ΔK (Evanno et al. 2005), a quantity based on the second-order rate of change with respect to K of the likelihood function, and using an alternative ancestry (Wang 2017) can help in identifying the correct number of clusters and assigning the individuals to the appropriate population. In addition, the use of spatially explicit Bayesian analyses (e.g., TESS-package in Program R Software), multivariate methods (e.g., discriminant analysis of principal components) can be used to increase the robustness of population differentiation results (e.g., Dutta et al. 2015). Few unique alleles were present only in individuals from TJF or BNP but not CNP, suggesting that future studies with increased sample size from sloth bears' habitats in the eastern and western parts of Nepal could produce more reliable estimates of population differentiation.

Haplotypes and phylogenetic relationship

Phylogenetic analysis based on the control region (CR) of mitochondrial DNA showed that sloth bear individuals in our study area formed a distinct clade. Mitochondrial DNA (mtDNA) is maternally inherited, so these patterns reflect historical female-mediated gene flow rather than contemporary population structure. We identified 4 haplotypes based on base substitutions and a variable number of T and C repeats. Samples from the eastern region, located approximately 200 km from CNP's central sloth bear population, clustered together, forming a unique (MUNEP-E1) haplotype. Similarly, the BNP population, located approximately 300 km from the central sloth bear population, belonged to a different haplotype (MUNEP-B1) based on the T and C repeat regions variation. The central population also showed variation in the T and C-repat regions, forming 2 different haplotypes (MUNEP-C1 and MUNEP-C2). Phylogenetic analysis using the maximum likelihood method for sequences of the mtDNA control region indicated very low genetic divergence among these lineages. It suggests that sloth bear populations of BNP, CNP, and TJF shared common ancestry and have undergone only slight genetic differentiation since divergence. However, because mtDNA reflects only female-lineage history, these patterns may not fully represent overall gene flow. Thus, while these mtDNA results provide insights into historical female dispersal, additional nuclear markers would help clarify contemporary population connectivity.

Conservation implications

Our study provides the first evidence of conservation genetics to understand the status of sloth bears in Nepal. Camera-trap methods have been used for identifying individuals and estimating the population of big cats such as tigers; however, a similar approach for studying bears is challenging because of the lack of easily identifiable patterns. We show that noninvasive sampling can be implemented as a viable method for monitoring the sloth bear population in Nepal. At least 37 sloth bear individuals characterized by low heterozygosity were genetically identified using noninvasive samples collected from approximately 1,000 km^2. Fecal sample collection and the establishment of tree-rub traps for the noninvasive collection of DNA can provide useful information for park managers that can be used as a standard monitoring method for sloth bears.

Considering that a large population with adequate genetic variation is better suited to adapt to changing environmental conditions and resist diseases, low heterozygosity within sloth bear populations can be of concern. Although some bear populations have survived for years despite their low genetic diversity (e.g., Kodiak bears [*Ursus arctos middendorffi*]), it may accelerate extinction when the population is already small and faces threats such as habitat loss and human–bear conflicts. Low genetic diversity can be a concern for long-term conservation of bears in Nepal; however, other factors, including population decline due to poaching, conflict with humans, disease outbreak, habitat degradation, and climate change, may pose bigger and immediate threats, particularly for small and fragmented populations.

Minimizing the immediate conservation threats and increasing habitat connectivity is crucial to facilitating gene flow, maintaining genetic variation, and sustaining the sloth bear population (Dutta et al. 2015, Thatte et al. 2020). Studies on tigers suggest that 'Churia' landscape may facilitate dispersal and thus contribute to maintaining metapopulation dynamics in Nepal (Thapa et al. 2017, 2018; Subedi et al. 2021b). The functional connectivity provided by a corridor can be species-specific and influenced by its dispersal ability and density (Thatte et al. 2020). Rigorous assessments of sloth bear habitats, particularly elucidating the potential of Churia landscape as a corridor and a habitat for sloth bears, may provide further insights into functional connectivity and gene flow in sloth bears of Nepal. The long-term viability of sloth bears in Nepal

depends on ensuring gene flow, safeguarding the existing population, and provisioning habitats with adequate resources (termites, ants, fruiting plants) and safety (forest cover, minimal risk from humans, road, traffic, and other infrastructures). Translocating sloth bear individuals as a management intervention from genetically diverse and high occupancy areas can reinforce small populations with low diversity; however, comprehensive protocols incorporating conservation genetics must be prepared before undertaking such interventions (Pathak et al. 2022). Noninvasive sampling using fecal and hair samples can be a useful tool to support such conservation planning.

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Competing interest

The authors do not have any conflicts of interest to declare.

Authors' contribution

Rajan Prasad Paudel: Conceptualization (equal); data curation (lead); formal analysis (equal); funding acquisition (equal); writing—original draft (lead); writing—review and editing (equal). **Michito Shimozuru:** Conceptualization (equal); formal analysis (equal); writing—review and editing (equal); supervision (equal). **Rabin Kadariya:** Funding acquisition (supporting); writing—review and editing (supporting). **Naresh Subedi:** Conceptualization (equal); funding acquisition (supporting); supervision (supporting). **Toshio Tsubota:** Funding acquisition (equal); supervision (lead).

Data availability

All relevant data are within the paper and its supporting tables and figures. Additional data or information from the current study are available from the corresponding author upon reasonable request.

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Supplemental material

Text S1. PCR conditions of microsatellite and mitochondrial analysis.

Table S1. Primers used for microsatellite genetic analysis.

Table S2. Primers used for mitochondrial genetic analysis.

Table S3. Summary and raw STRUCTURE Harvester outputs for population differentiation analysis.

Fig. S1. Structure results of 37 individual sloth bears (*Melursus ursinus*) from 3 locations in Nepal. The mean of estimated Ln probability of data is higher when population subcluster $K = 1$. Y-axis values are fixed from −790 to −690 for a clear presentation of the graph.

Fig. S2. Population structure plots for sloth bears (*Melursus ursinus*) in Nepal without prior location information. The vertical bar represents individual bears, and the color green represents the membership coefficient (q) of each individual, which was <0.70 when $K = 2$. Numbers 1–37 represent the individual identified from microsatellite analysis.