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Evidence of tiger population structure and dispersal in the montane conservation landscape of Bhutan

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ABSTRACT

Bhutan is a part of the second-largest Tiger Conservation Landscape (TCL), The Northern Forest Complex-Namdapha-Royal Manas, and is home to a tiger population of global conservation priority. With its well-connected protected area network and a vast expanse of forested landscape, Bhutan has a unique potential to spatially connect tiger populations in the Terai TCL of India and Nepal on the western side to the north-eastern part of the Indian subcontinent. However, information on genetic structure and connectivity among tiger populations is lacking in the Eastern Himalayan region. Due to the large and contiguous forested landscape with a network of wellconnected protected areas and strong environmental protection measures in Bhutan, tiger populations in Bhutan are expected to have high genetic variation and gene flow. We made the firstever attempt at the genetic sampling of the tiger population in Bhutan, where we genotyped 24 tiger individuals using thirteen microsatellite loci. Genetic analyses revealed three genetic clusters and found high expected heterozygosity (mean $H_E = 0.73$) and moderate-to-high genetic differentiation ($F_{ST} = 0.135$) within this pool of sampled tigers. We also were able to assign poached tigers to our inferred genetic clusters. Two individual wild tigers showed distinct multilocus genotypes, similar to each other but highly divergent from all other sampled tigers, suggesting their origin from outside the sampled areas. Our study provides the first insights into the population genetic structure of tigers in this important region and suggests that gene flow is less than suggested by dispersal. A larger landscape-level genetic study with implications for the transboundary conservation of tigers in this highly biodiverse landscape is warranted.

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1. Introduction

Landscape connectivity is crucial for biodiversity conservation, particularly for mammalian carnivores known to occur in low densities and disperse over long distances. Carnivore species such as the mountain lion *Puma concolor*, leopard *Panthera pardus*, wolf *Canis lupus*, wolverine *Gulo gulo*, and tiger *Panthera tigris* are known for long-distance dispersals that are also vital for maintaining gene flow and metapopulation connectivity (Andersen et al., 2015; Fattebert et al., 2013; Hawley et al., 2016; Packila et al., 2017; Sarkar et al., 2021; Schwartz et al., 2002). Anthropogenic activities have increased habitat fragmentation, thereby causing population isolation, decreased effective population sizes, loss of genetic variation due to genetic drift, and increased vulnerability to local extinction (Haddad et al., 2015; Mills, 2013; Kardos et al., 2021).

The tiger *Panthera tigris* is a charismatic flagship species for biodiversity conservation globally. Tigers once occurred widely in Asia but now occupy less than 7% of their historical range, which is currently fragmented into 76 metapopulations known as Tiger Conservation Landscapes or TCLs (Sanderson et al., 2010). The global tiger population declined from an estimated 100,000 individuals in the early 1900 s to less than 4000 as of 2015 (Goodrich et al., 2015). These remaining tigers are mostly confined to small populations scattered across their entire range, often surrounded by human-dominated landscapes and at risk of extinction (Dinerstein et al., 2007). On the Indian subcontinent, a drastic population decline from about 40,000 tigers to less than 3000 led to a loss of genetic variation in the Bengal tiger sub-species *Panthera tigris tigris* (Mondol et al., 2013). Fragmented extant populations in the Indian sub-continent account for more than 60% of the overall current genetic variation in tigers across the entire range (Mondol et al., 2013, 2009b).

Bhutan, a small land-locked biodiverse country in the Indian subcontinent, is an important landscape for tigers in Asia (Tempa et al., 2019). It is part of the Northern Forest Complex-Namdapha-Royal Manas, one of the biggest TCLs, with one of the most extensive, intact, and contiguous forest covers within the Indian sub-continent. Tempa et al. (2019) argue that Bhutan could qualify as a tiger "source site" given the presence of an estimated 90 tigers, with > 50 adult females, spatially distributed in various habitat types across an elevation gradient from 100 MSL to 4400 MSL. Seventy percent of Bhutan's geographical area is covered by forest, which has a well-connected protected area network spanning over 50% of the country. Strong environmental legislation and site-specific management actions provide the species with the highest level of legal protection. Tigers are also deep-rooted in Bhutanese folklore, culture, and tradition. Bhutan's main religion, Buddhism, reveres tigers with mysticism and as a symbol of invincibility (DoFPS, 2015). Although the tiger density estimate for Bhutan (0.23 tigers/100 km²) is low compared to other tiger populations in India and Nepal, a few protected areas such as Jigme Dorji National Park (JDNP), Royal Manas National Park (RMNP), and Jigme Singye Wangchuck National Park (JSWNP) have a comparatively higher density of 2–3 tigers/100 km² (DoFPS, 2015). Non-uniform tiger density in Bhutan can be attributed to various environmental and anthropogenic correlates that drive their distribution and abundance (Penjor et al., 2019).

The Bhutan government's Tiger Action Plan recognizes the importance of genetic variation and population connectivity for the long-term conservation of tigers (NCD, 2018). Tiger monitoring in Bhutan started in the late 1980 s and to date has focused on spatial distribution, habitat use, and abundance of tigers, with little assessment of genetic variation, genetic structure, or gene flow (Penjor et al., 2019; Tempa et al., 2019). A low human population density of 20 people per km² and contiguous forest habitat with relatively low anthropogenic disturbance compared to neighboring regions of the Indian subcontinent would be expected to foster high levels of gene flow. Tigers in Bhutan are found both within and outside protected areas. Tiger movement among these protected areas and occupancy in corridors indicates structural and genetic connectivity (Letro. et al., 2022); however, this has not been empirically examined. Maintaining functional genetic connectivity will be paramount for preserving the genetic variation of the tiger population and its long-term viability in Bhutan.

Spatial genetic structure may be created via isolation by distance (IBD) and topographical barriers (Allendorf et al., 2022). The effect of gene flow on patterns of IBD depends upon the mobility of the species, space use, and spatial scale of dispersal. Highly mobile species like the tiger, which moves over large distances and utilizes a broader range of habitat types, can interact with other populations and hence might show limited IBD over large spatial scales (Schwartz et al., 2002). However, population decimation due to poaching in areas with low population densities might also create gaps within a potentially contiguous tiger population, which can affect the spatial genetic structure. Tigers in Bhutan are vulnerable to an increasing threat of poaching for the illegal trade of skins and other body parts. Within three years (2015–17), 17 poaching and trade cases of tigers were reported and prosecuted by the authorities (NCD, 2018). Although the origin of half of these 17 seized skins remains unknown, this number accounts for nearly 17% of Bhutan's tiger population. Undetected poaching cases, if any, might further affect the demographic and genetic viability of Bhutan's tiger population. Apart from this, the deep riverine gorges and high-mountain ridges in Bhutan might act as a barrier to movement and gene flow, creating genetic differentiation.

In our study, we use a suite of 13 polymorphic microsatellite loci to study the genetic variation and spatial genetic structure of tigers in Bhutan. While the number of individual tigers examined was relatively low (n = 24), this sample represents a relatively large proportion of the estimated extant tiger population in Bhutan (26%). We estimated genetic variation for the total sampled population and examined how variation is partitioned within and among populations. Our findings have implications for tiger conservation and management in Bhutan and globally.

2. Materials and methods

2.1. Genetic sampling and laboratory analysis

Our samples consisted of scats and tissues. For the scats, we carried out a systematic collection in Royal Manas National Park

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(RMNP) following a single-occasion sampling between February 21 and March 17, 2018 (Fig. 1). RMNP is located in the southern part of Bhutan and is a stronghold of tigers in the country. We distributed search routes spatially along a representative set of trails in a few areas in the southern part of the park, and each search route was surveyed by a team of at least three individuals. We chose trails regularly used by tigers as inferred from previous camera trapping data and other occurrence records. A part of feces (diameter approximately the size of a thumbnail) from each scat was collected and preserved in labeled plastic tubes with silica desiccants as a preservative. We recorded the geographic coordinate readings for each scat.

To expand our inferences more broadly across Bhutan, we also conducted opportunistic fecal sampling in several other areas in Bhutan (Fig. 1). These included samples from Jigme Singye Wangchuck National Park (JSWNP) (n = 3) in central Bhutan, Jigme Dorji National Park (JDNP) (n = 7) in northwestern Bhutan, Biological Corridor no. 8 (which connects JSWNP and JDNP) (n = 8), and three territorial forest divisions, namely, Paro Forest Division (n = 2) and Thimphu Forest Division (n = 4) to the West and Bumthang Forest Division (n = 1) in the central part of the country (Fig. 1).

We also included 21 other samples (tissue and skin) in our pool of samples. These 21 samples include samples of two wild tigers that presumably died of natural causes, two tigers that died in snares set by poachers, and 17 samples from confiscated tiger body parts presumed to have originated from tiger populations in Bhutan. We could confirm the source of origin for nine of these 21 samples. We stored 2–3 g of these samples in an air-tight labeled plastic tube filled with silica desiccant. All samples (feces and tiger body parts) were then stored in a -20 °C freezer until laboratory analysis.

We extracted genomic DNA from scats using QIAamp Fast DNA Stool Mini Kit (QIAGEN Inc. Germany), following the manufacturer's instructions with the following modifications: A) incubation of 3–4 g of sub-sampled scat in 1 ml of InhibitEX buffer on a rotating shaker overnight at room temperature; B) addition of carrier RNA (Qiagen Inc. Germany) after the samples were digested with Proteinase K. Carrier RNA was added to increase DNA yield from fecal samples (Kishore et al., 2006; Mondol et al., 2009a); C) elution in 80 μ L AE buffer. DNA from tissue samples was extracted using QIAamp DNA Blood and Tissue Kit (QIAGEN Inc. Germany) following the manufacturer's instructions. Genomic DNA extraction and downstream Polymerase Chain Reaction (PCR) procedures were carried out in separate rooms to avoid cross-contamination. Negative controls were used in every batch of DNA extractions and PCR reactions. All PCR reactions were performed in a 10 μ L volume containing 5 μ L of QIAGEN Multiplex PCR master mix (QIAGEN Inc. Inc. Germany), 1 μ L of each 2 μ M forward and reverse primers, 0.8 μ L of 2 mg/ml BSA (Bovine Serum Albumin), 1.2 μ L of PCR-certified water and 2 μ L of genomic DNA template.



Fig. 1. Sampling collection locations on forest cover map of Bhutan. Red circles are scat samples confirmed to be of a tiger, while white circles are samples that were not tiger positive. Blue triangles are tiger-positive tissue and skin samples.

First, we attempted to confirm that these samples were from tigers using species identification ascertained by using two tigerspecific mitochondrial primers. The first one amplifies at 164 bp in the NADH5 region, and the second one amplifies at 210 bp in the Cytochrome-b (CYT-B) region (Mukherjee et al., 2007). We electrophoresed the amplified PCR products on a 2% agarose gel, and PCR products were visualized using SYBR Safe nucleic acid gel stain (Thermo Fisher Scientific).

Subsequently, we used a panel of 13 microsatellite loci (*FCA441*, *Pati09*, *FCA391*, *FCA628*, *E7*, *FCA672*, *FCA304*, *F53*, *FCA232*, *FCA90*, *FCA42*, *FCA279*, *FCA126*) for individual identification and further genetic analyses. These microsatellite loci were developed initially for the domestic cat (*Felis catus*) (Menotti-Raymond et al., 1999) and Amur tiger (*Panthera tigris altaica*) (Wu et al., 2009). They were chosen based on their high polymorphism, ease of amplification, and low error rate reported in previous tiger population genetic studies in various parts of the Indian subcontinent (Aziz et al., 2017; Borthakur et al., 2013, 2011; Mondol et al., 2009a; Reddy et al., 2012a; Sharma et al., 2013a; Singh et al., 2015). We used a marker developed on the Amelogenin (AMEL) gene to determine sex. The AMEL sex identification marker amplifies two alleles (194 and 214 bp) for females and one allele (194 bp) for males (Pilgrim et al., 2005). We used tiger tissue samples of known origin as reference samples for all the analyses for species identification, microsatellite amplification, and sex identification. The microsatellite loci were grouped into three multiplexes with eight PCRs based on annealing temperature, allele range, and amplification success during the optimization process. Thermocycling conditions for PCR are provided in Supplementary Tables 1 and 2.

We used a multitube approach to address inherent issues of failed amplification and genotyping error associated with non-invasive genetic samples such as scats (Miquel et al., 2006). In this approach, we performed four PCRs for each scat sample and two PCRs for each tissue sample to ensure reliable genotyping and minimize genotyping errors. After ascertaining species identification of the sample, the tiger-positive scat samples were run with the multiplex set no. I and II, and samples that amplified at fewer than three loci were not amplified further. We genotyped the scat samples that passed this first screening of amplification success with the remaining two sets of multiplexed loci, ensuring that only good quality samples were further genotyped, saving time and laboratory analysis costs. The PCR products were diluted to 1:50 concentration, and 1 µL of the diluted PCR solution was analyzed on a 3130 ABI genetic analyzer (Applied Biosystems; USA) for size fractionation. Raw data were scored with Geneious software (Geneious R11.1, https://www.geneious.com).

2.2. Genetic analyses

We used MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004) to test for microsatellite stuttering errors, null alleles, and excess homozygosity. Genotyping errors (Allelic dropout and false alleles) were quantified using GIMLET v 1.3.3 (Valiere, 2002).

After the multitube approach, we set stringent quality control to create consensus genotypes. The two replicates of tissue samples had to match at all loci to generate a consensus multilocus genotype, and at least three of the four replicates had to match for scat samples. We calculated the value of the probability of identity P_{ID} in GenAlEx v 6.5 (Peakall and Smouse, 2012). We had high discriminatory power to separate individuals even at six loci ($P_{ID} = 2.2 \times 10^{-6}$, P_{ID} (*sibs*) = 5.9×10^{-3}). So, we used a minimum of six out of thirteen locus matching criteria using the identity analysis module in CERVUS v 3.0 (Kalinowski et al., 2007) to match unique individuals from multilocus genotype data.

We used GENEPOP (Raymond and Rousset, 1995) to check for deviations from Hardy-Weinberg proportions and test for linkage disequilibrium. We used an Exact Test with 10,000 dememorization steps, 1000 batches, and 10,000 iterations per batch. Sequential Bonferroni corrections were applied with alpha= 0.05. Genetic variation (allelic richness, expected heterozygosity (H_E), and observed heterozygosity (H_O) were calculated using GenAlEX v 6.5 (Peakall and Smouse, 2012).

Prior to our study, it was unknown whether population subdivision was present within Bhutanese tigers. After testing for deviations from Hardy-Weinberg proportions, we found evidence for a heterozygote deficit, consistent with a Wahlund effect that appeared to be due to two individuals with unusual genotypes (see Results). Departure from Hardy-Weinberg proportions could lead bayesian clustering approaches (such as that implemented in program STRUCTURE Pritchard et al., 2000) to overestimate the number of clusters in population genetic structure analyses (Falush et al., 2003; Kaeuffer et al., 2007). Bayesian methods also struggle to differentiate populations when the sample sizes are limited and small number of markers are used, as in our case (Corander and Marttinen, 2006). Therefore, we first used Discriminant Analysis of Principal Components (DAPC) followed by STRUCTURE to compare assignments. DAPC is an individual-based analysis that does not require a population genetics model and is better at handling hierarchical structure or clinal variation caused by isolation by distance (IBD; (Jombart et al., 2010)). The method used output from principal component analysis of the genotype data as input for Discriminant Analysis (DA).

We conducted DAPC using the R package *adegenet* (version 2.0–0) (Jombart and Ahmed, 2011). We selected the optimal number of clusters using the Bayesian Information Criterion (BIC). The lowest BIC value was achieved for K = 4, but the difference between the lowest BIC and K = 3 was less than 1. Based on the principle of parsimony, we retained K = 3 as the optimal number of clusters to describe the genetic structure (Viengkone et al., 2016). The number of optimum principal components retained was determined using a cross-validation approach implemented by the function *xvalDapc* with 100 repetitions. We used the number of principal components associated with the lowest Root Mean Squared Error (RMSE) as the 'optimum' number of PC axes in the DAPC analysis and retained six principal components for subsequent DA analysis. We also used STRUCTURE v.2.3.4 (Pritchard et al., 2000) to identify genetic structure in sampled tigers. We tested K values from 1 to 6, using 200,000 steps for Markov Chain Monte Carlo (MCMC) runs and 50, 000 burn-ins and achieved convergence. We conducted ten independent runs for each value of *K*, used an admixture model, and assumed correlated allele frequencies. We used the ad hoc statistic ΔK , based on the rate of change in the likelihood of the data between successive *K* values (Evanno et al., 2005), to estimate the most appropriate number of clusters (*K*) in our dataset. We did not run models that included any prior information about the location of samples. Individuals were assigned to a specific population cluster if

the posterior membership probability to the assigned cluster was q > 0.7. If q < 0.7, then the individuals were considered to have mixed ancestry.

Following DAPC and STRUCTURE analyses, we used the most supported population assignments to determine subpopulation membership. We then calculated global and pairwise F_{ST} values (a measure of genetic differentiation) according to the Weir and Cockerham method (1996) using the *Hierfstat* package (Goudet, 2005) in the statistical program R version 3.2.4 (R Development Core Team, 2014).

3. Results

3.1. Individual identification and error rates

We collected 72 putative tiger scat samples and 21 tissue samples from Bhutan in the spring of 2018. Out of the 72 scat samples, 47 were collected from RMNP, and the remaining were collected opportunistically from other parts of the country. Forty-three scats tested as tiger positive, of which 20 amplified at more than five loci and were further analyzed. We identified eight unique individuals (five males and three females), all from RMNP, and full genotypes (all 13 loci) were obtained for 6 individuals, and the remaining two individuals were genotyped at 11 and 10 loci, respectively.

From the 21 tissue samples, 17 amplified at more than five loci and thus passed our initial screening step for DNA quality. Two tissue samples were found to be from the same individual, so we retained only one of these for further analyses. We obtained 16 unique individuals from the tissues (seven males and nine females), of which four were from the northwestern part of the country, one from RMNP in the south, and 11 individuals did not have information on their source of origin. We obtained full genotypes (all 13 loci) of all 16 individuals. Our total sample of 24 unique individuals represents approximately 26% of the estimated tiger population of 90 tigers in Bhutan (Tempa et al., 2019).

The mean allele dropout (ADO) for the tissue samples was 0.003%, with no false alleles (FA). The estimated mean ADO and FA rates in the scat samples were 0.059% and 0.01%, respectively (Table 1).

3.2. Genetic variation

All 13 microsatellite loci were polymorphic, and the number of alleles ranged from 4 to 10 across all samples. The mean number of alleles per locus was 6.7 ± 0.5 (Table 2). The mean H_O and H_E were 0.60 ± 0.04 and 0.73 ± 0.02 , respectively. There was a consistent signal of a deficit of heterozygotes (positive F_{IS}) across loci, and three loci (*FCA441*, *FCA304*, and *FCA391*) deviated significantly from Hardy-Weinberg proportions (significant heterozygote deficit) based on Fisher exact tests and following sequential Bonferroni correction (Table 2). The departure from the Hardy-Weinberg proportions appeared to be due to two individuals that had distinct genotypes due to unique alleles at 10 of the 13 loci. When we removed these two individuals, none of the loci deviated significantly from Hardy-Weinberg proportions after sequential Bonferroni corrections. Significant linkage disequilibrium after sequential Bonferroni corrections ($P < 6.4 \times 10-4$) was detected for a pair of loci (*FCA628* and *F42*) with or without the two putative migrants included.

3.3. Population Genetic Structure

Multivariate analysis using DAPC predicted three as the optimal number of genetic clusters (K = 3) within the sampled population according to the Bayesian Information Criterion (Fig. 2, Supplementary Fig. 1). Cluster 3 was divergent along the first discriminant axis and Clusters 1 and 2 were mostly divergent along the second discriminant axis (Fig. 2). All individuals collected from the northern part

Table 1

Details of thirteen microsatellite loci used in this study. For each locus, the repeat motif, size in base pairs (bp), and estimated allelic dropout and false allele rates (in percent) for tissue and scat samples are shown.

Locus	Repeat motifs	Size range (bp)	Tissues		Scats	
			Drop out	False allele	Drop out	False allele
FCA628	Di-nucleotide	84–114	0.000	0.000	0.000	0.000
FCA441	Tetra-nucleotide	153–183	0.000	0.000	0.250	0.019
Pati09	Tri-nucleotide	107-125	0.000	0.000	0.028	0.000
FCA391	Tetra-nucleotide	189–217	0.000	0.000	0.150	0.028
FCA672	Di-nucleotide	82–114	0.000	0.000	0.000	0.000
FCA304	Di-nucleotide	109–198	0.000	0.000	0.031	0.083
E7	Di-nucleotide	133–159	0.000	0.000	0.083	0.000
F53	Tetra-nucleotide	128–181	0.000	0.000	0.000	0.000
FCA279	Di-nucleotide	89–113	0.000	0.000	0.000	0.000
FCA232	Di-nucleotide	86-118	0.000	0.000	0.000	0.000
F42	Tetra-nucleotide	207-255	0.042	0.000	0.167	0.000
FCA090	Di-nucleotide	91–125	0.000	0.000	0.000	0.000
FCA126	Di-nucleotide	132–154	0.000	0.000	0.063	0.000
Mean			0.003	0.000	0.059	0.010
SD			0.012	0.000	0.081	0.024

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Table 2

Measures of genetic variation at 13 microsatellite loci for 24 sampled tigers from Bhutan. The following genetic variation metrics are included: A, number of alleles per locus; H_0 , observed heterozygosity; H_E , expected heterozygosity; F_{15} , inbreeding coefficient. The 24 samples represent the 'All-Bhutan' sample reported in the text. Asterisks indicate statistical significance (alpha = 0.05) based on exact tests and Sequential Bonferroni correction.

Locus	Α	H_O	H_E	F_{IS}
FCA628	8.00	0.71	0.80	0.11
FCA441	4.00	0.33	0.64	0.48 *
Pati09	5.00	0.54	0.58	0.06
FCA391	5.00	0.38	0.65	0.42 *
FCA672	10.00	0.83	0.86	0.03
FCA304	6.00	0.54	0.72	0.24 *
E7	6.00	0.58	0.78	0.25
F53	8.00	0.83	0.81	-0.02
FCA279	6.00	0.63	0.79	0.21
FCA232	7.00	0.50	0.59	0.15
F42	7.00	0.65	0.73	0.11
FCA090	6.00	0.50	0.71	0.30
FCA126	9.00	0.70	0.78	0.10
Mean	6.69	0.59	0.73	0.19
SE	0.47	0.04	0.02	0.04



Fig. 2. Scatterplots from discriminant analysis of principal components (DAPC) reveal three genetic clusters. Each ellipse represents a cluster, each dot represents an individual, and eclipses represent 95% confidence. The bar plot shows the eigenvalues of the first two principal components in relative magnitude. Numbers represent the different subpopulations identified by DAPC analysis. '1' corresponds to the North, '2' the South, and '3' the two individuals sampled in the South but that possess many unique alleles.

of Bhutan assigned to Cluster 1 (Fig. 3). Of the nine individuals collected in the south, three assigned to Cluster 1, four assigned to Cluster 2, and two assigned to Cluster 3. The two individuals assigned to Cluster 3 were those with many unique alleles and highly unusual multilocus genotypes. Seven individuals of unknown origin were assigned to Cluster 2 and four to Cluster 1 (Fig. 3). Our interpretation of these patterns is that Cluster 1 corresponds to a putative northern group since all individuals sampled in the north assign to this cluster. Cluster 2 appears to be a putative southern group, with more individuals assigned to Cluster 2 than either Clusters



Fig. 3. Output from discriminant analysis of principal components (DAPC) at K = 3. Each line in the bar plot represents an individual tiger. The y-axis shows posterior membership probabilities. Facets show whether individuals were sampled from the North (N), South (S), or unknown (U; i.e., seized animals of unknown provenance). Colors correspond with the three genetic clusters, corresponding with each value of K.



Fig. 4. Output from STRUCTURE at K = 3. Each line in the bar plot represents an individual tiger. The y-axis shows Q-value estimates. Facets show whether individuals were sampled from the North (N), South (S), or unknown (U; i.e., seized animals of unknown provenance). Colors correspond with the three genetic clusters, corresponding with each value of K.

1 or 3. There was a clear signal of gene flow from both Clusters 1 and 3 into Cluster 2 (Fig. 3).

We performed Bayesian cluster analysis in STRUCTURE (without LOCPRIOR) as an additional test of the DAPC results. STRUC-TURE also provided evidence for three genetic clusters (K = 3) based on interpreting mean likelihood (ln P(K)) across values of K and the ΔK value using the Evanno methodology (Fig. 4, Supplemental Fig. 2). Twenty-two tigers had a q value over 0.7 and were assigned to one of the three genetic clusters, leaving two individuals with mixed ancestry based on this assignment criterion (Supplementary Table 3, Fig. 4). Except for the two admixed individuals, the assignment was concordant with the DAPC analysis (Fig. 3 & 4).

The global F_{ST} value was estimated to be 0.189. Pairwise F_{ST} between Clusters 1 and 2 (n = 11 each) was 0.135 (P > 0.05). The cluster formed by the two putative migrants (Cluster 3) was excluded from this pairwise F_{ST} comparison due to the small sample size. Estimates of expected heterozygosity within Cluster 1 and Cluster 2 were 0.651 \pm 0.036 and 0.582 \pm 0.043, respectively. We did not recalculate estimates of genetic variation for Cluster 3 due to the small sample size.

4. Discussion

Our study is the first to assess the population genetic structure of tigers in Bhutan. We genotyped 24 individuals using a panel of thirteen polymorphic microsatellite loci; these 24 tigers represented about 26% of Bhutan's estimated tiger population of 90 individuals (Tempa et al., 2019). In response to the 2010 St. Petersburg declaration for the global recovery of tiger populations and subsequent calls for specific conservation measures in the 13 tiger range countries, tiger conservation efforts in Bhutan have been substantially focused on estimating tiger population and trends and less on fostering connectivity to maintain genetic variation. Our study finds high genetic variation and substantial population subdivisions within the tiger population in Bhutan, providing valuable information to identify conservation priorities for the species in this region.

Tigers in Bhutan have high levels of genetic variation (mean $H_E = 0.73$), likely due to moderate population size and high dispersal gene flow resulting from population connectivity due to continuous forest habitat. Differences in the number and combination of microsatellite loci used in different studies mean that direct comparisons must be made with care. With that caveat, our estimates of expected heterozygosity ($H_E = 0.73$) were generally similar to microsatellite-based estimates for Bengal tigers overall ($H_E = 0.72$) (Luo et al., 2004), on the Indian sub-continent ($H_E = 0.70$) (Mondol et al., 2009b), in the Western Ghats ($H_E = 0.76$) (Reddy et al., 2012b), and in Central India ($H_E = 0.81$) (Sharma et al., 2013a). Several genetic and connectivity modelling studies report that tiger populations in central India are connected via forest corridors (Dutta et al., 2018, 2015; Schoen et al., 2022), facilitating tiger movement and dispersal among them, thereby maintaining genetic variation through gene flow (Joshi et al., 2013; Sharma et al., 2013a).

We used two different approaches - individual-based Bayesian and multivariate analyses - to infer genetic differentiation and population substructuring of tigers in Bhutan. Results from both STRUCTURE and DAPC suggest the presence of three genetic clusters. The assignment of individuals to the three clusters was consistent across both methods for 22 of the 24 individuals. The remaining two were inferred to have mixed ancestry with STRUCTURE. Our interpretation of the northern (Cluster 1) and southern (Cluster 2) groups is the most parsimonious explanation of our results, with the strongest evidence coming from all four individuals sampled in the north assigned to one cluster. However, sample sizes are small, and the assignment of geographic regions to the genetic clusters remains preliminary. The fixation index (F_{ST}) results indicate a moderate-to-high level of genetic differentiation between two of the three clusters identified by DAPC ($F_{ST} = 0.135$) for a wide-ranging carnivore. It is possible that the limited sample size (N = 24) has influenced these results. Small sample size, in general, tends to overestimate genetic differentiation with the approaches we used (Balkenhol and Fortin, 2016). A complete characterization of the genetic subdivision and sub-population boundaries will require a more complete representation and larger sample sizes. However, we suggest that the strength of the genetic differentiation signal we observed makes it likely to be upheld by future work.

Regardless of uncertainty in the regional assignment of the observed genetic clusters, individual assignments prove that dispersal occurs between the two regional groups. We infer north to south dispersal based on our regional assignments; however, larger samples might reveal bi-directional dispersal. South-to-north dispersal has been observed by a tiger captured on camera traps in the JDNP in 2015 that was confirmed as having been captured in 2010 in RMNP, a straight-line distance of ca. 130 km south of JDNP (*unpublished data*). This single incident provides evidence of the long-distance dispersal of tigers in Bhutan. Evidence for dispersal in the face of moderate-to-high F_{ST} is consistent with dispersal with incomplete gene flow. If all or most dispersing individuals successfully reproduced, we would expect to observe a lower F_{ST} and more evidence of admixture in the DAPC and STRUCTURE analysis. The degree of potential disparity between dispersal and gene flow will need to be resolved with a larger sample size in future analyses.

Two individuals among our sampled tigers had unique genotypes, thus forming a separate cluster in both STRUCTURE and DAPC analysis. These two individuals were from the RMNP, which is connected to the Manas National Park (MNP) in India in the south and north-eastern population of tigers in Bhutan through the Zhemgang forest division and Phrumsengla National Park. A recent transboundary camera trapping study along the international border demarking these two national parks (RMNP and MNP) has recorded four tigers with transboundary occurrence and movement (Borah et al., 2013). The north-eastern part of Bhutan also has a moderately-high density tiger population, specifically in the Zhemgang forest division and Phrumsengla National Park (NCD, 2019). Bhutan also shares the border with several northeastern states of India, including Sikkim, West Bengal, Assam, and Arunachal Pradesh. The north-eastern landscape of India supports a total population of about 219 tigers spread across several protected areas (Jhala et al., 2011). Kolipakam et al. (2019) found that the tiger population of the northeastern part of India (27 samples collected from Namdapha, Dibang, Buxa, Manas, and Kaziranga, H_E = 0.821) forms a unique genetic cluster and evidently showed genetic separation from the rest of the Indian tiger populations in the Western Ghats, Western India, Himalayan foothill or Terai, and Central India. Even within the northeast Indian tiger population, further subdivisions were observed between highland populations of Arunachal Pradesh state and

the foothill populations from the plains of Kaziranga- Manas-Buxa situated in Assam state. Given the proximity of RMNP to MNP in India and the tiger populations in north-eastern Bhutan, we postulate that these two tigers may have originated from either a population in north-eastern Bhutan or an adjoining region in India.

Both DAPC and STRUCTURE analysis assigned the 11 individuals of uncertain origin from confiscated tiger body-part samples to Clusters 1 and 2 of Bhutan. From these results, we infer that these tigers were poached either within Bhutan or from an adjoining genetically similar, but still not fully defined population across the border in India. If these 14 individual tigers, whose skins and bones were confiscated in 2015–2017, are indeed from Bhutan, then this would imply increasing poaching pressure in Bhutan and an urgent need to improve monitoring and anti-poaching efforts. These may include increasing the frequency of Bhutan's current 5-year national tiger survey and increasing the focus on poaching and retaliatory killings. Also, it is very timely for the Bhutan government to plan for developing an extensive spatially referenced genotype library of tigers in Bhutan. This information will create baseline genetic data to serve as a reliable and judicially accepted law enforcement tool for combating the poaching and illegal trade of tigers and prosecuting the offenders. This library can infer the geographical sites of origin of confiscated and poached tigers (Wasser et al., 2018).

Chronic habitat loss and decreased connectivity over a century have resulted in the loss of genetic variation and increased genetic differentiation among tiger populations in the Indian sub-continent (Mondol et al., 2013). For example, the isolated population in Similipal Tiger Reserve in eastern India showed very low heterozygosity at 81 single nucleotide polymorphisms (mean heterozygosity = 0.28, SD = 0.27), evidence of inbreeding, and a high frequency of a pseudo melanistic trait (Sagar et al., 2021). Likewise, human-induced landscape fragmentation has resulted in the isolation of the tiger population in the entire habitat from the Terai Arc Landscape to northeast India along the foothills of the Himalayas (Sharma et al., 2011). It is possible that fragmentation effects are evident in our genetic structure results of Bhutanese tigers, but a loss of within-population genetic variation has yet to occur. Bhutan, due to its unique geographical location and extensive contiguous forest habitat inhabited by a large and genetically viable tiger population, could act as a stepping-stone in connecting tiger populations of the two neighboring landscapes in India. Finding evidence linking the Terai Arc Landscape to the Namdapha-Royal Manas TCL and beyond could prove crucial for landscape-wide tiger conservation in this region. We call for a transboundary and landscape-level conservation approach and specifically recommend developing standard protocols for sharing genetic data sets to understand tiger dispersal, threats, and other factors influencing dispersal events. This could also involve standardization of the scoring of microsatellites that have been used in multiple studies of tigers in the Indian sub-continent (Aziz et al., 2018; Bhagavatula and Singh, 2006; Joshi et al., 2013; Mondol et al., 2013, 2009a; Reddy et al., 2012a; Sharma et al., 2013b, 2013a; Singh et al., 2017, 2015; Thapa et al., 2018; Yumnam et al., 2014). This will help formulate better management strategies to maintain structural and functional connectivity.

Bhutan is one of the fastest-growing economies in South Asia (Asian Development Bank, 2017), with developmental activities such as the construction of large hydropower dams, roads, and other associated infrastructure being implemented at an unprecedented rate. However, the country is fortunate that the ethos of Gross National Happiness guiding Bhutan's development places equal importance on environmental preservation. Also, Bhutan's constitution mandates a 60% forest cover in perpetuity. Here, we show high genetic variation, substantial genetic subdivision, and evidence of dispersal and possibly gene flow in the Bhutanese tiger population. It will be essential to keep the habitat unfragmented and corridors functional to ensure a demographically and genetically viable tiger population in Bhutan.

Author contribution

T.D., L.S.M, and A.W. conceived of the study. T.D. collected the samples and obtained permits; T.D., A.W., and S.S. conducted the analyses; S.P. supervised the lab work; both S.P. and T.D. performed the laboratory procedures; and T.D. wrote the paper with input from all authors.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tashi Dhendup reports financial support was provided by National Geographic Society. Tashi Dhendup reports financial support was provided by Bhutan Foundation.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2023.e02459.

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