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Tashi Dhendup
University of Montana

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TIGER MONITORING IN BHUTAN USING NON-INVASIVE GENETIC TOOLS

By

TASHI DHENDUP

B.S., G. B. Pant University of Agriculture and Technology, Uttarakhand, India, 2012

Thesis

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Approved by:

Dr. Scott Whittenburg, Dean of The Graduate School
Graduate School

Dr. L. Scott Mills, Chair
Wildlife Biology Program, Department of Conservation and Ecosystem Sciences

Dr. Michael Mitchell, Committee member
Montana Co-operative Wildlife Research Unit

Dr. Andrew Whiteley, Committee member
Wildlife Biology Program, Department of Conservation and Ecosystem Sciences

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Chairperson: Dr. L. Scott Mills

ABSTRACT

Large carnivores are one of the most threatened group of animals in the world. They suffer from prey depletion, persecution by humans, and habitat loss and fragmentation which are extensively driven by anthropogenic activities. One such species is the tiger *Panthera tigris*. Tigers are found in thirteen countries in Asia and are protected across the range; however, tiger numbers have declined as an after effect of habitat loss, prey depletion and poaching. Human-induced changes have reduced the tiger's historical range to about 7% in which a little more than 3900 tigers are found. Most of these individuals currently exist in small and highly structured populations. Obtaining reliable estimates of population size and density and a solid understanding of connectivity between populations are critical to understanding important aspects of effective tiger conservation. Bhutan, with a vast expanse of contiguous pristine forest cover, abundant prey, and active conservation policies, form a very critical part of tiger conservation in South Asia. However, due to limited funds, monitoring is erratic. Camera traps are a sought-after tool for monitoring tiger population and density in Bhutan, but costs have been a limiting factor. Therefore, we evaluated non-invasive genetic sampling (NGS) as an effective alternative to camera trapping for monitoring tigers in Bhutan. We carried out systematic camera trap and scat surveys in Royal Manas National Park in Southern Bhutan in 2018 and compared density, variability, and costs between the two methods. The densities were estimated under a spatially-explicit capture-recapture framework, and camera trap and NGS produced a density of 2.38 tigers/100 km^2 (95% CI 1.11-4.02) and 3.6 tigers/100km^2 (95% CI 1.06-12.23) respectively. Density and other parameters were estimated more precisely using camera traps, but the field and equipment cost was high as compared to single-session genetic sampling. When controlled for sampling effort, NGS performed better.

There is also no information regarding population connectivity and gene flow in tigers within Bhutan. We genotyped 24 individuals using thirteen microsatellite loci and found that Bhutanese tigers overall have a high genetic variation (H$_e$=0.75). Individual-based and multivariate analyses indicated three genetic clusters within the sampled individuals; however, the overall genetic differentiation was low (F_{ST}=0.44). Our results indicate that Bhutanese tigers can be a source of genetic variation in the region and could play a crucial role in the long-term persistence of the species. We strongly recommend a transboundary and landscape-level conservation approach using common genetic data sets to understand tiger dispersal, threats and other factors influencing dispersal events.

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CHAPTER 1: INTRODUCTION

Large carnivores occupy a crucial position at the top of the food chain and are often known to have strong regulatory effects on ecological communities. At least 7 of the 31 large carnivores have been documented to exert trophic effects (Ripple et al., 2014). They structure not only the ecosystem but also provide a multitude of ecosystem services and various social and economic benefits to people (Mills et al., 2007). However, large carnivores are also one of the most threatened groups of animals in the world. About 61% are listed as threatened on the IUCN red list and 77% are experiencing a substantial decline in populations (Wolf and Ripple, 2016). They suffer from prey depletion, persecution by humans, and habitat loss and fragmentation which are extensively driven by anthropogenic activities.

The tiger is a charismatic species and an effective flagship for biodiversity conservation in many parts of Asia (Dinerstein et al., 2007). However, the species is a perfect example of a large carnivore threatened to extinction by habitat loss, prey depletion, and poaching. The historical range of tigers have reduced by 97%, and the population has declined dramatically from approximately 100,000 in the early 20th century to less than 4000 by the year 2000 (Dinerstein et al., 2007; Sanderson et al., 2010). Most of the remaining individuals currently exist in small and highly structured populations (Kenney et al., 2014). The problem is further exacerbated by the fact that a large segment of the tiger's remaining habitat is situated in developing countries where changes are happening at an extraordinary rate (Wang and Macdonald, 2009). India which is home to 60% of the worlds tiger population lost one tiger per week in the year 2018, and in the last three months, 27 deaths have been reported (WPSI 2019).

Tiger was listed as endangered in 1969, and since then, tiger range countries (TRCs), international organizations and civil society have been working tirelessly to save the species from the brink of extinction. Studies show that tigers are resilient and can rebound provided adequate habitat and protection, prey and connectivity between populations are ensured (Karanth et al., 2006; Wang et al., 2016). In 2010, the heads of the 13 TRCs met in St Petersburg in Russia for the International Tiger Forum and endorsed a declaration on tiger conservation. A Global Tiger Recovery Program (GTRP) was developed to address the broad spectrum of threats facing the species and also to work toward increased financial sustainability. An ambitious goal was set: TX2, doubling the tiger population by 2022- the next Year of the Tiger (Global Tiger Initiative, 2011).

Obtaining reliable estimates of population size and density is critical to understanding important aspects of effective tiger conservation. The estimates can be invaluable to assessments of conservation interventions, species persistence and prioritization of investments (Mills, 2015; Sugimoto et al., 2012a). However, obtaining reliable estimates can be a big challenge especially when the species of concern is elusive, rare and found in low densities. Camera-trapping technology has emerged as a powerful conservation tool and has helped to refine the precision of estimates making it more reliable and useful. Bhutan conducted its first estimation of tiger population and density using camera traps in Jigme Singye Wangchuck National Park in 2009 (Wang and Macdonald, 2009), followed by Royal Manas National Park in 2011 (Tempa et al., 2013a) and then in Jigme Dorji National Park in 2015 (Thinley et al., 2015). Also in 2014-15, Bhutan conducted the first nationwide tiger survey using camera traps and estimated the population size and density at 103 (89-124 95%CI) and 0.46 per 100km^2 respectively (DoFPS,

2015a). However, camera traps are logistically challenging and require much time, effort and money.

Non-invasive genetic sampling (NGS) is increasingly being used as a valuable supplementary method for monitoring tiger populations and has been recently used in China (Sugimoto et al., 2012b), India (Bhagavatula and Singh, 2006; Borthakur et al., 2013; Mondol et al., 2013; Reddy et al., 2012b; Sharma et al., 2011, 2009, 2013b; Singh et al., 2015; Yumnam et al., 2014), Nepal(Thapa et al., 2018) and Bangladesh (Aziz et al., 2017). The method is reliable and increasingly becoming cheaper and faster. NGS also provides other useful information including sex, population structure, and population demographic history. Assessing mtDNA haplotypes have been highly successful in elucidating population structure in Bengal tigers on a large landscape level and have reported strong differentiation in tigers in various regions. Specifically, recent studies in the Indian-subcontinent have reported distinct populations of tigers each in Chitwan in Nepal, in the Terai Arc Landscape and the north-eastern part of India (Sharma et al., 2011). Mondol et al. (2013) also reports a substantial loss of genetic diversity and increasing differentiation in Indian tigers over the last 50 years.

Bhutan, with a vast expanse of contiguous pristine forest cover, abundant prey, and strong conservation policies, forms an integral part of the global tiger conservation area in the region (DoFPS, 2015a). A study on the population size and density estimates of tigers using genetic sampling has never been tried in Bhutan. Information on the population structure of the species is also lacking for the country.

My master's thesis consists of two chapters. In the first chapter, I compare camera traps to non-invasive genetic sampling for deriving density estimates in terms of effort, cost, and ability

to detect and recapture individual tigers in Royal Manas National Park. I carry out the NGS in a single session scat sampling, and density is estimated under a spatially explicit capture-recapture model.

In the second chapter, I look at the status of genetic variation, dispersal, and connectivity among tigers in Bhutan. Because of Bhutan's relatively low human density and high tolerance for wildlife, and a well-connected network of protected areas, tiger population in the country is expected to be distributed continuously across the country and not sub-divided into discrete populations. Nonetheless, gene flow can be limited to relatively short distances, leading to increasing genetic differentiation as the geographic distance between individuals becomes greater, also known as isolation by distance (IBD) (Allendorf et al., 2012). Tiger density is not uniform across the country (Tempa, 2017), and with the possibility of IBD and natural barriers such as deep gorges, steep mountains, and large rivers, affecting the average dispersal of genes, I assess whether there is a spatial genetic structure in the tiger population within Bhutan.

In this thesis, I use "We" to signify the collective effort by various collaborators that went towards the successful completion of the thesis.

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CHAPTER 2: ESTIMATING DENSITY OF TIGERS IN THE EASTERN HIMALAYAS USING CAMERA TRAPS AND NON-INVASIVE GENETICS

ABSTRACT

Recent advancements like camera traps and non-invasive genetics allow robust estimation of density. However, these methods differ in terms of statistical efficiency, field cost advantage and logistics and these factors must be taken into consideration for species which requires continuous monitoring. In this study, we evaluate camera traps and non-invasive genetics (NGS) using DNA obtained from scats to estimate the density of an important tiger population in the eastern Himalayas: Royal Manas National Park in Bhutan. We used spatially explicit capture-recapture (SCR) models and estimated densities at 2.38 tigers/100 km^2 (95% CI 1.11-4.02) using camera traps and 3.6 tigers/100km^2 (95% CI 1.06-12.23) using NGS. Density and other parameters were estimated more precisely using camera traps, but the field and equipment cost were high as compared to single-session genetic sampling. When the camera trap survey was controlled for sampling effort, NGS performed better. NGS is a promising tool for monitoring endangered species, and its statistical performance can be improved by increasing effort and effective sampling area and also by ensuring genotyping success.

INTRODUCTION

Local abundance and density have a decisive influence on the potential viability of any species (Mills 2015) and are central to most wildlife management and conservation decisions (Cheng et al., 2017). For highly elusive and rare species, estimating abundance and other critical

demographic parameters can be a daunting task, often expensive and time-consuming (Balme et al., 2006; Sollmann et al., 2013b). The tiger (*Panthera tigris*) is a useful flagship species for the Asian forest ecosystem but is threatened due to poaching, habitat loss and prey depletion. The global tiger population has declined drastically within the last century, and currently, there are fewer than 4000 tigers left in the wild and occupying a mere 7% of their historical range (Dinerstein et al., 2007). The species has been listed as endangered on the IUCN red list since 1969, and despite concerted efforts over the past four decades, continues to decline with a massive contraction of geographic range (IUCN, 2015). Alarmed by the plight of tigers, the tiger range countries, international organizations and civil society came together during the historical tiger summit of St Petersburg in Russia in the year 2010 and initiated a Global Tiger Recovery Program aimed at reducing threats to the species; a key goal was to stabilize the population within 5 years and to double the numbers by the year 2022 (Global Tiger Initiative, 2011). Since then, monitoring trends in tiger abundance has become a fundamental exercise for evaluating the success of on-going conservation efforts. However, obtaining reliable estimates of abundance and density of tigers is difficult as the species is sparse, cryptic, mostly solitary and wide-ranging (Gopalaswamy et al., 2012). Therefore, while significant conservation efforts are being invested in increasing and monitoring tiger populations across its range, it is vital that survey methods generate reliable quantitative estimates of density and be time efficient, easily repeatable, and affordable (Janecka et al., 2011).

State-of-the-art camera traps have emerged as a gold standard for monitoring carnivore populations, particularly for tigers, snow leopards, and other felids that can be individually identified through their unique stripe or spot patterns (Jackson et al., 2006; Karanth, 1995). Recently, cameras have also been successfully used for estimating abundance and density in

animals without distinct markings such as wolves and bears (Mattioli et al., 2018; Nakashima et al., 2018; Rowcliffe J.M. et al., 2008). Data obtained from camera traps are used in statistically robust Capture-Mark Recapture (CMR) analyses, and photographs also serve as a potent medium to connect with and influence policymakers and the public (Riley et al., 2017). However, the method is logistically constrained and can be expensive, especially in areas where wildlife habitats are rugged and remote. Further, cameras have limited utility for evaluating past and present connectivity (e.g., dispersal and gene flow).

Non-invasive genetics (NGS) provides an alternative approach to estimating the abundance of rare or elusive species through CMR based methods. It has been successfully used to study bears (Ciucci et al., 2015), wolverines (Mulders et al., 2007), snow leopards (Janecka et al., 2011) and ocelots (Rodgers and Phillips, 2014). It has also been used successfully with tigers in India (Borthakur et al., 2013, 2011; Mondol et al., 2009a), Nepal (Thapa et al., 2018), Bangladesh (Aziz et al., 2017) and China (Sugimoto et al., 2014). For elusive species, NGS may allow collection of large sample sizes and be cost efficient as spatial capture histories for CMR analyses can be built from DNA collected data during a single site visit (Cheng et al., 2017; Janecka et al., 2011; Morin et al., 2018). NGS also provides information on genetic diversity and connectivity and has broad utility for forensics and solving wildlife crimes (Kelly et al., 2012; Wasser et al., 2018). However, NGS also has specific challenges. DNA extracted from the non-invasively collected samples can be low in quality and quantity which decreases amplification success and increases genotyping errors (i.e., allelic dropout and false alleles), both of which can affect density estimates (Mills et al., 2000; Waits and Leberg, 2000). In places like Bhutan, tigers live sympatrically with other carnivores such as common leopard, clouded leopard, dholes, and

snow leopard, requiring additional costs of genetic identification of scats to species (Monterroso et al., 2018).

Royal Manas National Park is home to an important population of Royal Bengal Tigers (*Panthera tigris tigris*) in the eastern Himalayas, and the landscape is also a part of an important tiger conservation landscape: Northern Forest Complex - Namdhapha -Royal Manas (Sanderson et al., 2010). The tiger population in the park was estimated at 22 using camera traps in 2016 with a density of 2.9 tigers/100 km^2 and is among the highest within Bhutan (DoFPS, 2017, 2015b; Tempa et al., 2013b). Tigers here are monitored using camera traps at intermittent intervals. However, for the long-term monitoring of tiger populations across the country, given the potential cost and logistic limitations for cameras, NGS may be a useful complement or alternative. This is also the first time that non-invasive genetics has been tried to monitor abundance of tigers in Bhutan.

Several studies have made direct comparisons between camera trapping and non-invasive genetics for acquiring reliable abundance estimates (Anile et al., 2014; Burgar et al., 2018; Garrote et al., 2014; Janecka et al., 2011; Mondol et al., 2009a; Randi et al., 2015; Riley et al., 2017; Rodgers and Phillips, 2014). Such comparative studies are vital to understand the performance of multiple methods in different landscapes and for different species (Barba et al., 2010; Garrote et al., 2014). Here, density from both camera trap and NGS data was estimated under the framework of spatially explicit capture-recapture models. The objective of the present study is to evaluate non-invasive scat sampling and camera trapping through comparison of point estimates and variability, incorporating field logistics and cost considerations.

MATERIALS AND METHODS

Study area

Royal Manas National Park (RMNP) was established in the early 1960s and is the oldest protected area in Bhutan. The national park is located in the southern foothills of the country (90° 35' 03.61" E to 91° 13' 28.51" E and 26° 46' 16.16" N to 27° 08' 38.70" N) and spans an area of 1057 km^2. The elevation ranges from 97 m in the south to 2914m in the north and has a moist sub-tropical to cool temperate climate. Summer temperatures range between 20 to 40°C, winter temperatures between 5 to 20 °C, and rainfall from 200 to 4400mm annually. Primary vegetation transitions from tropical monsoon forests (<500 m) and subtropical forests (500 m-1000 m) to warm broadleaved forests (1000 -2000 m) and cool broadleaved forests (2000 -2714 m). RMNP continues to Jigme Singye Wangchuck National Park in central Bhutan to the north, and biological corridors connect it to Phibsoo Wildlife Sanctuary (PWS) to the southwest, Phrumsengla National Park (PNP) in the northern center, and Jumotshangkha Wildlife sanctuary (JWS) in the southeast. Towards the south, it shares a boundary with the Manas National Park (MNP) in India via a contiguously forested landscape. Together with PWS, JWS and two biological corridors in Bhutan and MNP in India, it forms an important protected area complex in the region known as the Transboundary Manas Conservation Area (TraMCA).

The national park is one of the most biodiverse areas in the region and is home to 558 species of flora, 65 species of mammals, 493 species of birds, 69 species of fishes and more than 180 species of butterfly (RMNP, 2015). RMNP also boasts as one of the hotspots of felid diversity in the world recording up to 7 species of wildcats which are tigers, common leopards, clouded leopard, Asiatic golden cat, marbled cat, leopard cat, and Jungle cat (Dhendup et al., 2016). Other important carnivore species include the endangered Asiatic wild dog or Dhole,

Himalayan black bear, Binturong, civets, and others. Herbivores include Asiatic elephant, Asiatic water buffalo, gaur, sambhar, barking deer, serow, goral, and numerous others.

For management purposes, the park is divided into three ranges: Umling range, Manas Range and the Gomphu Range. There are currently 1183 households living within the jurisdiction of the park, and the total population is 8936.

Camera trap survey

We carried out the camera trap survey in the southern foothills of Royal Manas National Park (RMNP), using a camera trapping array that encompassed an area of approximately 400 km^2 (Figure 1). We laid 2km x 2km grid cells across the study site and set up 101 camera stations. In each grid cell, we chose optimal locations to set camera traps based on the presence of tiger signs such as pugmarks, scats, scent marks, scrapes and kill sites. We spaced the camera stations 2-2.5km based on small home range sizes of 15km^2 for female tigers in India (Karanth & Nichols 1998). Our spacing allowed 2-3 camera stations within a single home range to ensure that each animal in the survey area has a probability of being captured (Otis et al., 2006). At each station, we placed two cameras to capture both flanks of a passing animal. The cameras were tied to a tree, or when a tree was not available, to a wooden pole or a standing rock at approximately 50 cm above the ground, the two cameras were separated by at a few meters away to avoid mutual flash interference. The sensitivity of the cameras was set to high and programmed to operate 24 hours a day. We used four different passive infrared camera models (Panthera-flash, Cuddeback Attack-Infrared (IR) -flash, Bushnell (IR) and Reconyx). The cameras were monitored once every month where possible, although in some cases, monsoon, swollen rivers or security issues limited monitoring to once in two months. The cameras were kept in the field from January to May 2018. We lost cameras at 15 of the 101 stations due to theft and wild animals.

Scat sampling

We collected tiger scats in Royal Manas National Park between February 21 to March 17, 2018, from a scat survey area embedded within the camera trap array. We collected scat samples in a single occasion and followed the approach outlined by Gopalaswamy et al., (2012) for fecal DNA surveys for tigers. We distributed search routes spatially along a representative set of camera trap trails and surveyed each search route with a team of at least three individuals. Search routes typically included trails regularly used by tigers as inferred from previous camera trap data and other occurrence records. We inferred the scats as belonging to tigers in the field based on diameter, segmentation, content, and proximity to other signs such as scrapes and urine (We confirmed the species identification with genetic methods; see next section). We collected only a small amount of scat material and stored it in a plastic vial filled with silica desiccant. We filled out our scat collection data sheet with information such as location, date, size of scat and comments.

The scat samples were then transported to a facility with a -20 °C freezer. It took almost three weeks for the samples to reach from Bhutan to the Conservation Genetics Lab at the University of Montana and during which, the samples were kept at room temperature. The samples were then stored at -20 °C until DNA extraction.

Laboratory methods

We extracted DNA from the fecal samples using QIAamp Fast Stool Mini Kit (QIAGEN Inc., Germany) following manufacturer's instructions with slight modifications. Negative controls were included in every batch of DNA extraction and subsequent polymerase chain reactions (PCR), and care was taken to avoid contamination at every step. The PCR reactions were performed in a 10 μL volume containing 5 μL of QIAGEN Multiplex PCR buffer mix

(QIAGEN Inc. Inc. Germany), 1 µL of each 0.2 µmol/L forward and reverse primers, 0.8 µl of BSA (Bovine Serum Albumin), 1.2 µL of PCR-certified water and 2 µL DNA template. We screened each sample using two tiger species-specific primers: 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) (S1). Using tiger tissues as a reference, we optimized a panel of 13 microsatellite loci (*Fca441, Pati09, Fca391, Fca628, E7, Fca672, Fca304, F53, Fca232, Fca090, Fca42, Fca279 and Fca126*) and grouped them into three multiplexes with eight separate reactions (S2). The selected primers were initially developed in the domestic cat (*Felis catus*) (Menotti-Raymond et al., 1999), and Amur tiger (*Panthera tigris altaica*) (Wu et al., 2009) and have been used successfully in tiger studies on the Indian sub-continent (Mondol et al., 2009a; Sharma et al., 2012). The loci set was chosen based on their polymorphic information content, ease of amplification and low error rates (Dou et al., 2016).

We used a multitube approach (Taberlet et al., 1996) in which each sample was genotyped four times to ensure reliable genotypes and to minimize genotyping errors. The tiger positive samples were first screened against five polymorphic loci, and samples which amplified at two loci or less were discarded. We then proceeded with genotyping the selected samples with the remaining eight microsatellite loci. This was done to ensure that only good quality samples were further genotyped, and it also saved time and laboratory costs. Gender was determined using *Amelogenin* (Pilgrim et al., 2005). The PCR products were run in an ABI 3130 Genetic Analyzer (Applied Biosystems, USA) and the raw data was scored using the software Geneious.

We used MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004) to look for the presence of stuttering errors, null alleles, and excess of homozygotes. Allele dropout and false alleles were quantified using GIMLET v 1.3.3 (Valiere, 2002). Each sample had to match for at least three of

the four PCR replicates to create a consensus genotype. Genetic diversity and values of P_{ID} and $P_{ID(sibs)}$ were measured using GenAlEx (Peakall and Smouse, 2012). We used CERVUS (Kalinowski et al., 2007) to match unique individuals and used 6 out of the thirteen loci matching criteria as six microsatellites had enough power to identify unique individuals ($P_{ID}=2.2 \times 10^{-6}$, $P_{ID (sibs)}=5.9 \times 10^{-3}$). We used GENEPOP to check for deviations from Hardy-Weinberg proportions and Linkage Disequilibrium. We used an exact test with 10,000 dememorization steps, 1000 batches and 10,000 iterations per batch. Bonferroni corrections were applied to a significance value (alpha=0.05). The details of the thirteen microsatellite loci used in individual identification are given in Table 1.

Data analyses

Camera trap data were downloaded and archived in folders by camera station and species. We obtained a total of 470 tiger pictures of any body part (from both cameras at a station). We used 171 left-flank photos to identify tiger individuals based on their unique stripe patterns on the sides, head, tail, and limbs (Karanth and Nichols, 1998). The left flank was selected because it maximized the number of individuals captured. Two trained observers made the identification, and individual identities were confirmed upon consensus. We developed trap-specific encounter histories and camera operation histories for each site, and sampling occasion was defined as seven days. Gender identification was done when possible by the presence or absence of scrotum and was included in the capture histories as a covariate for the detection function (Satter et al., 2019).

For the genetic survey, we organized our data similar to Sollmann et al., (2013a): we divided the sampling area into 2×2 km^2 grids, and each grid was considered as a trapping device that accumulates DNA evidence and is represented by the coordinates at the center. Each scat is

assigned to the center of the grid it was found in. When the DNA evidence of an individual animal is caught in more than one detector, these are assigned as recaptures (Borchers, 2012). In the end, the data is aggragated into a standard individual capture history format amenable to a capture-mark-recapture analysis similar to that of camera traps.

We used a likelihood-based spatially explicit capture-recapture (SCR) approach for estimating the density of tigers for both cameras and scat data (Efford, 2018). SCR models incorporate the detection-location information with a detection history of individuals to eliminate the need to add post hoc buffers. We used the *secr* package in R and ran a conditional likelihood SCR model, assuming half normal detection function and proximity detectors, reflecting an independent Bernoulli encounter model (Efford, 2018). The half normal detection function is described by two detection parameters: baseline detection rate $g0$ which is the probability of capture when the distance between the animal's activity centre and the trap is zero, and the spatial scale movement parameter sigma which explains how detection probability declines as a function of the distance to an individual´s activity center. We defined the state space by buffering the trapping array by four times the estimated sigma, and the spacing was set at 2000m. For camera trap data, we fitted various combinations of predefined variables (trap specific behavioral response to capture (bk) on $g0$, and the sex effects on $g0$ or sigma) and tested eight candidate models. Sex was associated with each individual in the capture history when available and we accommodated individuals with unknown sex by fitting a hybrid mixture model. We used the Akaike Information Criterion corrected for small sample sizes as our model selection criterion (Anderson and Burnham, 2002). The small sample size and spatial recaptures in the genetic dataset prohibited the use of the predefined variables as in camera trap data analysis, so we could

run only the null model. The null model from the camera trap dataset was also estimated to make a direct comparison to the null model density estimate from NGS.

The camera trapping survey was more intensive than the genetic sampling over space and in terms of effort. To allow direct comparison between the two methods, we randomly subsampled the camera trap dataset a number of times (without replacement) to match the number of individuals and recaptures from the NGS data. We fitted a null model to the subsampled camera trap datasets, estimated the tiger density for each subset and took an average.

RESULTS

Density from camera trapping (CT) survey

We accumulated a total effort of 14,483 trap nights and obtained 350,397 images and 2813 videos. The effort resulted in 470 photos of tigers with 67 detections spread across 25 occasions, and a trap success rate of 0.70 detections/100 trap nights. We camera trapped 22 individuals. One was a cub. Of the adults, four were males, 11 were females, and six were of unknown sex. Based on the top model, density was 2.38 tigers/100 km^2 (95% CI 1.11-4.02) and the null model produced a density of 1.6 tigers/100km^2 (CI: 1.03-2.66) (Table 2). The baseline encounter rates increased with a trap-specific behavioral effect and the top model estimated the *g0* at 0.07. The parameter sigma varied between the sexes, with male sigma estimated at 5.06km (CI=3.61-7.10) and female 2.41km (CI=1.75-3.34).

Density from the non-invasive genetic survey

Our team walked 112 km in 14 days and collected 61 putative tiger scats. Thirty-four samples were identified as tiger positive in the lab using tiger species-specific primers. After using the panel of 13 microsatellite loci to assign unique individuals, only 18 samples amplified

at a sufficient number of loci to discern unique genetic profiles and recaptures. We counted eight individual tigers: five males, and three females. Only two individuals of the eight were recaptured and that too, just once. We obtained a density of 3.6 tigers/100km^2 (95% CI: 1.06 to 12.23). The spatial scale parameter sigma and the detection parameter *g0* for both the sexes were estimated at 1.99km ±0.87 (CI: 0.88-4.5) and 0.24 km^2 ± 0.21 (CI: 0.03-0.75).

Camera trap survey controlling for sampling effort

The subsampled data from the camera trap dataset contain the same number of individuals and recaptures as the NGS, but the individuals were randomly picked without replacement. The null model estimated a density of 4.05 tigers/100km^2 (95% CI: 1.06-15.47). The sex-specific sigma and capture probability *g0* was constant for both the sexes at 1.80 km ± 0.93 (95% CI 0.69-4.67) and 0.008 ± 0.0084 (95% CI: 0.001-0.056) respectively.

Cost Comparison

We had 101 camera trap stations with two cameras per station across the study area. On average, a decent camera trap cost around $250, for an initial investment cost of ~$50,000. An SD card costs around $8 per unit and a reliable rechargeable battery set cost $15 per 6 pack. Costs for 202 SD cards and 2424 batteries is $7676. The fieldwork was carried out by a team of rangers comprising of an average of 7 members paid $18 per diem. The entire survey took 80 days (set up=30 days, monitoring=25 days, retrieving=25 days) for a total fieldwork cost ~$10,080. Excluding costs of travel to and from the field site, shipment of cameras to Bhutan, vehicle renting and the salary of the PI, the total expenditure is $67,756.

For the scat survey, the initial investment comprised of buying field equipment which included tubes, silica, gloves, zip log bags, sharpies and calipers for measuring scats at the cost of $315. Scat collection in the field took 14 days with a seven members team, totaling $1764.

The laboratory expenses included costs for primers, extraction kits, tubes, optimization of multiplexes, and due to the degraded DNA in the scat samples, many re-extractions, amplifications, and re-runs. The costs came to about $7550. The total expenditure for the NGS amounted to $9314.

In the camera trap survey, the total cost per camera station averaged $670.85. The scat survey covered 45 grids of 4km^2 each. If we set up one station in each grid in the scat sampling array, then the total cost amounts to $30,188.25 ($670.85/station x 45 stations) which is almost 3.2 times more than the NGS for covering the same sampling area. Similarly, each camera station required 1.3 person days for set up. For 45 stations, it will require 56.8 days which is four times more than what NGS took to sample the same area.

DISCUSSION

The effective sampling area of the scat survey was smaller than the camera trapping array. Despite this unequal spatial extent between the two surveys, densities estimated with SCR approaches are still comparable since the model takes differences in sampling area into account (Rodgers and Phillips, 2014). SCR approach is now widely adopted over traditional CMR analyses, where density estimates suffer from edge effects and require the addition of ad hoc buffers around the trapping array (Bozarth et al., 2015; Efford et al., 2009). SCR overcomes these limitations by incorporating the spatial information (individual distribution and movement in space) related to the encounter process directly into density estimation.

In the current study, we fitted a likelihood-based SCR model on both the camera trap (CT) and the NGS dataset and found that the density estimated from the top model (2.38 tigers/100 km^2) and null model from the camera trap dataset (1.6 tigers/100km^2) were both

lower than NGS (3.6 tigers/100km^2) and more precise as determined from the narrower

confidence intervals (top CT 95% CI: 1.11-4.02, null CT 95% CI: 1.03-2.66, NGS: 95% CI 1.06

to 12.23) (Figure 2). Cameras picked up more individuals and recaptures, mostly because of the

intensive sampling effort and larger effective sampling area. Our genetic sampling also picked up

two individuals whose multilocus genotypes appeared to be highly divergent from the others

suggesting immigration during the sampling period. However, they were each captured only

once suggesting a random movement in the study area and studies show that such random

movements in and out of the study area doesn't introduce bias in the estimators for closed

populations models (Kendall, 1999). However, it may affect the precision.

Our minimum counts and density estimate from camera trapping compared favorably to

previous samplings conducted by the park in the same area. They used a similar camera trapping

methodology and a Bayesian-based SCR analysis framework. Although a Bayesian-based

approach may generate a lower density estimate than a maximum likelihood approach, the

differences are minimal (Noss et al., 2012; Tempa, 2017). For two previous years of 2015 and

2016, the park counted 15 and 17 minimum adults compared to our minimum count of 22 adult

tigers. After accounting for detectability of tigers, the park's estimates of density in 2015 and

2016 were 1.7 tigers/100 km^2 and 2.9 tigers/100 km^2 respectively (DoFPS, 2017) and our current

density estimate is similar to these two. These estimates of density in RMNP is higher than the

overall density of tigers in Bhutan, which is estimated at 0. 23 tigers /100km^2 (Tempa, 2017).

The higher density implies that RMNP has a high-quality tiger habitat and forms an important

cornerstone for tiger conservation in the country and the region.

Both the Bayesian-based and likelihood-based SCR models are known to perform poorly

when the data are sparse (Noss et al., 2012). We could not fit pre-defined variables into the

model for the genetic dataset due to lack of adequate sample size and spatial recaptures resulting in increased uncertainty with our density estimate. We successfully PCR amplified about 52% (n=18) of the tiger positive samples which yielded consensus genotypes for eight individuals. The amplification success rate was lower than those reported by Mondol et al., (2009) (93%) in India and Thapa et al., (2018) (83%) in Nepal but similar to a study in the Bangladesh Sundarbans (53%) (Aziz et al., 2017). Lower amplification success may be due to weather conditions (humid and hot) in Royal Manas National Park which may accelerate DNA degradation in the fecal samples. Although the samples were preserved using silica desiccant in the field, it was not until the survey was completed that we were able to transfer it to a -20-degree freezer. The time until storage in freezers was increased by the challenges of sampling extreme terrain with no road access. We extracted DNA from the samples only three months later in the U.S since Bhutan currently does not have a wildlife genetics lab.

We may be able to increase capture efficiency in the future by using hair snares and scat detection dogs to complement scat surveys. Hair snares are usually inexpensive, but scat detection dogs can be logistically challenging and expensive (Kelly et al., 2012). The choice of fecal DNA preservation also impacts PCR amplification success and genotyping accuracy rates especially when access to laboratory and cooling facilities are limited. Studies show that dimethyl sulfoxide saline solution (DETs buffer) performs significantly better in tropical environments when compared to silica desiccant or ethanol (Wultsch et al., 2015). Reddy et al., (2012) also found that for tiger fecal samples, a two step-method involving short-term storage in ethanol followed by desiccation with silica produced 2-3 times more DNA than when stored with either ethanol or silica alone.

We subsampled the camera trap survey data to control for the sampling effort for a more direct comparison of the camera and NGS methods. Under the null model, the sub-sampled dataset produced a higher density (4.05 tigers/100km^2) and a larger confidence interval (95% CI: 1.06-15.47) compared to the genetic dataset. This implies that the uncertainty about the density parameter from NGS could have arisen merely from restricted spatial scale, not because of the survey type. Therefore, we may be able to increase precision by increasing the sampling effort and improving the storage conditions for samples. Although the NGS cost increases with the increase in sample size, it is still relatively cheaper than cameras under the RMNP field conditions. Besides, maintenance and replacement of camera traps due to failure are also expected to incur additional costs. Notably, we lost cameras from 15 stations during the current survey.

Rigorous and cost-effective monitoring methods have become an essential part of managing wild animal populations, particularly of threatened species like the tiger. It is even more critical in developing countries where resources are limited, and biodiversity conservation is a race against time. Density is a parameter of vital importance, but it can be difficult to quantify. There are numerous studies comparing monitoring tools such as camera traps, live-traps, non-invasive genetics (DNA obtained from feces, and hair), track ID, scent dogs and also comparing statistical methods. Camera traps are known to be rigorous, and more researchers opt for cameras over other alternatives (Wearn and Glover-Kapfer, 2019). Several studies report lower sample size and recaptures from NGS when compared to camera traps (Anile et al., 2014; Janecka et al., 2011; Randi et al., 2015; Rodgers and Phillips, 2014). Researchers also caution against the risk of overestimating abundance and density when NGS is used alone. The uncertainty arises from our inability to exclude cubs and sub-adults from the analysis, and also

due to the smaller sampling area when collections are carried out along transects (Janecka et al., 2011). However, we were able to correctly identify the sex of all the individuals counted by the NGS while for camera traps, more than 30% were unknown. This is important to consider as sex influences detection probabilities and the resulting precision of parameters particularly in felids where home range movements differ between sexes (Sollmann et al., 2011).

Besides density and other population parameters, NGS also provides critical insights into the ecology of wild animals such as genetic diversity, gene flow, and kinship, among others. The technique is improving tremendously, addressing issues such as small size and violations of population closure. The costs have also reduced, and single session DNA sampling allows for a reduced field effort, and lower expenditure overall. For example, in the current study, NGS recorded all the nine individuals over 14 days while in the camera survey, it took 50 days to obtain the first picture of a tiger, 72 days to catch nine tigers and 114 days to catch all 22 tigers (including the cub).

CONCLUSION

The current study is the first in Bhutan and one of very few in the eastern Himalayas to derive population density estimates of tigers using non-invasive genetic sampling under a SCR framework. Our results demonstrate that NGS is a viable option for population monitoring of tigers and other endangered species. NGS gains advantage over camera traps as the genetic data required for estimating density and other population parameters can be obtained during a single site visit, i.e. fewer temporal sessions. As for camera traps, the costs and logistical challenges to setting up and maintaining cameras over large survey areas specially in the mountainous regions are substantial. If the primary objective is to obtain a more precise density estimate, then

combining data from multiple sources such as camera trapping, non-invasive genetics, and

telemetry can build a stronger inference, but the cost and effort will again be significant

(Gopalaswamy et al., 2012; Sollmann et al., 2013a).

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Figure 1. Camera trap stations in triangles and scat surveys in red lines in Royal Manas National

Park, Bhutan.
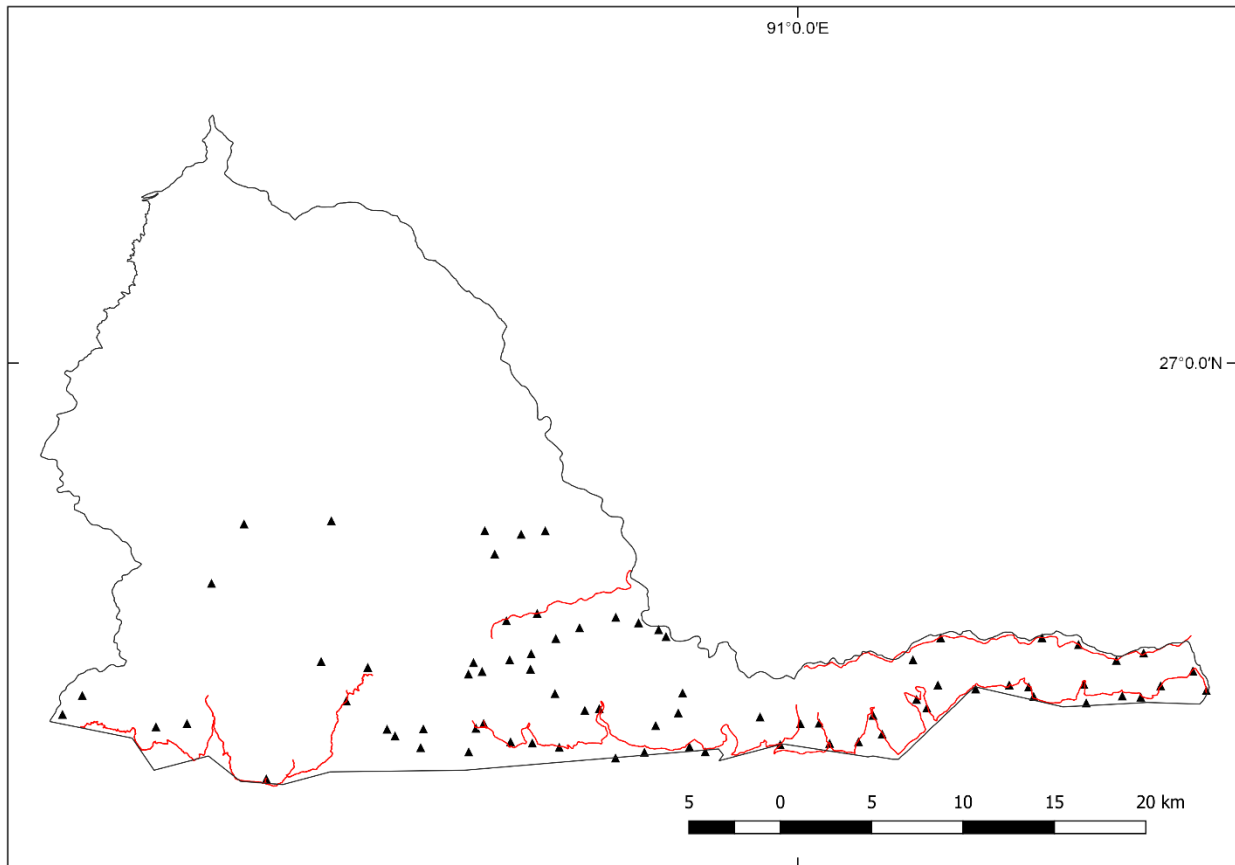
Figure 2. Density estimates and 95% confidence intervals for tigers estimated under different methods.
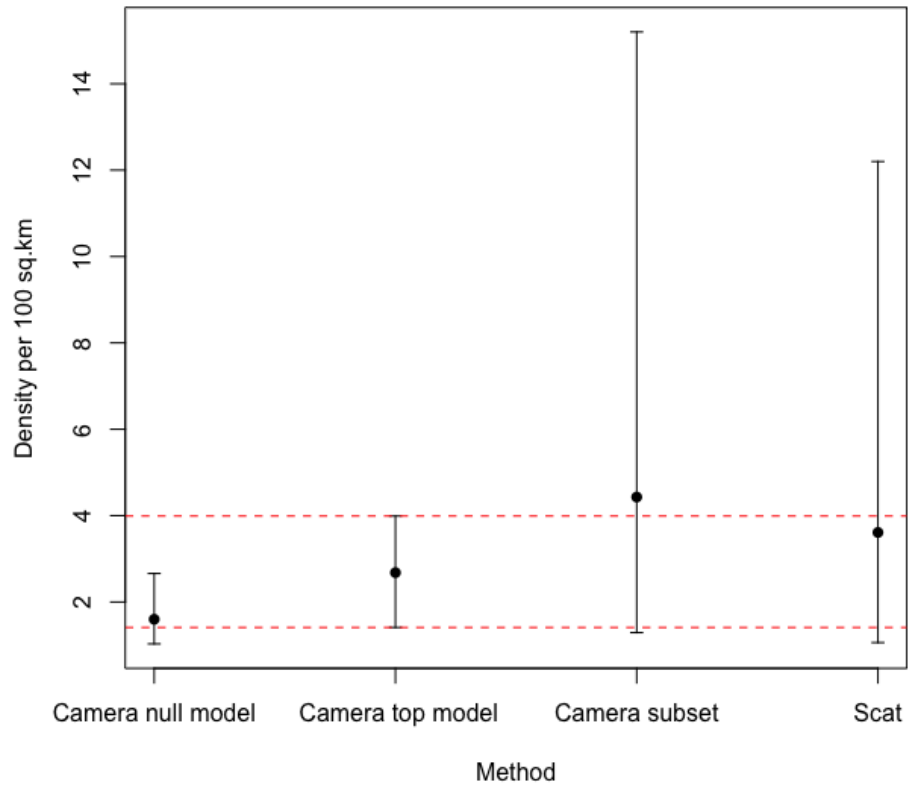
Table 1. Details of 13 microsatellite loci used in the current study

Locus	Allele range	No. of Alleles	Allele Dropout	False allele	Ho	He
FCA628	84-114	8	0	0	0.708	0.798
FCA441	153-183	4	0	0	0.333	0.645
Pati09	107-125	5	0.015	0	0.542	0.576
FCA391	189-217	5	0	0	0.375	0.649
FCA672	82-114	10	0	0	0.833	0.861
FCA304	109-198	6	0	0	0.542	0.715

E7	133-159	6	0.267	0	0.583	0.779
F53	128-181	8	0	0	0.826	0.809
FCA279	89-113	6	0	0.162	0.625	0.789
FCA232	86-118	7	0	0	0.500	0.591
F42	207-255	7	0	0	0.652	0.733
FCA090	91-125	6	0	0.069	0.500	0.713
FCA126	132-154	9	0	0	0.696	0.775

Table 2. Maximum likelihood models fit on camera trap data to estimate tiger density in Royal

Manas National Park, Bhutan

	model	detectfn	npar	logLik	AIC	AICc
g0bksigh2	g0~bk sigma~h2 pmix~h2	halfnormal	5	-358.2255	726.451	730.451
sigh2	g0~1 sigma~h2 pmix~h2	halfnormal	4	-360.9924	729.985	732.485
g0bkh2sigh2	g0~bk + h2 sigma~h2 pmix~h2	halfnormal	6	-357.9703	727.941	733.941
sigg0h2	g0~h2 sigma~h2 pmix~h2	halfnormal	5	-360.6914	731.383	735.383
g0bk	g0~bk sigma~1 pmix~h2	halfnormal	4	-363.0747	734.149	736.649
g0bkh2	g0~bk + h2 sigma~1 pmix~h2	halfnormal	5	-362.4873	734.975	738.975
null	g0~1 sigma~1 pmix~h2	halfnormal	3	-367.6635	741.327	742.739
g0h2	g0~h2 sigma~1 pmix~h2	halfnormal	4	-366.5879	741.176	743.676

SUPPLEMENTARY MATERIALS

S1. Thermocycling conditions for species identification

Steps	CYT B			NADH5		
	Temperature	Time	Cycling	Temperature	Time	Cycling
	95 °C	15 min	x 1			
Initial denaturation	95 °C	30s				
Denaturation	52.5 °C	45s	x 10	95 °C	15 min	x 1
	72 °C	30s				
	95 °C	30s		95 °C	30s	
Annealing	52 °C	45s	x 30	57 °C	30s	x 45
	72 °C	30s		72 °C	90s	
Extension	72 °C	7min	x 1	72 °C	10 min	x 1
Hold	4 °C	hold		4 °C	hold	

S2. Thermocycling conditions for individual and sex identification

Multiplex 1

Steps	FCA441, Pati09, FCA391- F/R			FCA628-F/R		
	Temperature	Time	Cycling	Temperature	Time	Cycling
Initial denaturation	95 °C	15'	x 1	95 °C	15'	x 1
Denaturation	94 °C	30 s		94 °C	30 s	
Annealing	54 °C	90 s	x 50	54 °C	90 s	x 50
Extension	72 °C	60 s		72 °C	60 s	
Final extension	60 °C	30'	x 1	60 °C	30'	x 1
Hold	4 °C	hold		4 °C	hold	

Steps	Amelogenin-F/R		
	Temperature	Time	Cycling
Initial denaturation	95 °C	15'	x 1
Denaturation	94 °C	30 s	
Annealing	51 °C	90 s	x 45
Extension	72 °C	30 s	
Final extension	60 °C	30'	x 1
Hold	4 °C	hold	

Multiplex 2

Steps	E7, FCA672- F/R			FCA304		
	Temperature	Time	Cycling	Temperature	Time	Cycling
Initial denaturation	95 °C	15'	x 1	95 °C	15'	x 1
Denaturation	94 °C	30 s	x 11 (-0.5 °C)	94 °C	30 s	x 50
	60 °C	90s				
	72 °C	30s				
Annealing	94 °C	30s	x 34	56 °C	90 s	
	55 °C	60s				
	72 °C	30s		72 °C	60 s	
Extension	60 °C	30'	x 1	60 °C	30 '	x 1
Hold	4 °C	hold		4 °C	hold	

Steps	F53		
	Temperature	Time	Cycling
Initial denaturation	95 °C	15s	

	94 °C	30s	
	55-50 TD (-1°C/CYC)		x 6
Denaturation	72 °C	60s	
	94	30s	
	50 °C	90s	x 44
Annealing	72 °C	60s	
Extension	60 °C	30s	x 1
Hold	4 °C	hold	

Multiplex 3

Steps	*FCA232, FCA090, F42*- F/R			*FCA279, FCA126*- F/R		
	Temperature	Time	Cycling	Temperature	Time	Cycling
Initial denaturation	95 °C	15'	x 1	95 °C	15'	x 1
Denaturation	94 °C	30 s	x 6 (-0.5 °C)	94 °C	30 s	x 11 (-0.5 °C)
	63 °C	90s		60 °C	90s	
	72 °C	60s		72 °C	30s	
Annealing	94 °C	30s	x 51	94 °C	30s	x 34
	52 °C	60s		55 °C	60s	
	72 °C	60s		72 °C	30s	
Extension	60 °C	30'	x 1	60 °C	30'	x 1
Hold	4 °C	hold		4 °C	hold	

CHAPTER 3. GENETIC DIVERSITY AND POPULATION STRUCTURE OF ROYAL BENGAL TIGERS IN BHUTAN

ABSTRACT

Bhutan is a part of the second largest Tiger Conservation Landscape (TCL): The Northern Complex Namdapha-Royal Manas and is home to a tiger population of global conservation priority. Using thirteen microsatellite loci, we genotyped 24 individuals to document for the first time the genetic characteristics of tigers in Bhutan. We found that Bhutanese tigers overall have a high genetic variation (H_e=0.75) and could play a crucial role in supplying genetic variation for the long-term persistence of tigers in the region. Population and individual-based analyses detected three genetic clusters; however, overall genetic differentiation was low. Multilocus genotypes of two individuals were similar to each other and highly divergent from all other individuals, suggesting they originated from outside of the sampled areas. Bhutan with its well-connected protected area network and a vast expanse of the forested landscape could be a connecting link between tiger populations in the Terai TCL to the North-eastern part of the Indian sub-continent. However, information on gene flow and connectivity is currently lacking. Therefore, we recommend a landscape level conservation approach using genetic advances to understand tiger dispersal and threats beyond political boundaries.

Keywords: Bhutan, eastern Himalayas, genetic diversity, gene flow, tigers

INTRODUCTION

Habitat loss and fragmentation threaten the persistence of species globally by creating small and isolated populations (Dirzo et al., 2014). Haddad et al., (2015) predict that fragmentation will reduce biodiversity by 13 to 75% in about 35 years, affecting essential

ecosystem functioning. Many mammals have already lost a substantial portion of their historical range (~50%), and vertebrate populations have declined by about 60% between 1970 and 2014 (WWF, 2018). Fragmentation events decrease the effective size and connectivity among wildlife populations causing loss of genetic variation due to genetic drift (Mills, 2015). Low genetic diversity has fitness consequences particularly in small and isolated populations making them highly vulnerable to local extirpation.

The tiger (*Panthera tigris*) is a large enigmatic carnivore inhabiting forest ecosystems in Asia and is a well-known flagship and umbrella species for biodiversity conservation (Seidensticker et al., 2010). Globally, tigers suffer from habitat loss and fragmentation, poaching and prey depletion. The species currently occupies 7% of its historical range, and less than 4000 tigers remain in the wild. Most wild tigers live in small, isolated areas within human-dominated landscapes and these local populations, mostly consisting of less than a 100, are often in danger of extinction due to demographic and genetic stochasticity (Dinerstein et al., 2007). A recent study shows that subpopulations of the Royal Bengal tiger (*Panthera tigris tigris*) in India have lost a substantial amount of genetic variation relative to historical times and reveal increased genetic differentiation among subpopulations (Mondol et al., 2013). Although tigers on the Indian sub-continent exhibit low to moderate levels of nuclear genetic variation, they hold more than 60% of the overall current genetic variation present in the species (Mondol et al., 2013, 2009c). The Indian sub-continent is also home to more than 70% of the global tiger population (Jhala et al., 2015). Therefore, Bengal tigers are very important for the future species recovery, both from demographic and genetics perspective.

Bhutan has an estimated 103 tigers, and the species is protected under the Forest and Nature Conservation Act (DoFPS, 2015b). They occur at elevations from less than 100 m in the

plains and valleys to more than 4000 m in the alpine mountains. Tigers in Bhutan are known to disperse over long distances; with the country having 72% forest cover and over 51% under protected area designation, the population is expected to be distributed continuously across the landscape and not sub-divided into discrete populations (Tempa, 2017). Though natural features such as large torrential rivers and deep gorges may impede dispersal in tigers to some extent, barriers to gene flow are minimal. Nonetheless, gene flow over limited distance can create isolation by distance (Allendorf et al., 2012) which may create a spatial genetic structure within Bhutan. Tigers are also increasingly threatened by poaching and illegal trade of skins and parts with 17 individual tiger cases recorded and prosecuted between 2015 and 2017 (NCD, 2018). Although the origin of half of the seized skins remains unknown, the numbers account for nearly 17% of the tiger population in Bhutan. With several others possibly going undetected, a further reduction in tiger numbers may affect the ability of the local population to keep inbreeding at a tolerable level. However, Bhutan is a part of the second largest Tiger Conservation Landscape (TCL)-the Northern Forest Complex Namdapha-Royal Manas, and some gene flow to and from the neighboring populations could provide a reasonable level of genetic variation for long-term persistence. A few tigers are already known to be 'transboundary' because they actively move between Manas National Park in India and Royal Manas National Park in Bhutan (Borah et al., 2013).

Information on the spatial distribution and ecology of tigers in Bhutan is now becoming available; however, there is a lack of knowledge about gene flow and structure in Bhutanese tigers. The current Tiger Action Plan of Bhutan recognizes the importance of genetic variation and population connectivity for long-term tiger conservation and to inform conservation

management decisions. Therefore, the present study aims to evaluate the amount of variation and genetic structure in the tigers of Bhutan using microsatellite markers.

MATERIALS AND METHODS

Study area and sampling

Between February and March of 2018, we carried out systematic scat sampling in Royal Manas National Park (RMNP) in southern Bhutan. Royal Manas National Park is one of the most biodiverse regions in the country. The park has an area of 1057 km^2 and the elevation ranges from <100m to 3000m (more details about RMNP in chapter 1). The samples were collected in a single session sampling by walking transects that totaled 112 km along trails. We collected the samples in plastic tubes with silica desiccants and recorded geographic coordinates.

Outside of RMNP, we also conducted opportunistic collection of fecal samples in a few pockets of Jigme Singye Wangchuck National Park (JSWNP) in the central region, Jigme Dorji National Park (JDNP) in the north-western region, biological corridor no 8 connecting JSWNP and JDNP, and three territorial forest divisions- Paro Forest Division and Thimphu Division to the west and Zhemgang Division in the south-eastern part of the country (Figure 1).

To supplement the scat samples, we also acquired 18 tiger tissue from the Department of Forest and Park Services. Sixteen were seized during illegal transactions within Bhutan, and two had died of natural causes. Only five had reliable geographic locations while it was unknown for the remaining 13 samples. We collected 2-3 g of the tissue in an air-tight plastic tube filled with silica desiccant. The samples were then stored in a -20 ° C freezer until extraction.

Laboratory methods

We extracted DNA from scats using QIAamp Fast DNA Stool Mini kit (QIAGEN Inc. Germany) following manufacturers' instructions with slight modifications. The modifications were: A) incubation of scat material in 1 ml InhibitEX buffer on a rotating shaker overnight at room temperature; B) addition of cRNA after the samples are digested with Proteinase K; C) elution in 80 µl AE buffer. cRNA was added to increase potential DNA yield from fecal samples (Kishore et al. 2006, Mondol et al. 2009, Sharma et al. 2014). We carried out DNA extraction from tissues using QIAamp DNA Blood and Tissue Kit (QIAGEN Inc. Germany) following standard kit protocols. Storage of samples, genomic DNA extraction and downstream Polymerase Chain Reactions (PCR) procedures were carried out in separate rooms to avoid cross-contamination. Negative controls were set up in every batch of DNA extraction and during setting up PCR. All the PCR reactions were performed in a 10 µL volume containing 5 µL of QIAGEN Multiplex PCR buffer mix (QIAGEN Inc. Inc. Germany), 1 µL of each 0.2 µmol/L forward and reverse primers, 0.8 µl of BSA (Bovine Serum Albumin), 1.2 µL of PCR-certified water and 2 µL DNA template.

Species identification was carried out using two tiger-specific 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 ran the PCR products on a 2% agarose gel by electrophoresis, and the bands were visualized using SYBR Safe nucleic acid gel stain (Thomas Fisher Scientific) under ultraviolet light.

We used a panel of 13 microsatellite loci (*Fca441, Pati09, Fca391, Fca628, E7, Fca672, Fca304, F53, Fca232, Fca 090, Fca42, Fca279* and *Fca126*) for individual identification and further genetic analyses. The locus set was chosen based on their polymorphic information

content, ease of amplification and low error rate reported from previous tiger studies. The primers were developed initially in the domestic cat (*Felis catus*)(Menotti-Raymond et al., 1999), and Amur tiger (*Panthera tigris altaica*) (Wu et al. 2008) and have been used successfully for studying Royal Bengal Tigers on the Indian sub-continent (Mondol et al., 2009b; Sharma et al., 2012). *Amelogenin (AMEL)* was used as the sex primer, and it amplified at 192/214-bp if it is a female and only 192-bp if it is a male (Pilgrim et al., 2005). We used tiger tissues as reference samples during the initial microsatellite standardization work. Based on their annealing temperature, allele range and amplification success during the optimization process, the microsatellite loci were grouped into three multiplexes with eight separate reactions. Thermocycling conditions for each PCR reaction are provided in the supplementary of chapter 1.

We used a multitube approach in which each scat sample was genotyped four times and each tissue sample two times to ensure reliable genotypes and to minimize genotyping errors. We ran the tissue samples across all the loci, but the tiger positive scat samples were first screened against one of the multiplexes, and samples which amplified at two loci or fewer were discarded. We then genotyped the scat samples that passed the first test with the other two multiplexes. This ensured that only good quality samples were further genotyped, saving time and laboratory costs. The PCR products were diluted to 1:50 concentration and one μL of the diluted PCR solution was run in a 3130 ABI genetic analyzer (Applied Biosystems; USA), and the raw data was scored with Geneious software.

Genetic Analyses

We used MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004) to look for the presence of stuttering errors, null alleles, and excess of homozygotes. Allele dropout and false alleles were quantified using GIMLET v 1.3.3 (Valiere, 2002).

The two replicates of tissue samples had to match at all loci to create a consensus multi-locus genotype, and in scats, at least three out of the four replicates had to match. We used Identity Test in CERVUS v 3.0 (Kalinowski et al., 2007) to match unique individuals and used a minimum of 6 out of the thirteen loci matching criteria because six microsatellites had enough power to separate individuals (P_{ID}=2.2 x 10^{-6}, $P_{ID (sibs)}$=5.9 x 10^{-3}). We used GENEPOP to check for deviations from Hardy-Weinberg proportions and 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 to a significance value (alpha=0.05). Genetic variation in terms of alleles per locus, expected heterozygosity (H_e) and observed heterozygosity (H_o) were calculated using GenAlEX (Peakall and Smouse, 2012). Pairwise F_{ST} for the two main sampling sites were estimated according to the method of Weir and Cockerham (ref from the 1990's, I think 1996) using *Hierfstat* package (Goudet and Jombart, 2006) in the statistical program R version 3.2.4 (R Development Core Team, 2014).

We used STRUCTURE (Pritchard et al., 2000), a Bayesian clustering approach to identify genetic structure in our dataset. We tested K values from 1- 6, using 100,000 steps for Markov Chain Monte Carlo (MCMC) runs and 50,000 burn-ins. We conducted ten independent runs for each value of K and 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 also conducted a Discriminant Analysis of Principal Components (DAPC) using the R package *adegenet* (version 2.0-0) (Jombart and Ahmed, 2011). 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). The method runs a principal component analysis on the genotype data and then uses it as an input for Discriminant Analysis (DA). We selected the optimal number of clusters using the BIC criterion. The lowest number of BIC was achieved for K=4, but the difference between the lowest BIC and K=3 was less than 1. So, based upon 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 to retain was determined using a cross-validation approach as implemented by the function *xvalDapc ()* with 100 repetitions. Based on the model validation literature, we used the number of PCs associated with the lowest Root Mean Squared Error (RMSE) as the 'optimum' n.pca in the DAPC analysis. For our dataset, we retained 6 PCs.

RESULTS

Individual identification and error rates

We collected 81 scat and 18 tissue samples from Bhutan in the spring of 2018. 43 scat samples were tiger positive of which 20 amplified at more than five loci. Of these, we identified eight unique individuals (five males, and three females). We obtained 16 unique individuals from the tissues (seven males and nine females) of which four were from the northwest, one from Royal Manas National Park in the south, and the remaining 11 individuals were of unknown origin. One tissue sample did not amplify and was discarded. For subsequent analysis, the population in Royal Manas National Park and the northwest part are considered separate and named as "South" and "North", respectively, and collectively as "All Bhutan." Our total sample of 24 unique individuals represents 23% of the estimated tiger population in Bhutan.

Within the tissue samples, the mean percent of allele dropout (ADO) rate and false allele (FA) were 0.15 and 0.09. The mean ADO and FA in the scat samples were 0.02 and 0.017 (Table

1). Analyses from MICROCHECKER suggested that several loci might contain null alleles, but less than 9% were detected on average.

Genetic diversity and population subdivision

The panel of 13 microsatellite loci had a high discriminatory power with the cumulative sibling probability of identity measuring 2.14×10^{-5}. All loci in the set were polymorphic, and the number of alleles ranged from 4-10 across all the samples with a mean of 6.69 ± 0.47 (Table 2). The mean H_o and H_e for the All Bhutan population were 0.6 ± 0.06 and 0.75 ± 0.03, and when all the samples were pooled together, H_o and the H_e were 0.59 ± 0.04 and 0.73 ± 0.02. Three loci (*Fca441, Fca304*, and *Fca391*) were found to be out of HWE with the Fisher exact test, after sequential Bonferroni corrections. One locus from South and three loci in the All Bhutan population significantly deviated from HW proportions. In each case, these significant departures appeared to be due to two individuals with highly distinct genotypes at all or most loci. For the loci where significant HW departures occurred, the cause of these departures was homozygosity within these two individuals for alleles that occurred in no other individuals. We did not observe any significant Linkage Disequilibrium in pairwise loci-comparisons. The overall mean F_{ST} value was found to be low (0.044 ± 0.24).

Genetic structure

We conducted a Bayesian clustering analysis using STRUCTURE without prior knowledge of sampling locations. The results indicated three genetic populations (K=3) by the mean likelihood ln P(K) and delta K value (Figure 2). We followed criteria of Singh et al., (2017) for assigning individuals to populations; individuals were assigned completely to a cluster if the posterior membership probability q>0.7 and if q<0.7, individuals were considered to be of mixed ancestry (S1). Nineteen tigers were assigned entirely and only 5 showed mixed ancestry. However, we did

not observe any specific geographic patterns from the assignment process (Figure 3). The multivariate DAPC also identified *K=3* as the optimal number of clusters according to the Bayesian Information Criterion (S2). DAPC results indicate directional gene flow from the North population to the South (Figure 4). Three individuals in the South of which one was poached, appear to have dispersed from the North. Seven unknown tissue samples were assigned to the South and four to North. Two males from the South population formed a separate cluster and suggested a recent arrival of immigrants from a population possibly from India. STRUCTURE assigned them to the South population.

There was some slight overlap in the spatial extent of the other two clusters although the centroids were separate (S3).

DISCUSSION

The present study is the first in Bhutan to assess genetic diversity and population structure of tigers in the country. Using a panel of thirteen microsatellite loci, we genotyped 24 individuals representing roughly 1/4 of the estimated tiger population in Bhutan. Although the difference in microsatellite markers and combinations used in different studies limit quantitative comparisons to other parts of Bengal tiger range, the amount of genetic diversity observed in our study (H_e=0.75) is higher than the genetic diversity in Bengal tigers overall ($H_e = 0.72$)(Luo et al., 2004) and the tigers on the Indian sub-continent ($H_e = 0.70$)(Mondol et al., 2009c). However, the landscapes in the Western Ghats ($H_e = 0.76$)(Reddy et al., 2012b) and Central India ($H_e = 0.81$)(Sharma et al., 2012) are known to have higher genetic diversity. The nearest Indian tiger populations to Bhutan for which genetic data exist is the Brahmaputra floodplains in Assam

(H_e=0.63) and the Buxa Tiger Reserve (H_e=0.67) in West Bengal (Borthakur et al., 2013, 2011). Our results suggest that tigers in Bhutan are genetically diverse.

We used individual-based and multivariate analyses to infer genetic differentiation and population sub-structuring in Bhutan. Results from both STRUCTURE and DAPC suggest the presence of 3 genetic clusters; however, the posterior membership probability of several individuals to the three clusters differed between the two methods. Our results in the present study could be influenced by sampling error due to sampling being mostly opportunistic, low sample size (n=24) and the presence of weak genetic differentiation (F_{ST} = 0.044). Limited dataset is also not ideal for identifying immigrants using Monte Carlo resampling methods such as in STRUCTURE (Paetkau et al., 2004). Low sample size tends to overestimate genetic differentiation and collecting samples too close or far away from each other fails to capture adequate genetic variation and heterogeneity in the landscape (Blakenhol and Fortin, 2016). Rule of thumb suggests sampling at least 25-30 individuals or 10% of the total population in individual-based studies using microsatellites to obtain adequate power, accuracy and precision (Hale et al., 2012).

The nationwide tiger survey using camera traps in 2015 observed apparent localization of tigers within Bhutan over a 151 day study period (DoFPS, 2015b). Tigers from the north were never camera trapped in the south and vice versa. Density also was found to differ across the landscape, suggesting heterogeneity in habitat quality. Studies suggest that low-density populations can have high migration rates and that tigers disperse from sites with low prey abundance to high-quality habitats (Joshi et al., 2013). Breeding populations in tigers are known to occur in higher quality habitats (Smith et al., 1998). The north and western part of the country reported 19 adults and 9 cubs in 2015 with a density as high as 3 tigers/100km^2 in Paro division

(DoFPS, 2015b). The presence of breeding tigers suggest that the emigration may not be necessarily influenced by habitat quality. The dispersal could be explained by a greater human presence in the area. As of 2005, 44% of the total population of Bhutan is concentrated in the western region constituting only 20% of the total land area, and around 65% of the urban population is in the western region (MoWHS 2016). The tigers cannot move northwards or further west due to the greater Himalayan mountains. Tigers are known to occupy large, remote, dense forest patches; with large ungulate abundance, and low human footprint, which best describes the landscape in the central and southeastern part of the country (Reddy et al., 2012b). Jigme Singye Wangchuck National Park, Royal Manas National Park, and Zhemgang territorial division have vast swathes of contiguous forest cover and is home to more than 50% of the entire tiger population in Bhutan. Camera trap records show evidence of breeding in this landscape as well (DoFPS, 2017, 2015b). Tigers dispersing into this region and reproducing suggest an abundance of habitat and prey. However, given our relatively small sample size, especially in the north, our results cannot be definitive that dispersal was only north to south. In fact, in 2015, a tiger from Royal Manas National Park was camera trapped in Jigme Dorji National Park to the north.

Decreased connectivity and habitat loss on the Indian sub-continent has resulted in the loss of genetic variants and increased genetic differentiation among tiger populations (Mondol et al., 2013). Over the years, human-induced landscape fragmentation has resulted in the isolation of the tiger population in the entire habitat from Terai Arc Landscape to North-East India along the foothills of the Himalayas (Sharma et al., 2011). Approximately 30% of total forest cover in Northeast India is currently under pressure of rapid land use changes, and studies report continuous forest cover loss during the last two decades (Kushwaha et al., 2018; Lele and Joshi,

2009). Bhutan shares a contiguous forested boundary with the Northern India Terai Arc landscape tiger population in Buxa Tiger Reserve and Manas National Park in North-east India. Bhutan has 72% forest cover and a well-connected protected area network. The presence of two unique individuals in the South population indicates that Bhutan could be a connecting link between tiger populations in the Terai Arc Landscape to the populations in the Northeastern part of the Indian sub-continent. Finding evidence linking the Terai Arc Landscape to the Namdapha-Royal Manas TCL and beyond could prove crucial for tiger conservation in this region. We strongly recommend a transboundary and landscape level conservation approach using common genetic data sets to understand tiger dispersal, threats and other factors influencing dispersal events. From a genetics perspective, this could involve standardization of the scoring of microsatellites that have been run for multiple studies of Bengal tigers (Aziz et al., 2018; Bhagavatula and Singh, 2006; Joshi et al., 2013; Mondol et al., 2009c, 2005; Reddy et al., 2012b; Sharma et al., 2013a, 2012; Singh, 2017; Singh et al., 2015; Thapa et al., 2018; Yumnam et al., 2014; Zachariah et al., 2017), or development of more powerful and more easily standardized Single Nucleotide Polymorphism (SNP) markers. The information shall help inform management strategies to maintain physical as well as functional connectivity in the landscape.

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Table 1. Locus-specific information on repeats, size range, and genotyping errors

| Locus | Repeat motifs | Size range (bp) | Tissues | | Scats | |
			Dropout	False allele	Dropout	False allele
FCA628	Di-nucleotide	84-114	0.1210	0.0000	0.0000	0.0000
FCA441	Tetra-nucleotide	153-183	0.0000	0.3030	0.0000	0.0000
Pati09	Tri-nucleotide	107-125	0.0000	0.4850	0.0150	0.0000
FCA391	Tetra-nucleotide	189-217	0.6060	0.0000	0.0000	0.0000
FCA672	Di-nucleotide	82-114	0.0000	0.0000	0.0000	0.0000
FCA304	Di-nucleotide	109-198	0.1820	0.0000	0.0000	0.0000
E7	Di-nucleotide	133-159	0.3640	0.0000	0.2670	0.0000
F53	Tetra-nucleotide	128-181	0.0610	0.0000	0.0000	0.0000
FCA279	Di-nucleotide	89-113	0.1210	0.0000	0.0000	0.1620
FCA232	Di-nucleotide	86-118	0.4850	0.0000	0.0000	0.0000
F42	Tetra-nucleotide	207-255	0.0610	0.0000	0.0000	0.0000
FCA090	Di-nucleotide	91-125	0.0000	0.1820	0.0000	0.0690
FCA126	Di-nucleotide	132-154	0.0000	0.2420	0.0000	0.0000
Mean			0.1539	0.0932	0.0217	0.0178
SD			0.1955	0.1534	0.0709	0.0455

Table 2. Measures of genetic diversity at 13 microsatellite loci

| | Whole Bhutan (n=13) | | | | South (n=9) | | | | Overall (n=24) | | | |
Locus	A	Ho	He	Fis	A	Ho	He	Fis	A	Ho	He	Fis
FCA628	8	0.85	0.83	0.02	8	0.89	0.83	-0.02	8	0.71	0.80	0.13
FCA441	4	0.15	0.59	0.76	4	0.22	0.65	0.69	4	0.33	0.64	0.50
Pati09	5	0.46	0.57	0.23	4	0.56	0.54	0.04	5	0.54	0.58	0.08
FCA391	4	0.38	0.69	0.47	4	0.44	0.69	0.41	5	0.38	0.65	0.44

FCA672	10	0.85	0.87	0.07	9	0.89	0.84	0.00	10	0.83	0.86	0.05
FCA304	6	0.69	0.77	0.14	5	0.56	0.72	0.28	6	0.54	0.72	0.26
E7	6	0.69	0.80	0.18	6	0.67	0.75	0.17	6	0.58	0.78	0.27
F53	8	0.92	0.84	-0.05	7	1.00	0.81	-0.17	8	0.83	0.81	0.00
FCA279	5	0.54	0.77	0.34	4	0.44	0.72	0.43	6	0.63	0.79	0.23
FCA232	6	0.54	0.63	0.18	5	0.56	0.62	0.17	7	0.50	0.59	0.17
F42	7	0.67	0.78	0.19	7	0.63	0.80	0.28	7	0.65	0.73	0.13
FCA090	5	0.36	0.74	0.54	4	0.43	0.68	0.44	6	0.50	0.71	0.32
FCA126	7	0.75	0.80	0.11	6	0.75	0.76	0.08	9	0.70	0.78	0.12
Mean	6.23	0.60	0.75	0.24	6.23	0.62	0.72	0.21	6.69	0.59	0.73	0.21
SE	0.48	0.06	0.03	0.22	0.48	0.06	0.02	0.23	0.47	0.04	0.02	0.14

A, Alleles per locus; Ho, Observed Heterozygosity; He, Expected Heterozygosity; Fis=Inbreeding coefficient

[2] Significant values for Hardy-Wienberg equilibrium following Bonferonni correction (alpha=0.05)

Figure 1. Map of Bhutan showing protected area network. Red dots show sample collection

sites. RMNP, Royal Manas National Park; BWS, Bumdeling Wildlife Sanctuary; JDNP, Jigme

Dorji National Park; JSWNP, Jigme Singye Wangchuck National Park; JWS, Jumotshangkha

Wildlife Sanctuary; PWS, Phibsoo Wildlife Sanctuary; SWS, Sakteng Wildlife Sanctuary; PNP,

Phrumsengla National Park; JKSNR, Jigme Khesar Strict Nature Reserve; WCNP, Wangchuck
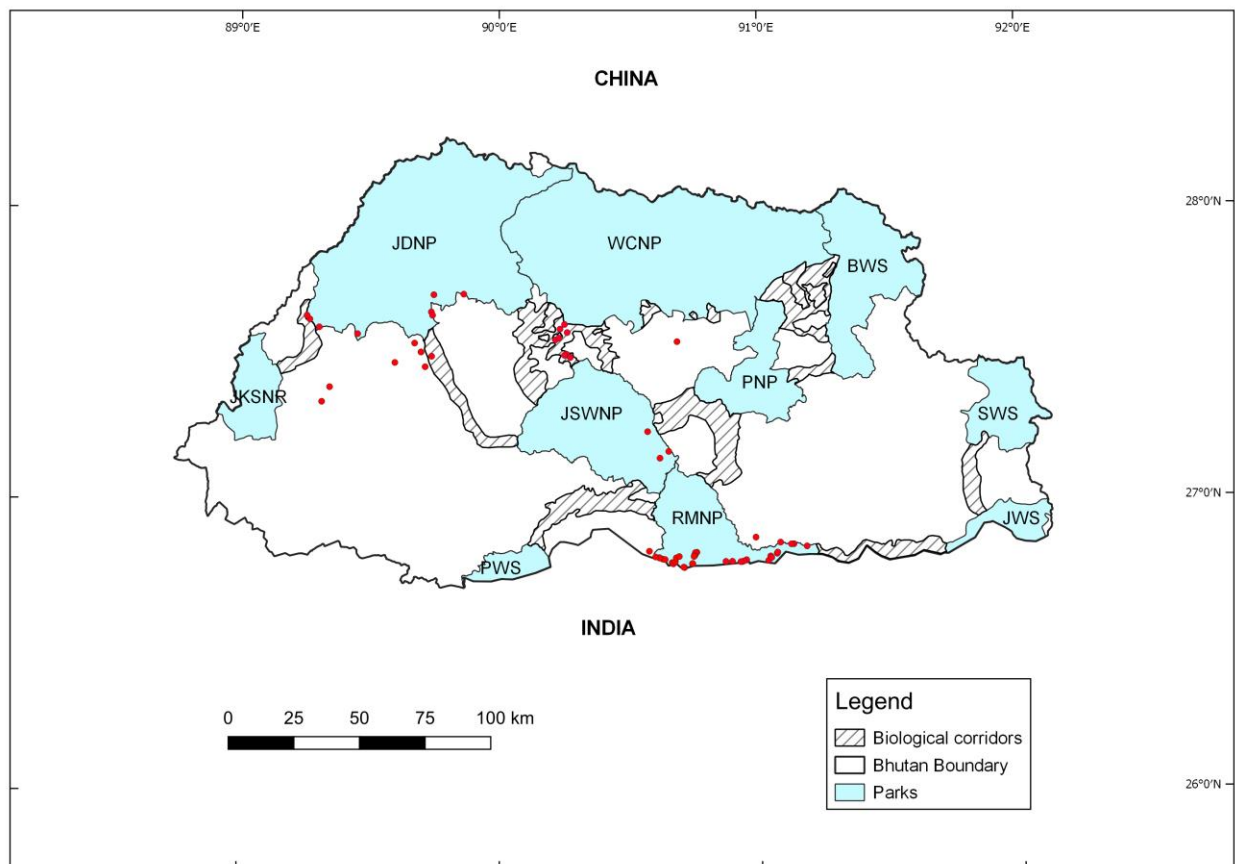
Centennial National Park

Figure 2. The average rate of change in the log-likelihood (ΔK) over 10 replicated runs, as a function of the number of clusters (K).

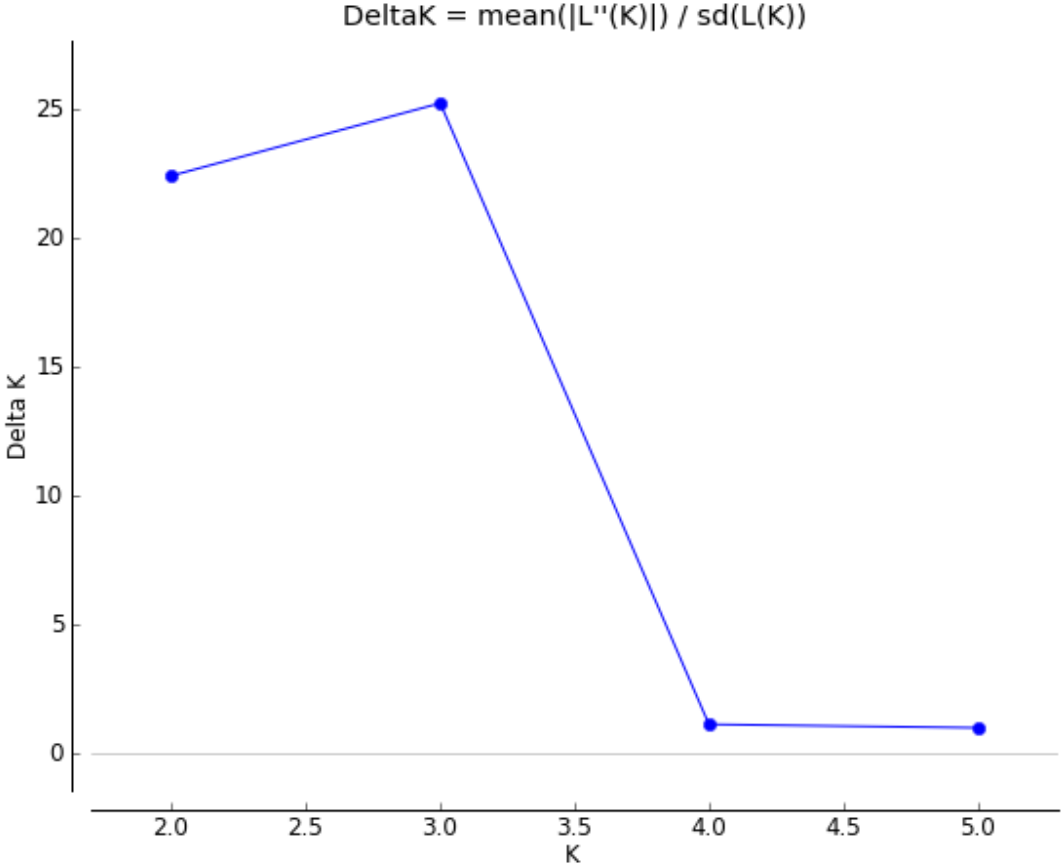DeltaK = mean(|L''(K)|) / sd(L(K))

Figure 3. The output from STRUCTURE at k=3. Each line in the bar plot represents an
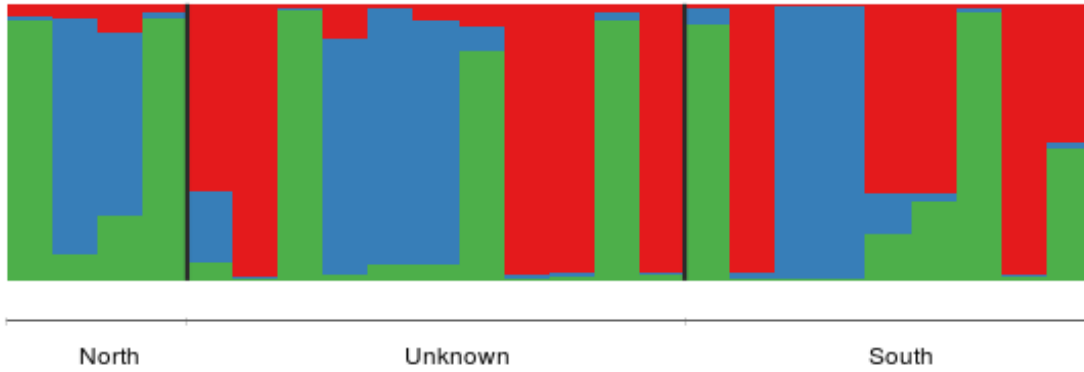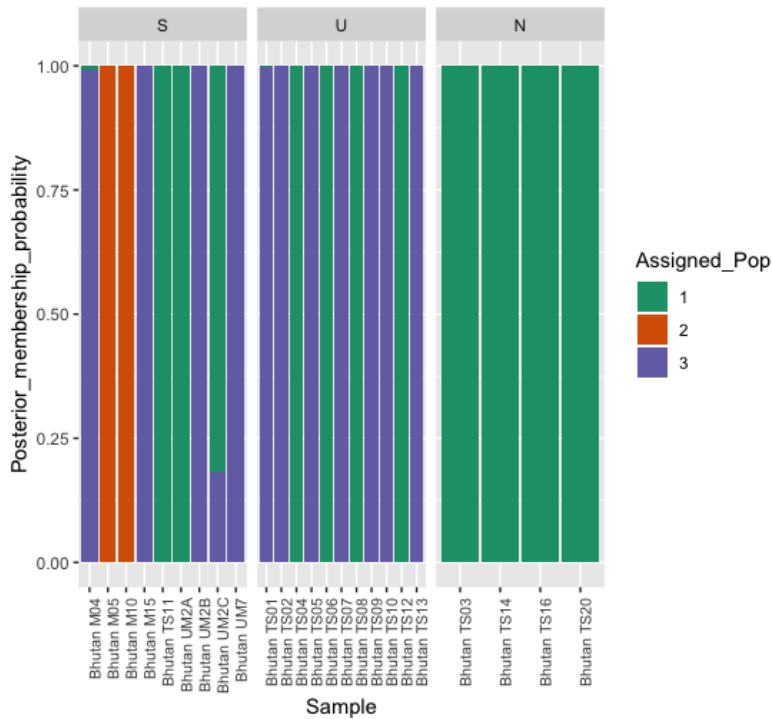
individual tiger

Figure 4. Individual assignments across populations (k=3). Each line in the bar plot represents

an individual tiger. S=South, N=North, U=Unknown

SUPPLEMENTARY MATERIALS

S 1. Table showing inferred ancestry of individuals from STRUCTURE at k=3. If the posterior

membership probability p<0.7, they are considered to be of admixed ancestry.

Sample ID	Posterior membership probability			Cluster
TS03	0.043	0.015	0.942	Cluster 3
TS14	0.051	0.853	0.096	Cluster 2
TS16	0.104	0.659	0.237	Admixed
TS20	0.033	0.017	0.95	Cluster 3
TS01	0.676	0.256	0.068	Admixed
TS02	0.983	0.009	0.008	Cluster 1
TS04	0.012	0.01	0.979	Cluster 3
TS05	0.126	0.853	0.022	Cluster 2
TS06	0.012	0.929	0.059	Cluster 2
TS07	0.062	0.879	0.059	Cluster 2
TS08	0.084	0.085	0.832	Cluster 1
TS09	0.981	0.01	0.008	Cluster 1
TS10	0.972	0.014	0.014	Cluster 1
TS12	0.03	0.029	0.941	Cluster 3
TS13	0.97	0.011	0.02	Admixed
TS11	0.015	0.058	0.928	Cluster 3
UM7	0.974	0.017	0.009	Cluster 1
M05	0.006	0.99	0.005	Cluster 2
M10	0.006	0.989	0.005	Cluster 2
M04	0.688	0.141	0.171	Admixed
M15	0.686	0.031	0.283	Admixed

UM2A	0.016	0.011	0.973	Cluster 3
UM2B	0.977	0.013	0.011	Cluster 1
UM2C	0.503	0.017	0.48	Admixed

S2. Bayesian Information Criterion (BIC) as a function of the number of clusters (K).
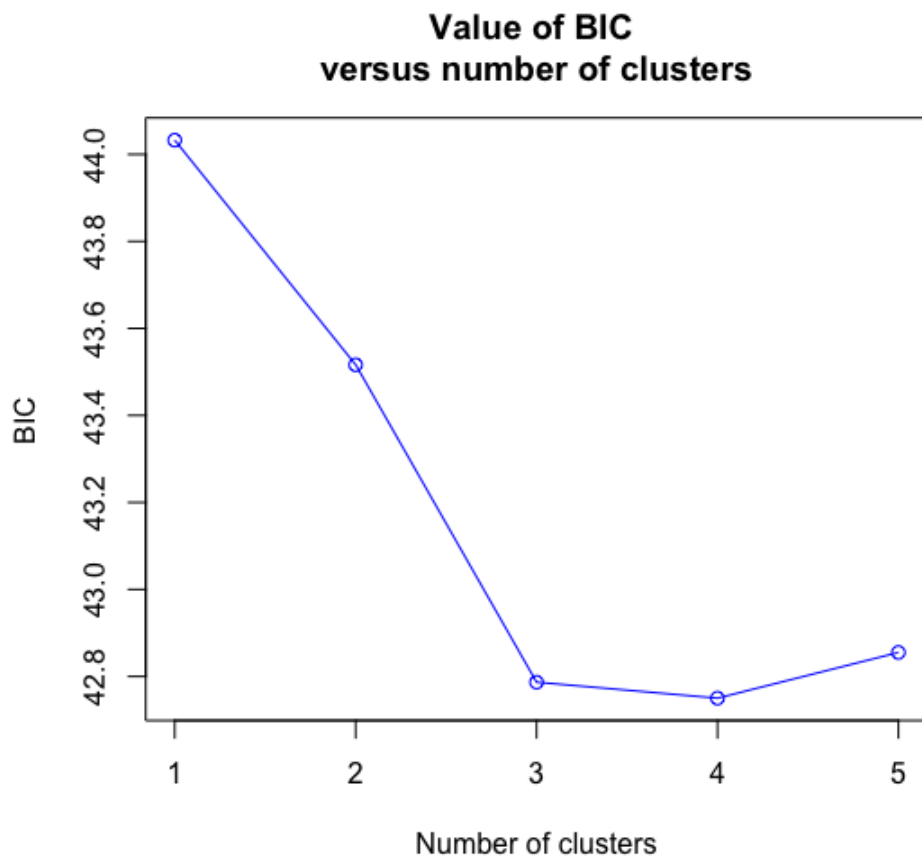
S3. Scatterplots from discriminant analysis of principal components (DAPC) reveal three genetic clusters. Each dot represents one individual.