Gene flow connects key leopard (*Panthera pardus*) populations despite habitat fragmentation and persecution

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Abstract

The leopard (*Panthera pardus*) is facing the threat of continued population decline across its range. In order to inform more effective conservation management programs, genetic information is needed from leopard populations that persist in previously unstudied, isolated and highly fragmented protected areas. The aim of this study was to explore the population structure and genetic diversity of leopard populations across the Mpumalanga province of South Africa. We collected a total of 33 leopard samples from four major locations along a west to east transect across the province. We analysed 17 polymorphic microsatellites and two regions of the mitochondrial genome (NADH-5 and Cytb) to determine the genetic structure of the leopard population in the province. We also calculated genetic diversity indices and explored gene flow in the region. We found that while there is gene flow occurring across the province, the population was genetically structured. We identified two major population units that we describe as 'West Mpumalanga' and 'East Mpumalanga'. Gene flow was moderate between the two populations and we found very high genetic diversity levels compared to other leopard populations previously studied in South Africa. From a conservation perspective, our results show that gene flow is still occurring across seemingly isolated leopard populations that exist in fragmented landscapes, highlighting the importance of all leopard populations in South Africa. Management authorities need to focus conservation efforts on maintaining corridors between regions that are suitable for leopard occupancy and work closely with human settlements to minimise human-leopard conflicts.

Keywords: Leopard; Gene flow; Connectivity; Mpumalanga; Population genetics; Conservation

Introduction

Large carnivores play an important role in maintaining and regulating healthy ecosystems (Miller et al. 2001; Ripple et al. 2014). They have direct effects on large herbivore species through predation and meso-carnivores through intraguild competition (Ripple et al. 2014). Currently, 64% of large carnivore species worldwide are threatened with extinction while 80% of species show declining population trends (Wolf and Ripple 2018). Large carnivores are particularly susceptible to the threat of extinction as they generally persist at low densities, have large home ranges and require large continuous areas of natural habitat to hunt and reproduce (Cardillo et al. 2005). As the human population continues to grow, natural habitats are being rapidly destroyed for new agricultural and urban developments. This has led to the loss of both suitable habitat and widespread habitat fragmentation. Habitat fragmentation impedes normal animal dispersal by introducing both physical (large distances between suitable habitat, farms, fences etc.) and anthropogenic barriers (lethal persecution/snares as a result from human wildlife conflicts) (Schlaepfer et al. 2018).

Animal dispersal is a critical factor in maintaining biodiversity as it is the process underlying gene flow and genetic exchange between populations. Gene flow is an important evolutionary process as it maintains or replenishes the genetic diversity of a population (Lenormand 2002). Restricted gene flow results in population differentiation, with the distribution of genetic variation becoming limited and compartmentalised across the landscape (Moodley et al. 2017). If a population experiencing restricted gene flow is also declining in size, detrimental genetic processes such as the loss of alleles due to genetic drift and the accumulation of deleterious mutations through inbreeding can occur (Andersen et al. 2004; Keyghobadi 2007). These processes will eventually lead to a net loss of genetic diversity and decreased population viability due to a reduction in fitness and adaptive potential (Keyghobadi 2007). Reduced genetic diversity can decrease a population's resistance to disease (Spielman et al. 2004) and reduced fitness can manifest itself as changes in birthweights, longevity and predation success rates (Keller and Waller 2002).

Population genetic studies examining the genetic structure of target species and gene flow between identified populations have thus become a crucial element in the development and implementation of successful conservation programs (Abdul-Muneer 2014; Kenney et al. 2014; Moodley et al. 2018). Defining the genetic structure of an animal population involves determining (i) the number of existing subpopulations, (ii) the connectivity between these subpopulations and (iii) the allele frequencies within these subpopulations (Chakraborty 1993; Mondol et al. 2013; Zanin et al. 2016). This information can be used to draw boundaries and delineate evolutionarily significant or management units, their levels of genetic diversity, degree of isolation or connectivity and inbreeding.

One country in which the genetics of large carnivores has been studied extensively is South Africa. Although this African republic still harbours much of its large carnivore biodiversity, its highly developed agricultural sector and growing human population mean that the ranges of these species are mostly confined to government and private conservation areas. It is therefore not surprising that species such as lion (*Panthera leo*), cheetah (*Acinonyx jubatus*) and African wild dog (*Lycaon pictus*) have already started to exhibit the detrimental genetic consequences of habitat fragmentation and subsequent isolation (Kotze et al. 2008; Marsden

et al. 2012; Miller et al. 2015). The South African leopard (*Panthera pardus pardus*) is a large carnivore that is unique in that only 8.4% of its extant range exists inside of protected areas (Jacobson et al. 2016). Due to their shy and elusive nature, leopards can live in close proximity with humans and move undetected across large distances (Fattebert et al. 2013). Therefore, it is possible leopards do not display the same genetic patterns of the other carnivores.

Several studies have explored the diversity and structure of both nuclear (microsatellite) and mitochondrial DNA (mtDNA) in southern African leopard populations (Anco et al. 2018; McManus et al. 2015; Ropiquet et al. 2015; Uphyrkina et al. 2001). Early mtDNA analysis revealed two maternal lineages among leopards across Africa, with both lineages observed in South Africa (Uphyrkina et al. 2001). Nuclear DNA studies using microsatellites reflect a degree of isolation-by-distance (IBD) across southern Africa (Ropiquet et al. 2015), with local structure observed between the Western and Eastern Cape Provinces (McManus et al. 2015). McManus et al. (2015) also estimated low to moderate gene flow connecting subpopulations within the Western and Eastern Cape. Yet, despite their importance to the conservation and management of leopards (Naude et al. 2020), gene flow and connectivity between populations have not been extensively studied in South Africa.

The province harbouring one of the largest leopard populations in South Africa, Mpumalanga, has not yet been studied in depth. Mpumalanga is located in north-eastern South Africa and contains some of the largest wild areas in the country, including the Kruger National Park, which also forms part of the Greater Limpopo Trans-frontier conservation agreement. Its main distinguishing feature is a major west–east altitudinal drop as the highveld plateau gives way to the low-lying plains (lowveld), via the Drakensburg mountain escarpment. Mpumalanga thus contains three major biomes, highveld grassland (61% of land area), lowveld savannah (39%) and escarpment forest (0.5%) (Ferrar and Lötter 2007). Human land use across this province predominantly involves mining, manufacturing and agriculture, and these industries have led to habitat fragmentation and the repurposing of 36% of the natural habitat (Ferrar and Lötter 2007). Detailed land use maps for Mpumalanga have been published in Ferrar and Lötter (2007); Lötter (2015); Simpson et al. (2019). Despite this, approximately 34% of Mpumalanga is still considered suitable habitat for leopard occupancy (Swanepoel et al. 2013), with a leopard population estimated between 338 and 1851 individuals (203–1111 mature individuals) (Swanepoel et al. 2014, 2016). This combination of anthropogenic pressure and large numbers of free-ranging large carnivores has brought humans and leopards into closer proximity, resulting in a higher occurrence of human-leopard conflicts (Balme et al. 2010), with real or perceived threats to livelihood (livestock and game) and human safety often resulting in persecution killings of leopards.

Given the persistence of significant leopard numbers in this diverse and fragmented landscape, one might expect reduced gene flow owing to either geographic barriers (e.g. altitude or isolation by distance) or anthropogenic factors, leading to increased population structuring due to genetic drift. Understanding the genetic properties of the current leopard population of Mpumalanga is essential for its conservation and management. The aims of this study were therefore to determine: (i) whether Mpumalanga leopards were genetically structured into subpopulations (ii) the genetic diversity of these subpopulations and (iii) whether subpopulations were connected by contemporary gene flow.

Materials and methods

Samples and study area

In order to characterise leopard population structure, diversity and gene flow across Mpumalanga's dramatic highveld-lowveld altitudinal gradient, 33 leopard samples were collected from four sampling locations along a roughly west–east transect across the northern part of the province (Fig. 1). In the highveld region, samples were collected from Loskop Dam Nature Reserve (LDNR) (n = 13) and the Lydenburg district (n = 3). LDNR has an altitude that ranges between 1285 and 1406 m while Lydenburg sits at approximately 1414 m. In the lowveld region, we sampled at Andover Nature Reserve (ANR) (n = 3) and Manyeleti Nature Reserve (MNR) (n = 12). While ANR (altitude 508 m) is fenced, nearby MNR (altitude 350–450 m) is a 23,000 hectare reserve that forms part of the Greater Kruger National park and is open to Kruger National Park (KNP).

All capture procedures were approved by the Animal Ethics Committees of the University of Adelaide (S-2016-023) and The University of Pretoria (V115-16) and permission to conduct the study was obtained from Mpumalanga Tourism and Parks Agency (MTPA, Project Approval TS3/11). Thirteen LDNR samples were collected from captured animals using single door cage traps which are activated by either a foot plate or a release mechanism placed on bait. Traps were approximately 90 cm wide, 110 cm tall and 1200 cm long. Traps were set up next to roads and game paths often utilised by leopards, based on the results of extensive camera trapping that was simultaneously conducted on the reserve. A South African Veterinary Council registered veterinarian anaesthetised the animals by darting using a combination of zolazepam and tiletamine, at 1–3 mg/kg (Zoletil, Virbac, Centurion, South Africa) and medetomidine at 0.05–0.09 mg/kg (Medetomidine, Kyron Laboratories, Johannesburg, South Africa). Reversal of the anaesthesia was accomplished by intramuscular injection of atipamezole at 0.25–0.45 mg/kg (Antisedan, Zoetis, Sandton, South Africa) (Kock and Burroughs 2012).

Whole blood was collected in EDTA tubes and stored by both freezing at approximately − 20 °C and on Whatman FTA cards (Merk, Darmstadt, Germany). Three additional whole blood samples from Lydenburg were collected opportunistically by the MTPA during standard management practices. In addition, twelve blood samples from MNR and three from ANR were collected as part of the annual census. Two skin samples were collected from post-mortems on recently deceased leopards found on Manyeleti NR and two skin samples were collected from confiscated leopard skins that were found in villages bordering ANR and MNR. The exact origins of the confiscated skins are unknown, but are very likely to have come from ANR, MNR or KNP.

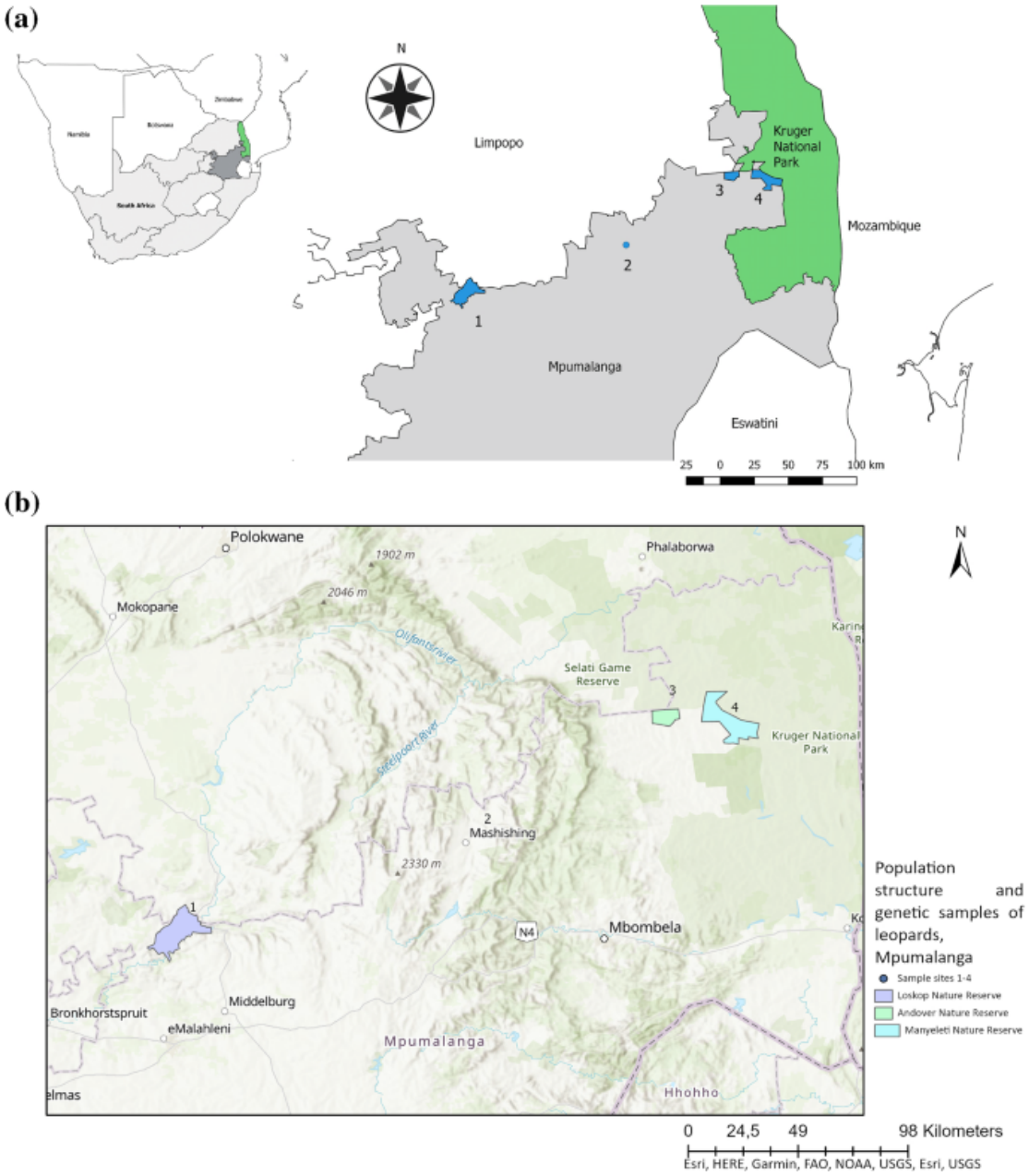

Fig. 1. The study area of Mpumalanga Province, South Africa. **A** Mpumalanga Province relative to the rest of South Africa. **B** Sampling areas along a west–east gradient across Mpumalanga's highveld and Lowveld regions. Blue shading represents the four sampling locations: Loskop Dam Nature Reserve (1), Lydenburg (2), Andover Nature Reserve (3) and Manyeleti Nature Reserve (4)

Molecular methods

DNA was extracted from the defrosted blood samples using a Quick-DNA Miniprep Plus Kit D4068 (Zymo Research, CA, USA). Eighteen polymorphic microsatellites were selected for analysis based on their use in previous leopard studies in South Africa (McManus et al. 2015; Ropiquet et al. 2015; Uphyrkina et al. 2001). The markers [FCA008, FCA032, FCA075, FCA077, FCA082, FCA085, FCA129, FCA133, FCA161, FCA191, FCA211, FCA224, FCA229, FCA261,

FCA310, FCA391, FCA441 & Y2F1-T3-4] were first isolated in the domestic cat (*Felis catus*) and were all found to be polymorphic in leopards (Menotti-Raymond et al. 1999).

Microsatellites were amplified via polymerase chain reaction (PCR) using the Q5 Hot Start High-Fidelity Kit (New England Biolabs, MA, USA) according to the manufacturer's specifications. Reactions were contained in a total volume of 25 µL comprising 1.25 µL each of forward and reverse primers, 1.0 µL of extracted template DNA, 12.5 µL master mix (M0494S) and 9 µL of nuclease free water (Amresco E476). Thermocycling included an initial denaturation for 30 s at 98 °C followed by 35 cycles of denaturation/annealing/elongation for 10 s at 98 °C, 30 s at 60 °C and 20 s at 72 °C followed by a final extension for 5 min at 72 °C. PCR products were genotyped on an ABI Prism 3500XL. Allele scoring was conducted on SoftGenetic GeneMarker (v 2.6.7) and double checked by eye.

Additionally, two mitochondrial DNA markers were sequenced for all samples. NADH dehydrogenase 5 (NADH-5) and Cytochrome-b (Cytb), were selected for amplification based on their successful use in previous leopard studies (Ropiquet et al. 2015). NADH-5 was amplified using the primers F/RL2 (Uphyrkina et al. 2001) and Cytb was amplified using the primers Leo-F/Leo-R (Ropiquet et al. 2015). PCR was performed in 25 µL reactions comprising 0.5 µL of each forward and reverse primer, 1.0 µL of extracted DNA, 12.5 µL OneTaq Quick-Load 2X Master mix with buffer M0486 (New England Biolabs, MA, USA) and 10.5 µL of nuclease free water (Amresco E476). Thermocycling included an initial denaturation for 30 s at 94 °C followed by 35 cycles of denaturation/annealing/elongation for 30 s at 94 °C, 30 s at 50 °C and 1 min at 68 °C followed by a final extension for 2 min at 68 °C. Amplicons were post-PCR purified with 2.5 µL exonuclease I (Exo I) and 5 µL shrimp alkaline phosphatase (rSAP) (New England Biolabs, MA, USA) according to the manufacturers instruction. The purified amplicons were then sequenced in both the forward and reverse directions using the BIG Dye Kit (Applied Biosystems, Thermo Fisher Scientific, CA, USA) and electrophoresed through an ABI PRISM3500xl Genetic Analyser using a 50 cm array and the POP-7 polymer (Applied Biosystems, Thermo Fisher Scientific, CA, USA). Sequences were checked by eye, then forward and reverse sequences were aligned into contigs, consensus bases were called and trimmed in BioEdit (v7.0.5.3) (Hall 1999). The final NADH-5 alignment was 426 bp in length (positions 12,632–13,058 on the complete mtDNA genome of *Felis catus*, Accession number: U20753) (Lopez et al. 1996), while the Cytb alignment was 1137 bp in length (positions 15,039–16,176 on the *Felis catus* mtgenome).

Microsatellite structure

Microsatellites were analysed in MICROCHECKER (v2.2.3) (Van Oosterhout et al. 2004) to quality check the data and ensure that there were no genotyping errors, allele dropout or null alleles present in the dataset. FSTAT (v2.9.4) (Goudet 1995) was used to test for linkage disequilibrium and deviations from Hardy–Weinberg equilibrium were detected in GENEALEX (6.503) (Peakall and Smouse 2012). The genetic structure of leopards across our Mpumalanga transect was initially analysed using the program STRUCTURE (v.2.3.4) (Pritchard et al. 2000). STRUCTURE uses a Bayesian approach to identify the most likely number of populations (*K*) observed within a dataset by clustering multi-locus genotypes together that share similar patterns of variation (Porras-Hurtado et al. 2013). We modelled up to 10 potential population clusters (*K*) with a Markov chain length of 1,000,000 iterations and a burn-in period of

100,000. We used the admixture model with correlated allele frequencies as the leopard populations across Mpumalanga could have been in recent genetic contact and likely share common ancestors. The number of populations was determined using the programs STRUCTURE HARVESTER (v.0.6.94) (Earl and Vonholdt 2012) and CLUMPAK (Kopelman et al. 2015), following *K*-selection methods described by Evanno et al. (2005). In addition, we used discriminate analysis of principal components (DAPC) to infer genetic structure (Jombart et al. 2010). This model-free multivariate method does not assume Hardy–Weinberg equilibrium nor linkage disequilibrium. The method was implemented in the R-package 'ADEGENET' (Jombart 2008) for $K = 1–10$. We also implemented a spatial approach to explore genetic structure across the Mpumalanga landscape using GENELAND (v4.0.7) (Guillot et al. 2005). Parameters in GENELAND were set to model $K = 1–10$, with 10 repetitions for each *K*, for 1,000,000 MCMC iterations. Finally, we also tested the microsatellite data for patterns of IBD using a Mantel test of geographic and genetic distance matrices, conducted in GENEALEX.

Mitochondrial DNA structure

The maternal genetic structure among Mpumalanga leopards was also examined. Since DNA sequence data for the same markers were also available for 16 samples from the nearby Kruger National Park (Ropiquet et al. 2015), we downloaded these data (GenBank accession NADH-5: JF720216-224, JF720234-236, JF720270-274; Cytb: JF720084-20092, JF720103-105, JF720139-142) and included them in our mtDNA analyses. The leopard samples we sequenced for these markers were uploaded to GenBank under the following accession numbers (NADH-5: OQ132962-992; Cytb: OQ117400-430). Initially, mtDNA genetic structure was examined by constructing minimum spanning haplotype networks from the concatenated mtDNA alignments in POPART (v.3.2) (Leigh and Bryant 2015). To further determine genetic structure, we reconstructed a phylogenetic tree using the program BEAST (Bayesian Evolutionary Analysis by Sampling Trees) (v.2.5.2) (Bouckaert et al. 2014). Three outgroup taxa were added to the sample set comprising three lion sequences (Genbank accession numbers KP001498, KP001502 & KP001506) and Asiatic leopard subspecies *P.p fuscia* and *P.p japonesis* (Genbank accession numbers EF199742, EF199743 & KJ866876). A nucleotide substitution model for each partition (alignment) was selected by first analysing the sequence data in JmodelTest (v2.1.10) (Darriba et al. 2012). The selected models were HKY + G for both mitochondrial genes alignments based on BIC (Bayesian information criterion) calculations. Heterogeneity in mutation rates was therefore modelled using a gamma distribution with four bins. A Relaxed Clock Log Normal model was selected because its posterior standard deviation for the mean clock rate did not include zero, implying that a strict clock was not appropriate for this data set. A Markov chain of length 100,000,000 was used to explore tree space, sampling a tree every 10,000 iterations, and discarding 15% as burn-in. TRACER (v1.7.1) (Rambaut et al. 2018) was used to check that the Markov chain had reached stationarity. Nodes that were not supported by a posterior probability > 50% were collapsed using TreeGraph 2 (v.2.15.0) (Stover and Muller 2010).

Genetic diversity

Genetic diversity for microsatellite loci was calculated using GENEALEX. Diversity was examined using the average number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E) and the inbreeding coefficient (F_{IS}). Pairwise estimates of

fixation index (F_{ST}) were calculated between the defined population groups to infer the degree of differentiation between them. MtDNA diversity indices included haplotype diversity, nucleotide diversity and the number of polymorphic sites and were calculated in DNASP (v.6.12.01) (Rozas et al. 2017). We also conducted neutrality tests, Tajima's D and Fu's fs statistic, to explore the likelihood of recent demographic changes.

Gene flow

Gene flow was inferred using microsatellite data. A rough measure of past levels of gene flow was calculated from F_{ST} using the formula $(1/F_{ST} - 1)/4$ (Wright 1984). Contemporary, and bidirectional gene flow between structured populations was inferred using a Bayesian approach implemented in BIMr (Bayesian Inference of Migration rates) (v1.0) (Faubet and Gaggiotti 2008). We ran the program using the F-model (correlated allele frequencies) using a Markov chain of 500,000 iterations and a burn-in period of 20,000 iterations. We ran five Markov chains in total, checked that they had converged in TRACER, then averaged the complementary gene flow between populations from each run as detailed in Epps et al. (2013).

Results

Null alleles were flagged at three loci [FCA224, FCA075, Y2-F1-T34], however only Y2-F1-T34 contained more than 25% null alleles and was omitted from any further analysis (Oosterhout > 0.25). Therefore, a total of 17 loci were included in all further analyses. No loci were found to be statistically linked nor significantly deviated from Hardy–Weinberg expectation ($p > 0.05$), after correction for multiple testing. MtDNA could not be amplified from the two dried skin samples and thus, were not included in the mtDNA analysis.

Microsatellite genetic structure

The highest number of population clusters (Evanno et al. 2005) detected by STRUCTURE was five ($K = 5$), suggesting that biologically meaningful population structure could potentially exist at $K2 \geq 5$ (Figs. 2, S6). At $K = 2$, the LDNR samples from the highveld separated from all the others into a western Mpumalanga group, with the eastern Mpumalanga group containing all other highveld and lowveld samples. At $K = 3$, the three Lydenburg samples from the highveld grouped together with one sample from ANR and another from MNR. At $K = 4$, the emergence of a 4th grouping comprised mostly of samples from MNR, but with one sample from LDNR (green). At $K = 5$, a further cluster was observed among the lowveld samples from ANR and MNR. In general, however, the STRUCTURE analysis highlighted several potentially admixed leopard genotypes at all locations as K increased (Figs. 2, S6).

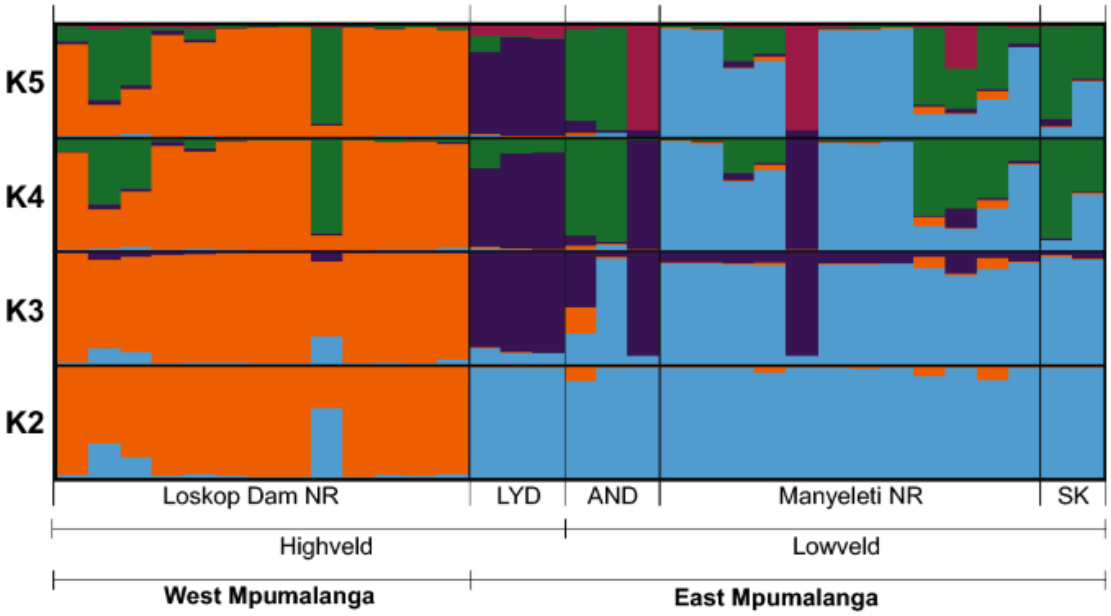

Fig. 2. The structure of genetic variation among leopards in a west–east transect across Mpumalanga Province, South Africa. Each genotype is represented by a single line in the plot and the contributions of different populations (*K*) are colour-coded. Models for population clustering range from 2 to 5 (*K*2–*K*5). Regions where samples were obtained are Loskop Dam Nature Reserve, Lydenburg (LYD), Andover Nature Reserve (AND), Manyeleti Nature Reserve (MNR) and confiscated leopard skin samples that were obtained from villages located on the border of the greater Kruger region (SK). A consensus of three methods suggested the greatest separation is between West Mpumalanga and East Mpumalanga nuclear genetic populations

In contrast, model free DAPC clustering fitted the samples most optimally into two populations, with individual grouping consistent with the STRUCTURE results of $K = 2$ (Figs. S1–S3). Our landscape analysis with GENELAND, which takes spatial information into account, suggested a best fit into three geographic populations ($K = 3$,) (Figs. S4, S5, Table S1). The only difference between this and $K = 2$ was the separation of the three Lydenburg samples, two ANR and three MNR samples into a third population, similar to the $K = 3$ result from STRUCTURE. The landscape analysis described a similar trend to both STRUCTURE and DAPC analyses, in that the LDNR population was always partitioned separately from samples collected in eastern Mpumalanga (Lydenburg, ANR, MNR). We also tested our microsatellite data for patterns of IBD, and found only a weak correlation between geography and genetics with a correlation co-efficient (Rxy) of 0.32, suggesting that IBD only accounts for 10% of the genetic structure observed among Mpumalanga leopards (Sample Size = 33, No. in Matrix = 528, p-value = 0.01) (Fig. S7). We, hereafter, considered the Mpumalanga landscape to be broadly divided into two main nuclear genetic populations inhabiting the eastern and western parts of the province. We define population 1 as 'West Mpumalanga' which consists of all thirteen LDNR samples and population 2 as 'East Mpumalanga' which contains all samples collected from Lydenburg, ANR and MNR.

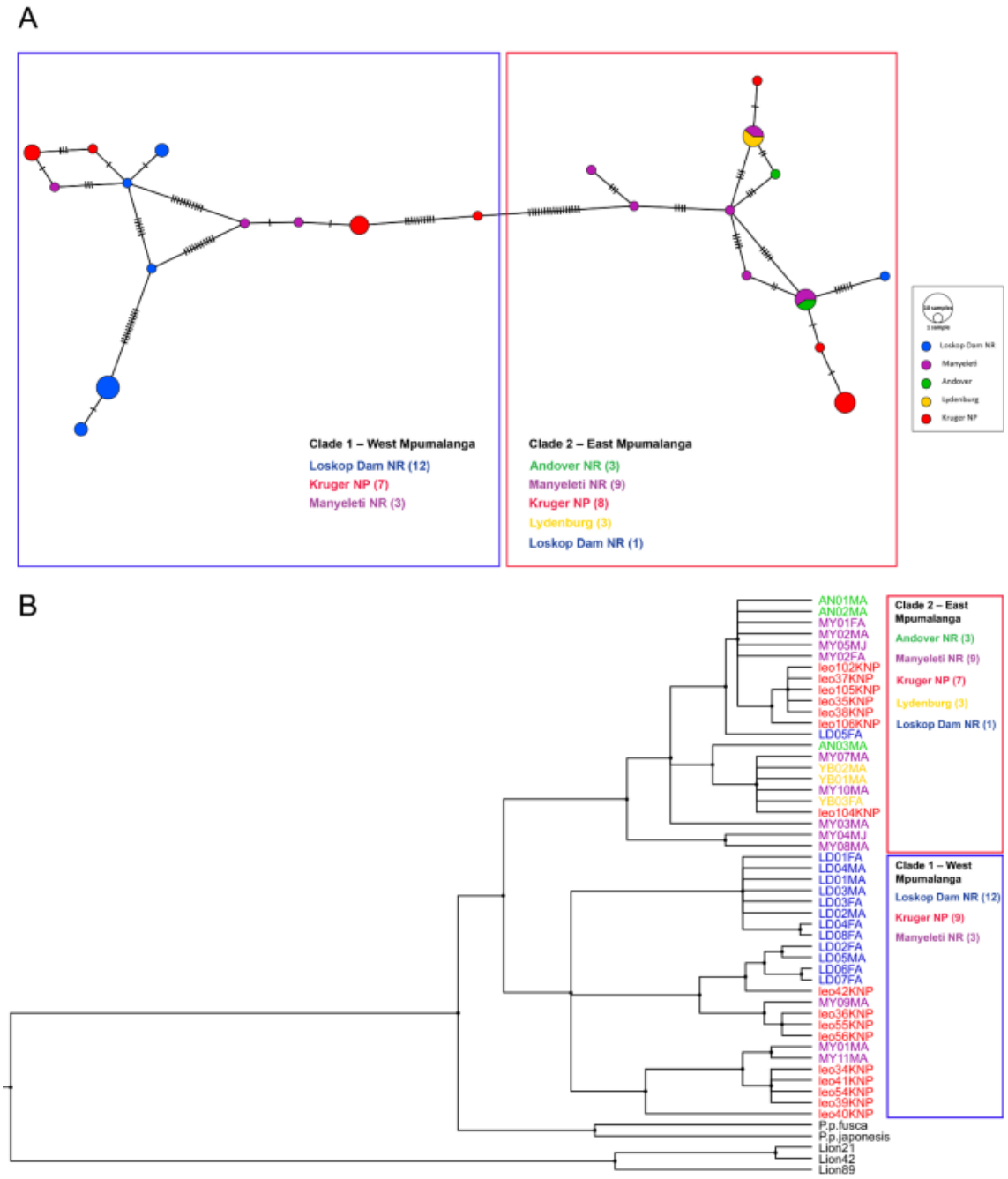

Fig. 3. Maternal genetic structure of leopards in Mpumalanga Province, South Africa. **A** Median spanning haplotype network based on NADH dehydrogenase 5 (NADH-5) and Cytochrome-b (Cytb) mtDNA sequences. Two major clades are identified in West Mpumalanga and East Mpumalanga. **B** Phylogeny showing the structure leopard mtDNA haplogroups. The tree was constructed in BEAST. Nodes with < 50% posterior probability were collapsed. The samples were coloured-coded according to sampled populations: Blue—Loskop Dam Nature Reserve, Yellow—Lydenburg, Green—Andover Nature Reserve, Purple—Manyeleti Nature Reserve, Red—Kruger National Park. Two major clades were identified, East Mpumalanga and West Mpumalanga

Mitochondrial genetic structure

The median spanning haplotype network of the concatenated NADH-5 and Cytb alignments showed considerable maternal structure across Mpumalanga (Fig. 3A). All haplotypes were structured into two prominent haplogroups, which we call here, East and West Mpumalanga. A Bayesian phylogenetic tree rooted with Asiatic leopard and lion confirmed this structure (Fig. 3B) and showed that while no sampling location constituted a monophyletic clade, all but one Loskop sample occurred in the East Mpumalanga clade. The East Mpumalanga clade is made up of all the samples from Lydenburg and the majority of the lowveld samples (61.3%). The West Mpumalanga clade consists of the remaining 38.7% lowveld samples and almost all of the samples from Loskop (92.3%).

Genetic diversity

Genetic diversity indices were calculated for populations 'West Mpumalanga' and 'East Mpumalanga' as defined by our genetic structure analyses. Nuclear genetic diversity was reasonably high, with observed and expected heterozygosity > 0.7 for both West and East Mpumalanga populations, as well as the entire data set, with mildly negative inbreeding coefficients (Table 1). West Mpumalanga displayed slightly fewer alleles per locus than East Mpumalanga.

Table 1. Genetic diversity of leopard populations in Mpumalanga, South Africa

	n	$H_o \pm SEM$	$H_e \pm SEM$	F_{IS}	$A \pm SEM$	PA	F_{ST}
Total	33	0.755 ± 0.025	0.735 ± 0.015	-0.027	6.44 ± 0.28	4.76	0.056
West Mpumalanga	13	0.787 ± 0.038	0.718 ± 0.023	-0.096	5.59 ± 0.38	3.15	–
East Mpumalanga	20	0.722 ± 0.031	0.751 ± 0.020	0.039	7.29 ± 0.31	5.80	–

Diversity indices are given for the entire provincial sample (total) and for the two major subpopulation samples identified in analyses of genetic structure (East and West Mpumalanga)

N number of samples, H_0 observed heterozygosity, H_e expected heterozygosity, F_{IS} inbreeding coefficient, *A* number of alleles averaged for all loci, *PA* private alleles, F_{ST} pairwise population comparison

We also explored genetic diversity using the mitochondrial data following the same population definitions (West and East Mpumalanga as above). However, for mtDNA diversity, the addition of the sixteen KNP samples were included in the 'East Mpumalanga' population grouping. The East Mpumalanga population was more diverse, containing 16 haplotypes compared to only six within the West Mpumalanga population. Similarly, haplotype diversity, nucleotide diversity and the number of polymorphic sites were also higher in the East Mpumalanga clade (Table 2). Tajimas D returned low and non-significant values.

Table 2. Table of mtDNA genetic diversity statistics for the concatenated NADH-5 and Cytb genes in leopard populations in Mpumalanga Province, South Africa

	No of samples	No. of haplo-types	Haplotype diversity (hd)	Nucleotide diversity (Pi)	Poly-morphic sites	Tajima's D	Fu's Fs
West Mpumalanga	13	6	0.782	0.00765	37	−0.010	4.332
East Mpumalanga	34	17	0.930	0.01073	44	1.596	1.430
Total	47	23	0.948	0.1266	52	2.301	0.919

Diversity statistics are given for the entire provincial population (total) and for the two major subpopulations indented (East and West Mpumalanga). Diversity analyses performed are *hd* haplotype diversity, *Pi* nucleotide diversity and the tests of neutrality Tajima's D and Fu's Fs

Gene flow

Both methods for calculating nuclear gene flow showed genetic connectivity between the West Mpumalanga and East Mpumalanga leopard populations. Using pairwise F_{ST}, which is a rough estimate of long-term rate of migration, the rate of exchange was approximately three individuals per generation (3.09). However, although bidirectional Bayesian estimates of gene flow were similarly high, there was a marked difference in directionality. Migration from western to eastern Mpumalanga (3.3%) was almost three times lower than the rate of migration in the opposite direction (9.7%), suggesting a greater net movement of leopards from east to west across Mpumalanga (Table S2).

Discussion

This is the first analysis of leopard population genetic structure occurring across the ecologically diverse landscape of Mpumalanga Province, South Africa. Our results show that while there is fine scale structuring occurring in the province, these subpopulations remain connected as there is gene flow occurring between them. This supports the findings of other studies that conclude the leopard population within Southern Africa comprises a continuous metapopulation but is subject to genetic structuring at a regional scale (McManus et al. 2015; Ropiquet et al. 2015; Spong et al. 2000). Leopard genetic structuring has also been found to occur at the wider African continental scale (Anco et al. 2018).

The structure of leopard populations across Mpumalanga

Our results provide compelling evidence for the structuring of leopard genetic variation across the landscape of northern Mpumalanga. In all analyses, nuclear genetic markers consistently partitioned the leopards of LDNR as separate to the rest of the province. The pairwise Fst between the two structured populations was moderate (0.056), implying some degree of differentiation, but with significant gene flow connecting the two populations. However, the partitioning of other subpopulations at greater values of *K*, only within East Mpumalanga, suggests that this region could harbour other pockets of leopard diversity. The fact that three highveld samples taken at Lydenburg always separated at $K = 3$ also implies some degree of distinctiveness around the escarpment region, at the interface between highveld and lowveld.

This nuclear population structure was further supported by even more pronounced maternal structuring of mtDNA genes. Both analyses of mtDNA structure partitioned the majority of LDNR samples (92.3%) into their own West Mpumalanga clade separate to the ANR and Lydenburg samples. A major difference however, between the mtDNA and microsatellite structure results, is that leopard samples from MNR and KNP shared haplotypes with both East and West Mpumalanga clades. This observation is consistent with other mtDNA studies of southern African leopards (Anco et al. 2018; Ropiquet et al. 2015).

Potential explanations for this observed structure of leopard populations across Mpumalanga include the large altitudinal gradient separating the two halves of the province. However, as samples from Lydenburg (highveld) consistently partitioned within the East Mpumalanga clade and not with LDNR (also highveld) samples in the West Mpumalanga clade, it is unlikely that genetic differences are attributable to altitude. The observation of lowveld samples from ANR and MNR portioning together with distant Lydenburg at *K*3 and within the East Mpumalanga mtDNA clade, strongly implies that the altitudinal difference between high- and lowveld does not pose a barrier to gene flow in leopards. Another explanation could be that localised dispersal and genetic drift resulted in a gradient of isolation by distance across the region. In this situation, patchy sampling across the landscape could give the illusion of two distinct population groups. However, we found that only 10% of the observed structure can be attributed to IBD. This finding, together with the high number of lowveld haplotypes from KNP and MNR clustering in the West Mpumalanga clade suggest that both populations could have diverged in historical isolation and have since come into more recent secondary genetic contact via gene flow across the Drakensberg escarpment. However, a larger nuclear and mitochondrial data set of South African and other African leopards would be required to test this hypothesis.

Connectivity of leopard populations across Mpumalanga

While mtDNA haplotype sharing could hint at population connectivity of leopard populations between western and eastern Mpumalanga, it is also possible that the lack of mtDNA regional monophyly stems from the presence of ancestral haplotypes, which are thus still present in both West and East clades because of incomplete lineage sorting. Microsatellites, on the other hand, are rapidly evolving and therefore more conducive for analyses of recent gene flow and contemporary population connectivity (Feulner et al. 2004; Teske et al. 2018). We found that, despite habitat fragmentation due to human activities, leopard populations in Mpumalanga remain connected to each other by contemporary gene flow. Leopard generation time is approximately 6–7 years (Balme et al. 2013), and at a minimum, one migrant between each population every generation is required order to maintain genetic diversity (Wang 2004). In the case of naturally occurring populations, which are subject to various environmental conditions and species-specific factors, up to ten migrants per generation has been suggested as a requirement to maintain maximal levels of genetic diversity (Mills and Allendorf 1996). Here, we show that more gene flow (9.7%) occurred from East Mpumalanga to West Mpumalanga than in the reverse direction (3.3%). A net influx of contemporary genetic diversity into West Mpumalanga from East Mpumalanga could be due to the high density of leopard occurring in the greater Kruger region and the distribution of leopard-suitable habitat in the province. The high density of leopards in KNP, which was estimated to be 12.7–30.9 (Bailey 1993; Maputla et al. 2013) individuals per 100 km^2, may be

what drives this east to west connectivity, as leopard density and competition is significantly lower at 8 individuals per 100 km^2 in Loskop Dam Nature Reserve (Morris et al. 2021). These microsatellite-derived gene flow estimates therefore complement the mtDNA results, demonstrating that non-monophyly in both West and East Mpumalanga mtDNA clades is likely due to gene flow, rather than incomplete lineage sorting.

Genetic diversity

The heterozygosity levels we recorded for Mpumalanga leopard populations are the highest reported in South Africa to date. All values, whether for the entire province or West or East Mpumalanga populations, returned observed heterozygosity values of between 0.72 and 0.78, which are significantly higher ($H_O \pm 2SE$) than reported values from the Western Cape (0.624–0.657), Eastern Cape (0.646–0.657) and KwaZulu-Natal (0.638–0.660) or Mozambique (0.687) (McManus et al. 2015; Ropiquet et al. 2015). The heterozygosity of Mpumalanga leopards was thus more in line with East African leopards (Ho Tanzania = 0.77) (Spong et al. 2000), than other southern African leopards. Although these values were compared against different, and in some cases significantly fewer loci, we are still confident that our data set of 18 polymorphic microsatellites is comparable, since loci were chosen specifically to overlap with all previous studies. The average number of microsatellite alleles was higher among leopards in East Mpumalanga, and consistent with the equivalent statistic in our mtDNA data set (number of haplotypes, Table 2), which was much higher in the East clade. The East Mpumalanga clade also displays more polymorphic sites and higher haplotype diversity comped to the West Mpumalanga clade. These similarities in patterns of genetic diversity between microsatellite and mtDNA data sets also strongly hints at the evolutionary equivalence of the observed microsatellite populations and mtDNA clades. Whether genetic structuring and patterns of diversity are similar in other parts of South Africa where leopards are still free-ranging (such as Limpopo, North-West and Northern Cape Provinces) is presently unknown, however, we show here that even opportunistically collected samples that are patchily distributed across the landscape can shed considerable light on the structure and diversity of leopard populations.

Mpumalanga leopards display relatively high genetic diversity in part due to the gene flow that is occurring between East and West Mpumalanga clades, but there is also likely to be gene flow occurring between neighbouring provinces. LDNR is closely located to large portions of leopard suitable habitat located in southern Limpopo that stretches and connects much of the Limpopo province (Swanepoel et al. 2013). The Eastern Mpumalanga population unit is highly connected to other regions in the lowveld such as KwaZulu-Natal, KNP, Mozambique and Limpopo, through conservation agreements such as the Greater Limpopo trans frontier conservation agreement. A detailed map of this suitable leopard habitat across South Africa is available to view in Swanepoel et al. (2013). We suggest that while there is still gene flow occurring across the east–west Mpumalanga transect, this connectivity is probably not the only contribution to the maintenance of high genetic diversity of Mpumalanga's leopards.

Conservation outlook and conclusion

While genetic diversity within the sampled Mpumalanga populations remains relatively high, it can also decrease rapidly within generations if gene flow is impeded (Kotze et al. 2019). Anthropogenic landscape use and human-leopard conflicts have the potential to impede gene flow. Currently only 14.8% of the land cover in Mpumalanga is protected (Ferrar and Lötter 2007). Additionally, 19.3% of the Mpumalanga land cover is made up of cultivated areas for farming purposes, 1.0% used for mining and 2.8% in urban areas (Lötter 2015). The land along the northern transverse of the province that links our studies' sample sites has been classified as predominantly grazing and poorly adapted cultivated land (Simpson et al. 2019). There is also highly adapted cultivated land found directly neighbouring LDNR and the again to the east just before the KNP region (Simpson et al. 2019). This brings leopards into direct contact with humans where conflicts, such as livestock predation, often occur. In South Africa, leopards accounted for between 40 and 89% of livestock attacks across the country (Constant et al. 2015; Thorn et al. 2012). Landowners, therefore, often view leopards negatively (Grey et al. 2017), with 98% of interview participants from communities that border the KNP perceiving predators as a major threat to their livestock (Lagendijk and Gusset 2008), and 67% of farmers have used lethal methods to resolve conflicts with leopards (Thorn et al. 2012). It is estimated that a maximum of 169 leopards are removed from the Mpumalanga province annually either from retaliatory killings or translocation of damage-causing individuals (Swanepoel et al. 2014). Anthropogenic leopard mortalities are also attributed to motor vehicle accidents, legal hunts and snares outside of protected areas (Swanepoel et al. 2015). In order to conserve or potentially increase gene flow throughout Mpumalanga, naturally occurring leopard dispersal corridors in Mpumalanga and to neighbouring provinces need to be identified and preserved. On a national scale, stringent regulation of the trophy hunting industry and the implementation of non-lethal control regulations for damage causing individuals, to maintain or improve enhance diversity, should be investigated.

In order to implement effective leopard conservation management on a national scale, genetic data for other unsampled parts of South Africa that have been identified as important leopard habitat regions (i.e. Limpopo, Northern Cape and North West provinces) (Swanepoel et al. 2013) are required. Furthermore, as each local study continues to conduct genetic research in isolation, microsatellite genetic data sets are often not directly comparable due to differences in markers used and the continued practice of scoring alleles by fragment size rather than repeat number.

We have also identified that these previously unstudied leopard populations contain a high level of genetic diversity and remain interconnected to neighbouring regions and reserves despite being surrounded by a high density of human settlements. This finding helps highlight, that even seemingly isolated leopard populations persisting in fragmented landscapes, are important to the overall conservation management of the species. These findings also have wider implications for other carnivore and large mammal species across Mpumalanga as it shows despite the challenges, animals are still moving between protected areas of the region.

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Contributions

All authors had input to the overall project research design. Field work research and sample collection was performed by DRM, JC, GS, FP & JW. JW contributed genetic samples for analysis. Data analysis was performed by DRM & YM. The paper was written by DRM with large input from YM, TJM & WSJB.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

Ethical approval was received from the University of Adelaide Animal Ethics Committee (S-2016-023) and the permit to conduct research on Loskop Dam Nature Reserve was given by the provincial municipality Mpumalanga Tourism and Parks Agency (TS3/11).

Consent for publication

All authors give their consent to the publication of this article.

Data availability

All mtDNA strands have been uploaded to Genbank under the following accession numbers; NADH-5 : OQ132962-OQ132992 & Cytochrome B : OQ117400-OQ117430. Microsatellite data were uploaded to Figshare (https://doi.org/10.6084/m9.figshare.21743852); There are no restrictions on data availability.

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