

# Genetic Restoration of Black Rhino In South Africa: Conservation Implications

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## Research Article

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# Abstract

Globally, wildlife populations are becoming increasingly small and isolated. Both processes contribute to an elevated risk of extinction, notably due to genetic factors related to inbreeding depression and a loss of adaptive potential. Wildlife translocation is a valuable conservation tool to reintroduce species to previously occupied areas, or augment existing populations with genetically divergent animals, thereby improving the viability of endangered populations. However, understanding the genetic implications of mixing gene pools is key to avoid the risk of outbreeding depression, and to maximise translocation effectiveness. In this study we used mitochondrial and microsatellite DNA collected from 110 black rhinos (*Diceros bicornis minor*) in Kruger National Park, South Africa, to determine levels of genetic diversity, inbreeding and relatedness. We compared this diversity with the two source populations (KwaZulu-Natal, South Africa and Zambezi River, Zimbabwe) using data from previously published studies, and assessed changes in the relative contribution of source lineages since their reintroduction in the 1970s. Our results show that Kruger's black rhinos are genetically more diverse than those from KwaZulu-Natal, with levels closer to those from the Zambezi Valley. Furthermore, our findings indicate a relative increase in the Zimbabwean lineage since reintroduction, suggesting a possible selective advantage. From a conservation perspective, our results demonstrate the benefits of mixing multiple source populations to restore gene flow, improve genetic diversity and thereby help protect small, isolated populations from extinction.

## Introduction

Compared to their historical counterparts, many wildlife populations remaining today are small and isolated with limited gene flow. These populations may exhibit an increased population differentiation and reduced genetic diversity as a function of genetic drift and/or inbreeding (Lacy 1987; Primack 2002). Negative genetic impacts are intensified in fenced populations, as mechanisms that evolved to reduce inbreeding, such as dispersal, are inhibited. Thus, a key challenge in wildlife conservation is to prevent or minimise the loss of genetic diversity to enable the long-term persistence of threatened species (Weeks et al. 2011; Keller et al. 2012).

Reintroductions and translocations are important conservation tools used to increase the effective population size, maintain genetic diversity (Hedrick and Fredrickson 2010; Heber et al. 2013), reduce inter-population differentiation (Thavornkanlapachai et al. 2019) and improve the geographic range of endangered species (Armstrong and Seddon 2008). Translocations that simulate the immigration of genetically divergent individuals (Tallmon et al. 2004) have successfully improved the demographic performance of populations of wildlife species such as adders (*Vipera berus*, Madsen et al. 1999), Florida panthers (*Puma concolor coryi*, Johnson et al. 2010), and bighorn sheep (*Ovis Canadensis*, Poirier et al. 2019). However, despite its potential benefits, mixing highly differentiated populations that lack local adaptations may render translocations counterproductive, as it may lead to reduced survival or fitness in the offspring (outbreeding depression; Templeton 1986; Edmands 2007; Frankham 2015; Ralls et al. 2018).

The black rhinoceros ('black rhino', *Diceros bicornis*) epitomises the threats facing many endangered species. The south-central black rhino (*D. b. minor*) historically occurred from Tanzania, through Zambia, Zimbabwe and Mozambique to the north-eastern parts of South Africa (Emslie and Adcock 2016). However, wide-scale poaching and unregulated trophy hunting reduced the south-central black rhino in southern Africa to two remnant populations, one in the KwaZulu-Natal region of South Africa, and the other in the Zambezi Valley and Sebungwe regions along the Zambezi River in Zimbabwe (Cumming et al. 1990; Emslie and Brooks 1999; Emslie 2012). The drastic reduction in population size and geographic isolation resulted in extremely low genetic diversity within the KwaZulu-Natal black rhinos, with only a single mitochondrial DNA haplotype remaining (Anderson-Lederer et al. 2012; Kotzé et al. 2014). The Zambezi River black rhino population retained substantially more genetic variation - possibly because of a milder bottleneck event - with six mitochondrial DNA haplotypes identified thus far (Kotzé et al. 2014). Black rhinos remain vulnerable due to relentless poaching for their horns (Amin et al. 2006; Ferreira et al. 2015, 2017; Knight 2016) and range expansion continues to be a conservation priority in South Africa (SANParks 2002; WWF-South Africa 2020).

Black rhinos were declared extinct in the Kruger National Park, South Africa (24°0'41"S, 31°29'7"E; Kruger), in the 1930s after decades of hunting eradicated the population (SANParks 2002). A new population was founded during the 1970s and 1980s with reintroductions from both the KwaZulu-Natal and Zambezi River source populations (Hall-Martin and Knight 1994; Ferreira et al. 2011). This founder population provides a unique opportunity to explore the outcome of mixing two source population gene pools. In this study, we used genetic data from 110 black rhinos to (i) quantify the genetic variation and relatedness and (ii) evaluate any changes in the proportion of source population ancestry present in the founded Kruger population. Finally, we compared the genetic diversity between the Kruger black rhinos with published estimates of both source populations. Describing the diversity and lineage composition of the Kruger black rhinos allowed us to evaluate the impact of this population admixture and provide information necessary for optimising the recovery and growth of this subspecies throughout its range.

## Methods

### *Study area and population history*

The Kruger National Park (Kruger) is situated on the eastern side of Limpopo and Mpumalanga provinces of South Africa and covers an area of 19,485km<sup>2</sup> (Ferreira et al. 2011), extending 360km from North to South and 90km from East to West at its widest part (Fig. 1; Foxcroft et al. 2008). Between 1971 and 1989 a total of 81 black rhinos were reintroduced into Kruger (SANParks 2002). These black rhinos originated from the Zambezi River, Zimbabwe (n=14) and KwaZulu-Natal, South Africa (n=67). In 2009, the population was estimated at 627 black rhinos living in southern Kruger (Ferreira et al. 2011), making it the largest south-central black rhino population in Africa.

### *Samples and genetic data*

Blood samples (n=110; female = 60, male = 50) were collected from black rhinos in southern Kruger (Fig. 1) from 2014–2019 during various management interventions, performed in accordance with South African National Parks (SANParks) Wildlife Capture Standard Operating Procedures. Sex, date and location were recorded at the time of capture. DNA was extracted using DNeasy Blood & Tissue Kits (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. A fragment of the mitochondrial DNA control region was sequenced by ZooOmics™ (Inqaba Biotec, Pretoria) using primers mt15996L (5'-TCCACCATCAGCACCCAAAGC-3'; Campbell et al. 1995) and mt16502H (5'-TTTGATGGCCCTGAAGTAAGAACCA-3'; Moro et al. 1998). Individuals were genotyped by ZooOmics™ using the standard rhino forensic panel in South Africa (Harper et al. 2013) comprised of 23 microsatellite markers (Supplementary Table S1).

### *Genetic analysis*

#### i. Quality control

Control region sequences were edited in Mega v 10.0.5 (Kumar et al. 2018) after visually inspecting individual chromatograms using Chromas v 2.6.6 (Technelysium Pty Ltd, Australia). The sequences were aligned using Clustal W (Larkin et al. 2007) as implemented in Mega. Published control region mtDNA sequences of south-central black rhino from the Zambezi River (Zambezi Valley and Sebungwe region), Zimbabwe and KwaZulu-Natal (South Africa) source populations were included in a combined dataset (GenBank accession numbers AF187825-AF187831, Brown & Houlden 2000; JN593089 (n=64); Anderson-Lederer et al. 2012; KM095529-KM095627, Kotzé et al. 2014; KY472322-KY472346, Moodley et al. 2017).

Genotype profiles were checked for genotyping errors, allele dropout and null alleles using MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004). Null allele frequencies were estimated using FREENA (Chapuis and Estoup 2007). Frequencies of null alleles may cause moderate (>0.08; Chapuis and Estoup 2007) to significant (>0.20; Dakin and Avise 2004) bias in F-statistics, and thus loci with null alleles with a frequency greater than 0.08 were removed from further analysis. Genepop v 4.7.2 (Raymond and Rousset 1995) was used to test for deviations from Hardy–Weinberg equilibrium (HWE) for each locus and genotypic linkage disequilibrium (LD) between loci. All probability tests were based on Markov Chain Monte Carlo (MCMC) default parameters (burn-in steps = 10,000; 100 batch runs; 1,000 iterations). Sequential Bonferroni correction was applied to determine significance thresholds for HWE and LD to account for multiple comparisons.

#### ii. Diversity

Genetic diversity within the mtDNA control region sequences was assessed by calculating nucleotide diversity ( $\pi$ ), haplotype diversity ( $h$ ) and the number of unique haplotypes present. These diversity metrics were calculated in DnaSP v 6 (Rozas et al. 2017). Microsatellite diversity was inferred from the range and number of alleles per locus ( $N_a$ ), observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ; Nei 1978). The effective number of alleles per locus ( $A_e$ ), i.e., the number of alleles one would expect in a

population with the same heterozygosity but with an equal distribution of allele frequencies (Crow and Maruyama 1971), was also calculated. All calculations were performed in GenAEx 6.5 (Peakall and Smouse 2012).

### iii. Relatedness and inbreeding

Average pairwise relatedness ( $r$ ) for the population was calculated using the package 'related' (Pew et al. 2015) in R version 3.6.1 (R Core Team 2019). The estimator of relatedness chosen for this analysis was based on a simulation analysis comparing different estimators using the 'compareestimators' function. Given similar performance, the Wang (2002) estimator (having the highest correlation between observed and expected values) was chosen for further analysis. Pairwise relatedness was calculated using the 'coancestry' function. Wright's inbreeding coefficient ( $F_{IS}$ ) was calculated using GENETIX 4.05.2 (Belkhir et al. 2004). Confidence intervals for inbreeding coefficient values for each locus and over all loci in each population were obtained by bootstrapping 1000 times.

#### *Source and founder population comparison*

The combined mtDNA sequence dataset was used to compare the nucleotide ( $\pi$ ) and haplotype ( $h$ ) diversity between source (Zambezi River and KwaZulu-Natal) and founder (Kruger) populations. The genetic structure among source and founder haplotypes in the combined dataset was visualised by constructing a median-joining network (Bandelt et al. 1999) in PopART (Leigh and Bryant 2015). Finally, the relative maternal lineage contributions of the source populations were determined and compared to the initial ratio of Zambezi River and KwaZulu-Natal founder females obtained from historic records (SANParks, unpublished data).

## Results

### *Quality control*

No evidence of genotyping errors or allelic dropout was found. Signatures of null alleles were detected at seven loci, namely SR74, IR12, SRS262, 7C, BIRh1B, DB44 and DB66; loci with null allele frequencies greater than 0.08 (SR74, IR12, SRS262, 7C and BIRh1B) were removed from subsequent analysis. ZF1 (Zinc Finger locus; Peppin et al. 2010) was used to confirm individual sex. Seven of the total of 254 combinations of paired loci were in linkage disequilibrium after applying a Bonferroni correction ( $P < 0.0001$ ; Supplementary Table S3). Loci IR12 and SR74 were removed as they appeared to be sex-linked. Locus 12F (originally isolated from white rhino) and locus BIRh37D were removed from further analysis. Loci 7B and 32A, both originally isolated from white rhino, were monomorphic in this study and were also removed. Finally, individuals with more than 30% missing data ( $n=1$ ) were removed from further analysis.

#### *Diversity, relatedness and inbreeding*

The final mtDNA dataset contained 103 sequences of 469 bp in length. Four haplotypes were identified. These haplotypes were characterised by five polymorphic sites, all containing transition nucleotide substitutions (G↔A and/or C↔T). Haplotype diversity ( $h$ ) and nucleotide diversity ( $\pi$ ) were 0.48 ( $\pm$  0.05 SD) and 0.29 ( $\pm$  0.20 SD), respectively. The final microsatellite data set comprised 109 animals. All 13 microsatellite loci retained for analyses were polymorphic, with two to 14 alleles each (Table 1). The effective number of alleles ( $A_e$ ) ranged from 1.3 to 5.03 (mean =  $2.26 \pm 0.25$  SE) alleles per locus. Expected heterozygosity ( $H_e$ ) varied greatly among loci, ranging from 0.23 (SR281) to 0.80 (DB66) and averaged 0.51 across all loci. Mean pairwise relatedness ( $r$ ) was -0.03. Population level  $F_{IS}$  was 0.04 (95% CI -0.01- 0.07) (Table 1).

**Table 1** Genetic diversity of the Kruger black rhino population across 13 microsatellite loci

Locus	$N_a$	$A_e$	$H_o$	$H_e$	$uH_e$	$F_{IS}$ (95% CI)
<b>DB23</b>	3	2.01	0.54	0.50	0.50	-0.07 (-0.26-0.13)
<b>DB1</b>	2	1.99	0.44	0.50	0.50	0.11 (-0.09-0.29)
<b>DB52</b>	5	2.08	0.51	0.52	0.52	0.02 (-0.15- 0.17)
<b>32A</b>	5	1.88	0.41	0.47	0.47	0.12 (-0.02- 0.24)
<b>SR281</b>	2	1.30	0.25	0.23	0.23	-0.07 (-0.18-0.09)
<b>IR22</b>	2	1.98	0.47	0.50	0.50	0.05 (-0.15- 0.22)
<b>BIRh1C</b>	5	2.16	0.56	0.54	0.54	-0.04 (-0.14- 0.08)
<b>SR63</b>	4	2.53	0.59	0.60	0.61	0.04 (-0.11- 0.17)
<b>DB44</b>	6	1.71	0.35	0.41	0.42	0.16 (-0.03- 0.33)
<b>BR6</b>	8	2.66	0.68	0.62	0.63	-0.09 (-0.18-0.00)
<b>DB66</b>	14	5.04	0.73	0.80	0.81	0.10 (-0.01- 0.19)
<b>SR268</b>	5	2.30	0.53	0.57	0.57	0.06 (-0.09- 0.19)
<b>IR10</b>	3	1.71	0.41	0.41	0.42	0.02 (-0.12- 0.17)
<b>Mean</b>	4.92	2.26	0.50	0.51	0.52	0.04 (-0.01- 0.07)
<b>SE</b>	0.90	0.25	0.04	0.04	0.04	

$N_a$  number of alleles,  $A_e$  number of effective alleles,  $H_o$  observed heterozygosity,  $H_e$  expected heterozygosity,  $uH_e$  unbiased expected heterozygosity,  $F_{IS}$  inbreeding coefficient

*Source and founder population comparison*

The combined Kruger, Zambezi River and KwaZulu-Natal mtDNA control region dataset comprised 296 sequences of 363 bp after alignment. A total of seven mtDNA haplotypes were found across the three populations, containing seven polymorphic sites (Table 2). The nucleotide and haplotype diversities calculated in the Kruger population was approximately mid-way between those of the two source populations (Table 2). The relationship between haplotypes can be seen in Fig. 2. The Kruger population shared two haplotypes (H1 and H5) with the Zambezi River population; these two haplotypes together represented 28.15% of the Kruger black rhino in this study. The extant KwaZulu-Natal population is represented by only a single haplotype (H2), and this haplotype was shared with 67.96% of the Kruger population. Haplotype 3 (H3) was unique to Kruger, two mutational steps from both H2 and H5 (Fig. 2). Haplotypes 4, 6 & 7 were unique to the Zambezi River population. The current haplotype distribution among the three populations is illustrated in Fig. 3.

**Table 2** Mitochondrial genetic diversity among black rhino populations in Zambezi River, Zimbabwe, KwaZulu-Natal and Kruger National Park, South Africa

Population	n	#H	#P	HD (SD)	$\pi$ (SD)	Origin
Zambezi River, Zimbabwe	104	6	7	0.77	0.70	Kotzé et al. (2014)
KwaZulu-Natal, South Africa	95	1	1	0*	0*	Anderson-Lederer et al. (2012); Kotzé et al. (2014); Moodley et al. (2017)
Kruger National Park, South Africa	103	4	5	0.48 ( $\pm 0.05$ )	0.29 ( $\pm 0.20$ )	This study

n is the number of individuals sampled, #H number of haplotypes, #P number of polymorphic sites, h haplotype diversity,  $\pi$  nucleotide diversity, \*no variation

The ratio of female black rhino founders introduced into Kruger from 1971-1989 from the two source populations was 5: 1 (KZN: ZIM). The lineage ratio found in the current Kruger population was 2.41: 1 (KZN: ZIM), indicating approximately a two-fold increase in the proportion of Zimbabwean ancestry since reintroduction (Fig. 4).

## Discussion

The Kruger black rhino population offered a unique opportunity to evaluate the outcome of mixing the two remnant south-central black rhino source populations. Using mtDNA and microsatellite markers, we found that mixing gene pools substantially enhanced both the mitochondrial and nuclear diversity of the founded Kruger population relative to the source *D. b. minor* population in South Africa. Together with the low levels of relatedness and no evidence of non-random mating, our results confirm that the Kruger black rhino population is a diverse, outbred, panmictic population. This study provides a baseline for

informing black rhino metapopulation management strategies and indicates that Kruger black rhinos would be ideal candidates for translocation and reintroduction efforts aimed at improving diversity in other *D. b. minor* black rhino populations.

Maintaining adequate levels of genetic diversity is essential for ensuring both the short-term health and long-term survival of isolated populations of endangered species. Small population numbers, genetic drift and/or inbreeding may all contribute to a substantial loss in genetic diversity of such populations, and consequently may negatively impact their viability. For example, the small, reintroduced population of black rhinos in Addo Elephant National Park, South Africa has comparatively low genetic diversity and high relatedness relative to their source populations, resulting in low fitness, manifesting as low population growth rate and reduced male survival (le Roex et al. 2018).

Our results are consistent with several other studies across taxa that have reported improved genetic diversity, and ultimately genetic rescue, in bottlenecked populations through translocation, including Australia's mountain pygmy possum (*Burramys parvus*; Weeks et al. 2017), adders (*Vipera berus*; Madsen et al. 1999), Scandinavian wolves (*Canis lupis*; Åkesson et al. 2016) and New Zealand's South Island robins (*Petroica australis*; Heber et al. 2013). Similarly, Poirier et al. (2019) reported that outbreeding with a few translocated individuals significantly increased the low genetic diversity observed in a post-bottleneck population of bighorn sheep (*Ovis canadensis*). Following this recovery in genetic diversity, first-generation ( $F_1$ ) admixed lamb survival rates improved and population size consequently increased, i.e. genetic restoration resulted in genetic and evolutionary rescue.

The reconstructed mtDNA haplotype network in this study demonstrates that the Zimbabwean mitochondrial lineages are well established in the Kruger black rhino population. Genetic analyses of museum specimens identified at least four mtDNA haplotypes historically found in South African black rhinos (Moodley et al. 2017). Thus our results suggest that mixing the two source populations has restored a comparable level of mtDNA diversity to South African black rhinos. The single haplotype (H2) found in all KwaZulu-Natal black rhino (Anderson-Lederer et al. 2012; Kotzé et al. 2014) was the most common haplotype present in the Kruger population (67.96%). We also found two Zambezi River haplotypes (H1 and H5) within the Kruger population; the remaining Kruger haplotype (H3) was reported in a single captive Zimbabwean black rhino (Fernando et al. 2006). Thus at least two (or three, if including H3) of the six known Zimbabwean haplotypes (33-50%) have been retained in the Kruger black rhino population. It is also possible that with more extensive sampling, additional Zimbabwean haplotypes would be detected. Future studies that directly compare the nuclear contributions of the two source populations within the current Kruger population would also provide further insight into this genetic admixture.

While mixing individuals from different source populations may increase genetic diversity and reduce the likelihood of inbreeding depression, it may increase the risk of outbreeding depression (Edmands 2007). For example, if source populations are under unique environmental pressures (e.g., different climates or habitats), local adaptations may arise, especially in long-isolated populations. Thus, outbreeding with

genetically diverse individuals is counterproductive if the hybrid offspring face lowered fitness due to the loss of locally adapted genetic variants (Edmands 1999). A classic case of outbreeding depression occurred when two subspecies populations of Alpine ibex (*Capra ibex*) were translocated from the Sinai Peninsula and Turkey into the European Alps. Unfortunately, the introduced Ibex bred earlier in the season than their European counterparts, resulting in hybrid offspring born in midwinter, reducing survival and ultimately leading to the hybridised herd's extinction (Templeton 1986). However, this is an exceptional case, and outbreeding depression is seldom seen in practice (Frankham et al. 2011; Ralls et al. 2018).

Outbreeding depression from mixing KwaZulu-Natal and Zambezi River black rhinos is unlikely when considering primary risk factors, such as chromosomal differences, lack of gene flow for more than 500 years, and substantial environmental differences between populations (Frankham et al. 2011). The Zambezi River and KwaZulu-Natal black rhino populations were historically connected (Kotzé et al. 2014) and a healthy population of translocated KwaZulu-Natal black rhino in Malilangwe, Zimbabwe suggests that the different environment between populations is unlikely to contribute to outbreeding depression. Furthermore, the increase in Zimbabwean lineage proportion seen in the extant Kruger population (relative to the ratio of founder females) contradicts any potential loss of local adaptation; if anything, selection over the generations may have favoured the more diverse Zambezi River black rhino. Further research, however, is required to test whether a selective advantage or stochastic events are responsible for the lineage proportion increase seen in this study.

In conclusion, this study indicates that the admixture of black rhinos from different gene pools substantially enhanced both the nuclear and mitochondrial diversity of the founded Kruger population relative to the source *D. b. minor* population in South Africa. In the absence of threat alleviation, metapopulation management strategies (such as population supplementation through translocation) aimed at increasing the range and securing the genetic health of black rhino are critical. The improved genetic diversity found in the Kruger population is encouraging for the long-term survival of this subspecies as a managed metapopulation within South Africa, possibly improving its adaptive potential to respond to environmental change. Given the encouraging levels of diversity observed, this also makes the Kruger black rhino population an ideal source candidate for founding new populations or improving the genetic variation (and thus reducing extinction risk) for genetically depauperate *D. b. minor* populations in South Africa.

## Declarations

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### **Conflicts of interest**

The authors declare that they have no conflicts of interest.

### **Availability of data and material**

GenBank accession numbers OK376773-OK376875

Microsatellite data to be submitted to Dryad upon acceptance of the manuscript

### **Code availability**

Not applicable

### **Ethics approval**

Samples collection was performed by South African National Parks (SANParks) veterinary staff in accordance with national Wildlife Capture Standard Operating Procedures.

### **Consent to participate**

Not applicable

### **Consent for publication**

Not applicable

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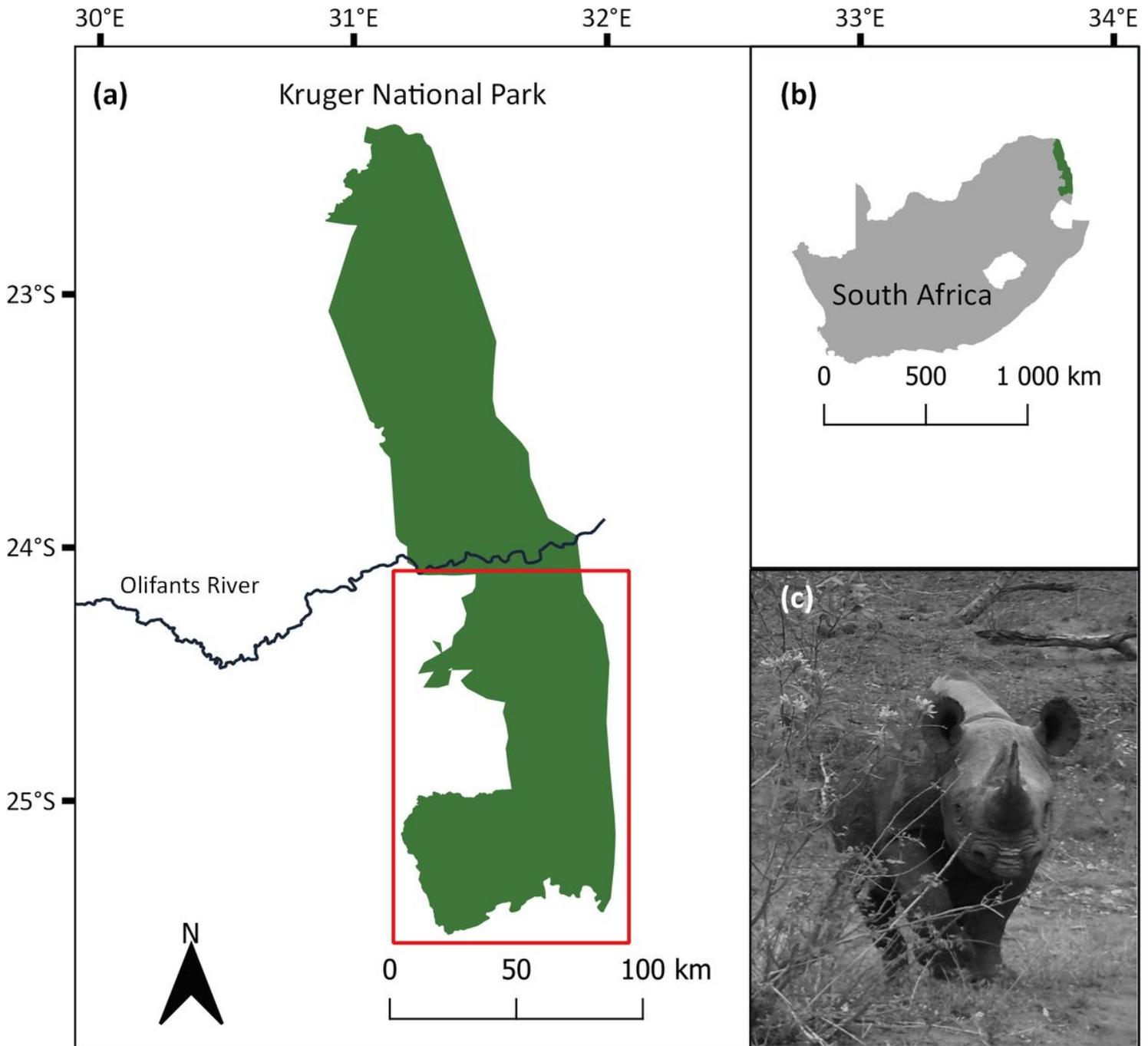
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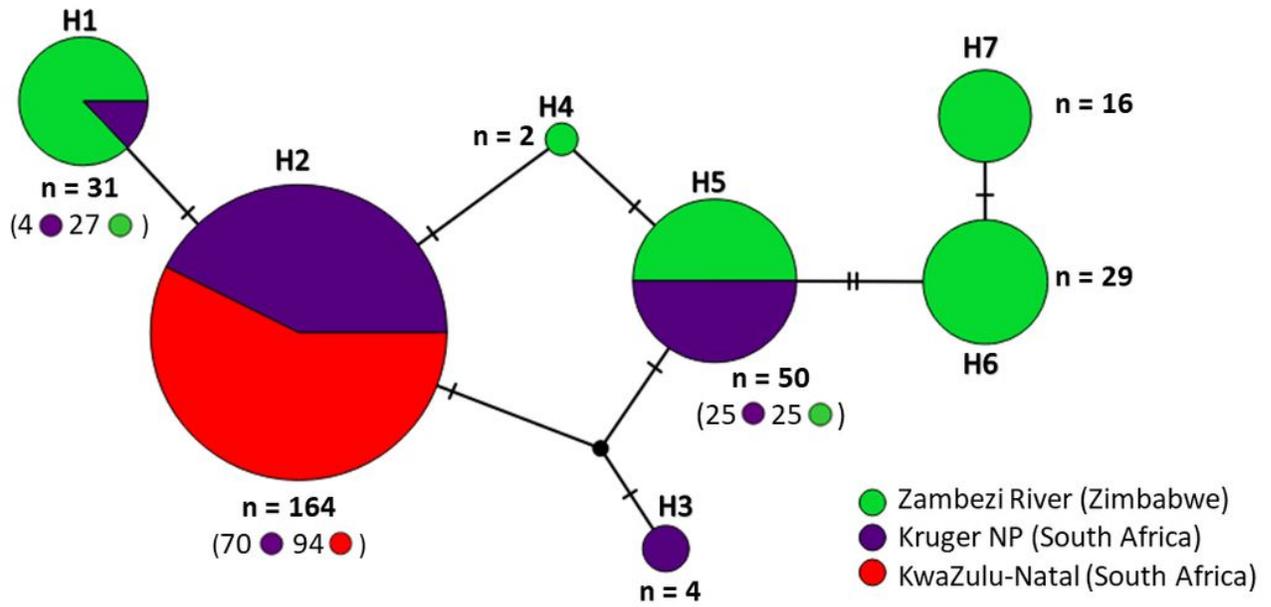
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## Figures



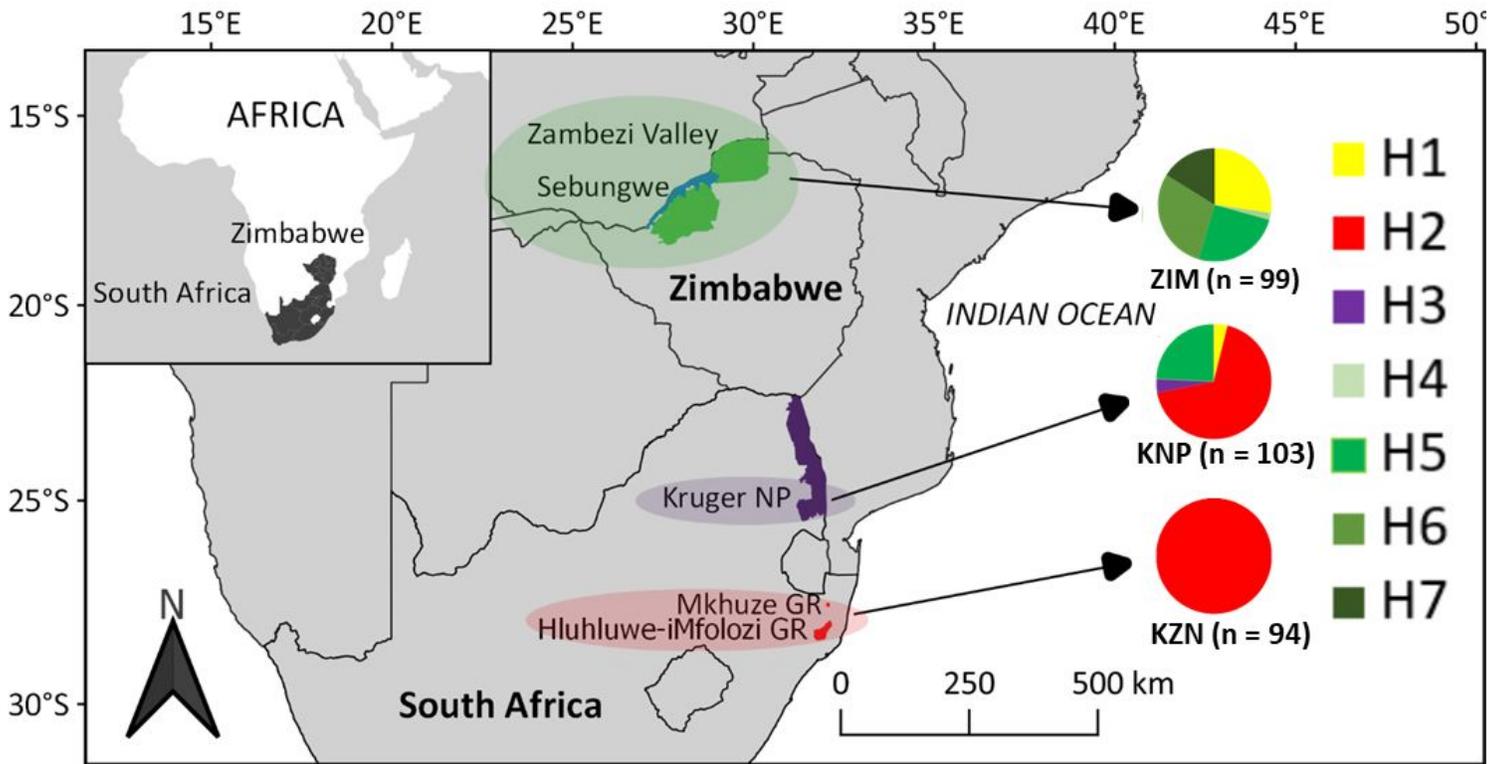
**Figure 1**

(a) Map showing the sampling area (red box) within the Kruger National Park (green polygon); (b) Kruger National Park's location and total extent within South Africa; (c) photograph of a black rhino taken during the 2019 census (photo credit: SANParks, C. Dreyer).



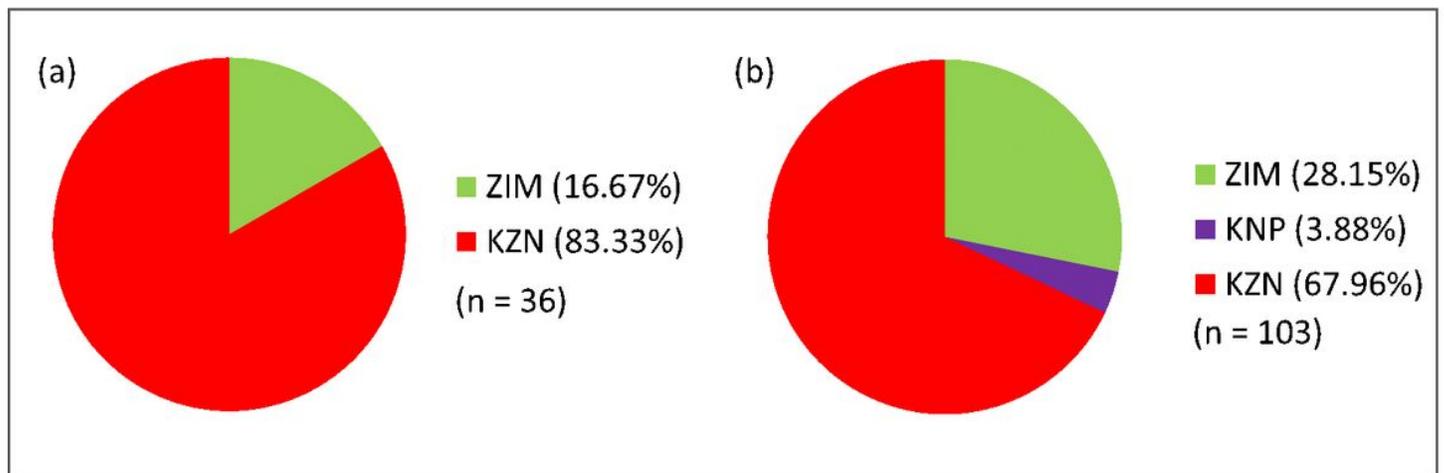
**Figure 2**

Median-joining haplotype network among 296 mtDNA sequences from three *D. b.* minor populations. The circle sizes are proportionate to the numbers of individuals representing each haplotype. Colours represent black rhino populations. Hatch marks represent the number of mutation steps between haplotypes. The label above or below the circles represent the haplotype number (H1 – H7). n=number of samples. Numbers in brackets indicate numbers of individuals from the colour-coded population within that haplotype.



**Figure 3**

Current mtDNA control region haplotype distribution in the Zambezi River, Zimbabwe (ZIM), KwaZulu-Natal (KZN) and Kruger National Park (KNP) source populations. n=sample size. H1–H7=haplotype number.



**Figure 4**

The proportion of Zambezi Valley (ZIM) and KwaZulu-Natal (KZN) animals in the (a) founder, and (b) current Kruger populations.

## Supplementary Files

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