Genetic admixture of Kruger National Park black rhino (*Diceros bicornis minor*): conservation implications

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Abstract

Black rhinoceroses (*Diceros bicornis*) have been extirpated from most of their historic range with the remaining individuals (ca. 5200) living in geographically isolated populations. Management priorities include creating new populations whilst maintaining genetic diversity and promoting gene flow between existing isolated populations. Such objectives are however currently hindered by a lack of comparative/reference data on levels of diversity, relatedness and inbreeding in a large, free-ranging black rhinoceros population. Here I attempt to address this gap in our knowledge by investigating the genetic diversity of the black rhinoceros *Diceros bicornis minor* within Kruger National Park (the largest free-ranging population of this subspecies) using nuclear and mitochondrial DNA. I compared the diversity of this founded population with the two source populations (KwaZulu-Natal, South Africa and Zimbabwe) using published studies, and evaluate the relative contribution of source lineages relative to the proportion of original founders. Analysis of the mtDNA control region revealed four haplotypes, with moderate haplotype and nucleotide diversity (h=0.48 (± 0.05 SD); π= 0.29%). Data from 13 microsatellite loci revealed moderate to high levels of genetic variation (number of alleles = 4.92 ± 0.90, effective number of alleles = 2.26 ± 0.25, observed heterozygosity = 0.50 ± 0.04, expected heterozygosity = 0.51 ± 0.04), low mean pairwise relatedness (r = -0.03), a low inbreeding coefficient (F_{is} = 0.04) and no evidence of genetic structuring. Diversity levels within the Kruger black rhinoceros population were high compared to levels reported in black rhinoceroses originating in KwaZulu-Natal and similar to those reported in individuals originating in Zimbabwe. Results show that 40-60% of the Zimbabwean lineages are represented in the Kruger population which is a noticeable increase in the relative contribution of the Zimbabwe founder population. The data provided by this study can be used to guide management and conservation decisions regarding maximising genetic variability across the subspecies. Furthermore, given the encouraging levels of genetic diversity observed, the Kruger black rhinoceros population would be an ideal source population for supplementation of genetically depauperate populations or creating new populations. Finally, these findings demonstrate a positive outcome in mixing the KwaZulu-Natal and Zimbabwe gene pools, with evidence that the founder Kruger black rhinoceros population has been genetically rescued from the low diversity seen in the KwaZulu-Natal black rhinoceroses in South Africa.

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Plagiarism declaration

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's own.
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Introduction

Extinction is as much a natural part of the evolutionary process as speciation (Raup 1981). Fossil records show that 99.9% of all species ever in existence, and at least 99.99% of all evolutionary lines, are now extinct (Raup 1981; Mayr 1991). Furthermore, the earth has experienced five periods of mass extinctions where vastly increased rates of species extinctions outnumbered the formation of new species (Raup 1994). Although the causes are complex, these mass extinctions have coincided with volcanic activity or major celestial impacts on the earth's surface that caused dramatic climate changes (Renne et al. 2013; Rampino et al. 2017). Today, substantial evidence suggests that we are in the midst of the sixth mass extinction (Dirzo et al. 2014), with unprecedented rates of biodiversity loss: species are disappearing at rates estimated to be 1,000-10,000 times greater than the natural extinction rate (de Vos et al. 2014), and 5,000–25,000 times that recorded in the fossil record (Frankham et al. 2010). This time, however, neither volcanism nor celestial impacts are to blame. Undoubtedly, modern anthropogenic activities and the ensuing habitat loss, invasive species, over-exploitation and pollution are the driving forces of current extinction rates. Combined with stochastic (demographic, environmental, genetic and catastrophic) factors, these activities are placing ever-increasing pressures on remaining natural populations (Primack 2002). Understanding the causes and trends of extinction risks, a key pursuit of conservation biology, is critical if we are to curb the current rate of species' loss and maintain biodiversity.

Since the 1970s, species' extinction has been associated with the loss of genetic variation (Frankel 1970, 1974). There are two explanations for this: 1) in the short term, population extinction risk is increased due to decreased individual fitness caused by inbreeding and genetic drift, and 2) in the long term, the evolution of populations, and thus their ability to adapt to changing environments, is impeded (Frankham 2005). While the role of genetics in extinction has been a controversial issue (Lande 1988; Caro & Laurenson 1994), compelling evidence on the contribution of inbreeding depression and loss of genetic diversity to extinction risk leaves little room for further debate (Frankham 2005). Inbreeding depression is the reduction in fitness of offspring resulting from mating between closely related individuals (Frankham 2005). It has a considerable effect on individual performance by reducing survival, reproduction and resistance to disease, predation and environmental stress (Crnokrak & Roff 1999; Keller & Waller 2002; Ross-Gillespie et al. 2007). A reduction in individual fitness also erodes population fitness, and thus increases the extinction risk. Likewise, a reduction in population heterozygosity (following higher levels of inbreeding), relates to reduced population fitness and the potential for adaptability (Reed & Frankham 2003). Without the ability to adapt to environmental change, populations seldom survive and typically go extinct

(Spielman et al. 2004). As such, international conservation policy now recognises genetic diversity, along with species and ecosystem diversity, as critical for successful biodiversity conservation (Convention on Biological Diversity 2007).

Inbreeding avoidance mechanisms typically prevent inbreeding depression in nature (Pusey & Wolf 1996; Perrin & Mazalov 1999; O'Riain et al. 2000), but for many populations inbreeding is unavoidable. This is particularly true of small, isolated populations that are more vulnerable to loss of evolutionary potential and extinction. In these populations, restricted mating opportunities increase the likelihood of inbreeding, leading to increased levels of homozygosity and the expression of recessive deleterious alleles (Charlesworth & Charlesworth 1987). If the population remains small and isolated over several generations, genetic drift will further reduce the genetic variation by random fixation and/or loss of alleles. Additionally, the interaction between environmental, demographic and genetic factors reinforce each other, propelling these populations in a downward spiral, known as the extinction vortex (Gilpin & Soulé 1986). The consequences of small population size are exacerbated in fenced populations, as natural prevention mechanisms such as dispersal are inhibited, therefore necessitating management intervention. Thus, to escape the extinction vortex, managers of these small, fenced populations may artificially simulate natural levels of gene flow through translocations or through the re-establishment of populations in their former ranges (Griffith et al. 1989).

Genetic rescue is the increase in population fitness of "at-risk" populations by crossing these populations with genetically distinct immigrants (Whiteley et al. 2015), thereby ameliorating the negative effects of inbreeding and low genetic diversity. Introducing genetic variation allows beneficial phenotypes to be expressed, thereby facilitating adaptation (Hedrick & Fredrickson 2010; Whiteley et al. 2015; Frankham 2015). A well-known example of successful genetic rescue is the Florida panther (*Puma concolor coryi*). Eight Texas panthers (*Puma concolor stanleyana*) were translocated to Florida to rescue the population that had plummeted to only 22 individuals, and was facing extinction. Following the genetic rescue, the Florida panther population increased to over 100 genetically robust individuals within a decade (Pimm et al. 2006; Johnson et al. 2010). However, a major concern with genetic rescue is its potential to promote outbreeding depression, as it risks introducing poorly adapted genes, thereby reversing fitness in locally adapted populations (Weeks et al. 2011). An example of outbreeding depression occurred when an overhunted Alpine ibex (*Capra ibex*) population in the European Alps was augmented by translocations from populations in the Sinai Peninsula and Turkey (Templeton 1986). The introduced ibex bred earlier in the season, resulting in hybrid young

born in the middle of the alpine winter. This inadvertent reduction in fitness ultimately led to the extinction of the hybridised herd.

Although the current biodiversity crisis affects species across a wide range of taxa, it is evident that some groups are disproportionately impacted (Ripple et al. 2015). The most common threats faced by vertebrates include poaching and hunting, land-use change (including habitat loss, agricultural cropping, and deforestation) and invasive species (Ripple et al. 2017). Slow reproductive rates and large range requirements make large herbivores particularly vulnerable to anthropogenic threats, increasing their extinction risks (Cardillo et al. 2005; Ripple et al. 2015). Ripple et al. (2019) found that 70% of extant megafauna species have decreasing populations and 59% are threatened with extinction. While ~42 terrestrial megafauna species (>1000kg) were present during the late Pleistocene (126,000 to 11,700 years ago), only eight remain today (Owen-Smith 1987; Grayson 2001), seven of which are considered threatened and four of these are critically endangered (Ripple et al. 2015). Megaherbivores play a significant role as ecosystem engineers (Gill et al. 2009; Rule et al. 2012; Sandom et al. 2014), dispersing seeds, aiding in nutrient cycling and influencing fire regimes (Ripple et al. 2015), and thus their removal from the ecosystem may lead to trophic cascades (Everatt et al. 2016). The loss of large herbivores also has direct effects on humans, particularly when the decline of flagship species translates into a loss of income from reduced tourism.

Rhinoceroses (Family: Rhinocerotidae), the second-largest living land animals after elephants, are emblematic of the threats facing megaherbivores (Kingdon 1997; IUCN 2019). All five of the remaining extant species are threatened to varying degrees: the white rhinoceros (*Ceratotherium simum*), is near threatened, the Indian rhinoceros (*Rhinoceros unicornis*) is vulnerable, and the black rhinoceros, Sumatran rhinoceros and Javan rhinoceros (*Diceros bicornis, Dicerorhinus sumatrensis, Rhinoceros sondaicus* respectively) are critically endangered (IUCN 2019). While there has been a slow increase in select rhinoceros populations, they continue to face profound threats, including habitat loss, alteration and fragmentation (Amin et al. 2006) and poaching for their horns (Amin et al. 2006; Knight 2016).

The black rhinoceros (*Diceros bicornis*), hereafter referred to as black rhino, is the sole survivor of a genus whose ancestral origins extend back to around four to five million years ago, when it diverged from the white rhinoceros (Geraads 2005). Historically, black rhinos were widely distributed across sub-Saharan Africa with numbers estimated to be greater than 850,000 individuals pre-20th Century (Emslie 2012). However, large-scale hunting and land-clearance for human settlement and agriculture

led to a precipitous decline in global population size to ~100,000 individuals by the 1960s (Knight 2016). Between 1972 and 1992, relentless poaching and habitat destruction caused a 96% reduction in numbers (Emslie & Brooks 1999). By 1995, only ~2,410 black rhinos remained (Knight et al. 2013). Since then, concerted conservation efforts have halted the decline, and black rhino numbers have steadily increased. By the end of 2010, the numbers in Africa had doubled to ~4,880 and by the end of 2015 they had reached ~5,250 (Emslie & Adcock 2016). Today, the majority (98%) of black rhino populations are concentrated in four countries: South Africa, Namibia, Kenya and Zimbabwe (African Wildlife Foundation 2019). However, these remaining populations are small and isolated, with fenced boundaries and/or human settlements restricting dispersal and consequently, gene flow.

The International Union for the Conservation of Nature (IUCN) recognises three extant subspecies of black rhinos based on slight morphological differences and their geographic and ecological separation across central and southern Africa (Emslie 2012). These subspecies include the south-western *D. b. bicornis*, with Namibia as its stronghold, the eastern *D. b. michaeli*, found predominantly in Kenya, and the south-central *D. b. minor,* the most numerous of the subspecies, with South Africa as its stronghold. Figure 1 illustrates the inferred historical and extant distributions of all black rhino populations

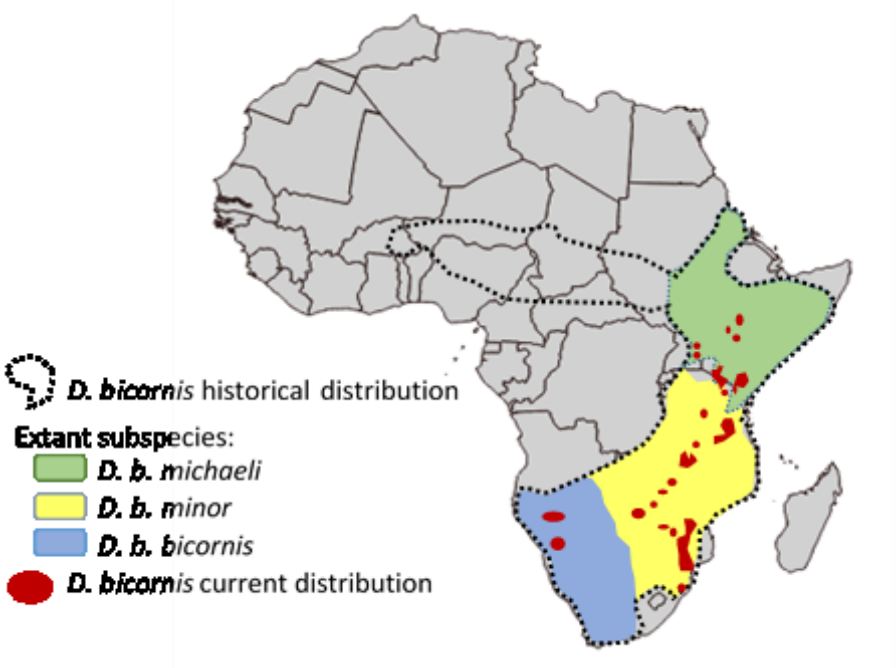

Figure 1. Map of current and historical distributions of black rhinos. Subspecies mapped are according to du Toit (1987). Map adapted from Moodley et al. (2017).

D. b. minor's historical range is thought to have extended from Southern Tanzania, through Zambia, Zimbabwe, and Mozambique to the northern, north-western and north-eastern parts of South Africa (north of the Mtamvuna River) (Emslie & Adcock 2016). Currently, in addition to South Africa, this subspecies is found in Zimbabwe (its second most important range state), and Southern Tanzania. *D. b. minor* has also been reintroduced to areas within its original range, including Swaziland, Botswana, Malawi and Zambia (Emslie 2012). Having borne the brunt of poaching during the 1970's and 1980's, as well as the recent resurgence in poaching, *D. b. minor* has suffered a global population decrease exceeding 80% over the last three generations and consequently is listed by the IUCN as critically endangered (Emslie 2012). However, in South Africa, numbers have increased since 1930 when only 110 individuals remained in two reserves: Hluhluwe-iMfolozi and Mkhuze nature reserves in KwaZulu-Natal (Emslie 2012). By the mid-1990s, the only other surviving *D. b. minor* population in southern Africa occurred in the Zambezi Valley, Zimbabwe (Emslie & Brooks 1999)

Since 1930 the translocation of black rhinos has led to the numbers and populations of *D. b. minor* steadily increasing. By the end of 2010, South Africa had 1684 individuals of which an estimated 480 (29%) resided in KwaZulu-Natal (Emslie 2012). Furthermore, an estimated 627 D. b. minor were living in southern Kruger in 2009 (Ferreira et al. 2011) making it the largest *D. b. minor* population in Africa. However, these large populations remain under intense pressure from poaching by criminal syndicates that is impeding the recovery of this subspecies. Most of the remaining *D. b. minor* black rhino populations are small and isolated. Consequently, movement between populations requires human intervention as part of a metapopulation management program, i.e. managing several discrete populations collectively as one herd (Emslie & Brooks 1999). Traditionally, metapopulation management of black rhinos focused on population size and growth with little consideration of genetic diversity between individuals and both donor and receiving populations (Emslie et al. 2007; Kim 2009). As the species recovers, to ensure its long-term survival, it is important for the focus to shift to population quality measured through indicators such as genetic variation (Western 1982; Tatman et al. 2000; Walpole et al. 2001; Mills et al. 2006).

In recent years, advancements in molecular tools for population genetics has allowed for accurate assessments of genetic parameters, such as within-population heterozygosity, relatedness, inbreeding, gene flow between populations and the genetic distinctiveness of taxonomic units (Moritz 1994; Avise 1995; Lyrholm et al. 1999). Common molecular approaches use mitochondrial DNA (mtDNA) and nuclear microsatellites (Lyrholm et al. 1999; Brown et al. 2005) to determine population genetic measures. Due to its high copy number, lack of recombination and high mutation

rates, mtDNA is an effective marker to measure genetic variation in recently declining populations, to define taxonomic units, and to determine the evolutionary or phylogenetic conservation value of populations (Moritz 1994). Although the rapid evolution of next-generation sequencing approaches has radically transformed the development of molecular markers (Vartia et al. 2016; De Barba et al. 2017; Kleinman-Ruiz et al. 2017), mtDNA remains a valuable tool due to its maternal inheritance, relatively technical ease-of-use and cost effectiveness. Microsatellites, which consist of short, tandemly repeated nucleotide sequences (generally 2-6 basepairs [bp] long), are typically non-coding and are not influenced by natural selection (Slatkin 1995). These nuclear markers exhibit high reproducibility, codominance, hypervariability, Mendelian inheritance and high mutation rates, all features that make them a popular and versatile choice for conservation genetics. Microsatellites can be used to assess heterozygosity and detect differences among individuals, population and subspecies. In combination, these marker types can address a myriad of conservation questions including population history and phylogeographic structure, genetic diversity, individual fitness and mating systems as well as sex-specific patterns of gene flow between populations (Slade et al. 1998; Abdul-Muneer 2014).

Black rhinos are classified into distinct subspecies, and it has been proposed that each subspecies may have genetic or behavioural adaptations to their local environment (Emslie & Brooks 1999; Harley et al. 2005). High levels of differentiation between the three extant subspecies, observed in both mtDNA and autosomal DNA analyses, support this hypothesis (Ashley et al. 1990; O'Ryan & Harley 1993; O'Ryan et al. 1994; Swart & Ferguson 1997; Brown & Houlden 1999, 2000; Harley et al. 2005; Nielsen et al. 2008; Karsten et al. 2011; Muya et al. 2011). As a consequence of the differences between local populations, it has been suggested to maintain locally adaptive traits and minimise the risk of outbreeding depression (O'Ryan et al. 1994; Brown & Houlden 2000; Harley et al. 2005). However, previous studies of black rhino genetics have provided a varied, and often conflicting, range of genetic variation depending on the molecular method. For example, studies using allozyme data (Swart et al. 1994; Swart & Ferguson 1997) and microsatellite data (Brown & Houlden 1999; Garnier et al. 2001; Harley et al. 2005) report moderate to high levels of diversity in some wild black rhino populations, while low diversity relative to other large vertebrates is seen in mtDNA (Ashley et al. 1990; O'Ryan & Harley 1993; O'Ryan et al. 1994; Brown & Houlden 1999). These varied results could be explained by variations in sample size, examining fewer than 50 loci, small genetic distances between the subspecies, or a combination of these limitations, that together underestimate genetic diversity and heterozygosity (Nei 1978). Where there is consensus, however, is that not all rhinos are equally genetically diverse. Within the black rhino subspecies, both mtDNA and microsatellite studies show *D.*

b. michaeli, the most endangered of the subspecies, to have the highest diversity (Harley et al. 2005; Scott 2008; van Coeverden De Groot et al. 2011). *D. b. minor* exhibits the lowest levels of genetic diversity compared to the other black rhino subspecies, even though they have the largest number of individuals remaining (Harley et al. 2005; Karsten et al. 2011).

A recent study of a small population of black rhinos within Addo Elephant National Park, a fenced reserve, found low population growth rates with low genetic diversity and high relatedness (le Roex et al. 2018). This suggests the beginnings of density-dependent growth regulation that may be enhanced by negative genetic factors in black rhinos. However, inferring the management implications is difficult as there is no current framework to evaluate levels of diversity, relatedness and inbreeding, and the definition of "acceptable levels" of genetic variation differ between studies. To successfully manage small populations using genetic indicators, one needs to know the levels of diversity and relatedness that occur in large, free-ranging populations. Currently the only such black rhino population is in Kruger, estimated in 2017 at 507 (95% CI: 427-586; Ferreira et al. 2019), with the highest density in the region south of the Olifants River (comprising 47% of Kruger's total area of 19,485km^2 (Ferreira et al. 2011)).

D. b. minor were extirpated from Kruger in the 1930's, but between 1971 and 1982 a total of 81 black rhinos were reintroduced into Southern Kruger. These black rhinos originated from both the two remaining *D. b. minor* populations: KwaZulu-Natal (Hluhluwe-iMfolozi and Mkhuze nature reserves), South Africa and the Zambezi Valley, Zimbabwe. Subsequently, studies using microsatellite data have shown that these two source populations exhibit a degree of genetic differentiation (F_{ST} = 0.08; Kotzé et al. 2014). Even though the Kruger National Park holds one of the country's largest black rhino populations, little is known about its genetic composition. As this population is free-ranging, with few space constraints and minimal active management, gaining insight into these parameters would provide the baseline data against which other populations can be compared and managed. This information would also help to devise strategies to secure the evolutionary potential of black rhinos.

Genetic diversity within *D. b. minor* is reported to be highly variable, with the Zimbabwe *D. b. minor* population exhibiting higher genetic diversity than the KwaZulu-Natal, South African population (Kotzé et al. 2014). The Zimbabwean population appears to have retained its historic genetic diversity (possibly due to the location being mostly sheltered from the ravaging effects of poaching), while the South African population has lost a substantial proportion of variation (Moodley et al. 2017). This difference may be explained by two hypotheses: 1) the low diversity seen in KwaZulu-Natal is a

consequence of the severe population bottleneck (most of these black rhinos are descendants of a relatively small number of animals) or 2) the populations may have separated earlier than thought and the low diversity seen in the KwaZulu-Natal population is pre-bottleneck and a result of local adaptation (Anderson-Lederer et al. 2012). These two hypotheses promote different management strategies with hypothesis 1 suggesting the need for genetic rescue or restoration (Storfer 1999; Weeks et al. 2011; Frankham 2015) through restocking and outbreeding with more genetically diverse individuals. By contrast, hypothesis 2 argues for preserving local adaptation and the separate management of genetically distinct populations (Templeton 1986; Avise 1989). The KwaZulu-Natal and Zimbabwe populations are currently the only two original source populations of *D. b. minor*, and therefore all future re-established populations will be founded with black rhinos from either one, or both. These populations are therefore important for the recovery of the subspecies throughout its range, and understanding the consequences of mixing animals from these populations is critical. Describing the diversity and lineage composition of the Kruger National Park black rhinos will allow us to evaluate the impact of this population admixture and inform future black rhino management decisions. Furthermore, translocating black rhinos across international borders is often politically sensitive, and thus the Kruger population may play a potentially crucial role in the future of black rhino conservation in South Africa.

Research objectives

My goal in this study was to answer two key questions: firstly, what is the population genetic composition of the Kruger black rhino population (a large, free-ranging population under no active management) that has grown over more than 30 years from a healthy founder population of 81 animals? More specifically, I aimed to: 1) determine the baseline nuclear and mitochondrial genetic diversity, 2) determine the population-level pairwise relatedness and inbreeding coefficient (as a means to test for non-random mating), and 3) test for evidence of any within-population structure.

Secondly, how does the genetic diversity of the founded Kruger population compare to the two source populations, and what is the genetic relationship between them? Since the Kruger population is founded from both Zimbabwe and KwaZulu-Natal populations, this study capitalises on a unique opportunity to explore evidence of a genetic cost versus gain of mixing these two gene pools. The specific goals were to 1) compare Kruger's baseline metrics with published Zimbabwean and KwaZulu-Natal metrics, 2) to calculate the genetic distance between the populations, and 3) to determine whether the relative contributions of the Zimbabwe and KwaZulu-Natal founders reflect the initial translocation ratios. As the Zimbabwean population shows higher levels of diversity than the KwaZulu-Natal population, we expect the Kruger population to have been 'rescued' from the low diversity seen in the KwaZulu-Natal animals. As such, genetic diversity of the Kruger population should be higher than the KwaZulu-Natal population and closer to that seen in the Zimbabwean population. Achieving these objectives will help formulate management and conservation strategies that will aid in the long-term survival of *D. b. minor* black rhino populations in southern Africa.

Methods

Study area

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^2 (Ferreira et al. 2011), extending 360km from North to South and 90km from East to West at its widest part (Figure 2a; Foxcroft et al. 2008). The 110 samples (female = 60, male = 50) used in this research were collected from the black rhino subspecies *D. b. minor* in southern Kruger (Figure 2b) during various management interventions, performed in accordance with South African National Parks (SANParks) Wildlife Capture Standard Operating Procedures. The current black rhino population is predominantly found in southern Kruger (south of the Olifants River) – this is likely because all re-introduction sites occurred in the south and the population has never reached a size that has forced dispersal further north of the Olifants River. Sex, date and location were recorded at the time of capture (Appendix Table A1). Samples were collected during the period 2014-2019, from locations shown in Figure 2b. Although the number of samples were not evenly distributed across southern Kruger, the distribution is representative of the number of black rhinos in each of these areas.

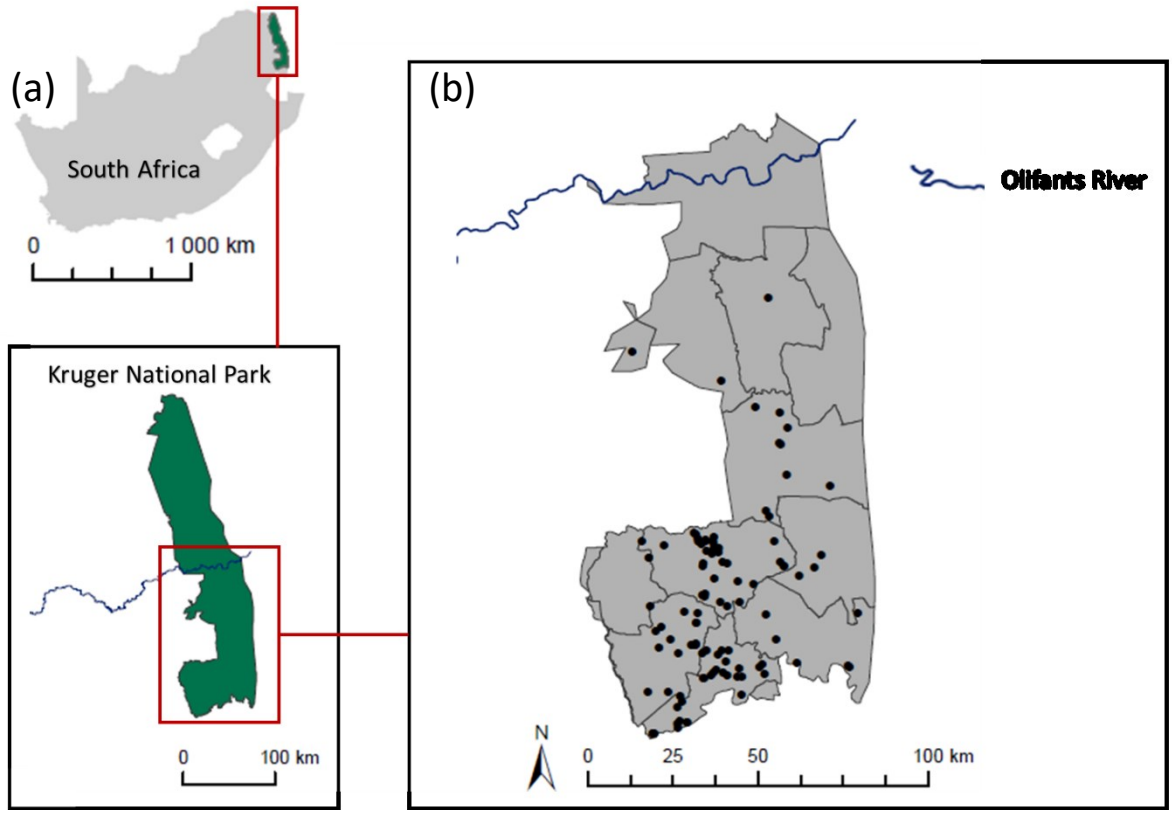

Figure 2. Map of black rhino sampling locations within Kruger National Park. (a) Kruger National Park's location within South Africa is shown in dark green (b) Southern Kruger National Park. Black dots indicate black rhinos sampled in this study.

Samples & genetic data

DNA was extracted from blood samples using DNeasy Blood & Tissue Kits (Qiagen), following the manufacturer's instructions. A fragment of the mitochondrial DNA control region was sequenced by ZooOmics using primers mt15996L (5'-TCCACCATCAGCACCCAAAGC-3'; Campbell et al. 1995) and mt16502H (5'-TTTGATGGCCCTGAAGTAAGAACCA-3'; Moro et al. 1998). DNA samples were genotyped by ZooOmics (Inqaba Biotech, Pretoria) using the standard rhino forensic panel in South Africa (Harper et al. 2013) comprised of 23 microsatellite markers (Table 1). 13 of the 23 markers are applicable to black rhinos and were retained for analysis.

Table 1: List of 23 microsatellite loci isolated from rhino DNA. Origin = taxonomic origin of microsatellite locus.

Locus	Origin	Reference
BlRh1B	Black rhino	Nielsen et al. 2008
BlRh1C	Black rhino	Nielsen et al. 2008
BlRh37D	Black rhino	Nielsen et al. 2008
BR6	Black rhino	Cunningham et al. 1999
DB1	Black rhino	Brown & Houlden 1999
DB23	Black rhino	Brown & Houlden 1999
DB44	Black rhino	Brown & Houlden 1999
DB52	Black rhino	Brown & Houlden 1999
DB66	Black rhino	Brown & Houlden 1999
IR10	Indian rhino	Scott 2008
IR12	Indian rhino	Scott 2008
IR22	Indian rhino	Scott 2008
SR63	Western Sumatran rhino	Scott et al. 2004
SR74	Western Sumatran rhino	Scott et al. 2004
SR262	Western Sumatran rhino	Scott 2008
SR268	Western Sumatran rhino	Scott 2008
SR281	Western Sumatran rhino	Scott et al. 2004
WR 7B	White rhino	Florescu et al. 2003
WR 7C	White rhino	Florescu et al. 2003
WR 12F	White rhino	Florescu et al. 2003
WR 32A	White rhino	Florescu et al. 2003
WR 32F	White rhino	Florescu et al. 2003
ZF1	White & black rhino	Peppin et al. 2010

Quality control

Mitochondrial DNA

MEGA was used to edit (align and trim) the mtDNA sequences after visually inspecting individual chromatograms using Chromas v 2.6.6 (Technelysium Pty Ltd, Australia). The sequences were then aligned using Clustal W (Larkin et al. 2007) as implemented in Mega v 10.0.5 (Kumar et al. 2018). Additional control region mtDNA sequences of *D. b. minor* black rhinos from the Zambezi Valley, Zimbabwe and KwaZulu-Natal, South Africa were included in a combined dataset. These published sequences were previously deposited in GenBank (Brown & Houlden 2000, accession numbers AF187825- AF187831; Anderson-Lederer et al. 2012, accession numbers JN593089- JN593089(64); Kotzé et al. 2014, accession numbers KM095529- KM095627; Moodley et al. 2017, accession numbers KY472322- KY472346).

Microsatellite DNA

Genotype profiles were checked for genotyping errors, allele dropout and null alleles using MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004). Allelic dropout is a common problem, usually as a result of low template DNA concentration. It occurs where the smaller allele of a heterozygous individual is preferentially amplified and the larger allele fails to amplify (Miller et al. 2002). Null alleles result from a mutation in the primer annealing region of one chromosomal copy of a locus, and give a false homozygous genotype as only one allele is amplified (van Oosterhout et al. 2004). Null allele frequencies were estimated using FREENA (Chapuis & Estoup 2007). Null alleles may affect genetic diversity parameters, by potentially decreasing population genetic diversity and increasing genetic differentiation among populations. Frequencies of null alleles may cause moderate (>0.08; Chapuis & Estoup 2007) to significant (>0.20; Dakin & Avise 2004) bias in F-statistics, and thus to avoid confusion and errors in the results, loci with null alleles with a frequency greater than 0.08 were removed from further analysis.

Genepop v 4.7.2 (Raymond & Rousset 1995) was used to test for deviations from Hardy-Weinberg equilibrium (HWE) for each locus and genotypic linkage disequilibrium (LD) between loci. Tests for genotypic linkage disequilibrium test the null hypothesis that genotypes at one locus are independent from genotypes at the other locus; if two loci are found too close together on a chromosome, they are considered linked and not independent. 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 in order to account for multiple comparisons.

Data analysis

Diversity

i. Mitochondrial DNA

Genetic diversity within the mtDNA control region sequences was estimated from the nucleotide diversity (π) (the number of nucleotide differences between sequence pairs; Tajima 1983), the number of unique haplotypes identified and the haplotype diversity (h) (the probability that two randomly chosen haplotypes in a population are different; Nei 1987). These diversity metrics were calculated using DnaSP v 6 (Rozas et al. 2017).

ii. Microsatellite DNA

Microsatellite diversity was inferred from the range and number of alleles per locus (Na), the mean observed heterozygosity (H_o) and the mean 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 & Maruyama 1971), was also calculated. The effective number of alleles will be significantly less than the actual number if some alleles within a locus have negligible frequencies. All calculations were performed in GenAlEx 6.5 (Peakall & Smouse 2012).

Relatedness and inbreeding coefficient

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). Relatedness may range from −1 to +1, where a positive value indicates that individuals share more alleles that are identical by descent than expected by chance (i.e., more related), while a negative value indicates they share fewer alleles identical by descent than expected by chance (i.e., less related). If a population is in Hardy−Weinberg equilibrium, 1st-degree relatives (e.g., parent–offspring or full siblings) should have relatedness values of 0.5, while pairs of unrelated individuals should have relatedness values of approximately 0. The estimator of relatedness chosen for this analysis was based on a simulation analysis comparing different estimators using the 'compareestimators' function of the 'related' package in R (Pew et al. 2015). Given similar performance (Pearson's correlation coefficients between observed and expected values: Wang (2002) = 0.734, Li et al. (1993) = 0.723, Lynch & Ritland (1999) = 0.705, Queller & Goodnight (1989) = 0.730)), the Wang (2002) estimator (having the highest correlation) was chosen for further analysis (Appendix Figure A1). Pairwise relatedness was calculated using the 'coancestry' function.

Microsatellite data was used to calculate Wright's inbreeding coefficient (F_{IS}) at the population level using GENETIX 4.05.2 (Belkhir et al. 2004). F_{IS} measures the departures from HWE in a population by

the mean reduction in heterozygosity of an individual due to non-random mating and is calculated as follows:

$F_{IS} = (H_e - H_o) / H_e$

where H_e is the heterozygosity expected from HWE and H_o is the observed level of heterozygosity. A positive F_{IS} indicates a deficiency of heterozygotes compared with the HWE expectation, while a negative F_{IS} indicates an excess. Confidence intervals for inbreeding coefficient values for each locus and over all loci in each population were obtained by bootstrapping 1000 times.

Genetic structure

In order to explore the genetic distances among individuals and how these relate to population membership, codominant genetic distances (Nei 1972) were calculated among individuals and the results visualised using principal coordinate analysis (PCoA) (Peakall & Smouse 2012). Individuals were grouped by sex and by sampling location within the various geographical sections of Southern Kruger. Additionally, a PCoA was used to visualise the genetic distance between individuals grouped by their sampling location being either North or South of the Sabie River. This tests if the river was restricting movement within the black rhino population. PCoA summarises multivariate data, using dissimilarities in the data to represent the relationship between individuals. The genetic structure of maternal black rhino haplotypes within Kruger (with individuals grouped by their sampling locations being either North or South of the Sabie River) was explored by constructing a median-joining network (Bandelt et al. 1999), using PopART (Leigh & Bryant 2015).

Source and founder population comparison

The combined *D. b. minor* mtDNA sequence dataset was used to compare the nucleotide (π) and haplotype (h) diversity between source (Zimbabwe and KwaZulu-Natal) and founder (Kruger) populations. Furthermore, the relative maternal lineage contributions of the source populations were determined and related to the initial ratio of Zimbabwe and KwaZulu-Natal founder females, obtained from historic records. Finally, the genetic structure among source and founder haplotypes was visualised by constructing a median-joining network (Bandelt et al. 1999) in PopART (Leigh & Bryant 2015).

Results

Quality control

Micro-Checker analysis found no evidence of genotyping errors or allelic dropout. Seven loci with possible signatures of null alleles were detected, namely SR74 (maximum frequency 0.1940), IR12 (0.1838), SRS262 (0.1318), 7C (0.1679), BlRh1B (0.1163), DB44 (0.0758) and DB66 (0.0624). Loci with null allele frequencies greater than 0.08 (i.e., SR74, IR12, SRS262 and 7C) were removed from subsequent analysis. The loci showing significant deviations from HWE due to heterozygote deficits corresponded to the seven loci suspected of null alleles, a well-known cause of heterozygote deficit. ZF1 (Zinc Finger locus; Peppin et al. 2010) is a sex-determining locus, and was therefore used to ensure that the sex of all individuals sampled had been correctly assigned. Thereafter this locus was removed and not included in further analysis.

Analysis of linkage disequilibrium found 7 of the total of 254 combinations of paired loci to be tightly linked ($P<0.0001$), after applying a Bonferroni correction (Appendix Table A2). IR12 and SR74 were found to be in linkage disequilibrium with the sexing marker ZF1. Further inspection of IR12 and SR74 revealed that all males were homozygous for these two loci, whilst females were both homozygous and heterozygous, supporting the conclusion that these markers are sex-linked. After removing the five loci with potential null alleles (SR74, IR12, SRS262, 7C and BlRh1B), two combinations of loci in LD remained: BlRh1C and 12F, and BlRh37D and DB66. Since locus 12F was originally isolated from white rhinos and BlRh1C from black rhinos (Nielsen et al. 2008), locus 12F was removed, and locus BlRh1C retained in the downstream analysis. Locus BlRh37D was also removed from further analysis, while DB66 was retained. Although both BlRh37D and DB66 have been used in previous studies, BlRh37D may be inconsistently amplified (Dicks 2014; Moodley et al. 2018), while locus DB66 is highly polymorphic and has been successfully used in numerous studies (Garnier et al. 2001; Harley et al. 2005; Muya et al. 2011; le Roex et al. 2018). Loci 7B and 32A, both originally isolated from white rhinos, were monomorphic in all samples in this study and thus were also removed from further analysis. Finally, individuals with more than 30% missing data (n=1) were removed from further analysis.

Data analysis

Diversity, relatedness and inbreeding coefficient

i. Mitochondrial DNA

Evidence of potential heteroplasmic single nucleotide polymorphisms was observed in three individuals (BR35, BR36 and BR96), with overlapping chromatograms occurring at a single location in each of their mtDNA sequences (site 77 for BR96 and site 387 for BR35 and BR36). Since mtDNA is maternally inherited, genomes within one individual are assumed to be identical. However, heteroplasmy (the presence of more than one mtDNA type) has been reported in studies of several taxa (Kondo et al. 1990; Crochet & Desmarais 2000; Maté et al. 2007). Common causes of heteroplasmic single nucleotide polymorphisms include de novo mutations, single nucleotide polymorphisms, oocyte heteroplasmy and paternal leakage, but may also be due to contamination of samples. Since the results could not be confirmed or corrected within the time frame of this study, these three samples were removed from further analysis. The final dataset of mtDNA contained 103 sequences of 469 bp in length after editing. Four haplotypes were identified in Kruger black rhinos. These haplotypes are characterised by five polymorphic sites, all containing transition nucleotide substitutions (guanine ↔ adenine and/or cytosine ↔ thymine). The haplotype diversity (h) was 0.48 (± 0.05 SD) and the nucleotide diversity (π) was 0.29% within the Kruger population.

ii. Microsatellite DNA

The final microsatellite data set, after removing one individual (BR05) during quality control, comprised 109 animals. All 13 microsatellite loci retained for analyses were polymorphic, with two to 14 alleles each. The effective number of alleles (A_e) ranged from 1.3 to 5.03 with a mean of 2.26 (± 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 for the whole Kruger population. These diversity metrics are summarized in Table 2. Mean pairwise relatedness (r) within the Kruger population was -0.03, (Appendix Figure A2) with 17.45% of r values greater than 0.25, indicating that a proportion of the black rhinos were closely related (r > 0.25). However, the vast majority (70.88%) of pairs had r values between -0.25 and 0.25. The distribution of the mean pairwise relatedness values is illustrated in Figure 3, where a slightly right-skewed distribution can be observed. Inbreeding coefficients ranged from -0.09 to 0.16 with a slightly positive overall F_{is} value of 0.04 (95% CI -0.01- 0.07) (Table 2).

Table 2. Summary of the diversity metrics of the Kruger black rhino population using 13 microsatellite loci. N_a = No. of alleles, A_e = No. of effective alleles, H_o = Observed heterozygosity, H_e = Expected heterozygosity, uH_e = Unbiased expected heterozygosity, F_{is} = Inbreeding coefficient.

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

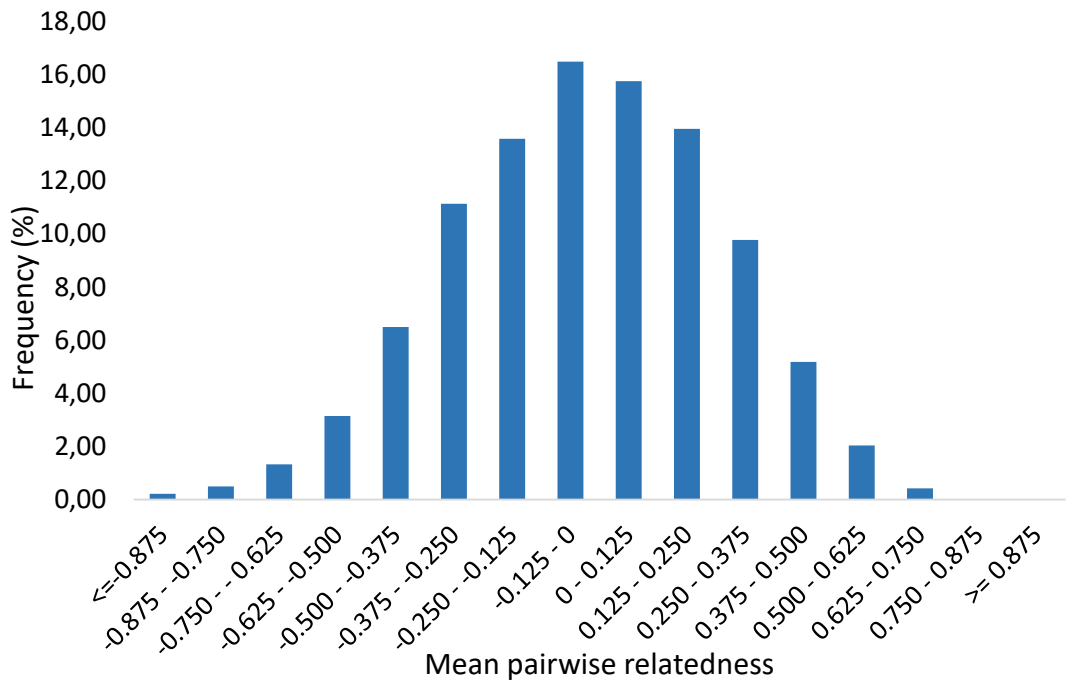

Figure 3. Distribution of mean pairwise relatedness values (r) (Wang et al. 2002) for black rhinos within Kruger National Park.

Genetic structure

i. Mitochondrial DNA

The distribution of the haplotypes shows a complex mixture among individuals located North or South of the Sabie River with no strong geographical pattern (Figure 4). Haplotype 1 was represented only in individuals sampled North of the Sabie River. Haplotypes 1 and 3 each represented four animals. Haplotypes 2, 3 and 4 were represented in individuals located both North and South of the Sabie River. Haplotype 2 represented 67.96% of black rhinos, while 24.27% of black rhinos shared haplotype 4.

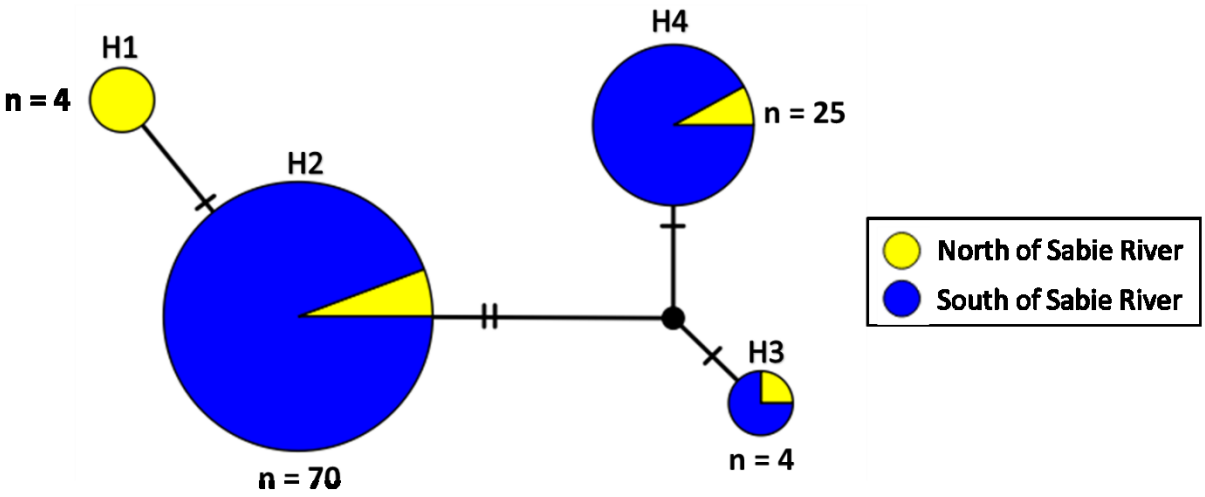

Figure 4. Median-joining network of *D. b. minor* haplotypes within Kruger National Park. The circle sizes are proportional to the numbers of individuals belonging to that haplotype. The colours in the circles mark the location from which the samples originate relative to the Sabie River and are described in the legend. Hatch marks represent the number of mutation steps leading to the next haplotype. The label above the circles represent the haplotype number (H1 – H4). n = number of samples.

ii. Microsatellite DNA

Principle coordinate analysis shows minimal discernible clustering of individuals in the first two coordinates, when grouped by sampling location (by sections and by location relative to the Sabie River) or sex (Figure 5). This suggests that genetic distance between individuals is not correlated with broad geographic location, and little differentiation seems to exist at the population level.

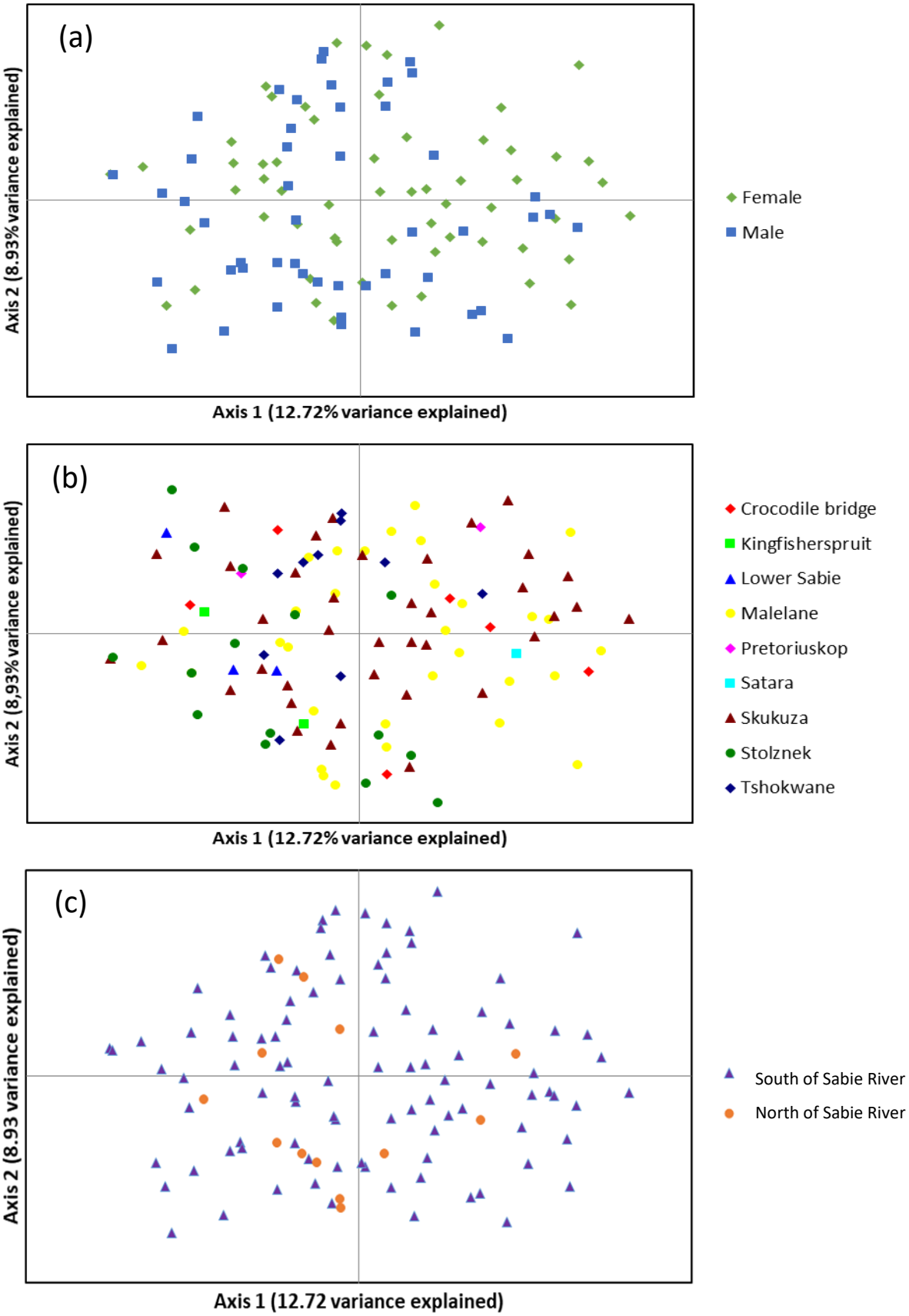

Figure 5. Principal coordinates analysis of genetic distances for all individuals (a) grouped by sex (b) grouped by sampling location according to sections within Kruger NP (c) grouped by sampling location being North or South of the Sabie River. Individuals are positioned in space according to the first two coordinates from a summarized transformation of a pairwise genetic distance matrix. Coordinate 3 (not shown) explained an additional 8.17% of the variation.

Source and founder population comparison

The combined dataset of the Kruger, Zimbabwe and KwaZulu-Natal mtDNA comprised 296 sequences of 363 bp in length after editing and alignment. A comparison of the mitochondrial genetic diversity among the combined black rhino populations is summarised in Table 3. A total of seven haplotypes were found across the three populations, containing seven polymorphic sites. The relationship between haplotypes can be seen in figure 6. Only haplotype 2 exists within the KwaZulu-Natal population, and is shared with 67.96% of the Kruger population. The Kruger population shares two haplotypes (haplotype 1 and 5) with the Zimbabwe population. These two haplotypes together represent 28.15% of Kruger's black rhinos in this study. Haplotype 3 was only found in Kruger, midway between haplotypes 2 and 5 (Figure 6).

Table 3. Mitochondrial genetic diversity among black rhinoceros populations in Zimbabwe, KwaZulu-Natal and Kruger National Park.

Population	N	#H	#P	HD (SD)	π (%)	Originally published
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 (±0.05)	0.29	This study

N = number of individuals sampled; #H = number of haplotypes; #P = number of polymorphic; h = haplotype diversity; π = nucleotide diversity; * no variation

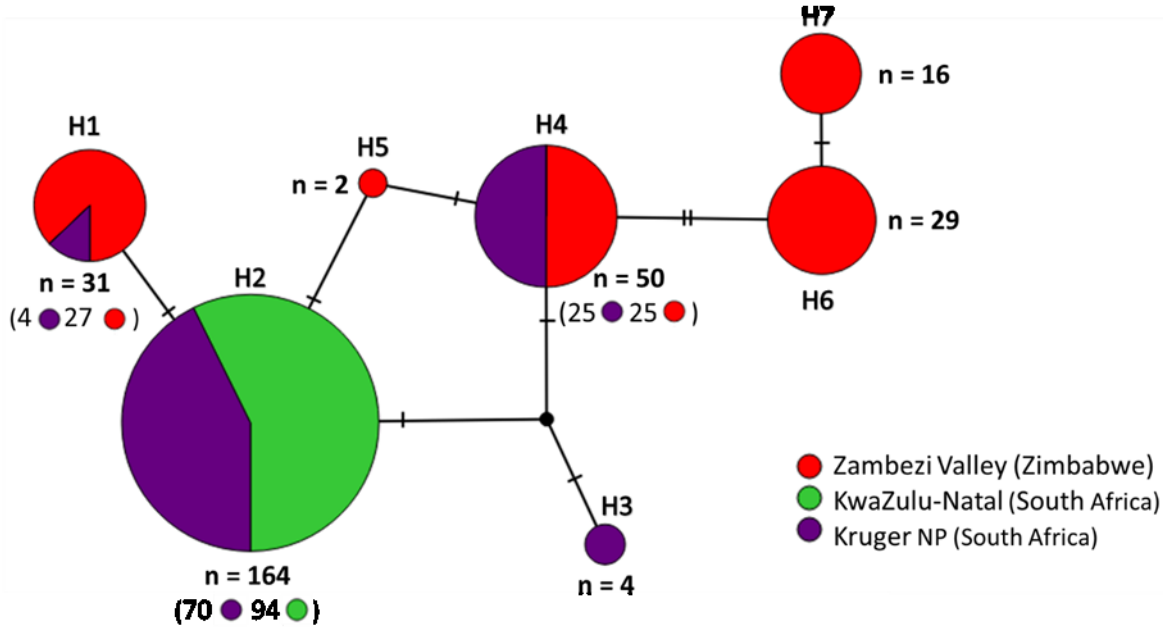

Figure 6. Median-joining haplotype network among 296 mtDNA sequences originating from three *D. b. minor* populations (Zambezi Valley, Zimbabwe; KwaZulu-Natal, South Africa; Kruger National Park, South Africa). The circle sizes are proportional to the numbers of individuals belonging to each haplotype. 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.

The current haplotype distribution amongst the three populations is illustrated in Figure 7. The Kruger founder population comprised 37 females: 30 (83.33%) originating from KwaZulu-Natal and 6 (16.67%) from Zimbabwe (excluding one female that died on release). Considering the current representation of KwaZulu-Natal and Zimbabwe haplotypes within the Kruger population (67.96% and 28.15%, respectively), we see a noticeable increase in the relative contribution of the maternal Zimbabwe founder lineages (Figure 8).

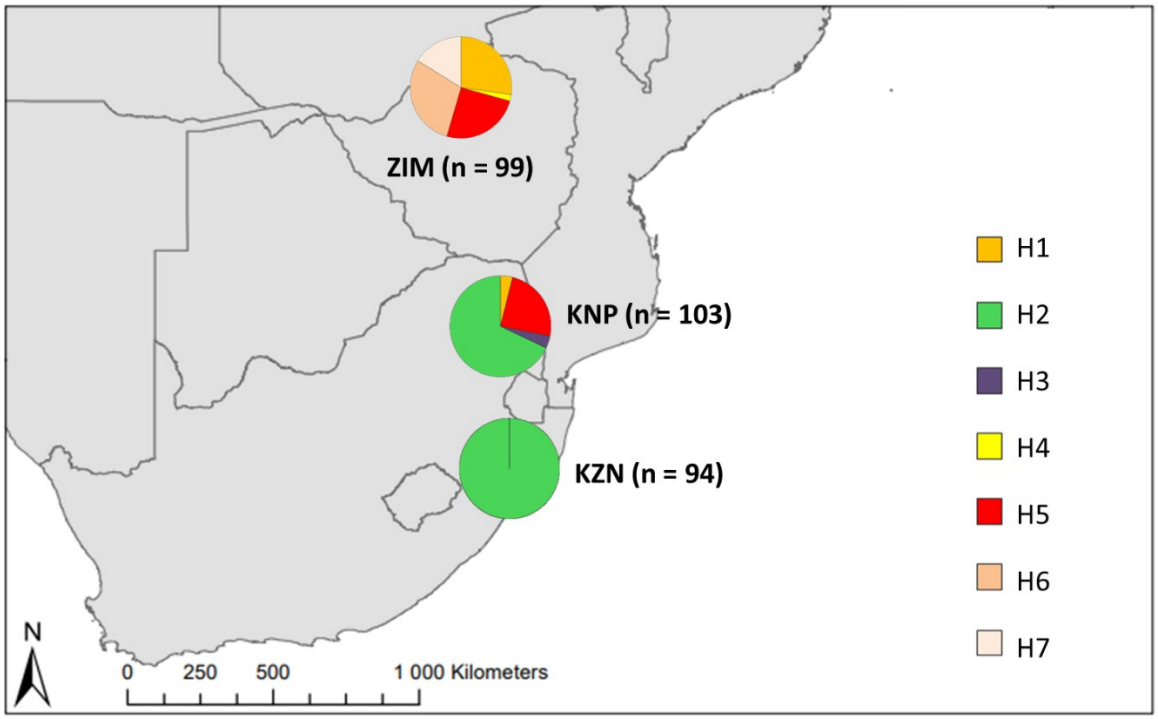

Figure 7. Current MtDNA control region haplotype distribution frequencies in the Zambezi Valley, Zimbabwe (ZIM), KwaZulu-Natal (KZN) and Kruger National Park (KNP) populations. n = sample size.

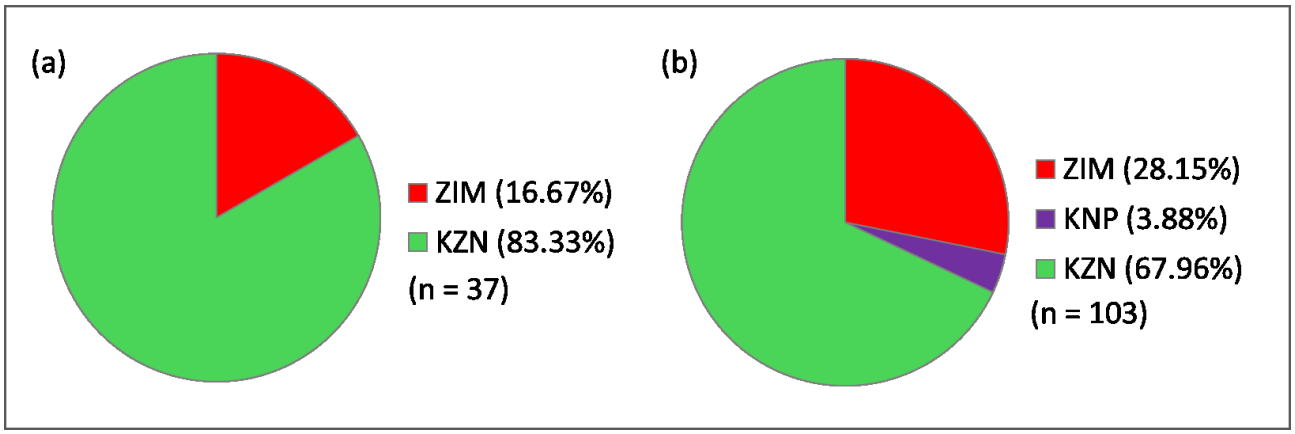

Figure 8. The proportion of Zimbabwe (ZIM) and KwaZulu-Natal (KZN) animals in the (a) founder Kruger (KNP) population, and (b) current Kruger population.

Discussion

Successful metapopulation management of remnant black rhino populations is a crucial part of their future conservation (Garnier et al. 2001). Central to metapopulation management is the promotion of gene flow through translocations, as well as re-establishing populations within their former range (Garnier et al. 2001; Emslie et al. 2009). Maximising the evolutionary benefits and minimising the associated risks of translocations requires knowledge of the genetic composition (including diversity, relatedness, and inbreeding levels) in a self-sustaining, unmanaged black rhino population, as well as an understanding of the genetic implications of mixing gene pools (Weeks et al. 2011; Frankham et al. 2017). The broad goal of this thesis was thus to characterise the genetic composition of the *Diceros bicornis minor* subspecies within Kruger National Park, (the largest free-roaming population in South Africa), using both mtDNA and microsatellite markers and to evaluate how this population's genetic make-up compares to its two source populations, namely KwaZulu-Natal and Zimbabwe.

Genetic diversity levels within the Kruger black rhino population were moderate to high, compared to diversity levels in Zimbabwe (Anderson-Lederer et al. 2012; Kotzé et al. 2014) and KwaZulu-Natal (Kotzé et al. 2014; Moodley et al. 2017) respectively. Additionally, low levels of relatedness with no evidence of non-random mating, nor of within-population substructure, was found within Kruger. These findings indicate that the Kruger black rhino population is a diverse, outbred, panmictic population. Examining the mtDNA control region revealed four haplotypes within the Kruger population, compared to only one reported within KwaZulu-Natal (Anderson-Lederer et al. 2012; Kotzé et al. 2014) and six within Zimbabwe (Anderson-Lederer et al. 2012; Kotzé et al. 2014). Together these results support the hypothesis that the mixing of the KwaZulu-Natal and Zimbabwe gene pools can produce a genetically diverse population. However, further insight into this admixture may be gained from directly comparing nuclear contributions of the two source populations with the current Kruger population. If confirmed, this admixture between KwaZulu-Natal and Zimbabwe gene pools could also contribute to the genetic restoration of other genetically depauperate populations of *D. b. minor* within South Africa. These findings are important to informing metapopulation management strategies aimed at securing the genetic health of the black rhino subspecies, *D. b. minor.*

In 2013, the South African Government gazetted a Biodiversity Management Plan (BMP) for the black rhino (Government Gazette vol. 571 no. 36096) in terms of section 43 of the National Environmental Management: Biodiversity Act (NEMBA). The BMP aims for a South African black rhino population growth rate of 5% per annum, with a target population of 2800 individual *D. b. minor* by the end of 2020 (Knight et al. 2013). Achieving these population parameters will mean prioritising the genetic

health of individuals within the population by maximising the genetic diversity of fragmented populations. Additionally, the BMP recommends that source populations remain below their ecological carrying capacity to maintain habitat quality, and avoid density dependent reduced performance such as slow population growth rates (Emslie et al. 2009). This will necessitate annual minimal harvesting of 5% for established populations where the goal is a zero growth population density. Managing black rhino populations, particularly small ones, may cause donor populations' sex ratios to be skewed in favour of males, as founder populations are ideally skewed towards breeding females (Knight et al. 2013). This can negatively impact the breeding performance and genetic status of the donor population, necessitating surplus males to be translocated (or harvested). However, both these objectives (population growth and removing surplus individuals) require knowledge of the genetic status of the species and subspecies.

Diversity, relatedness and inbreeding coefficient

Genetic diversity can be measured on two temporal scales: 1) the historical diversity of female (maternal) lineages is obtained by investigating mitochondrial DNA sequence variation (Boore 1999; Frankham et al. 2005), and 2) current diversity in the population is revealed by examining the nuclear variation (Schlötterer & Pemberton 1998). Levels of mtDNA haplotype and nucleotide diversity observed in the Kruger black rhino population (0.48 and 0.29% respectively), along with the four distinct mtDNA haplotypes identified, are moderate compared to levels reported for *D. b. minor* originating from Zimbabwe but substantially higher than those of KwaZulu-Natal. For example, Brown and Houlden (2000) found high levels of mtDNA haplotype diversity (h=0.86), nucleotide diversity (0.43%) and five distinct mtDNA haplotypes in captive *D. b. minor* (n=8) that originated from Chete National Park, Zimbabwe. Kotzé et al. (2014) reported similarly high levels of haplotype diversity (h=0.77), an even higher nucleotide diversity (π=0.70%), and six mtDNA haplotypes from 104 *D. b. minor* inhabiting the lowveld region in Zimbabwe. In contrast, Anderson-Lederer et al. (2012) revealed a single haplotype, and therefore no haplotype or nucleotide diversity, amongst 65 *D. b. minor* originating from Itala Game Park, Mkhuze Game Park, and Hluhluwe-iMfolozi Reserve in KwaZulu-Natal. Their finding was supported by Kotzé et al. (2014) who also found only a single mtDNA haplotype to exist within 6 *D. b. minor* sampled in Malilangwe, Zimbabwe (but that originated in KwaZulu-Natal). The intermediate level of diversity of the Kruger black rhino population, shown in this study, indicates that within the Kruger population, historical gene flow between its two source populations has been restored, and that the Zimbabwe mitochondrial lineage is well established in the Kruger black rhino population.

This study revealed that the level of nuclear variation shown in Kruger black rhinos was high (H_o=0.50; H_e=0.51) relative to the microsatellite diversity observed in studies exploring diversity in *D. b. minor* populations in KwaZulu-Natal. For example, Nielsen et al. (2008) reported low diversity levels (H_o=0.32; H_e=0.37) in samples from six black rhinos within the Hluhluwe-iMfolozi Reserve in KwaZulu-Natal. Karsten et al. (2011) examined 77 black rhino samples sourced from various locations within KwaZulu-Natal (including Hluhluwe-iMfolozi Reserve and Mkhuze Game Park), with slightly improved levels of microsatellite diversity (H_o=0.38; H_e=0.44) across the samples, but still lower than what I have reported for the Kruger population. Kotzé et al. (2014) also found similarly low levels of nuclear microsatellite diversity to occur within the KwaZulu-Natal-derived Malilangwe population (H_o=0.40; H_e=0.41; n=44). In contrast, Kotzé et al. (2014) reported significantly higher levels of diversity (H_o=0.54; H_e=0.52) across 236 individuals that had origins in the Zambezi Valley and Sebungwe, Zimbabwe. These levels are similar to those observed within the Kruger black rhino population reported here.

A direct comparison of microsatellite diversity between populations is not possible due to the different number and type of microsatellite loci used between studies. Despite this results suggest that the genetic diversity of the black rhino population in Kruger is close to the average reported for this subspecies (H_o=0.44, Harley et al. 2005; H_o=0.52, Kotzé et al. 2014). Similarly, the mean number of alleles per locus of 4.97 (uncorrected for differences in sample size) observed within Kruger correspond to the mean number of alleles per locus (Na=5.09) reported across the subspecies by Kotzé et al. (2014). A particularly high number of alleles were found in Locus DB66 (Na=14), however this locus has an A_e of only 5.04, indicating that a high proportion of these alleles are present at a low frequency. Having a high number of rare alleles is a possible indication of gene flow between subpopulations (Slatkin 1985; Barton and Slatkin 1986), and therefore this result is further evidence of gene flow amongst Kruger's black rhino population.

The genetic diversity described above shows that the Kruger black rhino population has high variation, within both the nuclear and mitochondrial DNA, consistent with aggregate levels reported across the subspecies distribution. This is an example of how admixture of individuals from different genepools can enhance the genetic diversity of the admixed population, relative to its source populations. While the future of the Kruger black rhino population remains precarious because of illegal harvesting, these levels of variation within Kruger's *D. b. minor* population provide a promising outlook for long-term survival of this subspecies as a managed metapopulation. Although the low diversity seen in KwaZulu-Natal *D. b. minor* has not been red-flagged as a conservation concern (Karsten et al. 2011), the ramifications, such as reduced population fitness, resilience and long-term adaptability (Lacy 1997),

may only become apparent in times of environmental stress and thus should not be discounted (Ross-Gillespie et al. 2007). Furthermore, the rate of recovery following a severe reduction in population size (i.e., a bottleneck) is slower for genetic diversity compared to population numbers and thus the risk of extinction may persist long after the population size has recovered (Bickham et al. 2000). This is especially true in the absence of gene flow as increased genetic variation is then reliant on new mutations that accrue very slowly, particularly as their long-term persistence is dependent on drift and natural selection (Wright 2005).

The remaining *D. b. minor* black rhinos within South Africa are largely confined to small, isolated populations (Knight et al. 2013; le Roex et al. 2018) and are thus at risk of reduced genetic diversity and ultimately local extinction (Frankham et al. 2005). The levels of genetic variation presented in this study offer managers a baseline against which they can compare the genetic health of populations with the goal of working towards restoring natural diversity levels and so buffering small populations against novel pathogens or rapid environmental change. Monitoring genetic diversity within isolated populations will also facilitate the process of identifying suitable populations for future translocation or supplementation, as part of the overall metapopulation management strategy (O'Ryan et al. 1998).

Genetic markers can be used to infer the relatedness as well as the degree of increase in homozygosity due to non-random mating between individuals in a population. In this study, the low mean pairwise relatedness value (r=-0.03) together with the low inbreeding coefficient (F_{is}=0.04), indicate that the Kruger black rhino population is at a low risk of inbreeding depression. Large, positive F_{is} values are seen in the case of positive-assortative mating (i.e. a pattern of non-random mating where individuals mate with phenotypically (and possibly genotypically) similar individuals (Hedrick 2016). Thus the low F_{is} value observed in the Kruger population suggests that the Kruger black rhinos are well mixed, showing no evidence of assortative mating by the founding lineage. In small populations, positive-assortative mating carries the risk of inbreeding (and consequently inbreeding depression), as phenotypically similar individuals are likely to be close kin. Inbreeding depression is a conservation concern, especially for endangered species, as it reduces individual fecundity and increases juvenile mortality (Frankham et al. 2010). While positive-assortative mating facilitates speciation (Bolnick & Kirkpatrick 2012), it may incur some fitness costs as it yields increased homozygosity within loci and so can result in the loss of overall genetic diversity within a population (Campbell et al. 2017). A further loss of genetic diversity for endangered species, that already have depleted genetic diversity, increases their vulnerability to environmental stochasticity such as disease outbreaks (Roelke et al. 1993; Willi et al. 2006; Siddle et al. 2007). Thus monitoring the levels of relatedness and inbreeding

coefficients within small, threatened populations is critical for their management. Although the distribution of the mean pairwise relatedness values across all sampled individuals was slightly right-skewed, this elevated proportion of closely related individuals is likely to be as a result of the management capture procedure. If black rhinos are found together, they are both immobilised and sampled. Pairs of black rhinos are often the mother and her older calf, and therefore the sample set may be biased toward showing more closely related animals than by chance. Despite the presence of mother-calf pairs, only a slight skew in the distribution was observed, and therefore this bias is unlikely to have influenced the results.

Genetic structure

A population is considered panmictic when each individual has an equal chance of mating with another individual, regardless of its location within that population (Dawson & Belkhir 2001). At one extreme, a genetically homogenous population with high levels of gene flow can be indicative of total panmixia, while at the other extreme, strong genetic structuring indicates low levels of gene flow between discrete populations. Understanding complex, fine-scale genetic structuring is important from a management perspective, as identifying genetically independent groups within a population has practical implications, such as determining appropriate management units. Discrete management units are important in maintaining local adaptations, yet optimal management may favour larger groups, particularly in terms of optimising limited resources, a common consideration in conservation today.

In this study, principle co-ordinate analysis revealed no underlying structure amongst black rhinos in Kruger based on sampling location or sex. This indicates high levels of gene flow within the population, which is further supported by the low relatedness coefficient (r=-0.03), low inbreeding coefficient (F_{is}=0.04), as well as the comparatively high level of genetic variation. Furthermore, the complex mixture of maternal haplotypes observed in the mtDNA across both sides of the Sabie River showed no strong geographical pattern. Although one haplotype (H3) was found in individuals located only north of the Sabie River, this may be a result of some localisation of founders originally released north of the river. This overall absence of strong mtDNA structure concurs with the lack of genetic structure observed within the nuclear DNA, and suggests that the Kruger black rhinos are dispersing throughout southern Kruger. Although reliable home range sizes of black rhinos are lacking due to inconsistent statistical methods and study areas reporting highly variable estimates (3-218km^2; Plotz et al. 2016), black rhinos do have localised, well-defined home ranges (le Roex et al. 2019). Additionally, le Roex et al. (2019) found home ranges for the black rhinos in Kruger National Park to be dependent on season and resource availability (with surface water being the limiting factor), i.e. the average black rhino

home range sizes contracted considerably in size during the dry season (33.36 km^2) in comparison to the wet season (46.45 km^2).

In contrast, Göttert et al. (2010) recorded substantially larger average home range sizes of 89.9 km^2 for a black rhino population in semi-arid Namibia during the first season post-reintroduction (dry season), and the following wet season. A recently-reintroduced population is unlikely to exhibit typical behaviour until the population acclimatises to its new surroundings, and therefore the results reported by Göttert et al. (2010) more likely demonstrate atypical, post-reintroduction behaviour (le Roex et al. 2019). The high gene flow observed in this study may therefore be a consequence of initial high rates of dispersal of the reintroduced black rhino population. Furthermore, as the reintroductions of black rhinos into Kruger was staggered over an 18-year period, newly released animals may have encountered already established black rhinos near to their release site. Intraspecific competition for resources, such as food, mates and/or territory, may have prevented the newcomers from settling near their release sites, thus encouraging their dispersal (Matthysen 2005) and consequently promoting gene flow within the Kruger black rhino population. Post-translocated black rhinos are vulnerable to mortality, particularly due to intraspecific fighting (Brett 1998; Linklater et al. 2011), and therefore understanding their range and resource requirements will aid in ensuring the success of future translocations and reintroductions. Wide-ranging mate searching and polygamous behaviour likely maintain gene flow across Kruger despite the highly localised ranges found within the population.

The lack of significant population structure within the Kruger black rhino population means that individuals for translocations can be selected from any areas within Kruger, and selection criteria can therefore be focused on demographic factors (such as age or sex). This is particularly useful in black rhinos as translocation and reintroduction successes vary greatly with different sex ratios and across age classes (Linklater et al. 2011). Together with other management considerations, such as disease management, ecological integrity or logistical coordination, the results presented in this study indicate that the Kruger black rhino population will offer the best source population of *D. b. minor* currently residing in South Africa. Furthermore, using the Kruger population to supplement KwaZulu-Natal reserves, as well as other small reserves in South Africa, is likely to rescue diversity in those populations.

Source and founder population comparison

Species translocations and reintroductions have become an important conservation strategy used to mitigate current extinction rates by restoring gene flow between small, isolated populations or

repopulating areas where species have been extirpated (Weeks et al. 2011). In addition to improving population growth rates and facilitating genetic conservation, translocating black rhinos offers a tactical benefit to rhino managers through reducing the impact of poaching and stochastic events on single populations (Emslie et al. 2009). Despite the benefits the overall success rates of translocations and reintroductions are low (Griffith et al. 1989; Armstrong & Seddon 2008; Germano & Bishop 2009). Additionally, these already low success rates may be an over-estimation of the true success rate due to reporting biases where failed reintroduction and translocation projects are seldom published (Fischer & Lindenmayer 2000; Miller et al. 2014). A reintroduction is considered successful if the released organisms are able to survive, successfully reproduce and increase their numbers in their new habitat (Armstrong & Seddon 2008; Seddon et al. 2012).

Factors contributing to the success or failure of translocations and reintroductions include a complex combination of socio-ecological factors (e.g., habitat quality and reserve size, cohort size, sex and age structure, genetics, translocation logistics, experience of those involved and legal considerations) which vary with both source and receiver populations (Linklater et al. 2011) as well as between taxa (Germano & Bishop 2009). Fischer and Lindenmayer (2000) reviewed the outcomes of 180 published animal relocation cases (mostly mammals and birds) and found that the re-introductions were more likely to succeed with wild (as opposed to captive) source populations, when the founder population was relatively large (n>100), and when the cause of the original decline was eliminated. Biebach & Keller (2012) have shown that success rates of reintroductions and translocations of Alpine ibex populations were improved with fewer, but more genetically diverse animals, compared to those with more animals, but with less genetic variation. Therefore, when considering reintroduction and translocations as a conservation strategy, understanding the factors contributing to a success for a particular species and population is important.

As far back as 1962, translocations and reintroductions of black rhinos in South Africa were carried out with varying degrees of success (Knight & Kerley 2009). For example, in 1997, 27 *D. b. minor* were translocated from KwaZulu-Natal to Malilangwe Private Game Reserve in Zimbabwe (Kotzé et al. 2014). This re-established population is thriving in its new habitat, and is considered successful from a biological and financial perspective. However, no outbreeding with the local Zimbabwe black rhinos has been allowed due to concerns of outbreeding depression (Anderson-Leder et al. 2012). When crossing divergent populations, outbreeding depression (i.e., a reduction in reproductive fitness and juvenile survival) may occur through the break-up of genetic combinations that may have facilitated local adaptation (Templeton 1986). Frankham et al. (2011) describe three factors that can help predict

the likelihood of outbreeding depression in supplemented populations of the same species: 1) if they have different karyotypes, 2) if they have been separated for more than 500 years, and 3) if they occupy different environments. Although future studies on *D. b. minor* in Zimbabwe and KwaZulu-Natal may confirm, or refute, any chromosomal differences (Anderson-Lederer et al. 2012), the different environment between these two population is unlikely to contribute to outbreeding depression, as evidenced by the healthy status of the translocated KwaZulu-Natal *D. b. minor* in Malilangwe, Zimbabwe. Furthermore, studies have shown that the Zimbabwe and KwaZulu-Natal gene pools were historically connected (Kotzé et al. 2014) and thus belonged to the same population only a few generations previously. Together these factors suggest that the likelihood of outbreeding depression between these two populations is low. While previous black rhino translocations largely occurred under conditions of crisis management, with little concern for the genetic consequences (Braude & Templeton 2009), improved management and genetic data provide useful tools for more strategic forward planning. We can now explore the genetic outcome in extant populations that are the progeny of these re-established populations.

The current Kruger black rhino population is such an admixed population, having been re-established with founder members from both KwaZulu-Natal and Zimbabwe source populations. This study compared the mtDNA control region of the Kruger founder population and its two source populations (KwaZulu-Natal and Zimbabwe), 37 years after the successful translocation and re-introduction of 81 black rhinos into Kruger. Results presented here reveal that at least four haplotypes exist within the Kruger black rhino population. Genetic analyses of museum specimens showed four mtDNA haplotypes to have historically existed within South African black rhinos (Moodley et al. 2017), thus the four haplotypes observed within the Kruger population in this study demonstrate that mixing the Zimbabwe and KwaZulu-Natal gene pools has restored the historic mtDNA diversity of South African black rhinos. While it is possible that the low diversity in the KwaZulu-Natal black rhinos may be due to local adaptation, the relatively recent historical gene flow between the Zimbabwe and KwaZulu-Natal gene pools, together with evidence that historically South African black rhinos contained at least four mtDNA haplotypes (Moodley et al. 2017), oppose the theory of local adaptation. It is more plausible that the current low diversity in the KwaZulu-Natal population, a recent occurrence, is a consequence of the genetic bottleneck that this population experienced. Fears of loss of local adaptation through mixing gene pools may also be a moot point in the face of future climatic changes as the environmental conditions driving this adaptation are themselves unstable. Furthermore, it may be a more responsible strategy to encourage greater variation in re-established populations, providing

the evolutionary potential for these critically endangered animals to survive rapidly changing conditions.

The most common Kruger haplotype (H2) is shared with the single haplotype seen in the KwaZulu-Natal samples. This single haplotype is consistent with other studies investigating the mtDNA within the KwaZulu-Natal black rhino population (Anderson-Lederer et al. 2012; Kotzé et al. 2014). Two Kruger haplotypes (H1 and H5) were shared with Zimbabwe black rhinos; the remaining Kruger haplotype (H3) was reported in a captive Zimbabwe black rhinos (Fernando et al. 2006), but without any further samples, date or locality information, I did not include this sample in my analyses. Thus a minimum of two (or three, if including H3) of the five Zimbabwean haplotypes are present in Kruger, i.e. at least 40-60% of the Zimbabwean lineages are represented in the Kruger population. It is also possible that with more sampling additional Zimbabwean haplotypes would be detected in Kruger. The comparison of the contribution of founders as observed in the current Kruger mtDNA haplotype distribution versus the original ratio of Zimbabwe: KwaZulu-Natal females, shows a 68.87% increase in the Zimbabwe contribution relative to the initial translocation ratio. This percentage increase is even more remarkable when including haplotype 3 (92.14%). One possible explanation is that this is an indication of natural selection favouring black rhinos containing the Zimbabwe mtDNA haplotypes. However, this interpretation is speculative as the result observed may also be due to random chance. For example, if stochastic events caused mortalities within the founder females from KwaZulu-Natal soon after reintroduction, the initial ratio of KwaZulu-Natal: Zimbabwe females contributing to the current population would differ from what are considered to be the initial ratios. Additionally, reintroductions were staggered between 1971 and 1989. This may mean that the initial Zimbabwe contribution is underestimated, as Zimbabwe females introduced in the early years were already contributing to the gene pool at the time of later introductions from KwaZulu-Natal. Further investigations are needed to provide more insight into this finding.

When determining the success or failure of a reintroduced population, geneticists may debate whether the new population is an accurate reflection of the original gene pool it replaces. However, since the original population no longer exists, determining the historical accuracy may be difficult (Falk et al. 2001). Perhaps a more pertinent conservation question to ask is whether the reintroduced population is functional (i.e., will it persist, is it resilient and is it stable?). As a means to implement the BMP, the Black Rhino Range Expansion Project (BRREP), a partnership between the World Wildlife Foundation (WWF), provincial conservation agencies (Ezemvelo KwaZulu-Natal Wildlife and Eastern Cape Parks and Tourism Board) and private landowners (Sherriffs 2003), has facilitated the founding

of 13 new black rhino populations, translocating over 200 individuals since 2003 (WWF-South Africa). Evidenced by the 21% increase in the KwaZulu-Natal black rhino population since 2003 (WWF-South Africa), BRREP has been considerably successful in the short term. Results presented in this study may present an exciting opportunity to consider supplementing these populations with additional genetic diversity that will also aid in securing their long term resilience.

It must also be borne in mind that genetic rescue should not be considered a panacea for all species threatened with extinction. As a relatively novel management and conservation strategy, its long-term effects may be overstated and misunderstood. For example, populations of the Florida panther which has served as the benchmark for successful genetic rescue, remain small, isolated and therefore vulnerable to demographic and stochastic forces (van de Kerk et al. 2019). Without addressing the factors leading to the decline of an endangered population in the first instance (such as poaching and habitat loss in the case of black rhinos), these populations will continue to be vulnerable.

Conclusions

An accurate understanding of the contemporary genetic composition in a free-roaming *D. b. minor* black rhino population is key to preserving the genetic health of this subspecies across isolated protected areas. This study found relatively high levels of genetic diversity, low levels of relatedness and a low inbreeding coefficient within the Kruger black rhino population. The population was found to be panmictic, with minimal genetic structure, indicating high levels of gene flow. A substantial proportion (40-60%) of the reported Zimbabwean black rhino haplotypes were represented. The results presented here have several important implications for the conservation and management of *D. b. minor* populations. First, this study offers baseline metrics for the genetic monitoring and restoration of small black rhino populations. Second, given the encouraging levels of genetic diversity observed, the Kruger black rhino population is an ideal candidate for founding new populations or improving the genetic variation (and thus reducing extinction risk) for isolated, genetically depauperate populations. Finally, the results presented in this study provide empirical evidence that mixing the KwaZulu-Natal and Zimbabwe gene pools has served to genetically rescue the Kruger black rhino population over time. This is an example of how a management strategy (the reintroduction of black rhinos into Kruger National Park from two different source populations) can have successful conservation implications.

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Appendix

Table A1: Summary of the study black rhino individuals from Kruger National Park, South Africa. Sampling location and sex and is reported for each individual.

Sample Name	ID	Section (Origin)	Sex
ZM190553	BR88	Crocodile bridge	Female
ZM190554	BR90	Crocodile bridge	Female
ZM190587	CB1	Crocodile Bridge	Female
ZM190600	BR13	Crocodile Bridge	Female
ZM190621	BR49	Crocodile Bridge	Male
ZM190625	BR89	Crocodile bridge	Female
ZM190576	C5 (Thor)	Kingfisherspruit	Male
ZM190584	K1	Kingfisherspruit	Female
ZM190585	L1	Lower Sabie	Female
ZM190586	L2	Lower Sabie	Female
ZM190591	BR04	Lower Sabie	Female
ZM190105	BR34	Malelane	Male
ZM190106	BR35	Malelane	Female
ZM190107	BR36	Malelane	Male
ZM190108	BR37	Malelane	Male
ZM190109	BR38	Malelane	Male
ZM190110	BR39	Malelane	Female
ZM190111	BR40	Malelane	Male
ZM190112	BR41	Malelane	Female
ZM190113	BR42	Malelane	Female
ZM190114	BR43	Malelane	Female
ZM190515	BR44	Malelane	Female
ZM190516	BR45	Malelane	Male
ZM190517	BR46	Malelane	Male
ZM190518	BR47	Malelane	Male
ZM190519	BR48	Malelane	Male
ZM190521	BR51	Malelane	Female
ZM190522	BR52	Malelane	Male
ZM190523	BR53	Malelane	Female
ZM190524	BR54	Malelane	Female
ZM190525	BR55	Malelane	Male
ZM190526	BR59	Malelane	Female
ZM190527	BR60	Malelane	Male
ZM190579	C30 (Phoebe)	Malelane	Female
ZM190582	M1	Malelane	Male
ZM190583	M2	Malelane	Female
ZM190601	BR14	Malelane	Female
ZM190602	BR15	Malelane	Female
ZM190607	BR20	Malelane	Female
ZM190614	BR27	Malelane	Female
ZM190617	BR30	Malelane	Female

ZM190622	BR56	Malelane	Male
ZM190623	BR57	Malelane	Female
ZM190580	P1 (N17/27)	Pretoriuskop	Male
ZM190581	P2	Pretoriuskop	Male
ZM190520	BR50	Satara	Female
ZM190529	BR62	Skukuza	Male
ZM190530	BR63	Skukuza	Male
ZM190537	BR70	Skukuza	Female
ZM190546	BR80	Skukuza	Female
ZM190578	C13 (Marcules)	Skukuza	Male
ZM190588	BR01	Skukuza	Female
ZM190589	BR02	Skukuza	Female
ZM190590	BR03	Skukuza	Female
ZM190595	BR08	Skukuza	Female
ZM190596	BR09	Skukuza	Female
ZM190597	BR10	Skukuza	Female
ZM190598	BR11	Skukuza	Female
ZM190599	BR12	Skukuza	Female
ZM190603	BR16	Skukuza	Female
ZM190605	BR18	Skukuza	Female
ZM190606	BR19	Skukuza	Female
ZM190608	BR21	Skukuza	Female
ZM190609	BR22	Skukuza	Female
ZM190611	BR24	Skukuza	Female
ZM190613	BR26	Skukuza	Male
ZM190618	BR31	Skukuza	Male
ZM190624	BR81	Skukuza	Male
ZM190531	BR64	Skukuza	Female
ZM190532	BR65	Skukuza	Male
ZM190533	BR66	Skukuza	Male
ZM190534	BR67	Skukuza	Female
ZM190535	BR68	Skukuza	Female
ZM190536	BR69	Skukuza	Female
ZM190538	BR71	Skukuza	Male
ZM190539	BR73	Skukuza	Male
ZM190540	BR74	Skukuza	Male
ZM190541	BR75	Skukuza	Male
ZM190542	BR76	Skukuza	Female
ZM190543	BR77	Skukuza	Male
ZM190544	BR78	Skukuza	Male
ZM190545	BR79	Skukuza	Male
ZM190635	BR101	Skukuza	Male
ZM190612	BR25*	Skukuza/Skukuza or Skukuza/Malelane	Male

ZM190528	BR61	Stolznek	Male
ZM190547	BR82	Stolznek	Female
ZM190548	BR83	Stolznek	Male
ZM190549	BR84	Stolznek	Male
ZM190550	BR85	Stolznek	Male
ZM190551	BR86	Stolznek	Female
ZM190552	BR87	Stolznek	Male
ZM190592	BR05	Stolznek	Female
ZM190593	BR06	Stolznek	Female
ZM190594	BR07	Stolznek	Female
ZM190604	BR17	Stolznek	Female
ZM190610	BR23	Stolznek	Female
ZM190615	BR28	Stolznek	Female
ZM190616	BR29	Stolznek	Male
ZM190619	BR32	Stolznek	Female
ZM190620	BR33	Stolznek	Male
ZM190577	C12 (Odin)	Tshokwane	Male
ZM190626	BR91	Tshokwane	Male
ZM190627	BR92	Tshokwane	Male
ZM190628	BR94	Tshokwane	Male
ZM190629	BR95	Tshokwane	Female
ZM190630	BR96	Tshokwane	Male
ZM190631	BR97	Tshokwane	Male
ZM190632	BR98	Tshokwane	Male
ZM190633	BR99	Tshokwane	Male
ZM190634	BR100	Tshokwane	Female

*Born in Rhino orphanage, parents from Kruger National Park, South Africa

Table A2: Linkage disequilibrium for 23 microsatellite loci

	DB23	DB1	BIRh37D	DB52	ZF1	SR74	32A	SR281	IR22	BIRh1C	SR63	IR12	7B	SRS262	7C	DB44	BIRH1B	BR6	DB66	SR268	12F	IR10	32F
DB23																							
DB1	-																						
BIRh37D	-	-																					
DB52	-	-	-																				
ZF1	-	-	-	-																			
SR74	-	-	-	-	+																		
32A	-	-	-	-	-	-																	
SR281	-	-	-	-	-	-	-																
IR22	-	-	-	-	-	-	-	-															
BIRh1C	-	-	-	-	-	-	-	-	-														
SR63	-	-	-	-	-	-	-	-	-	-													
IR12	-	-	-	-	+	+	-	-	-	-	-												
7B	-	-	-	-	-	-	-	-	-	-	-	-											
SRS262	-	-	-	-	-	-	-	-	-	-	-	-	-										
7C	-	-	-	-	-	-	-	-	-	-	-	-	-	-									
DB44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								
BIRH1B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+							
BR6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
DB66	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-					
SR268	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
12F	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-			
IR10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
32F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

+ Significant linkage disequilibrium (P < 0.001 after the Bonferroni correction was applied)

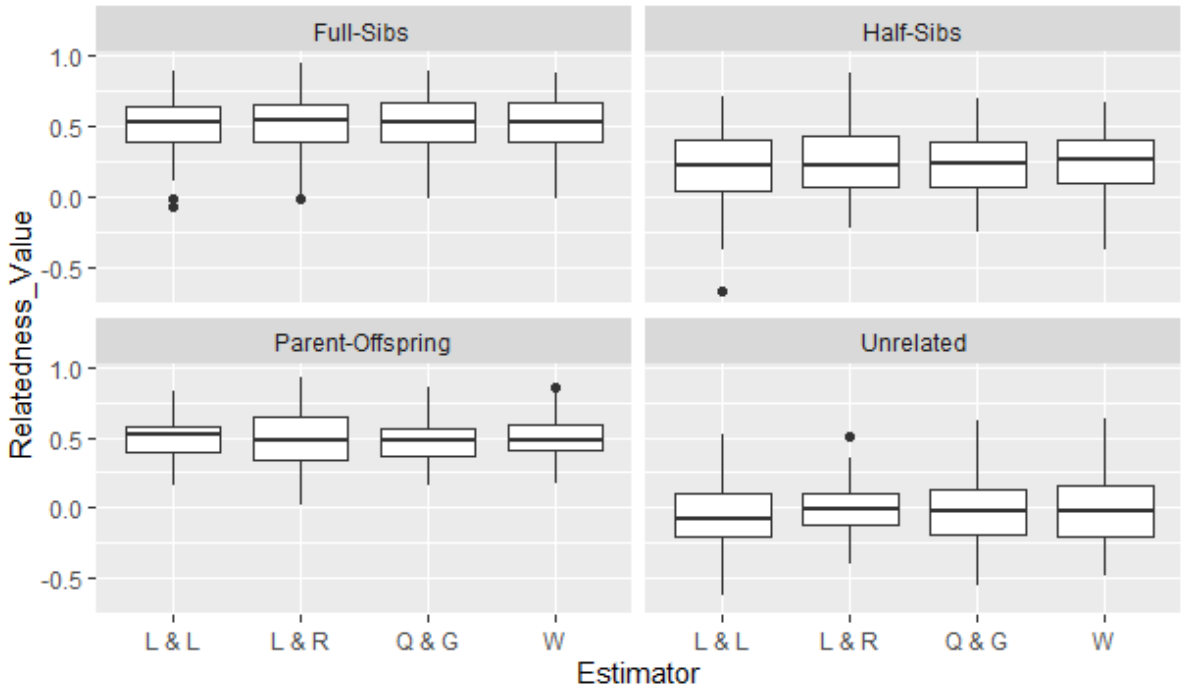

Figure A1. Performances of four non-likelihood relatedness estimators (L&L – Li et al. 1993; L&R – Lynch & Ritland 1999; Q&G – Queller& Goodnight 1989; and W – Wang 2002) on simulated data sets. The 'related' package in R statistics (Pew et al. 2015) was used to perform the simulations with 100 simulated pairs of individuals for each type of relationship.

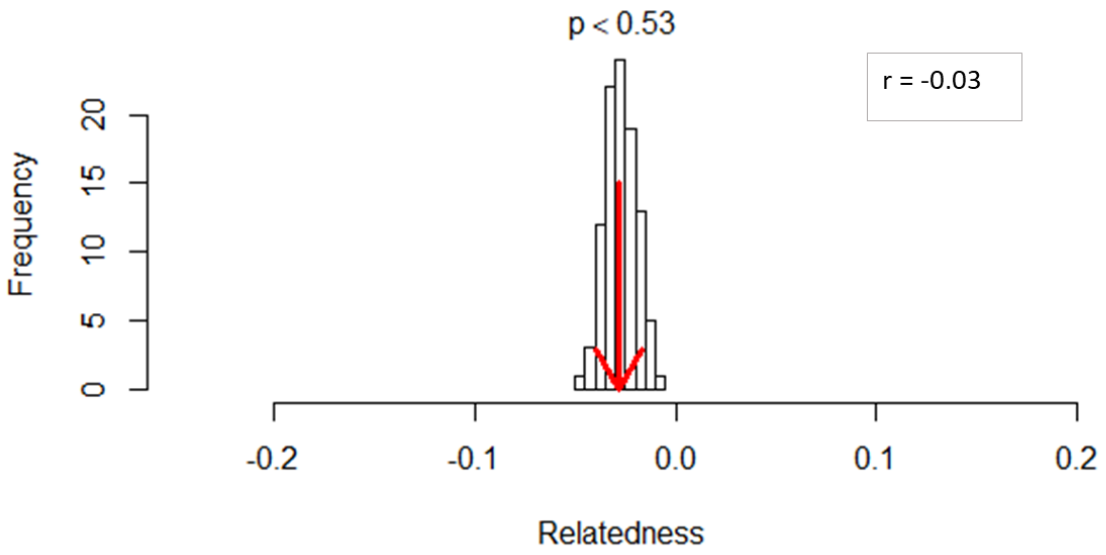

Figure A2. Histogram of the expected relatedness values within Kruger National Park, using the 'related' package of R (Pew et al. 2015). The red arrow indicates the observed value. The p-value indicates the percentage of randomized iterations where the expected values were greater than or equal to the observed value. The r value indicates the overall mean pairwise relatedness.