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Limited mitochondrial DNA variation within South Africa's black rhino (*Diceros bicornis minor*) population and implications for management

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Abstract

The taxonomy of African black rhinoceros (*Diceros bicornis*) remains unresolved. Maintaining levels of genetic diversity and species rescue by reintroduction and restocking requires its resolution. We compared the sequences of the mitochondrial DNA (mtDNA) control region for a total of 101 *D. bicornis* from three subspecies: *D. b. minor*, *D. b. michaeli* and *D. b. bicornis*. A single unique haplotype was found within the 65 *D. b. minor* samples from KwaZulu-Natal (KZN) Province, South Africa, 55 of which came from Hluhluwe-iMfolozi Game Park (HiP) and Mkuzi Game Reserve (MGR) source populations. However, six different haplotypes were represented in eleven *D. b. minor* samples from Zimbabwe. Similarly, published autosomal microsatellite data indicate low levels of diversity within the KZN *D. b. minor* populations. The low levels of mtDNA diversity within the KZN metapopulation point to the possible need for genetic supplementation. However, there is a need to determine whether the low levels of genetic variation within KZN *D. b. minor* are a result of the recent bottleneck or whether KZN historically always had low diversity.

Key words: conservation genetics, control region, *Diceros bicornis*, D-loop, genetic rescue

Résumé

La taxonomie du rhinocéros noir d'Afrique (*Diceros bicornis*) n'est pas encore résolue. Pour préserver le taux de diversité génétique et pouvoir sauvegarder l'espèce par des réintroductions et des repeuplements, il faut résoudre cette question. Nous avons comparé les séquences de la région de contrôle de l'ADN mitochondrial (ADNmt) d'un total de 101 *D. bicornis* appartenant aux trois sous-espèces *D. b. minor*, *D. b. michaeli* et *D. b. bicornis*. Nous avons trouvé un seul et unique haplotype pour les 65 échantillons venant de *D. b. minor* de la province du KwaZulu-Natal (KZN) en Afrique du Sud, dont 55 venaient de populations sources du Parc de Hluhluwe-iMfolozi (HiP) et de la Réserve de Faune de Mkuzi (MGR). Par contre, il y avait six haplotypes différents dans 11 échantillons de *D. b. minor* venant du Zimbabwe. Les données publiées sur les microsatellites autosomaux indiquent, elles aussi, un faible taux de diversité au sein des populations de *D. b. minor* du KZN. Le faible taux de diversité de l'ADNmt dans la métapopulation du KZN indique un éventuel besoin de supplémentation génétique. Cependant, il faut d'abord déterminer si le faible taux de variation génétique chez les *D. b. minor* du KZN est un résultat des réductions récentes ou si le KZN a toujours eu une faible diversité.

Introduction

Species conservation depends on identifying genetically distinct groups or management units and implementing strategies to retain genetic variation. Genetically distinct populations can contain unique genetic variation, and/or they can be locally adapted to their habitat. Mixing them with other populations may break up genetically complex traits and, in some cases, lead to outbreeding depression (Templeton, 1986; O'Ryan, Flamand & Harley, 1994). Alternatively, genetic differences between populations can also result from strong genetic drift caused by population fragmentation and declining

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population sizes (Frankham, Ballou & Briscoe, 2002; Allendorf & Luikart, 2007). When the genetic structure and historic pattern of gene flow of a species has been described, reintroduction methods can be used to secure locally adapted populations or restocking used for genetic supplementation.

Variation in mitochondrial DNA (mtDNA) is a particularly useful metric for determining population structure and history (Moritz, 1994). The control region of mtDNA is highly variable, and it can often be used to resolve phylogenetic relationships between closely related taxa or for describing the genetic structure within species (Moritz, Dowling & Brown, 1987; Kidd & Friesen, 1998). MtDNA is maternally inherited and so does not recombine (Hayashi, Tagashira & Yoshida, 1985), which means it reflects a quarter the effective population size (N_e) compared with nuclear loci and hence it is more sensitive to changes in population demography.

The black rhinoceros (*Diceros bicornis:* Perisodactyla) once ranged across the African continent and numbered in the hundreds of thousands (Western & Vigne, 1985). By 1969, their numbers had declined to ~65,000 (Muya & Oguge, 2000), and during the last century, the species disappeared faster than any other large mammal (Hitchens, 1975; Western & Vigne, 1985). The major causes for their decline have been anthropogenic, primarily illegal hunting (Western & Vigne, 1985; Emslie & Brooks, 1999; Amin *et al.*, 2006). Nevertheless, conservation efforts have seen *in situ* black rhino numbers increase from a low of 2475 individuals in 1993 to approximately 4880 in 2010 (Emslie, 2011).

Three extant black rhino subspecies are recognized across Africa, including approximately 742 *D. b. michaeli* (Eastern black rhino), 1922 *D. b. bicornis* (South–western black rhinoceros) and 2216 *D. b. minor* (South–central black rhinoceros) (Emslie, 2011). Appraisal of the black rhino subspecies was initially based on skull measurements (Zukowsky, 1964; Groves, 1964; du Toit, 1987); however, uncertainty regarding taxonomy remained (du Toit, 1987). Although there are apparently no impervious geographic boundaries or reproductive barriers between the subspecies, they occupy different areas with distinct habitats and climates (Harley *et al.*, 2005; Emslie & Brooks, 1999). With no historical records of migration and the extent of gene flow between the subspecies unknown, some authors have speculated that each subspecies may have genetic or behavioural adaptations to their local environments (Emslie & Brooks, 1999; Harley *et al.*, 2005). Their suggestion regarding genetic differences was confirmed through recent mtDNA and autosomal DNA analyses (Merenlender *et al.*, 1989; Ashley, Melnick & Western, 1990; O'Ryan & Harley, 1993; O'Ryan, Flamand & Harley, 1994; Swart & Ferguson, 1997; Brown & Houlden, 1999, 2000; Nielsen *et al.*, 2008; Karsten *et al.*, 2011; Muya *et al.*, 2011). Thus, current black rhino management policy is for each subspecies to be managed separately in order to maintain possible local adaptive traits and minimize the risk of outbreeding depression (Templeton, 1986; O'Ryan, Flamand & Harley, 1994; Brown & Houlden, 2000; Harley *et al.*, 2005).

The largest remnant population of the critically endangered (IUCN, 2008) *D. b. minor* subspecies is in Hluhluwe-iMfolozi Game Park (HiP) (n = ~220 Clinning *et al.*, 2009) in KwaZulu-Natal (KZN) Province, South Africa (Fig. 1). KZN black rhinos have been separated from other populations to the north (e.g. Zimbabwe) since at least the latter half of the 19th century (Swart *et al.*, 1994). HiP and the smaller remnant in Mkuze Game Reserve (MGR) (n = ~45 D. Kelly, personal communication) have been sources for metapopulation expansion and genetic management by reintroduction and restocking. Translocations from HiP to other KZN reserves first began in 1962, expanded to other South African provinces and later to other African nations (e.g. Zimbabwe, Zambia) (Hitchens, 1984; Emslie, Amin & Kock, 2009). The potential now exists for KZN *D. b. minor* to be mixed with *D. b. minor* in or from other smaller African populations, especially those in Zimbabwe, if they are not too genetically divergent. Although the KZN population will likely be strategic to the subspecies recovery throughout the African continent (Emslie & Brooks, 1999), no study has yet compared the mtDNA sequences of the KZN *D. b. minor* metapopulation with populations outside South Africa.

The aim of our study was to use mtDNA control region sequences (406 bp) to determine the level of variation within the *D. b. minor* source population at HiP (n = 50) and compare it with the KZN metapopulation (n = 15) and *D. b. minor* populations outside South Africa (n = 11) and the other black rhino subspecies (*D. b. michaeli* n = 21, *D. b. bicornis* n = 4). We considered the implications of our findings for the long-term management of *D. b. minor* and make recommendations for possible future research.

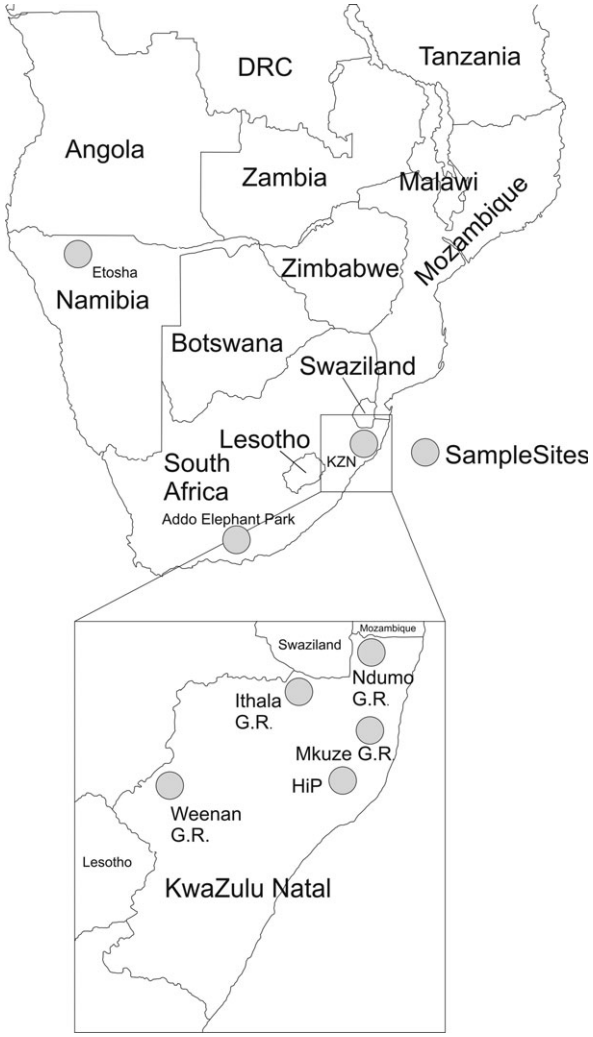

Fig 1 Map of southern Africa showing black rhinoceros sample sites. Inset showing KwaZulu-Natal study Game Reserves (Ndumo G.R., Ithala G.R., Mkuze G.R., Hluhluwe-iMfolozi Game Park (HiP) and Weenan G.R.).

Materials and methods

Sampling

Samples of blood and pinna ear tissue were collected from individuals of *D. b. minor* in the KZN province in South Africa (n = 65), *D. b. michaeli* in Addo Elephant National Park, South Africa (n = 1), and *D. b. bicornis* in Namibia's northern region (n = 4) (Fig. 1). The samples were acquired opportunistically during routine translocation and ear notching (for identification) events from 2002 to 2009. Blood samples were stored in cryovials containing 1 ml of DMSO/EDTA/Tris/salt solution (Seutin, White & Boag, 1991).

DNA sequencing and analysis

DNA extraction. Seventy micro-litres of the preserved blood solution or a 3 × 3 mm piece of pinna ear tissue was digested in an SDS/proteinase-K solution. After dissolution, a standard phenol–chloroform DNA extraction and ethanol precipitation was conducted following the procedure of Sambrook, Fritsch & Maniatis (1989).

PCR and DNA sequencing. A fragment of the mitochondrial DNA control region (406 bp) was amplified using the primers mt15996L (5′-TCCACCATCAGCACCCAA AGC-3′) (Campbell *et al.*, 1995; Brown & Houlden, 2000) and mt16502H (5′-TTTG-ATGGCCCTGAAGTAAGAAC CA-3′) (Moro *et al.*, 1998; Brown & Houlden, 2000). PCR amplifications using 1–2 μl of DNA template were carried out in 25 μl volumes with 67 mM Tris pH 8.8, 16 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.4 μg ml^{-1} BSA, 0.4 μl of each of the forward and reverse primer, 200 μM of each dNTP and 0.5–1 units of BIOTAQ DNA polymerase (Bioline USA Inc., Taunton, MA, USA). Thermal cycling was carried out using an Eppendorf Mastercycler at 94°C for 2 min, (94°C for 3 min, 50–54°C for 30 s, 72°C for 2 min), repeated for 30–40 cycles, followed by a final step of 72°C for 3 min.

PCR products were electorphoresed in agarose gel, and a molecular weight standard was used to determine the size of amplified products. Products of the correct size were purified using column purification (Roche Corporate Communications, Basel, Switzerland) or ExoSAP-IT (GE Healthcare, Waukesha, WI, USA), and their DNA sequence was determined using an ABI 3730 Genetic Analyzer (Massey Genome Service, Palmerston North, New Zealand).

For comparison, eleven *D. b. minor* sequences stored in GenBank (accession numbers AF187825 - AF187827 & AF187829 - AF187831, Brown & Houlden, 2000; AY742832 & AY742833, Fernando *et al.*, 2006) originally sampled from Zimbabwe and zoos in Australia and the United States were added to the data set, in addition to 20 *D. b. michaeli* samples (accession numbers AF187834 & AF187835, Brown & Houlden, 2000; AY742830 & AY742831, Fernando *et al.*, 2006; FJ227484 - FJ227498, Muya *et al.*, 2011) originally

sampled from Kenya and zoos in Australia and the United States.

Data analysis. The 101 mitochondrial DNA sequences were edited by eye and then aligned using Clustal W (Larkin *et al.*, 2007). Homogeneity of base compositions was tested using PAUP 4.10b (Swofford, 2002). DnaSP v 5.10.1 (Rozas *et al.*, 2003) was used to calculate haplotype diversity (h), nucleotide diversity (π) and standard deviation (SD) within the subspecies. The level of sequence divergence within and between populations was estimated using a pairwise distance analysis in MEGA 5.1 (Tamura *et al.*, 2011), and standard errors were calculated using a bootstrap procedure. A statistical parsimony haplotype network was calculated using NETWORK 4.610 (Bandelt, Forster & Rohl, 1999).

Results

The sequence of the mtDNA control region was determined for a total of 70 individual black rhinos as follows: *D. b. minor* samples: 50 from HiP, eight from Itala, five from MGR, one from Ndumo Game Reserve, one from the Johannesburg Zoo (accession number JN593089) and eleven sequences from GenBank (accession numbers AF187826 - AF187831, AY742832 - AY742833 & AF187832 - AF187833); *D. b. michaeli* samples: one from Addo Elephant Park (Accession number JN5930090) and 20 from GenBank (accession numbers FJ227483 - FJ227498, AY742830 - AY742831 & AF187834 - AF187835) and four samples for *D. b. bicornis* from Namibia's northern region (accession numbers JN593091-JN593094) (Table 1).

The 101 aligned sequences were 363 bp long with 31 polymorphic sites, and there was an average pairwise difference of 4% (±1%) between *D. b. michaeli* and *D. b. minor*, 4.5% (±1.1%) between *D. b. michaeli* and *D. b. bicornis* and 2.3% (±0.8%) between *D. b. minor* and *D. b. bicornis*. No insertions or deletions were observed.

Considering each subspecies separately, the greatest level of diversity was recorded in *D. b. michaeli* (n = 21), which contained thirteen haplotypes and showed comparatively high nucleotide diversity ($\pi = 0.011 \pm 0.00106$) and haplotype diversity ($h = 0.958 \pm 0.026$) (Table 2). The lowest level of diversity within a subspecies was seen in the Namibian *D. b. bicornis* samples

Table 1 Rhinoceros subspecies and sources analysed for mtDNA variation

Subspecies	Sample size	Sample/sequence source	References
Diceros bicornis minor	50	Hluhluwe-iMfolozi Game Park, KNZ Region, Accession number JN593089	This study
	8	Itala Game Park, KNZ Region	This study
	5	Mkuze Game Park, KNZ Region	This study
	1	Ndumo Game Park, KNZ Region	This study
	1	Johannesburg Zoo	This study
	6	Chete National Park, Zimbabwe, Accession numbers AF187825-AF187827 & AF187829-AF187831	Brown & Houlden, 2000;
	2	Zambezi Valley, Zimbabwe, Accession numbers AY742832-AY742833	Fernando *et al.*, 2006;
	1	Captive born, San Diego Zoo Accession number AF187832	Brown & Houlden, 2000;
	1	Captive born, Milwaukee Zoo, Accession number AF187833	Brown & Houlden, 2000;
	1	Captive born, Western Plains Zoo Accession number AF187828	Brown & Houlden, 2000;
D. b. michael	16	Kenya, Accession numbers FJ227483-FJ227498	Muya 2011
	2	Solio Game Reserve, Kenya, Accession numbers AY742830-AY742831	Fernando *et al.*, 2006;
	1	Cincinnati Zoo, Accession number AF187834	Brown & Houlden, 2000;
	1	Taronga Zoo, Accession number AF187835	Brown & Houlden, 2000;
	1	Addo Elephant Park, South Africa Accession number JN593090	This study
D. b. bicornis	4	Etosha, Namibia, Accession numbers JN593091-JN593094	This study
Ceratotherium simum	4	HiP & London Zoo, Accession numbers AF187836-AF187839	Brown & Houlden, 2000

Table 2 MtDNA D-loop sequence variability within subspecies

		Genetic variability				
	n	H	h	(SD)	π	(SD)
Subspecies						
Diceros bicornis minor (aggregate)	76	7	0.267	0.067	0.0023	0.001
KZN metapopulation	65	1	–	–	–	–
Zimbabwe samples	11	6	0.855	0.085	0.0074	0.001
D. b. michaeii	21	13	0.952	0.024	0,0112	0.001
D. b. bicornis	4	1	–	–	–	–

Sample size (n), Number of haplotypes (H), haplotype diversity (*h*), Nucleotide diversity (π), Standard deviation (SD).

(n = 4) where only one unique haplotype was found; however, this was based on a small sample size and might not represent the total amount of genetic variation within the population. The pooled KZN samples and GenBank sequences of all *D. b. minor* individuals (n = 79) contained seven haplotypes, and haplotype diversity (*h*) was 0.267 ± 0.067 and a nucleotide diversity (π) of 0.002 ± 0.00063. The eight *D. b. minor* Zimbabwe sequences from Brown & Houlden (2000) and two from Fernando *et al.* (2006) had shared haplotypes (Table 3); however, there were no shared haplotypes with the KZN samples.

The haplotype network (Fig. 2) shows a clear pattern of the separation amongst the three currently recognized subspecies with the KZN population falling out with the *D. b. minor* populations of Zimbabwe. Our finding of no more than three base pair substitutions between adjacent haplotypes within the *D. b. michaeli* subspecies is consistent with the finding by Muya *et al.* (2011). There is a significant separation between *D. b. minor* and *D. b. bicornis* with eight base pair substitutions as well as between *D. b. minor* and *D. b. michaeli* with nine base pair substitutions.

Discussion

We showed that the KZN population of *D. b. minor* is fixed for a single mtDNA haplotype, like most Sumatran rhino (*Dicerorhinus sumatrensis*) populations (Morales *et al.*, 1997). However, unlike the Sumatran rhino populations that have occupied separate land masses for more than 10,000 years (Morales *et al.*, 1997), it has been widely assumed that the KZN *D. b. minor* population has been separate from other *D. b. minor* populations only recently (i.e. caused by anthropogenic settlement and habitat modification during the 19th century, Swart *et al.*, 1994). The single mtDNA haplotype in KZN *D. b. minor* raises the question of whether the KZN remnant population lost genetic variation recently because of the population bottleneck or has been a genetically separate lineage for longer than previously thought.

MtDNA has a smaller effective population size (N_e) compared to nuclear loci and is one of the first genetic markers to show the genetic signature of a demographic decline. The likelihood of two or more mtDNA haplotypes persisting within an isolated population is reduced to $P < 0.1$ over $4N_{ef}$ generations, and the population is expected to become monophyletic after $4N_{ef}$ generations (Avise, Neigel & Arnold, 1984; Mucci *et al.*, 1999). If this holds true for the KZN *D. b. minor*, then recent population decline and fragmentation would have increased the rate of drift and might be responsible for the lack of haplotype diversity within the KZN black rhinoceros. Examples of monomorphic haplotypes occurring from severe bottlenecks are well documented in several species. For instance, the Whooping Crane (*Grus americana*) once found throughout North America had six haplotypes in ten prebottleneck museum samples, but only one haplotype persisted in the remnant postbottleneck population of 14 (Glenn, Stephan & Braun, 1999). Such rapid declines in genetic variation have also occurred amongst southern Africa's other large mammals. For example, three small remaining remnant populations of Cape mountain zebra (*Equus zebra zebra*) each contain a single, unique haplotype but larger Namibian populations of closely related Hartmann's mountain zebra (*E. z. hartmannae*) have as many as eleven different haplotypes (Moodley & Harley, 2006; Watson & Chadwick, 2007). Another case in point is the loss of genetic diversity at mitochondrial and Y-chromosome loci observed in small, managed populations of Cape buffalo in Kenya and Uganda, which was attributed to restricted gene flow into protected areas (Van Hooft, Groen & Prins, 2002).

Table 3 Summary statistics for the mtDNA control region sequence variablity in each subspecies and haplotype identifiers used in Fig. 2

		Genetic variability					
Subspecies & population	n	H	h	(SD)	π	(SD)	S
Diceros bicornis minor	76	A-G	0.267	0.067	0.002	0.00063	6
Pooled D. b. minor samples							
Hluhluwe-iMfolozi Game Park (KZN) Accession number JN593089	50	A	–	–	–	–	–
Itala Game Park (KZN)	8	A	–	–	–	–	–
Mkuze Game Park (KZN)	5	A	–	–	–	–	–
Ndumo Game Park (KZN)	1	A	–	–	–	–	–
Johannesburg Zoo	1	A	–	–	–	–	–
Chete National Park, Zimbabwe, Accession numbers AF187825-AF187827 & AF1878329-AF187831	6	B, C & D	0.733	0.155	0.004	0.00056	4
Zambezi Valley, Zimbabwe, Accession numbers AY742832-AY742833	2	E & G	1	0.5	0.008	0.00413	3
Captive born, San Diego Zoo, Accession number AF187832	1	E	–	–	–	–	–
Captive born, Western Plains Zoo, Accession number AF187828	1	B	–	–	–	–	–
Captive born, Milwaukee Zoo, Accession number AF187833	1	F	–	–	–	–	–
D. b. michaeli	21	H-T	0.958	0.026 0.011	–	0.00106	15
Pooled *D. b. michaeli* samples							
Kenya, Accession numbers FJ227483-FJ227498	16	1-S	0.952	0.031	0.011	0.00119	15
Solio Game Reserve, Kenya, Accession numbers, AY742830-AY742831	2	J-T	1	0.5	0.011	0.00413	3
Cincinnati Zoo, Accession number AF187834	1	H	–	–	–	–	–
Taronga Zoo, Accession number AF187835	1	1	–	–	–	–	–
Addo Elephant Park, South Africa, Accession number JN593090	1	T	–	–	–	–	–
D. b. bicornis	4	U	0	–	–	–	–
Pooled *D. b. bicornis* samples							
Etosha, Namibia, Accession numbers JN593091-JN593094	4	U	–	–	–	–	–

Sample size (n), Haplotypes, labelled A–U (H), haplotype diversity (ft), Nucleotide diversity (π), Standard deviation (SD), Number of segregating sites (S).

Low genetic variation is not always a consequence of recent anthropogenic fragmentation. An alternative hypothesis is that low levels of mtDNA and autosomal variation are a result of long-term demographic separation, historically small population sizes and local adaptation. For example, despite having lower mtDNA and autosomal DNA variation, there was no evidence of a genetic bottleneck in the Yellowstone National Park, U.S.A. grizzly bear (*Ursus arctos*) population compared to surrounding grizzly bear populations (Miller & Waits, 2003). Although Yellowstone's large population is embedded within the species' range, Miller & Waits (2003) attribute the lower genetic variation to restricted gene flow into the area from the north. The common impala (*Aepceros melampus melampus*) of KZN also exhibited population differentiation from populations in the Limpopo Province just 490 km north. Schwab *et al.* (2012) attributed the genetic divergence to a narrow zone of unsuitable habitat below the eastern escarpment of the Drakensberg Mountains that impeded dispersal between the two provinces.

Genetic replenishment by restocking and outbreeding is recommended in cases where anthropogenically induced fragmentation has caused a loss in genetic diversity and an increase in genetic divergence. For example, 'genetic rescue' has been recommended for the Cape zebra (Moodley & Harley, 2006; Watson & Chadwick, 2007). However, where differences amongst genetically depauperate populations might be of natural origin, population management may need to take into account local adaptation and the possibility of outbreeding depression.

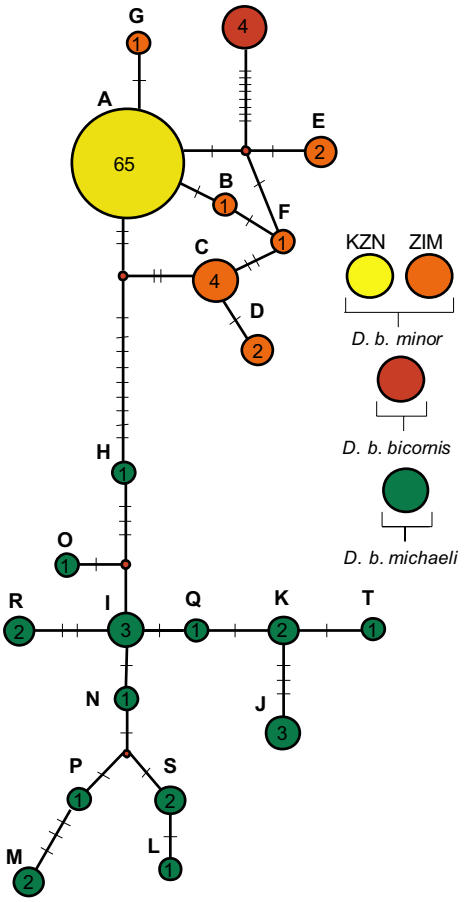

Fig 2 Statistical parsimony haplotype network calculated with Network Software for *Diceros bicornis*. KZN refers to the pooled *D. b. minor* samples within KwaZulu-Natal (KZN) (Ndumo Game Reserve, Ithala Game Reserve, Mkuze Game Reserve, Hluhluwe-iMfolozi Game Park (HiP) and Weenan Game Reserve as well as the sample from the Johanesburg Zoo). ZIM refers to the *D. b. minor* samples from Zimbabwe. Circles with numbers refer to the number of sample with corresponding haplotype from Table 3. The small dots denote the mutational step, and the cross bars are the number of base pair differences.

Resolving the question regarding KZN *D. b. minor* mtDNA and autosomal DNA genetic structure being a recent or old event is important for guiding management plans (Rookmaaker, 2005).

Microsatellite DNA markers were previously used to assess the levels of genetic variation amongst *D. b. minor* populations. Harley *et al.* (2005) found appreciable amounts of variation within the *D. b. minor* subspecies using nine microsatellite loci (Table 4). They recommended that as long as heterozygosity and allele numbers stayed at 'current' levels, no management policy change was necessary.

Based on a survey of ten microsatellites (Table 4), Karsten *et al.* (2011) found low levels of genetic variation within the KZN *D. b. minor*, but concluded that it was not cause for concern. They reached their conclusion based on (i) the similarity of allelic diversity and heterozygosity between the KZN *D. b. minor* population and the other subspecies; and (ii) a higher level of diversity within the black rhinoceros metapopulation compared to those found in other large African mammals. In their study, H_E estimates for the *D. b. bicornis* and *D. b. michaeli* subspecies (each based on only four samples) were substantially lower than those reported by Harley *et al.* (2005) (Table 4). Thus, estimates for *D. b. bicornis* and *D. b. michaeli* in the study by Karsten *et al.* (2011) are probably underestimates. Moreover, comparisons with other large African mammals should be made cautiously. Lions in the Serengeti Plains and Ngorongoro Crater have an H_E of 0.54 and 0.46, on par with black rhinos in the study by Harley *et al.* (2005), yet unlike the Serengeti Plains lions the Ngorongoro Crater lions have a marked decrease in their reproductive rate attributed to inbreeding depression caused by an anthropogenic bottleneck (Brown & Houlden, 1999; Driscoll *et al.* 2002).

HiP *D. b. minor* may be exhibiting signs of inbreeding depression owing to low reproductive rates that are not meeting the 0.25 fecundity rate desired by black rhino managers (Clinning *et al.*, 2009). If historic gene flow is verified and inbreeding is detected, there may be a need for genetic supplementation. Supplementation would also be supported based on genetic distances between the subspecies, low levels of genetic diversity and differentiation within and amongst the KZN metapopulation reported by this mtDNA study and published autosomal microsatellite data.

The likelihood of outbreeding depression in supplemented populations of the same species is low if they have the same karyotype, have been isolated for less than 500 years and occupy similar environments (Frankham *et al.*, 2011). Houck *et al.* (1995) identified the variation in chromosome morphology (number of submetacentric elements) between *D. b. minor* and *D. b. michaeli* zoo samples and recommended further studies to investigate possible differences in geographically separated populations of each subspecies in the wild. Furthermore, twenty-seven KZN *D. b. minor* were translocated to Malilangwe, Zimbabwe in 1997 where

Table 4 Microsatellite results from Harley *et al.*, 2005 and Karsten *et al.*, 2011. Expected heterozygosity (H_E), Observed heterozygosity (H_o)

Study	Microsatellites used	Subspecies	Geographic region	Sample size	H_e	H_o
Harley	BR4, BR6, BR17	*Diceros bicornis* minor	South Africa and	46	0.46	0.436
et al., 2005	(Cunningham *et al.* 1999)	*D. b. bicornis*	Zimbabwe	53	0.51	0.523
	DB1, DB14, DB44, DB49, DB52, DB66	*D. b. michaeli*	Namibia	19	0.68	0.731
	(Brown & Houlden, 1999)		South Africa			
Karsten	BR4, BR6, BR17	*D. b. minor*	South Africa	77	0.44	0.38
et al., 2011;	(Cunningham *et al.* 1999)	*D. b. bicornis*	Namibia	4	0.43	0.46
	DB1, DB14, DB49, DB66	*D. b. michaeli*	South Africa and	4	0.54	0.54
	(Brown & Houlden, 1999)		Tanzania			
	AY606078, AY606080, AY606083					
	(Nielsen *et al.*, 2008)					

they were managed separately and not outbred with any Zimbabwe populations. The translocated population thrived with a growth rate of 8.3% per annum (R. du Toit, personal communication) cf. 3.4% over a 10-year period (1999–2008) in HiP (Clinning *et al.*, 2009). The success of the translocated KZN *D. b. minor* in Zimbabwe alleviated concerns about the adaptability of KZN rhino to Zimbabwe. The only remaining concern is whether the populations have been genetically isolated for longer than previously considered.

We recommend five research tasks to assist in resolving the genetic structure of southern Africa's black rhino as a guide to future management: (i) determine historic levels of genetic variation using museum or collection samples; (ii) investigate whether there is evidence of inbreeding depression within the HiP and KZN metapopulation; (iii) conduct a karyotype analysis on *D. b. minor* in KZN and Zimbabwe to determine whether chromosomal differences exist; (iv) increase the mtDNA sample size of the Zimbabwe *D. b. minor* population. Considering the high level of variation in the small sample size of the Zimbabwe sequences, a larger sample size of *D. b. minor* from that region might show that the KZN haplotype (A) is also there; and (v) lastly, genetic supplementation experiments should be implemented cautiously and systematically. A mixed population should be founded with at least 20 animals as suggested by du Toit (2006), perhaps using the Malilangwe, Zimbabwe translocation event as a template or more recent guidelines (Linklater *et al.*, 2011, 2012). The translocated KZN *D. b. minor* rhinos in Malilangwe have not yet been outbred with the Zimbabwe rhinos (R. du Toit, personal communication) but might be with the F1 and F2 offspring carefully monitored for signs of reduction in reproductive fitness (outbreeding depression). If the research tasks we have recommended are completed and there is evidence of historic gene flow between KZN and Zimbabwe *D. b. minor* and no signs of outbreeding depression in the experimentally mixed population, then KZN *D. b. minor* is a candidate for genetic supplementation using progeny from Zimbabwe populations.

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