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RESEARCH ARTICLE

Genetic structure of the black rhinoceros (*Diceros bicornis*) in south-eastern Africa

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Abstract Despite an on-going struggle to conserve the endangered black rhinoceros (*Diceros bicornis*) since the 1980s, huge capital investment and several genetic surveys, the level of genetic structure and connectivity among populations in southern Africa is not well understood. Here, we undertake a major population genetic study of black rhinoceros in the Zimbabwe Lowveld, an area inhabited by over half of that country's original Zambezi descendants plus one large population sourced from the relict KwaZulu stock of South Africa. Using nuclear microsatellite and mitochondrial DNA data, we found much higher levels of genetic diversity in the indigenous Zimbabwean populations, where observed multilocus heterozygosity was 0.54 versus 0.40 in KwaZulu, and maternal haplotype diversity was 0.77 versus 0.03. We show, for the first time, that both gene pools can be differentiated from each other on the basis of nuclear markers. This, along with the discovery of recent gene flow between all Lowveld populations, suggests that Zimbabwean and South African gene pools were prehistorically connected.

Keywords *Diceros bicornis* · Black rhino · Structure · Connectivity · Conservation

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Introduction

The black rhinoceros (*Diceros bicornis*) was once distributed in large numbers across most of sub-Saharan Africa but suffered a massive reduction in numbers and range during the 20th century due to human pressure and intensive poaching (Leader-Williams 1992; 2002). After a species-wide low of about 2,400 in the mid 1990s (Emslie and Brooks 1999), black rhinoceros numbers have recovered slowly to just over 5,000 today (Emslie 2013), but their range has been severely contracted into isolated protected areas. Only five African countries still maintain aboriginal black rhinoceros populations.

One of these countries is Zimbabwe. Here, the black rhinoceros was once widespread (Fraser 1958; Roth 1967; Milliken and Thomsen 1993) throughout colonial Southern Rhodesia (Zimbabwe) until unregulated sport hunting, poaching and snaring, shooting of "problem animals" in areas required for agriculture and settlement, and controversial tsetse control hunting campaigns carried out from 1919 to 1958 virtually eradicated it from most of the colony,

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except from the valley of the Zambezi River, where control hunting never occurred (Child and Riney 1987). Today the Zambezi River forms an international border between Zimbabwe to the south and Zambia to the north (Fig. 1a). Heavy military presence in the Valley during Zimbabwe's guerrilla war of independence from 1966 to 1979 suppressed rising rhinoceros poaching activity that was then exterminating the even larger population in the nearby Luangwa Valley of Zambia. At independence in 1980, the southern (Zimbabwean) bank of the Zambezi river held one of the world's largest surviving black rhinoceros populations, and perhaps as many as 750 individuals were still present in the lower part of the valley, with additional significant sub-populations further upstream in the Sebungwe region (Cumming et al. 1990). Cross-border poaching from Zambia arose shortly after Zimbabwe's independence as a consequence of the decline in military presence in the Zambezi Valley and the virtual extinction of the Luangwa Valley population by the mid-1980s. This poaching onslaught increased to such an extent that by 1992 there were only 425 black rhinoceros remaining in Zimbabwe (Emslie and Brooks 1999). Most of these remnant individuals comprised two recently-isolated populations along the Zambezi River: Sebungwe, including the Chete Safari Area on the southern shore of Lake Kariba and the broken country to its south; and the Zambezi Valley, that lower portion of the river between Kariba Gorge and the Mozambican border, including the world heritage Mana Pools National Park (Fig. 1a). For ease of reference, we use the term "Zambezi" when referring collectively to Sebungwe and Zambezi Valley stocks. Through a series of major translocation operations in the late 1980s, ~300 Zambezi black rhinoceros were removed from these two regions to "breeding nuclei" away from the cross-border poaching pressure (Cumming et al. 1990). Two main recipient areas for these translocated rhinoceros were Save Valley Conservancy and Bubiana Conservancy in Zimbabwe's Lowveld region, which respectively received approximately 31 and 38 surviving rhinoceros either directly or through two-step translocations from the Zambezi Valley and Sebungwe, over the period 1986–1993. Owing to highly suitable habitats, these newly established populations bred rapidly, achieving population growth rates of between 7.1–8.1 % per annum, not including poaching losses (unpublished data, Lowveld Rhino Trust). By the beginning of 2008 the total population of Zambezi-derived black rhinoceros in these two Lowveld populations reached 280 (unpublished data, Lowveld Rhino Trust), just under half the national population of 546 (Milliken et al. 2009), although by that time the Bubiana population had come under severe poaching pressure and its survivors were largely moved to the adjoining, safer Bubye Valley Conservancy (but for the purposes of this paper will still be referred to as the "Bubiana population"). In addition, in 1997 a group of 27 individuals was introduced to the nearby Malilangwe Wildlife Reserve from KwaZulu (Fig. 1a) in neighbouring South Africa, via a private purchase.

Taxonomically, the Zimbabwean black rhinoceros comprises one of three remaining gene pools within *D. b. minor* (Drummond 1876), the south-central African subspecies that was once thought to be continuously distributed from KwaZulu in the south to northern Tanzania in the north (see Fig. 1b, Groves 1967; Groves and Grubb 2011). Genetic information about Zimbabwean populations is limited. Swart and Ferguson (1997) reported that these rhinoceros showed higher allozyme heterozygosity than any other southern African population, while microsatellite (Harley et al. 2005; Scott 2008) and mitochondrial (Brown and Houlden 2000; Anderson-Lederer et al. 2012) DNA surveys suggest that the East African black rhinoceros shows highest levels of genetic variation. Given the considerable south–north range of *D. b. minor*, its true level of genetic diversity may be higher than previously thought. It is also possible that the three isolated *D. b. minor* gene pools in South Africa, Zimbabwe and Tanzania were connected by prehistoric gene flow via intermediately located populations, such as those that until at least the 1880s still inhabited the Zimbabwean Lowveld (Cumming et al. 1990). Only a limited number of Zimbabwean individuals were available to previous studies (nmax = 8, Harley et al. 2005), and these have always been grouped with South African samples to report general indices of genetic variation for *D. b. minor* as a whole (Harley et al. 2005; Scott 2008). Therefore, the structure of nuclear genetic variation between the Zambezi and KwaZulu gene pools has never been addressed. To date, only the Anderson-Lederer et al. (2012) have demonstrated a degree of mtDNA structuring within *D. b. minor*, but the exclusive KwaZulu haplotype they observed was nested among other Zimbabwean haplotypes. Without precise knowledge of how related these extant relict populations are to each other, conservation authorities in Zimbabwe have so far deemed it prudent to manage the newly introduced KwaZulu animals at Malilangwe separately from the indigenous Zimbabwean populations at Bubiana and Save Valley.

Africa, especially Zimbabwe and South Africa, is presently experiencing a massive resurgence of poaching due to the exponential increase in the value of rhinoceros horn. Therefore, quantification of remaining diversity and knowledge of the level of ancient connectivity between now-isolated populations has never been more critical. Using both nuclear microsatellite and maternally-inherited mitochondrial (mt)DNA, we conducted a population genetic study of 280 black rhinoceros individuals from three populations in Zimbabwe's Lowveld conservancies. We aimed to (1) obtain a definitive estimate of genetic

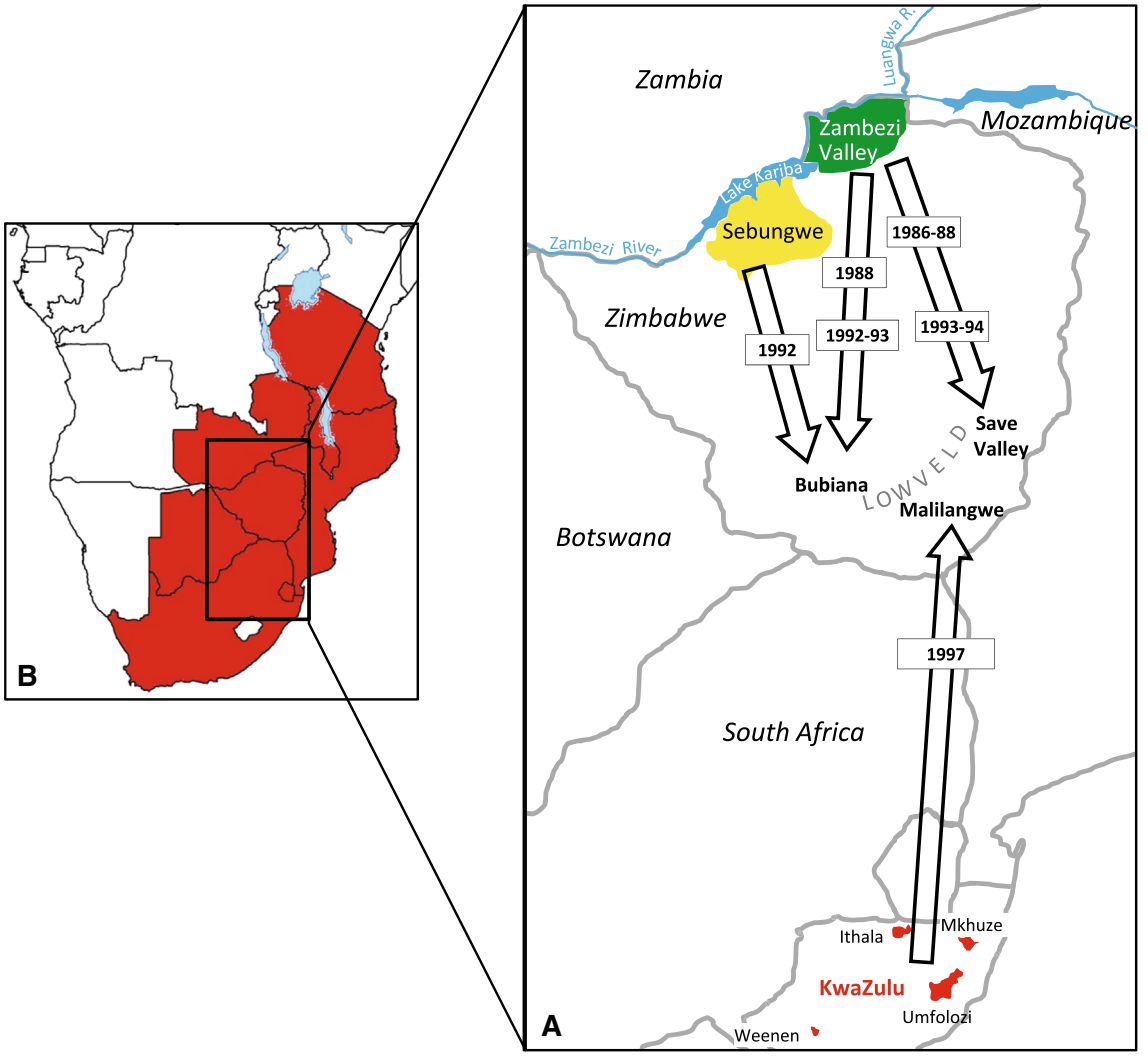

Fig. 1 **a** Map of the Republic of Zimbabwe and neighbouring countries depicting the sources of two of the five remaining aboriginal black rhinoceros populations in Africa. All extant Zimbabwean black rhinoceros are derived from two formerly contiguous populations (Sebungwe and Zambezi Valley) that once ranged in large numbers along southern bank of the Zambezi River. All South African black rhinoceros are descended from survivors located in KwaZulu (*red*). Individuals of both Zambezi and KwaZulu stocks were translocated to the Lowveld region of Zimbabwe during the period 1986–1997. Note that exact locations and dimensions of the recipient Lowveld reserves are intentionally not given for security reasons. **b** South-central Africa with *Diceros bicornis minor* range states (after Groves and Grubb 2011) coloured *red*. (Color figure online)

diversity among Zimbabwe's Lowveld black rhinoceros populations and (2) to infer the level of extant structure and prehistoric connectivity between Zambezi and KwaZulu *D. b. minor* populations. The scale of this study is the largest ever undertaken for this highly endangered species.

Materials and methods

Samples

Samples were collected from as many individuals as possible during the course of on-going rhinoceros management operations in Zimbabwe from the late 1980s to the end of 2007. 301 individual black rhinoceros samples were collected from the Save Valley, Bubiana and Malilangwe populations (Table 1) under the supervision of a qualified veterinary professional using standard rhinoceros immobilization procedures. Blood samples were collected and stored in ethylenediaminetetraacetic acid (EDTA) tubes. Pinna ear tissue was collected during ear notching for identification purposes. All samples were stored in liquid nitrogen and numbered in accordance with the Zimbabwe's National Rhinoceros Numbering System. Collection was carried out by the World Wide Fund for Nature–Zimbabwe Programme and the Lowveld Rhino Trust, with the

Table 1 Microsatellite genetic diversity in Zimbabwe's Lowveld black rhinoceros populations

Population	Source	N (F:M)	NA (F:M)	F (F:M)	FA″ (F:M)	A (C)	H_O (CI 95)	H_E (CI 95)	F_{IS} (CI 95)	Global F_{ST} (CI 95)
Save Valley	Zambezi Valley	130 (68:62)	123 (65:58)	31 (16:15)	24 (11:13)	4.27 (3.87)	0.542 (0.519, 0.564)	0.504 (0.481, 0.526)	−0.076 (−0.126, −0.034)	–
Bubiana	Zambezi Valley/Sebungwe	122 (56:66)	113 (51:62)	38 (18:20)	27 (14:13)	4.36 (3.99)	0.545 (0.519, 0.571)	0.519 (0.494, 0.544)	−0.051 (−0.096, −0.015)	–
Malilangwe	KwaZulu	49 (20:29)	44 (16:28)	27 (14.13)	18 (8:10)	3.18 (3.18)	0.398 (0.332, 0.463)	0.412 (0.353, 0.470)	0.034 (−0.079, 0.118)	–
Save Valley and Bubiana	Zambezi Valley & Sebungwe	252	236	65	51	4.82 (3.99)	0.543 (0.527, 0.559)	0.517 (0.501, 0.533)	−0.051 (−0.085, −0.021)	0.023 (0.008, 0.043)
Total	Zambezi Valley/Sebungwe/ KwaZulu	301	280	87	69	5.09 (4.13)	0.522 (0.508, 0.535)	0.523 (0.509, 0.537)	0.003 (−0.030, 0.032)	0.080 (0.057, 0.105)

N number sampled, F:M relative number of females to males, *NA* number analysed, *F* number of founders, *FA* number of founders analysed; *″*, differs from F either because of death at or soon after release, too much missing data or the sample failed to amplify, *A* mean number of alleles/locus, *A(C)* mean number of alleles/locus corrected for differences in sample size with 10,000 jack-knife replicates of size 44, H_O Heterozygosity observed in the data, *CI* 95 is the 95 % confidence interval, H_E Heterozygosity expected under Hardy–Weinberg equilibrium, F_{IS} Inbreeding co-efficient

cooperation of the Zimbabwean Government (Zimbabwean Department of National Parks and Wildlife Management). One further sample, from the Kruger National Park, was obtained from the National Zoological Gardens of South Africa. Animal ethics approval for the genetic analysis of this project was obtained from the Ethics and Scientific Committee (ESC) of the National Zoological Gardens of South Africa (P06/01). CITES export/import permits (export permit number 069267, import permit number 089294), Veterinary permit (permit number 13/1/17/2/10/1-113) and an Ordinary permit (permit number 020823) were obtained for the exportation of samples from the Republic of Zimbabwe and their importation into the Republic of South Africa.

DNA extraction and amplification

DNA was extracted from 96 blood and 205 skin samples using the Qiagen© DNA isolation kit, following the manufacturer's protocol. Twelve microsatellite loci: BR4, BR6, BR17 (Cunningham et al. 1999), DB1, DB14, DB23, DB44 (Brown and Houlden 1999), RHI32A (Florescu et al. 2003), SW35 (Rohrer et al. 1994), AF129734 (Nielsen et al. 2008) B1RH37D and B1RH2B (QUMEL, unpublished) were used (see Table S1 for primer sequences and multiplex conditions). The PCR optimization for each locus was as follows: 2 ng of template DNA, 1.5–2.5 mM $MgCl_2$, 2 mM dNTP's, 1 µM forward and 1 µM reverse primer, 0.10 U *Taq* DNA polymerase and ddH_2O to a final volume of 15 µl. PCR cycles were as follows: initial denaturing stage at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, annealing for 30 s, extension at 72 °C for 30 s and a final step of 72 °C for 20 min. Products were electrophoresed on an ABI Prism 3130 DNA sequencer (Applied Biosystems). Allele sizes were estimated by comparison with a Genescan™ 500 LIZ™ internal size standard (ABI, Foster City, CA) using the ABI programs GENESCAN (version 1.2.2.1) and GENOTYPER (version 1.1). Samples were repeated if the profiles were low, of low quality, if no amplification or only a homozygote amplification had occurred. In addition, PCR was repeated a third time in the case of non-amplification at 5 °C lower than the prescribed annealing temperature.

We also amplified and sequenced a fragment of the mtDNA control region in a subsample of 100 individuals using primers mt15996L (5′-TCCACCATCAGCACCCAAAGC-3′ Campbell et al. 1995) and mt16502H (5′-TTTGATGGCCCTGAAGTAAGAACCA-3′ Moro et al. 1998). PCR conditions were the same as those for microsatellites above, and cycle sequencing in both directions was carried out using the BigDye Terminator Kit (Applied Biosystems). Sequencing reactions were also run through an ABI Prism 3130 DNA sequencer.

Table 2 Mitochondrial genetic diversity among black rhinoceros populations in Zimbabwe and South Africa

Population	Source	N	#H	#P	HD (CI 95)	π (%) (CI 95)	Originally published
Save Valley	Zambezi Valley	32	4	6	0.700 (0.683–0.717)	0.494 (0.466–0.522)	This study
Bubiana	Zambezi Valley/Sebungwe	61	5	6	0.736 (0.729–0.743)	0.726 (0.718–0.734)	This study
Chete Safari Area	Sebungwe	7	3	4	0.667 (0.519–0.815)	0.367 (0.219–0.515)	Brown and Houlden (2000)
Zoo population (in captivity)	Zambezi Valley	4	3	4	0.833 (0.480–1.000)	0.597 (0.319–0.875)	Fernando et al. (2006); Brown and Houlden (2000)
Kruger National Park	KwaZulu/Sebungwe	1	1	0	0 One sample	0 One sample	This study
Malilangwe	KwaZulu	6	1	0	0 No variation	0 No variation	This study
Hluhluwe-iMfolozi Game Reserve	KwaZulu	50	1	0	0 No variation	0 No variation	Anderson-Lederer et al. (2012)
Ithala Game Reserve	KwaZulu	8	1	0	0 No variation	0 No variation	Anderson-Lederer et al. (2012)
Mkhuze Game Reserve	KwaZulu	5	1	0	0 No variation	0 No variation	Anderson-Lederer et al. (2012)
Ndumo Game Park	KwaZulu	1	1	0	0 One sample	0 One sample	Anderson-Lederer et al. (2012)
Johannesburg Zoo (in captivity)	KwaZulu	1	1	0	0 One sample	0 One sample	Anderson-Lederer et al. (2012)
Zimbabwe (Save, Bubiana, Chete, Captive)	Zambezi Valley/Sebungwe	104	6	7	0.765 (0.743–0.787)	0.695 (0.689–0.701)	This study
South Africa (KNP, Malilangwe, H-iM, Ithala, Mkzuze, Ndumo, Captive)	KwaZulu/Sebungwe	72	2	2	0.028 (0.000–0.071)	0.015 (0.011–0.019)	This study
Total	Zambezi Valley/Sebungwe/ KwaZulu	176	7	7	0.755 (0.752–0.758)	0.611 (0.607–0.615)	This study

N number of samples sequenced, *#H* number of haplotypes, *#P* number of polymorphic sites, *HD* Haplotype diversity, π nucleotide diversity, *CI* 95 95 % confidence interval, *KNP* Kruger National Park, *H-iM* Hluhluwe-iMfolozi Park

Genetic analyses

Allele profiles were checked for genotyping errors, allele dropout and null alleles using MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004). We also performed analyses of linkage disequilibria using the software Genepop 1.2 (Raymond and Rousset 1995). We uploaded all microsatellite genotypes to the Dryad repository (number doi:10.5061/dryad.0d3v5). Control region DNA sequences were checked by eye, assembled and trimmed of primer sequence to 477 bp in CLC DNA Workbench (CLC Biotech). This mtDNA data set was aligned to a further 76 previously published, but shorter, sequences (see Table 2) for a final alignment size of 363 bp. All DNA sequences generated in the study were deposited in Genbank (accession numbers KM095529-KM095628).

Diversity

Levels of genetic variation at microsatellite loci were inferred from the mean number of alleles per locus (A), the observed heterozygosity (H_O) and Nei's (1978) unbiased expected heterozygosity (H_E) all of which were calculated using the software GENETIX 4.05 (Belkhir et al. 2004). To correct for the effect of differing sample size on A, jackknife resampling was carried out using AGaRst (Harley 2002) with 100,000 replicates and resampling size equal to that of the smallest population. We also used GENETIX to calculate the population inbreeding co-efficient F_{IS}, and for groups of two or more populations, we also calculated global F_{ST}. Genetic diversity indices for control region sequences were calculated in DNAsp 5 (Librado and Rozas 2009).

Structure

We determined the number of distinct populations (K) within our Lowveld microsatellite data set using Bayesian assignment analysis which was carried out in STRUCTURE 2.3.3 (Pritchard et al. 2000). We ran five simulations for $1 \leq K \leq 5$ of 1 million iterations each, using the admixture model and discarding the initial 10 % as burn-in. We restricted K to five or fewer populations since little to no phenotypic differentiation has been observed across the range of the subspecies *D. b. minor*, although its range extends from South Africa, north until a division somewhere in Tanzania between this subspecies and *D. b. michaeli* (Groves and Grubb 2011). Since populations were relatively small and isolated since founding, we corrected for the possibility of genetic drift artificially inflating between-population differences, thereby overestimating K, by re-running the simulation above including only the founding members of each population. We then used the method outlined in Evanno et al. (2005), in which the mean rate of change of the likelihood distribution and delta K are used to determine the true value of K for the data set. We also tested allele frequencies of pairs of populations against a null hypothesis of non-differentiation using the more conservative exact test in Arlequin 3.5.1.3 (Excoffier and Lischer 2010).

We determined maternal genetic structure among 176 control region sequences by constructing a median joining network, using Network 4.6.1.2 (Bandelt et al. 1999). We used the default settings of epsilon = 0 and an equal transition/transversion ratio.

Connectivity

To evaluate the level of prehistoric connectivity between two of the three remaining black rhinoceros populations in southern Africa, we employed the Bayesian approach of Rannala and Mountain (1997), which has been shown to outperform frequency- and distance-based methods when population differentiation is moderate, as may be the case for populations within the same subspecies. We conducted the test only on founder animals as they best represent the aboriginal gene frequencies of Zambezi and KwaZulu stocks. To detect first generation migrants, that is individuals whose multilocus genotypes have more in common with populations other than their population of origin, we computed the likelihood (L) of an individual belonging to its population of origin (home population) relative to the likelihood of belonging to any other population (L_home/L_max) and determined significance using Monte Carlo simulations (Paetkau et al. 2004).

Results

MICRO-CHECKER analysis found no evidence of genotyping errors or allelic dropout. Despite the presence of several individuals with missing data, frequencies of null alleles in the data set were non-significant for all loci (with frequencies <0.05). However, an analysis of linkage disequilibrium found loci DB23 and AF129734 to be tightly linked ($p < 0.001$). We therefore removed locus AF129734 prior to any further analyses.

Genetic diversity

All nuclear markers used in this study were polymorphic, ranging from two to ten alleles each. The final microsatellite data set after removing individuals with more than 33 % missing data consisted of 280 individuals. Nuclear genetic diversity among the populations in Zimbabwe's Lowveld black rhinoceros was surprisingly high (H_O, 0.522; A, 5.09). The two populations originating from

indigenous Zimbabwean stock-Save Valley and Bubiana-were significantly more diverse than the KwaZulu-derived Malilangwe (Table 1). This pattern held true even when the mean number of alleles was corrected for Malilangwe's smaller sample size. However, unlike Malilangwe, the heterozygosity observed in Save and Bubiana was higher than expected from allele frequencies. Diversity was of similar magnitude among indigenous Zimbabwean populations, with Bubiana slightly more diverse than Save Valley, and both populations had negative inbreeding coefficients, whereas Malilangwe returned a positive F_{IS} value.

Control region mtDNA sequences reflected similar but more pronounced patterns of genetic diversity in a data set expanded to account for variation among several South African reserves, all of which, like Malilangwe, were originally derived from remnant KwaZulu source populations Hluhluwe-iMfolozi and Mkhuze. The entire data set of 176 sequences contained only seven polymorphic sites distributed among seven haplotypes, resulting in moderate levels of haplotype and nucleotide (π) diversity (Table 2). Among indigenous Zimbabwean populations, Bubiana was significantly more diverse than Save Valley, and moderate diversity was also detected in a previously published collection of captive individuals sourced directly from Sebungwe (Chete Safari Area) and the Zambezi Valley, although smaller sizes for these latter two samples mean that their true mean values could be considerable larger or smaller than reported here. In complete contrast to the moderate levels of diversity in populations sourced from the Zambezi Valley and/or Sebungwe, we found that Malilangwe and all other KwaZulu-derived individuals ($n = 71$, Table 2) comprised a single haplotype and were therefore devoid of mtDNA variation. This situation was slightly improved at the national level for South Africa as the only individual we sampled from the Kruger National Park returned a typical Zimbabwean sequence (haplotype 5).

Structure

Although the origin of each Lowveld population is known, the extant and ancient genetic relationships among them are unknown, despite being highly relevant to their effective conservation. Global FST values calculated from multilocus microsatellite allele frequencies for the group containing only Zambezi-derived individuals was significantly lower than for the group containing all three populations (Table 1), implying greater nuclear genetic structuring in the total data set, that within the Zambezi-derived stock. Both mean rate of change of the likelihood distribution and delta K statistics showed unambiguous support for a two-population scenario for both complete and founder data sets (Fig. 2a). At $K = 2$, individual assignment was consistent with all 280 Lowveld individuals being derived from two source populations—Zambezi and KwaZulu (Fig. 2b). On the other hand, the more conservative exact tests of differentiation based on the distribution of allele frequencies among each population pair showed that the null hypothesis of non-differentiation could not be rejected in any pair-wise comparison ($p > 0.05$ in all cases).

Structuring at the mitochondrial level appeared even less pronounced as all observed haplotypes were closely related, within a maximum of six mutational steps from each other. Within Zimbabwe, there was considerable haplotype sharing (four shared haplotypes) between individuals sourced from the Zambezi Valley and Sebungwe, with the former population containing two more haplotypes than the latter (Fig. 3). Haplotype 2 was shared exclusively by all 71 KwaZulu-derived black rhino, but was nested within Zambezi Valley and Sebungwe haplotypes, only one mutational step away from haplotypes 1 and 4 (Fig. 3).

Connectivity

Individual population assignment probabilities showed that for 14 individuals (Save = 2, Bubiana = 10, Malilangwe = 2), six of whom were founders, the proportion of ancestry inferred from their home population was less than 80 %. We investigated this further by estimating the number of first-generation migrants, a proxy for the level of gene flow between populations, among the founding members of each population using GENECLASS 2. Four individuals, all of whom showed lower than 80 % home-ancestry (female 2,020 from Save, males 1,008 and 1,091 from Bubiana and male 1,136 from Malilangwe), were found to be more similar to members of the alternative population, rather than the population from which they were sampled ($p < 0.05$).

Discussion

Diversity in context

We found Zimbabwe's Lowveld black rhinoceros populations, especially those derived from Zambezi Valley or Sebungwe stock, to possess moderate levels of microsatellite genetic diversity compared to the KwaZulu-derived Malilangwe. Although a direct comparison is not possible given the differing number of loci used in previous studies, our finding is consistent with studies showing particularly low microsatellite diversity in South African populations (Harley et al. 2005; Karsten et al. 2011). Considering variation within the subspecies *D. b. minor* in southern

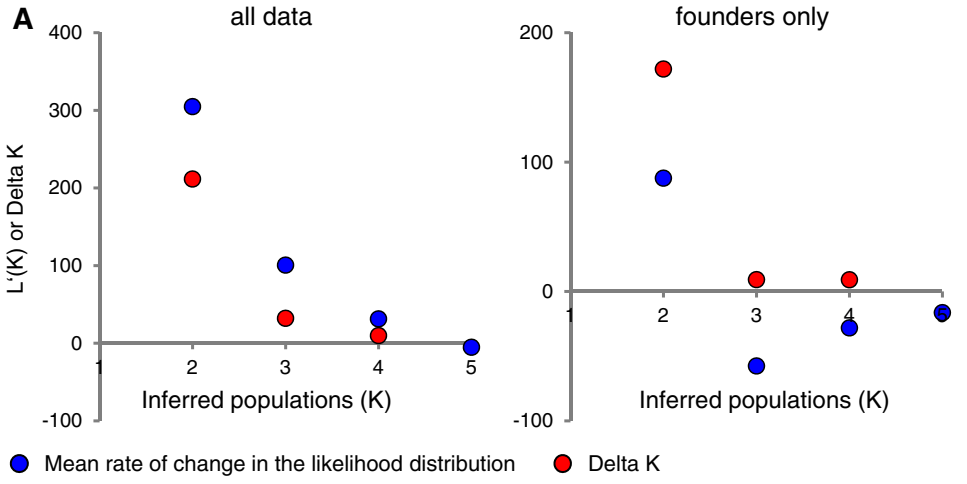

Fig. 2 Nuclear microsatellite population structure among 280 black rhinoceros in Zimbabwe's Lowveld Region. **a** Rates of change of the likelihood distribution and delta K averaged over 5× STRUCTURE runs for $1 \leq K \leq 5$ performed on founder and complete data sets. **b** DISTRUCT (v 1.1) plot (Rosenberg 2004) showing individual probabilities of assignment assuming a two-population ($K = 2$) scenario

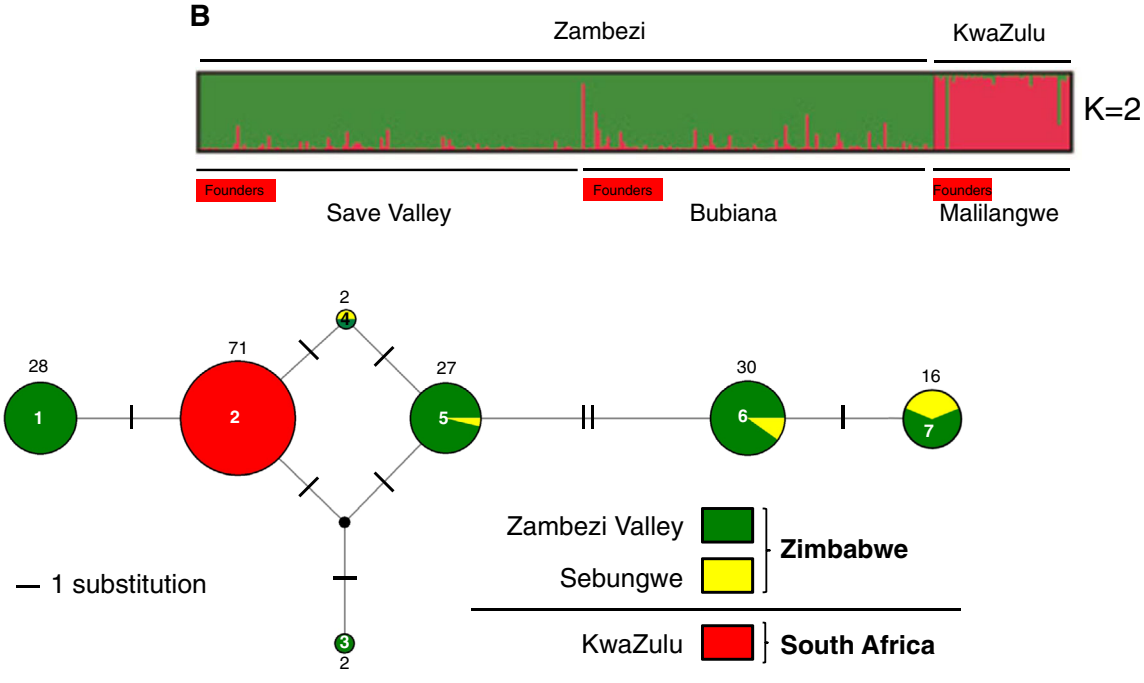

Fig. 3 Median joining network of maternal mitochondrial DNA structure among 176 black rhinoceros control region sequences from Zimbabwe and South Africa. Each haplotype (*circle*) is scaled by its frequency in the total data set. The number of individuals belonging to each haplotype is also given above or below, whereas haplotype identification numbers are shown within each circle. Source populations Zambezi Valley, Sebungwe and KwaZulu are colour coded as in Fig. 1

Africa, we detected an average of over five alleles per locus, comparable to levels among Kenyan black rhinoceros (Harley et al. 2005; Muya et al. 2011), but higher than populations in Namibia (Harley et al. 2005; van Coeverden de Groot et al. 2011).

The mitochondrial control region sequencing allowed for a more direct comparison with other studies. Interestingly, our addition of 100 new control region sequences to the 76 that have previously been published (Brown and Houlden 2000; Fernando et al. 2006; Anderson-Lederer et al. 2012) did not identify any new haplotypes. Therefore, we can be reasonably certain that the combined 176-sequence data set analysed here is an accurate description of the extant mtDNA variation in Zimbabwean and South African black rhinoceros populations. Since mtDNA is maternally inherited, effective population sizes for this locus are four times smaller than for nuclear DNA, potentially increasing the erosive effect of population demographic forces such as genetic drift on genetic diversity. Consequently, the patterns of genetic diversity

observed at microsatellite loci are more clearly defined by mtDNA. The significantly higher variation observed in Bubiana relative to the Zambezi Valley-derived Save Valley or Sebungwe-derived Chete may have resulted from its mixed Zambezi Valley/Sebungwe ancestry (see schematic in Fig. 1). The discovery of a typically Zimbabwean haplotype in the Kruger National Park is not surprising, since 12 Zimbabwean (ex-Sebungwe) rhinoceros had been introduced there in 1972 to complement the 20 animals already resident from a reintroduction from Hluhluwe-iMfolozi (KwaZulu) the previous year (Hall-Martin 1979). This suggests that the mtDNA diversity in South Africa must, in reality, be higher than the single haplotype reported by Anderson-Lederer et al. (2012). The extant mtDNA diversity in Zimbabwe (Zambezi Valley and Sebungwe) as well as the combined extant diversity of Zimbabwe and South Africa is comparable to average levels reported for the same locus in Kenya (HD = 0.73, $\pi = 0.7$, Muya et al. 2011), although it should be noted that levels for some Kenyan populations such as the Maasai Mara and Tsavo East were much higher (HD $\geq$ 0.9, $\pi \geq 0.8$).

Differentiation and connectivity and within *Diceros bicornis* minor in southern Africa

We were able to partition our large microsatellite data set reliably into two clusters, implying a degree of distinctiveness between Zambezi and KwaZulu, two of the three remaining black rhinoceros gene pools in southern Africa. Whether this distinctiveness is evolutionarily significant or whether it signifies the allele frequencies at opposite ends of a gradually changing geographic cline is unclear since populations from intervening regions have been eradicated. One yardstick by which one may attempt the inference of evolutionary significance are pair-wise exact tests of population differentiation, however, in our case the null hypothesis of non-differentiation was rejected in all pair-wise comparisons, suggesting that while Bayesian clustering is able to differentiate between the Zambezi and KwaZulu gene pools, they are remain very closely related to each other.

The distinctiveness between Zimbabwean and South African gene pools observed at microsatellite loci is less obvious when structure among the seven observed mtDNA haplotypes is considered. Using a subset of these mtDNA data Anderson-Lederer et al. (2012) hypothesised that low mtDNA variation in KwaZulu-derived populations may have resulted either from recent anthropogenic fragmentation, followed by elevated genetic drift or from long-term demographic separation and perhaps local adaptation. In the present study, we argue that if the first hypothesis is correct and the South African gene pool contained a greater diversity of mtDNA haplotypes in the past, then they must have shared at least some of those additional haplotypes with Zimbabwean individuals because haplotypes 1 (Zambezi Valley) and 4 (Zambezi Valley/Sebungwe, see Fig. 3) are only one mutational step away from KwaZulu's exclusive haplotype 2. Greater haplotype sharing would thus imply greater overlap and historical connectivity between the two southern African *D. b. minor* gene pools. If on the other hand, we consider the second hypothesis, that diversity within South African gene pool was always low, then it appears that a large proportion of the extant Zambezi stock (27 %—28/104 individuals, haplotype 1, Fig. 3) are more closely related to the KwaZulu haplotype 2 than they are to any of the other Zimbabwean haplotypes, also implying potential historical connectivity between the two gene pools. It is possible that the differing patterns of structure observed between molecular markers may reflect the inherent qualities of the markers themselves. Highly polymorphic microsatellites may be better able to distinguish recently differentiated populations, whereas haplotype sharing at more slowly evolving mtDNA may signify incomplete lineage sorting.

Strong evidence of recent gene nuclear flow was also detected in multilocus microsatellite data, among the founders of Bubiana/Save Valley and Malilangwe. Some of the individuals identified as first generation migrants (e.g. Bubiana founders 1008 and 1091) appear to have achieved much breeding success, since eight individuals in post-founder cohorts have obtained at least 20 % of their genetic material from one or both of them. However, since both Zambezi and KwaZulu gene pools have been isolated since colonial times, a timeframe representing several generations and the dates of birth for two of the "migrant" individuals are known (1,091 in 1985 and 2,020 in 1990), we interpret these results as relatively recent gene flow into these gene pools from adjacent areas, from which populations have now disappeared. However, despite the demonstration of distinctiveness and connectivity between southern African *D. b. minor*, a study including other extant populations will certainly shed more light on levels of gene flow within this subspecies.

Conservation implications

In this study, we use multilocus microsatellite and mtDNA data to demonstrate that southern African *D. b. minor* comprise two very closely related gene pools. We were also able to identify potential migrant individuals among the founders of all three study populations and so demonstrate the existence of prehistoric connectivity between these gene pools in south-eastern Africa. Low nuclear genetic diversity and the widespread, exclusive occurrence of a single mtDNA haplotype in all KwaZulu-derived

individuals poses a serious dilemma to conservation management authorities: to continue managing KwaZulu separately or to attempt to restore historic connectivity between the Zambezi River and KwaZulu? In this instance, the black rhinoceros population in the Kruger National Park, approximately intermediate between the Zambezi River and KwaZulu, makes for an interesting case study in the restoration of historical connectivity, since more than a third of this population is of Sebungwe origin (Hall-Martin 1979). Indeed, our only sample from this park belonged to haplotype 5, typical of Zambezi black rhinoceros, thereby increasing the mtDNA genetic diversity across all of South Africa to a number slightly above zero. Similarly controlled mixing of the Zambezi and KwaZulu gene pools in geographically intermediate areas may further help to approximate a degree of ancient connectivity. Despite the evidence presented here, historic data from prior to the 20th century population crashes are still needed to quantify prehistoric levels of genetic diversity and the degree of overlap between Zimbabwean and South African populations.

Given the proximity of the Zambezi to other range states, such as Namibia, Botswana, Angola, Zambia and Mozambique, in which the black rhinoceros previously occurred until as recently as the early 1980s (Emslie and Brooks 1999), it is possible that similar or even higher levels of gene flow existed in the past. However, we are unable to carry out a fully comparative study, even of extant populations, due to the use of different genetic markers in previous studies of other gene pools. A fully comparative study is still required to assess the relatedness of populations in Zimbabwe and South Africa to those in Namibia, Tanzania and Kenya. Such a framework would allow for a modern reappraisal of subspecies/population classification for this species.

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