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RESEARCH ARTICLE

Conservation genetics of the black rhinoceros, *Diceros bicornis bicornis*, in Namibia

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Abstract Poaching and habitat destruction across sub-Saharan Africa brought the black rhinoceros (*Diceros bicornis*) close to extinction. Over the past few decades, however, one of four subspecies, *D. b. bicornis*, has experienced a significant population increase as a consequence of its protection within Etosha National Park (ENP), Namibia. We report here on the level and spatial distribution of black rhinoceros genetic diversity within ENP. Using nine microsatellite loci, genetic variation was assessed from 144 individuals. Our results are consistent with the observation of lower levels of genetic diversity in *D. b. bicornis*, when compared to *D. b. michaeli,* but greater diversity when compared to *D. b. minor*. We also showed that ENP's black rhino genetic diversity is well represented in Waterberg National Park, originally founded with ENP individuals. We found no genetic signature of a recent bottleneck in ENP, however, suggesting that the genetic diversity within ENP has not been adversely affected by the recent severe population decline. Using Bayesian clustering methods, we observed no significant population structure within ENP, but positive spatial genetic correlation is observed at distances up to 25 km. This relationship exists in females but not males, suggesting reduced dispersal among females, the first evidence of limited female dispersal or philopatry in any species of rhinoceros.

Keywords Conservation genetics · *Diceros bicornis* · Microsatellite · Spatial autocorrelation

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Introduction

Human-mediated habitat destruction, disease and poaching have drastically reduced the population sizes of numerous large African mammals over the past two centuries (Cardillo et al. 2005). The historic range of one of these large mammals, the critically endangered black rhinoceros (*Diceros bicornis*), included most of sub-Saharan Africa. Although some have suggested that the population size stood at 100,000 individuals as recently as 1960 (Emslie and Brooks 1999), the black rhinoceros is now found only in fragmented populations, with an estimate of ~4,000 individuals throughout Africa (CITES 2009). Despite the international ban on trade in rhinoceros products and their listing on Appendix 1 by the United Nations Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), poaching and habitat destruction remain serious threats to the black rhinoceros, with a recent increase in poaching activity in South African rhinoceros populations (African Rhino Specialist Group; http://www.rhinos-irf.org/afrsg).

To counter dwindling black rhinoceros numbers, some southern African countries, an impressive example of

which is Namibia, have invested significantly in their conservation. Although likely to be a conservative estimate, a 1966 survey concluded there were 90 black rhinoceros left in Namibia, with 15 in Etosha National Park (ENP; Joubert 1971). To increase black rhinoceros numbers in ENP, 43 rhinoceros were translocated from western Namibia into ENP between 1970 and 1972. By 1973 the estimated population within ENP had grown to 80 individuals by (Hofmeyr et al. 1975), and it has continued to expand, with more recent estimates approaching 700 individuals (Martin 1994). Black rhinoceros numbers are also improving across the rest of Namibia, with total estimates of ~1,400 individuals country-wide (CITES 2009). Namibia currently holds the greatest number of any black rhinoceros sub-species (CITES 2009), and ENP individuals are frequently translocated out of the park to seed new populations in Namibia and parts of southern Africa.

The black rhinoceros in Namibia is considered to be one of four sub-species, referred to as the Southwestern subspecies, or *D. bicornis bicornis* (Du Toit et al. 1987). *D. bicornis bicornis* is the only sub-species found in Namibia. The other sub-species and their current ranges are *D. b. michaeli* (Kenya and northern Tanzania), *D. b. minor* (South Africa to southern Tanzania), and *D. b. longipes* (Cameroon; Harley et al. 2005). Nuclear and mitochondrial DNA analyses have revealed appreciable genetic differentiation among these four sub-species (O'Ryan et al. 1994; Brown and Houlden 1999; Harley et al. 2005; Fernando et al. 2006).

The successful population expansion of *D. b. bicornis* in Namibia presents new opportunities and challenges for their management and conservation, especially within the 22,270 km^2 Etosha National Park (ENP; Fig. 1). Earlier population declines may have resulted in reduced genetic diversity, which can affect long-term population fitness (Allendorf and Leary 1986; Keller and Waller 2002). Another consideration is that the ENP population continues to be used as a source for a number of *D. b. bicornis* founder populations for other locations, the largest of which is Waterberg Plateau Park (WPP), Namibia (Fig. 1). The long-term fitness of the Waterberg and other new populations may be improved if the founders of these populations possess most of the ENP population's genetic diversity (Allendorf and Leary 1986). In addition, examining patterns of genetic spatial autocorrelation may provide some insight into the dispersal patterns in this poorly studied species. The existence of population genetic structure in ENP would have direct management implications with respect to conducting park-wide rhino censuses and subsampling to create founder groups with a full array of ENP genetic diversity.

Here, we use genetic data from nine polymorphic microsatellite loci to examine genetic diversity and structure of black rhinoceros in Etosha National Park, Namibia. We explore the data set for a genetic signature of a bottleneck to determine whether the recent *D. b. bicornis* decline has resulted in a loss of genetic diversity. We also evaluate whether the Waterberg Plateau Park population, the largest population founded from ENP, contains a representative sample of ENP black rhinoceros genetic diversity. By incorporating spatial data into our analyses, we investigate broad scale genetic pattern within Etosha National Park, and quantify fine scale genetic spatial autocorrelation for all individuals and separately for the two sexes.

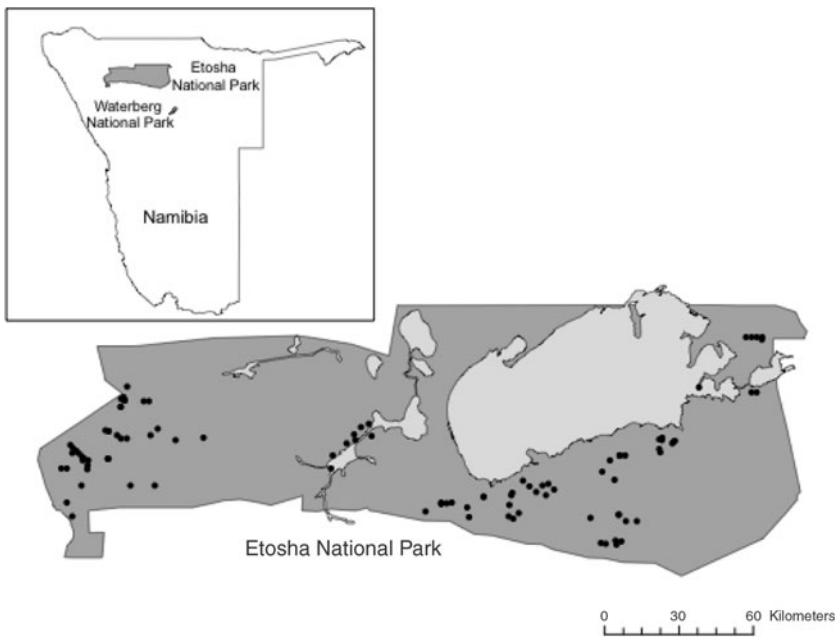

Fig. 1 Map of Etosha National Park showing locations of sampled *D. bicornis* individuals. *Shaded circles* represent sampled individuals. The large *light grey* polygon represents the Etosha salt pan. The *inset* map of Namibia identifies the location in *grey* of Etosha National Park and Waterberg National Park

Table 1 Microsatellite marker loci used to genotype *D. b. bicornis* in Etosha National Park

Locus	Repeat motif	Primers	T_m (°C)[a]	Size range (bp)[b]
BlRh2B	$(CA)_{19}$	F: CCCTTTTCTCCCTTTATCTAG	58	239–253
		R: ATACTGTGAAATCCTGTTCC		
BlRh37D	$(TG)_6(AG)_{17}$	F: CCACTCAGAATGAGAAATGG	58	163–165
		R: TCTCCCTACTTAATCCCACC		
BR4	$(CA)_{19}$	F: CCCCTAAATTCTAGGAACAC	55	143–147
		R: CCAAAGACCACCAGTAATTC		
BR6	$(CA)_{15}$	F: TCATTTCTTTGTTCCCCATAGCAC	58	134–156
		R: AGCAATATCCCACGATATGTGAAGG		
BR17	$(AT)_6(GT)_{18}$	F: ACTAGCCCTCCTTTCATCAG	58	123–133
		R: GCATATTGTAAGTGCCCCAG		
DB01	$(CA)_{14}$	F: AGATAATAATAGGACCCTGCTCCC	58	120–130
		R: GAGGGTTTATTGTGAATGAGGC		
DB44	$(CA)_4g(CA)_{16}$	F: GGTGGAATGTCAAGTAGCGG	58	174–178
		R: CTTGTTGCCCCATCCCTG		
DB52	$(CA)_{21}$	F: CATGTGAAATGGACCGTCAGG	63	232–240
		R: ATTTCTGGGAAGGGGCAGG		
DB66	$(CA)_7ta(CA)_{16}$	F: CCAGGTGAAGGGTCTTATTATTAGC	55	204–224
		R: GGATTGGCATGGATGTTACC		

Also given are the PCR annealing temperature and the size range of each microsatellite. The PCR conditions are described in the text

[a] Annealing temperature

[b] Range of allele sizes compared to commercial standard

Methods

Samples

Ear notch tissue or blood samples were collected from 144 adult *D. b. bicornis* of various ages in Namibia, in the years 1989 ($N = 13$), 1993 ($N = 18$), 1994 ($N = 10$), 1995 ($N = 10$), 1996 ($N = 6$), 1997 ($N = 10$), 1999 ($N = 22$) and 2000 ($N = 55$). The samples were collected during rhino translocations out of and within Etosha National Park, and during routine census work. For 109 of these sampled animals, we have explicit geographic locations (Fig. 1). Only these 109 georeferenced samples were included in spatial population genetic structure and autocorrelation analyses. Fifteen of the 144 samples were from rhinoceros translocated out of ENP and into Waterberg Plateau Park, ~250 km south of ENP (Fig. 1). These represent 15 of the 29 founders of the WPP population. Genetic samples are not available for the other 14 WPP founders, which came from Namibia's Kunene region, west of ENP.

DNA extraction and microsatellite amplification

For all samples, DNA was extracted with a DNeasy kit (Qiagen Inc.) and using polymerase chain reaction (PCR), nine variable microsatellite loci were amplified. Primers for seven polymorphic microsatellite loci BR4, BR6 and BR17 (Cunningham et al. 1999) and DB01, DB44, DB52 and DB66 (Brown and Houlden 1999) have been used in previous black rhino surveys (Harley et al. 2005). Primers for two additional loci, 2B (Accession # AY606080.1, GI: 47933799) and 37D (Accession #AY606083.1, GI: 47933802), were developed specifically for this project. Primer sequences and PCR conditions for these loci are described in Table 1. All PCR products were generated using a Biometra T-Gradient Thermocycler. Conditions for each PCR optimization were as follows: 6 ng of template DNA, 1× QIAGEN PCR buffer (Tris–HCl pH 8.7, KCl, $(NH_4)SO_4$, 15 mM $MgCl_2$), 1 mM dNTPs, 1 μM forward primer, 1 μM reverse primer, 0.05 U *Taq* DNA polymerase and sterile ddH_2O to a final volume of 10 μl. An initial denaturing cycle of 3 min at 94°C was followed by 35 cycles of 94°C for 15 s, annealing T_m for 30 s, 72°C for 30 s and a final 10 min extension at 72°C. Microsatellite products were electrophoresed on a Licor 4200 automatic sequencer (Licor, USA). Amplicon sizes were scored, relative to known standards, using Gene ImagIR v.4.05 (Scanalytics, USA).

Microsatellite variation

Many methods for assessing population genetic structure assume populations are in Hardy–Weinberg and linkage

equilibrium. We computed allele frequencies, conducted tests of Hardy–Weinberg equilibrium (HWE; Guo and Thompson 1992) and linkage equilibrium (Goudet et al. 1996), as well as computed observed and expected heterozygosity (H_O and H_E; (Guo and Thompson 1992)) using default settings in GENEPOP ver.4 (Rousset 2008). Tests for Hardy–Weinberg equilibrium used Markov Chain Monte Carlo (MCMC) simulations with 100,000 steps and a burn-in length of 10,000. To determine if a representative sample of extant genetic diversity in Etosha National Park is present in Waterberg Plateau Park founders, we compared microsatellite diversity of the 15 animals translocated to WPP with that of the entire 144 Namibian *D. b. bicornis* data set, which includes the 15 translocated rhinoceros. Allelic diversity measurements were adjusted to account for differences in sample size using the software FSTAT 2.9.3 (Goudet 2000).

Bottleneck analyses

Because *D. b. bicornis* suffered a precipitous decline in population numbers within the past century, genetic evidence of a bottleneck may be expected. As historic levels of genetic variation are unknown, summary statistics of the data, i.e. current levels of diversity and the allele frequency distribution, were compared with simulated datasets under non-bottleneck HWE conditions. For several generations after a bottleneck, transient heterozygote excess is often observed (Cornuet and Luikart 1996). This is because populations that have experienced a recent reduction in effective population size (N_e) exhibit a more rapid reduction in allelic diversity than of heterozygosity at polymorphic loci. Because the allele number is used to infer mutation-drift equilibrium (Nei 1987) in the short-term, a bottlenecked population will have larger heterozygosity than expected when compared to simulations under equilibrium. We further investigated whether population decline produced genetic signatures of a bottleneck in the allele frequency distribution. A transient mode-shift in the allele frequency distribution is also characteristic of a population bottleneck (Luikart et al. 1998), because a loss of rare alleles compared to alleles of intermediate frequency is expected, compared to coalescent simulations of a non-bottlenecked population (Maruyama and Fuerst 1985). A two-phase model of mutation is suggested to be most appropriate for microsatellites (Di Rienzo et al. 1994; Ellegren 2004). Deviation from mutation-drift equilibrium under a model in which 90% of mutations follow a strict step-wise mutation model (SMM) and 10% follow an infinite allele model (IAM) was explored using the program BOTTLENECK v.1.2 (Cornuet and Luikart 1996; Luikart et al. 1998; Piry et al. 1999). This program uses coalescent simulations to test for recent bottlenecks ($\sim$2 to 4 N_e generations ago) by comparing the allele frequency distribution of 10,000 simulations to the observed data.

Spatial genetic structure

Very little is known about the spatial structure and dispersal behavior of the black rhinoceros under pre-twentieth century densities. Males and females are considered to be territorial. Home-range sizes of *D. bicornis* subspecies vary between 2.5 and 500 km^2, depending on resource availability, with a much smaller core range (Goddard 1966; Kiwia 1989; Lent and Fike 2003; Owen-Smith 2004; Hutchins and Kreger 2006). In addition, a study of *D. bicornis minor* found that home ranges appear to shift from year to year, and females appear to have a closer clustering and overlap of home ranges than do males (Lent and Fike 2003). Given that the limited literature suggests flexible home range size in this species and that there is a general absence of dispersal data for any *D. bicornis* species, as a staging point we chose to use sampling locations as points for inter-individual pairwise geographic distance estimates in our autocorrelation analyses.

Genetic structure and individual population assignment was inferred using the model-based Bayesian clustering algorithm TESS v. 2.3 (François et al. 2006; Chen et al. 2007). TESS incorporates prior information about spatial structure in the identification of population clusters (K) that are in Hardy–Weinberg equilibrium by using a dependence parameter (ψ) that defines the intensity that the effect of neighboring individuals belonging to the same cluster have on each other. Spatial priors derived from the geographic coordinates of the sampled individuals inform the model. TESS has been used to identify population structure that results in isolation by distance, clines, admixture, and clustering (Fedy et al. 2008; François et al. 2008; Tishkoff et al. 2009). To estimate the maximum number of population clusters (K_{max}) we used both admixture and no-admixture models. The advantage of the no-admixture model is that it is not affected by isolation by distance (Pritchard et al. 2000; Chen et al. 2007). For the no-admixture model we used $\psi = 0$ as a non-informative spatial prior for sequential values of K ranging from 2 to 10 population clusters; this analysis is similar to the model underlying the software program STRUCTURE (Pritchard et al. 2000). For each model, the algorithm was run 100 times, with a burn-in of 50,000 cycles and 50,000 additional cycles to obtain estimates. Likelihood graphs showed that removal of 50,000 samples provided sufficient burn-in. Deviance Information Criterion (DIC) values for $K > 2$ were stabilized, indicating that a maximum of two clusters, $K_{max} = 2$, were likely. DIC scores, commonly used in Bayesian analyses, are a measure that penalizes model-complexity to estimate how well various TESS models fits

Table 2 Estimates of heterozygosity from 9 microsatellite loci in the black rhinoceros, *D. b. bicornis*

Locus	Subset[a]				All individuals[b]			
	Allele sample size	Number of alleles per locus	Heterozygosity[c] (Observed)	Heterozygosity (Expected)	Allele sample size	Number of alleles per locus	Heterozygosity (Observed)	Heterozygosity (Expected)
B2	107	8	0.68	0.74	141	8	0.65	0.71
37D	104	2	0.32	0.43	135	3	0.36*	0.47
BR4	104	5	0.41	0.41	136	6	0.40	0.41
BR6	106	3	0.16	0.18	140	4	0.22	0.25
BR17	104	3	0.54	0.49	134	2	0.53	0.48
DB01	100	4	0.58	0.54	130	4	0.57	0.53
DB44	102	3	0.34	0.33	136	4	0.37	0.40
DB52	108	7	0.75	0.71	142	7	0.78	0.71
DB66	98	8	0.74	0.73	131	8	0.71	0.71
Mean	103.67	4.78	0.50	0.51	136.11	4.89	0.51	0.52

Nei's unbiased estimates of expected heterozygosity are also shown (Nei 1987)

[a] The subset of 109 individuals with spatial data

[b] All 144 genotyped individuals

[c] Observed heterozygosity values are not significant compared to expected heterozygosity in an exact test (Guo and Thompson 1992) after a Bonferroni correction for multiple tests

*$P > 0.05$

the data. Models with smaller DIC values are considered a better fit for a given data set (Spiegelhalter et al. 2002; Durand et al. 2009). Admixture models were then tested with $K_{max} = 2$ to $K_{max} = 10$ using three values of ψ (0.5, 0.75, 0.99) to test the spatial dependence of K on individual clustering assignments from weak to very strong dependence. All values of ψ gave similar results and $\psi = 0.75$ is reported here. The 10% of runs with the smallest DIC were used to estimate average matrices of membership proportions for individuals using Clumpp v. 1.1.2 (Jakobsson and Rosenberg 2007), a software program that accounts for individual label switching occurring at each run. Graphical distributions of membership proportions were portrayed using a Kriging method in R scripts, available at http://membres-timc.imag.fr/Olivier.Francois/admix_display.html.

We investigated the genetic autocorrelation of multilocus genotypes at multiple spatial scales using GenAlEx 6.3 (Peakall and Smouse 2006). Non-standardized pairwise inter-individual genetic distances were calculated first. We then performed spatial autocorrelation using distance classes set so that approximately equal numbers of pairwise comparisons were analyzed for each binning class size. In addition, spatial autocorrelation was examined within males and females to test for sex-specific differences in autocorrelation. The 95% confidence interval around the point estimate of correlation (r) was obtained by bootstrapping with replacement. A value of r was considered statistically significant if its 95% confidence interval was above the null hypothesis of $r = 0$.

Results

Microsatellite variation

Allele frequencies in the total (144 individuals) and in a subset (109 individuals with spatial data) of the samples are reported for the nine loci in Table 2. The observed number of alleles across loci ranged from 2 to 8 in the two populations of 109 and 144 (average was 4.78 and 4.89, respectively). After Bonferroni correction for multiple tests, exact tests did not indicate statistically significant deviation from Hardy–Weinberg equilibrium within the nine loci. These results are consistent with the smaller sampling of *D. b. bicornis* from Harley et al. (2005). In the subset of 109 individuals, four of 35 locus-pair tests were significantly in linkage disequilibrium, however, these pairs were not statistically significant after sequential Bonferroni correction for multiple tests. We concluded that these loci were sufficiently independent for the application of population genetic structure analyses. In addition, using a two-tailed exact test, the observed number of heterozygotes did not significantly depart from the Hardy–Weinberg expected number at any loci after Bonferroni correction. Overall, these results meet the assumptions for the ensuing population structure analyses.

No locus was statistically significant for an excess of heterozygotes, one signature of a recent population bottleneck, in either population of 144 or 109 individuals. In the set of 144 individuals, Wilcoxon signed-rank tests for

heterozygote deficiency and excess across all loci were not significant ($P = 0.10$ and $P = 0.92$, respectively) when compared to simulations of expected heterozygosity, although in individual tests a single locus, BR4, did display a significant heterozygote deficiency ($P = 0.04$). The allele frequency distribution across all loci showed the typical L-shape, thus no mode-shift was observed. Together, these results suggest a lack of evidence for a recent severe population bottleneck.

Fifteen of the 144 individuals sampled in this dataset were translocated as founders of the Waterberg Plateau Park population. These individuals contain 87% of the alleles present in the other 129 ENP individuals sampled. It is interesting to note that the 15 WPP individuals possessed 4 rare alleles that were not present in the remaining ENP individuals. We tested for allele frequency heterogeneity using a log-likelihood based exact test (G-test, (Sokal and Rohlf 1995; Goudet et al. 1996) to determine whether the WPP alleles are drawn from the same distribution as the total ENP samples ($N = 144$). This test was not statistically significant for population differentiation at all loci. Comparisons of allelic diversity between ENP and WPP, after correcting for sample size differences, were not statistically significant ($N = 144$, Wilcoxon-sign rank test, $P = 0.43$). The alleles not represented in WPP founders are predominately rare or low frequency alleles, occurring in less than 10% of the total population. An exact test for heterozygote deficiency (Mann–Whitney *U*-test, Raymond and Rousset 1995) within the WPP group of 15 was not statistically significant for any locus, after a Bonferroni correction.

Spatial genetic structure

The smallest DIC values were obtained for $K = 2$ in the TESS analysis for both the admixture and no-admixture models. All individuals with spatial coordinates ($N = 109$) were assigned with greater than 70% probability to a single genetic cluster. This clustering pattern held true for all spatial interaction values. Figure 2 shows the graphical representation of the posterior estimates of membership proportions and the predictive map. The posterior probabilities of assignment to a single population cluster decreased in the southeastern individuals, compared with all others. These individuals, however, were assigned to a second population cluster with no greater than a 30% probability.

The spatial autocorrelation results suggest that genetic and geographic distances among individuals are not random. There was positive and significant ($r = 0.04$, $P = 0.001$) spatial autocorrelation for distances extending to ~21 km when binning classes were distributed so that each distance had ~750 pairwise comparisons (Fig. 3). Males and females were analyzed separately to examine if

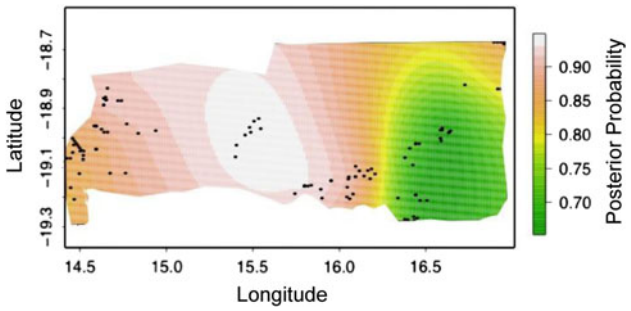

Fig. 2 A spatially explicit predictive map of admixture coefficients within Etosha National Park for *D. b. bicornis* from TESS analyses. *Black circles* represent sampled individuals with known geographic locations. The *color scale* represents the posterior probabilities of individuals having membership in one population, as opposed to several. All individuals have >70% probability of belonging to a single genetic cluster

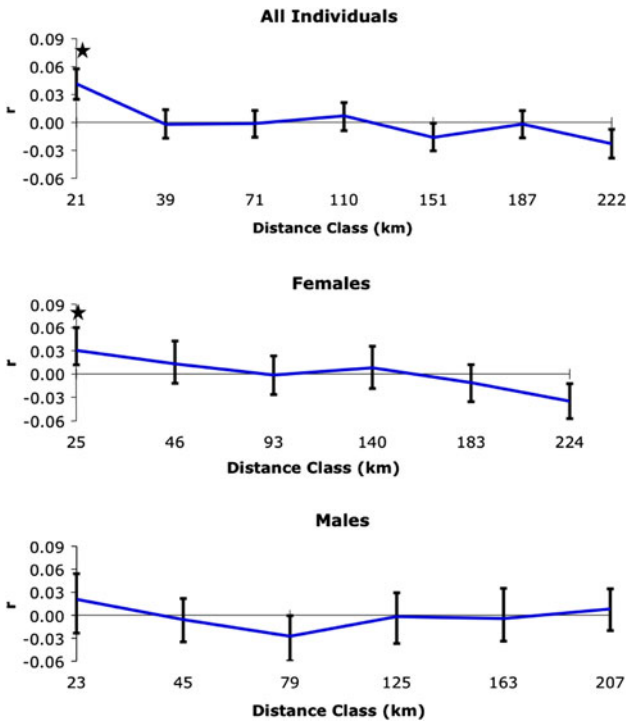

Fig. 3 Spatial autocorrelation correlograms for Etosha National Park *D. b. bicornis* in all individuals, females, and males. *Error bars* represent 95% confidence intervals for the point estimate of the autocorrelation coefficient, *r*, in each distance bin. Statistical significance ($P < 0.05$) *r* is indicated with an *asterisk*

grouping patterns differ between the sexes. No positive autocorrelation in males was detected in the first distance class of 23 km ($r = 0.02$, $P = 0.06$, Fig. 3). In females, however, significant positive spatial autocorrelation ($r = 0.03$, $P = 0.01$) was identified at the first distance class of 25 km. The divergence in autocorrelation pattern between males and females suggests more limited dispersal in females in ENP, following the typical mammalian

pattern (Greenwood 1980). Partitioning the data using equal distance classes of 5, 10, and 25 km (with between 150 and 900 pairwise comparisons per class), instead of equal pairwise comparisons yields similar statistically significant results. These results show statistical significance in the first class of 25 km for females, 20 km for all data together, and no significant spatial autocorrelation for males.

Discussion

Our main findings support and expand upon the first multilocus population genetic study of *D. b. bicornis* (Harley et al. 2005). Two of the nine microsatellite loci differed from those reported in Harley et al. (2005) and 91 more samples were used. We find similar levels of genetic variation in Namibian *D. b. bicornis*, with a mean heterozygosity of 0.51 compared to 0.52 reported in Harley et al. (2005). We also found no evidence of significant loss of genetic variation resulting from a recent population bottleneck. The subset of individuals sampled from Etosha National Park to establish a black rhinoceros population in the smaller Waterberg Plateau Park contain a representative sample of genetic diversity present in *D. b. bicornis*. While there was some loss of rare alleles in the translocation, if both populations continue to expand in numbers the loss of the remaining rare alleles because of genetic drift may be minimized. Within Etosha National Park, there does not appear to be any statistically significant geographic structure. Females do exhibit modest (though significant) genetic autocorrelation at distances up to 25 km, but males do not show a genetic autocorrelation at any scale. It is important to note that while statistically significant, the estimated values of genetic spatial autocorrelation are small. Nonetheless, this is the first suggestive evidence for sex-biased dispersal in black rhinoceros. These findings and their conservation implications are discussed below.

Mean microsatellite diversity and heterozygosity can be used as baseline information for longitudinal studies within the park and guidance for maximizing diversity in translocation efforts outside the park. Our results are consistent with earlier estimates of microsatellite variation in *D. b. bicornis*. For example, the average observed number of alleles across loci was 4.78, and mean microsatellite heterozygosity was 0.50, very similar to a smaller ($N = 53$) sample of *D. b. bicornis* reported by Harley et al. (2005). At nuclear microsatellite markers *D. b. bicornis* appears to have greater mean heterozygosity than *D. b. minor* (0.44) but less than *D. b. michaeli* (0.73; Harley et al. 2005). Although the appropriate scaling factor for comparing effective population size between nuclear and mitochondrial markers is not known for black rhinoceros, Brown and Houlden (2000), reporting on mtDNA genetic diversity, π, also showed that *D. b. michaeli* ($\pi = 0.86$) exhibited substantially higher levels than *D. bicornis minor* ($\pi = 0.43$). While these rhinoceros loci were selected because they are hypervariable, potentially leading to an overestimation of genome-wide diversity (Ellegren et al. 1997; Beaumont 1999), the relative differences among the subspecies are likely to be real as the microsatellite loci were developed in different *D. bicornis* subspecies. Although relative levels of genetic diversity cannot determine absolute genetic health (Zhang et al. 2002), conservation managers in ENP can use these diversity estimates as a benchmark to compare with future studies and gauge the outcome of management decisions.

Diceros b. bicornis have been managed as a metapopulation within Namibia, and no other *D. bicornis* subspecies exists within the country. Translocations from ENP to other smaller national parks or game reserves have aimed to maintain genetic diversity and increase population numbers to buffer against problems associated with small population sizes (Hofmeyr et al. 1975; Martin 1994; Emslie and Brooks 1999). Waterberg Plateau Park now contains the second largest population of black rhinoceros within Namibia's protected areas. We found the rhinoceros in WPP to be a reasonable representation of the genetic diversity of *D. b. bicornis*. The WPP sample ($N = 15$) is broadly representative of ENP *D. b. bicornis* genetic diversity; with 87% of all the ENP alleles are present. Future WPP translocation choices may need to include individuals with rare ENP alleles, so as to increase the allelic richness in this park—although it is possible that some of these alleles may be present in the WPP founders not sampled here. It is important to note that one of the stated objectives of the metapopulation model of *D. b. bicornis* management in Namibia—preserving genetic diversity—has been significantly advanced by creating the WPP rhino population (CITES 2007).

Examining allele frequency distributions and tests for heterozygote excess and deficits, no genetic evidence for a bottleneck was identified. Harley et al. (2005) similarly did not find strong genetic evidence of a bottleneck in *D. b. bicornis*. As there is no obvious evidence of inbreeding in the thriving ENP population, inbreeding depression from a detrimental reduction in allelic diversity resulting from a small number of founders is not likely. Our findings of no appreciable genetic signatures of a bottleneck in *D. b. bicornis* are consistent with the expectation that changes in genetic diversity depends on a bottleneck's length and severity, as well as the mutation rate of the loci (Piry et al. 1999). The long generation time ($\sim$12 years), length of the reproductive lifespan, admixture from translocation, and rapid population recovery may have limited the effect of a recent bottleneck on *D. b. bicornis* (Swart

et al. 1994). A lengthy lifespan and a large variance in effective population size, prior to severe population declines, has been offered as the explanation for high genetic variation in a broad set of organisms that experienced recent bottlenecks, from the greater one-horned rhinoceros (*Rhinoceros unicornis*; Dinerstein and McCracken 1990) to white-tailed eagles (*Haliaeetus albicilla*; Hailer et al. 2006). In addition, protein electrophoretic assays of *D. bicornis minor* found levels of genetic diversity, after the severe population bottleneck in southern Africa, to be similar to levels in a larger population of *D. b. minor* that was not known to have undergone a bottleneck (Swart et al. 1994; Swart and Ferguson 1997). Ancestral population size variation among the subspecies of black rhinoceros, which is not accounted for in the bottleneck analyses used here and in Harley et al. (2005), may also result in the differences in current genetic variation and the lack of genetic evidence for recent bottleneck effects in these taxa (Tajima 1989; Marth et al. 2004). A more detailed study of the genetic effects of the recent bottleneck awaits the analysis of archived bone specimens from museums, along with the large Namibian black rhinoceros skull collection that predates the recent population decline.

While our broad scale Bayesian clustering analyses (TESS; François et al. 2006, Chen et al. 2007) assigned all individuals to one single population cluster with over 70% probability, there was a trend for decreasing posterior probability of assignment to one cluster from west to east across the length of ENP (Fig. 2). It is possible that the 11-year span of DNA sample collection may be partly responsible for this pattern. However, analysis of a subset of 69 individuals that were sampled between the years of 1998–1999 supports the findings from the larger more inclusive dataset. An interpretation of these results is the emergence of genetic structure within ENP which may become more pronounced with time. Another possibility is that the cline in population clustering results from differences in allele frequencies that existed before translocation decades ago.

With the introduction of scale in autocorrelation analyses we found significant spatial genetic autocorrelation at distances up to 25 km for females, but not for males, lending support to a hypothesis of burgeoning population structure owing to male biased dispersal. To our knowledge there have been no studies on equilibrium dispersal behavior of any rhinoceros species, as most populations consist of recently introduced individuals in small fragmented populations. Our study provides the first suggestion of female-limited dispersal in any rhinoceros population. While female-limited dispersal is common in many large mammals (Greenwood 1980), dispersal in all rhinoceros species has proved difficult to study (Lent and Fike 2003). In this regard it is important to note that our findings appear contrary to the purported female-biased dispersal in the white rhinoceros (*Ceratotherium simum*; Owen-Smith 1975). However, this conclusion was based only on the observation that females have larger home ranges than males, which is not sufficient evidence for sex-biased dispersal alone. More comprehensive and repeated genetic sampling of ENP (and other rhinoceros populations) may provide a more accurate estimate of dispersal for these taxa as has been detailed in other mammals for example bush rats (*Rattus fuscipes;* (Peakall et al. 2003) and the bush-tailed rock-wallaby (*Petrogale penicillata*; Hazlitt et al. 2004).

It is plausible that positive spatial genetic autocorrelation in *D. b. bicornis* females may result from the number of waterholes placed throughout ENP since the 1970's and not from an innate limited female dispersal within *D. b. bicornis*. It is thus possible that increased spatial genetic autocorrelation in females at the scale of the park would be lessened if the artificial water sources were removed, as distances to find suitable water would be greater. Although females may be more affected by the presence of watering holes as seen in Grevy zebra where females prefer ranges closer to water holes compared with males, especially during reproductive years (Rubenstein 1986), the affect of watering holes on dispersal and spatial autocorrelation will be best answered through a longitudinal genetic analysis of the historic bone collection housed in ENP where many samples predate the sinking of waterholes.

Conservation implications

This study presents comprehensive baseline population genetic data that will aid the genetic monitoring of ENP and WPP black rhinoceros populations in the future. The subtlety of genetic structure in the population at present indicates the population may continue to be managed as one large metapopulation including the way aerial census efforts are applied throughout ENP. Using genetic diversity estimates from the large *D. b. bicornis* population in ENP, we also have empirical genetic diversity targets for new founder populations. In addition, the data presented in this study suggest animals destined for translocation should be drawn from a wide geographic range within ENP, in order to maximize the genetic diversity of any founder group created to seed a new population. As black rhino populations in South Africa are once again subject to serious poaching pressure, the black rhinoceros of ENP and other smaller Namibia populations take on greater importance and should be the focus of redoubled efforts to protect them as well as manage them demographically and genetically.

One of this study's conservation implications may relate to estimating carrying capacity of black rhinoceros in ENP. Studies of density dependence and interspecific aggression in *D. bicornis minor* found that once the density increased to

$\sim$0.1 rhino/km^2, the percentage of females achieving reproductive success began to fall (Hrabar and Du Toit 2005) and often fatal injuries from aggression increased (Linklater and Swaisgood 2008). While these data come from both *D. b. bicornis* and *D. b. minor* across environments with a variety of elevations and rainfall, they imply ecological carrying capacity is an important target in the conservation management of any black rhinoceros population. The estimation of the rhino carrying capacity for ENP is complex, but an estimate based the size of the park alone is $\sim$2,000 rhinoceros which is much greater than the $\sim$700 currently in ENP. Carrying capacity estimates must take into consideration that a significant portion of ENP is a saltpan that does not provide suitable rhinoceros habitat. On the other hand, the presence of many artificial waterholes will likely increase rhino carrying capacity for ENP. One possible way to indirectly monitor the ENP rhino population's approach to an ecological carrying capacity is to calculate autocorrelation patterns of the same population on a more frequent basis. The existence of positive female autocorrelation driven by female philopatry may indicate this population is not near carrying capacity. However, as the population approaches and exceeds carrying capacity and females disperse further genetic spatial autocorrelation may disappear.

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References

Allendorf FW, Leary RF (1986) Hetozygosity and fitness in natural populations of animals. In: Soule ME (ed) Conservation biology: the science of scarcity and diversity. Sinauer Associates, Sunderland, MA, pp 57–76

Beaumont MA (1999) Detecting population expansion and decline using microsatellites. Genetics 153:2013–2029

Brown SM, Houlden BA (1999) Isolation and characterization of microsatellite markers in the black rhinoceros (*Diceros bicornis*). Mol Ecol 8:1559–1561

Brown SM, Houlden BA (2000) Conservation genetics of the black rhinoceros (*Diceros bicornis*). Cons Genet 1:365–370

Cardillo M, Mace GM, Jones KE et al (2005) Multiple causes of high extinction risk in large mammal species. Science 309:1239–1241

Chen C, Durand E, Forbes F, Francois O (2007) Bayesian clustering algorithms ascertaining spatial population structure: a new computer program and a comparison study. Mol Ecol Notes 7:747–756

CITES (2007) Black rhinoceros conservation and management in Namibia. Available at: http://www.cites.org/common/cop/14/inf/E14i-43.pdf. Accessed 29 July 2010

CITES (2009) Black Rhinoceros Conservation and Management in Namibia. Available at: www.cites.org/common/cop/13/inf/E13i-21.pdf. Accessed 29 July 2010

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144:2001–2014

Cunningham J, Harley EH, O'Ryan C (1999) Isolation and characterization of microsatellite loci in black rhinoceros (*Diceros bicornis*). Electrophoresis 20:1778–1780

Di Rienzo A, Peterson AC, Garza JC et al (1994) Mutational processes of simple-sequence repeat loci in human populations. Proc Natl Acad Sci USA 91:3166

Dinerstein E, McCracken GF (1990) Endangered greater one-horned rhinoceros carry high levels of genetic variation. Conserv Biol 4:417–422

Du Toit R, Foose TJ, Cummings DHM (1987) Small population management of black rhino. In: Proceedings of African Rhino Workshop, Cincinnati, October 1986. 9:31–34

Durand E, Jay F, Gaggiotti OE, François O (2009) Spatial inference of admixture proportions and secondary contact zones. Mol Biol Evol 26:1963–1973

Ellegren H (2004) Microsatellites: simple sequences with complex evolution. Nat Rev Genet 5:435–445

Ellegren H, Moore S, Robinson N et al (1997) Microsatellite evolution—a reciprocal study of repeat lengths at homologous loci in cattle and sheep. Mol Biol Evol 14:854–860

Emslie R, Brooks M (1999) African Rhinoceros status and conservation action plan. IUCN/SSC African Rhino Specialist Group. IUCN, Gland, Switzerland and Cambridge, UK

Fedy BC, Martin K, Ritland C, Young J (2008) Genetic and ecological data provide incongruent interpretations of population structure and dispersal in naturally subdivided populations of white-tailed ptarmigan (*Lagopus leucura*). Mol Ecol 17:1905–1917

Fernando P, Polet G, Foead N et al (2006) Genetic diversity, phylogeny and conservation of the Javan rhinoceros (*Rhinoceros sondaicus*). Cons Genet 7:439–448

François O, Ancelet S, Guillot G (2006) Bayesian clustering using hidden Markov random fields in spatial population genetics. Genetics 174:805–816

François O, Blum MG, Jakobsson M, Rosenberg NA (2008) Demographic history of european populations of *Arabidopsis thaliana*. PLoS Genet 4:e1000075

Goddard J (1966) Mating and courtship of the black rhinoceros. Afr J Ecol 4:69–75

Goudet J (2000) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available at: http://www.unil.ch/izea/softwares/fstat.html. Accessed 29 July 2010

Goudet J, Raymond M, de Meeüs T, Rousset F (1996) Testing differentiation in diploid populations. Genetics 144:1933–1940

Greenwood PJ (1980) Mating systems, philopatry and dispersal in birds and mammals. Anim Behav 28:1140–1162

Guo SW, Thompson EA (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 48:361–372

Hailer F, Helander B, Folkestad AO et al (2006) Bottlenecked but long-lived: high genetic diversity retained in white-tailed eagles upon recovery from population decline. Biol Lett 2:316–319

Harley EH, Baumgarten I, Cunningham J, O'Ryan C (2005) Genetic variation and population structure in remnant populations of black rhinoceros, *Diceros bicornis*, in Africa. Mol Ecol 14:2981–2990

Hazlitt SL, Eldridge MD, Goldizen AW (2004) Fine-scale spatial genetic correlation analyses reveal strong female philopatry within a brush-tailed rock-wallaby colony in southeast Queensland. Mol Ecol 13:3621–3632

Hofmeyr JM, Ebedes H, Fryer REM, De Bruine JR (1975) The capture and translocation of the black rhinoceros (*Diceros bicornis*) in South West-Africa. Modoqua 9:35–44

Hrabar H, Du Toit JT (2005) Dynamics of a protected black rhino (*Diceros bicornis*) population: Pilanesberg National Park, South Africa. Anim Conserv 8:259–267

Hutchins M, Kreger MD (2006) Rhinoceros behavior: implications for captive management and conservation. Int Zoo Year 40:150–173

Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23:1801–1806

Joubert (1971) The past and present distribution and status of the black rhinoceros (*Diceros bicornis*) in southwest Africa. Modoqua 4:33–43

Keller LF, Waller DM (2002) Inbreeding effects in wild populations. Trends Ecol Evol 17:230–241

Kiwia HYD (1989) Ranging patterns of the black rhinoceros (*Diceros bicornis* (L.)) in Ngorongoro Crater, Tanzania. Afr J Ecol 27:305–312

Lent PC, Fike B (2003) Home ranges, movements and spatial relationships in an expanding population of black rhinoceros in the Great Fish River Reserve, South Africa. South Afr J Wildlife Res 33:109–118

Linklater WL, Swaisgood RR (2008) Reserve size, conspecific density, and translocation success for black rhinoceros. J Wildl Manage 72:1059–1068

Luikart G, Allendorf FW, Cornuet JM, Sherwin WB (1998) Distortion of allele frequency distributions provides a test for recent population bottlenecks. J Hered 89:238–247

Marth GT, Czabarka E, Murvai J, Sherry ST (2004) The allele frequency spectrum in genome-wide human variation data reveals signals of differential demographic history in three large world populations. Genetics 166:351–372

Martin EB (1994) Rhino Poaching in Namibia from 1980 to 1990 and the Illegal Trade in the Horn. Pachyderm 18:39–51

Maruyama T, Fuerst PA (1985) Population bottlenecks and nonequilibrium models in population genetics. II. Number of alleles in a small population that was formed by a recent bottleneck. Genetics 111:675–689

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York

O'Ryan C, Flamand JRB, Harley EH (1994) Mitochondrial DNA variation in black rhinoceros (*Diceros bicornis*): conservation management implications. Conserv Biol 8:495–500

Owen-Smith RN (1975) The Social Ethology of the White Rhinoceros *Ceratotberium simum* (Burchell 1817*). Zeitschrift für Tierpsychologie 38:337–384

Owen-Smith RN (2004) Rhinoceroces. In: Hutchins M, Kleiman DG, Geist V, McDade M (eds) Grzimek's animal life encyclopedia. Gale Group, Farmington Hills, MI, pp 249–262

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Peakall R, Ruibal M, Lindenmayer DB (2003) Spatial autocorrelation analysis offers new insights into gene flow in the Australian bush rat, *Rattus fuscipes*. Evolution 57:1182–1195

Piry S, Luikart G, Cornuet JM (1999) Computer note. BOTTLENECK: a computer program for detecting recent reductions in the effective size using allele frequency data. J Hered 90:502

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Hered 86:248

Rousset F (2008) GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol Ecol Resour 8:103–106

Rubenstein DI (1986) Ecology and sociality in horses and zebras. In: Rubenstein DI, Wrangham RW (eds) Ecological aspects of social evolution. Princeton University Press, Princeton, NJ

Sokal RR, Rohlf FJ (1995) Biometry. WH Freeman and Company, New York, NY

Spiegelhalter DJ, Best NG, Carlin BP, van der Linde A (2002) Bayesian measures of model complexity and fit. J R Stat Soc B Methodol 64:583–616

Swart MKJ, Ferguson JWH (1997) Conservation implications of genetic differentiation in southern African populations of black rhinoceros (*Diceros bicornis*). Conserv Biol 11:79–83

Swart MK, Ferguson JW, du Toit R, Flamand JR (1994) Substantial genetic variation in southern African black rhinoceros (*Diceros bicornis*). J Hered 85:261–266

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595

Tishkoff SA, Reed FA, Friedlaender FR et al (2009) The genetic structure and history of Africans and African Americans. Science 324:1035–1044

Zhang Y, Wang X, Ryder OA et al (2002) Genetic diversity and conservation of endangered animal species. Pure Appl Chem 74:575–584