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Genetic variation and population structure in remnant populations of black rhinoceros, *Diceros bicornis*, in Africa

ERIC H. HARLEY,* INGRID BAUMGARTEN,* JESSICA CUNNINGHAM† and COLLEEN O'RYAN†
**Department of Clinical Laboratory Sciences, University of Cape Town, Anzio Road, Observatory 7925, South Africa, †Department of Molecular Biology, University of Cape Town, Rondebosch, 7701, Cape Town, South Africa*

Abstract

Black rhinoceros (*Diceros bicornis*) are one of the most endangered mammal species in Africa, with a population decline of more than 96% by the end of the last century. Habitat destruction and encroachment has resulted in fragmentation of the remaining populations. To assist in conservation management, baseline information is provided here on relative genetic diversity and population differentiation among the four remaining recognized subspecies. Using microsatellite data from nine loci and 121 black rhinoceros individuals, and comparing the results with those of other African species affected in similar ways, *Diceros bicornis michaeli* retained the most genetic diversity (heterozygosity 0.675) compared with *Diceros bicornis minor* (0.459) and *Diceros bicornis bicornis* (0.505), suggesting that the duration of the known bottlenecks in these populations has only had a limited impact on diversity. Comparable and moderate degrees of population differentiation were found between *D. b. minor*, *D. b. bicornis* and *D. b. michaeli*. Results from the single sample available of the most endangered subspecies, *Diceros bicornis longipes*, showed the least diversity of all individuals examined. This information should assist conservation management decisions, especially those affecting population viability assessments and selection of individuals for translocations, and will also facilitate subspecies identification for *ex situ* individuals of uncertain origin.

Keywords: bottleneck, conservation, genetics, microsatellite, rhinoceros

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Introduction

The African black rhinoceros, *Diceros bicornis*, once widespread across Africa has suffered a massive reduction in both numbers and range during the 20th century. The largest contributing factor to this decline in numbers has been the intensive poaching of rhinoceros for their horns (Leader-Williams 1992, 2002). More than 96% of the black rhinoceros population was lost between 1970 and 1992 and most black rhinoceros are currently distributed in small, isolated populations. These populations number approximately 3610 individuals found principally in South Africa, Namibia, Kenya, Tanzania and Zimbabwe, with about 1775 of these animals being in South Africa. Seven subspecies of *D. bicornis* were originally described (Groves 1967); however, only four geographical/ecological groupings (Table 1) are currently recognized, which effectively correspond to the subspecies *Diceros bicornis michaeli*, *Diceros bicornis minor*, *Diceros bicornis bicornis* and *Diceros bicornis longipes* (Du Toit *et al*. 1987), and all of which are classified as either critically endangered or vulnerable.

Four countries currently make up the Rhino Management Group (RMG): South Africa, Namibia, Swaziland, and Zimbabwe. In 1997, these countries were together reported to have conserved about 2100 (81%) of Africa's remaining black rhinoceros, including 100% of Africa's *D. b. bicornis*, 97% of Africa's known *D. b. minor*, and 7% of Africa's *D. b. michaeli* (Emslie & Brooks 1999), and with effective management policies now being implemented these figures have now improved to 3085 (85%) (http://www.rhinos-irf.org/rhinoinformation/populationtable.htm).

Correspondence: Eric H. Harley, PhD, MD, Division of Chemical Pathology, Department of Clinical Laboratory Sciences, University of Cape Town, Faculty of Health Sciences, Anzio Road, Observatory 7925, South Africa. Fax: 021 4488150; E-mail: harley@chempath.uct.ac.za

Table 1 Remaining black rhinoceros populations and their subspecies, ecological designation and IUCN Red List Categories of Threat, with numbers rounded to the nearest five animals (Emslie & Brooks 1999) and http://www.rhinos-irf.org/rhinoinformation/populationtable.htm

Subspecies designation	Ecological region	Geographical region	Threat category	No. of animals
D. b. minor	South central	Extending from South Africa to Zimbabwe, Zambia and southern Tanzania	Critically endangered	1775
D. b. bicornis	Southwestern	Namibia	Vulnerable	1310
D. b. michaeli	Eastern	Extending from Kenya to northern Tanzania	Critically endangered	520
D. b. longipes	Northwestern	Cameroon and possibly Chad	Critically endangered	*c.* 10

The national South African black rhinoceros conservation plan (Brooks & Adcock 1997) sets national target numbers for each subspecies of black rhinoceros and is structured to help field managers in decision making. The plan's objectives are (i) to develop and conserve in the long term, genetically viable populations of at least 2000 *D. b. minor* and 200 *D. b. bicornis* within their former range in South Africa; (ii) to develop and conserve in the long term, genetically viable populations of *75 D. b. michaeli* in the wild in South Africa; and (iii) to support captive breeding programmes, both within and outside South Africa, provided they play a significant role in maintaining or improving the conservation status of the species.

As a result of habitat destruction and the resulting fragmentation of the populations, the ecological groupings of black rhinoceros have become physically separated for at least the last century and possibly longer. There are no defining boundaries separating these subspecies or ecological groupings, but there are differences between habitat types and climates in the core geographical habitats (Emslie & Brooks 1999). The amount of historical migration between the groupings and therefore the extent of gene flow limiting differentiation is uncertain. It is possible that specific genetic and behavioural adaptations have developed to adapt to both climatic differences and food preferences, and in this case the populations would be on different evolutionary trajectories and there would be a stronger argument for maintaining physical separation. Molecular measures of genetic diversity will assist in deciding what constitutes a genetically viable population, and measures of population differentiation can contribute to placing value on the different ecological groupings.

Small, isolated populations are vulnerable to local extinctions as a result of stochastic factors (Gilpin & Soule 1986). In addition, small populations will also lose genetic variation as a result of random genetic drift, and this loss of genetic variation can result in a decrease in adaptive fitness (Charlesworth & Charlesworth 1987; O'Brien *et al*. 1987; Westemeier *et al*. 1998), which further exacerbates the problems of population decline. The current population bottlenecks that are affecting all the ecological groupings of black rhinoceros will be causing progressive loss in genetic diversity. However, this loss can be minimized by means of managed translocations (O'Ryan *et al*. 1998), and this is already taking place within ecological groupings. At a rhinoceros conservation symposium held in Cincinnati in 1986, it was agreed that the four black rhinoceros subspecies should be conserved and managed separately until their genetic status has been examined. This strategy has been supported by the African Rhino Specialist Group. However, if one of these groupings falls to extremely low numbers, as has already happened for *D. b. longipes*, where numbers are reported to be less than 10 animals, the question arises as to whether under such circumstances admixture with another grouping becomes an appropriate strategy. Such an action would not be undertaken lightly since it would result in gene flow across subspecies designations and the ensuing population would no longer constitute an ecological grouping as originally described.

Comparative mitochondrial DNA (mtDNA) restriction maps and control region sequences have revealed fixed differences among the subspecies of black rhinoceros, and imply some population differentiation (Ashley *et al*. 1990; O'Ryan *et al*. 1994; Brown & Houlden 2000). The restriction maps were monomorphic within each subspecies with estimated values of about 0.4% for overall mtDNA sequence divergence between any two species, and the more variable control region sequence showed a divergence of 2.6% between some captive individuals of *D. b. michaeli* and *D. b. minor* (Brown & Houlden 2000). However, a more comprehensive population genetic analysis is required to define more accurately the extent of population subdivision and to document the relative amount of genetic diversity remaining in the extant *in situ* populations of black rhinoceros. This will provide a baseline for continued monitoring of genetic diversity in black rhinoceros in general and in the management units in particular. It will assist in conservation management decisions of declining rhinoceros populations *in situ*, particularly with respect to potential translocations between conservation areas, protection zones, sanctuaries and conservancies and should contribute to the management of *ex situ* breeding programmes. It will also provide insight into the rate of loss of genetic diversity in a large mammal approaching or arriving at a population bottleneck,

Table 2 Origin and numbers of individuals sampled

Subspecies	Geographical region	Total no. of samples
D. b. minor	*Umfolozi-Hluhluwe NP (38)	47
	*Kruger NP (1)	
	Zimbabwe (8)	
D. b. bicornis	†Damaraland (1)	53
	†Vaalbos NP (11)	
	†Etosha NP (25)	
	†Waterberg NP (5)	
	†Tswala Desert Reserve (2)	
	*Addo NP (2)	
	*Augrabies NP (6)	
	*Karoo NP (1)	
D. b. michaeli	*Addo NP (18)	19
	*Kimberly (1)	
D. b. longipes	Cameroon	1
D. b. chobiensis	†Caprivi	1

*South Africa; †Namibia, NP, national park.

especially in comparison to comparable studies on the Indian rhinoceros (Dinerstein & McCracken 1990) and other African mammalian species.

We have therefore undertaken a microsatellite analysis with representative samples from each of the *D. b. minor*, *D. b. bicornis*, and *D. b. michaeli* subspecies currently found in southern Africa. We have also included a single *D. b. longipes* individual, valuable because of the rarity and inaccessibility of the Cameroon population, as well as a single individual from southern Angola attributed to Groves's (1967) *D. b. chobiensis* subspecies.

Materials and methods

Samples

Wild black rhinoceros samples were collected over a period of 16 years in the form of skin biopsies taken from the pinna of the ear (Table 2), primarily for marking purposes, during veterinary or translocation intervention procedures. Cell cultures were established from each ear biopsy, providing a renewable source of DNA for each sample (O'Ryan *et al*. 1994). These cultures are maintained in long-term liquid nitrogen storage as part of the University of Cape Town Wild Life Cell Bank.

DNA extraction and microsatellite loci amplification

Total DNA was extracted from cell cultures as previously described (O'Ryan *et al*. 1994). The resulting DNA pellets were air dried and resuspended in 100 μL TE, pH 8. The genomic DNA was then diluted to a final concentration of between 10 and 50 ng/μL for polymerase chain reaction (PCR) amplifications. Variation at nine polymorphic microsatellite loci isolated from the *Diceros bicornis* was investigated using the following primers: BR4, BR6 and BR17 (Cunningham *et al*. 1999), and DB1, DB14, DB44, DB49, DB52 and DB66 (Brown & Houlden 1999). These all contain dinucleotide (CA) repeats of between 13 and 21 repeat units in the original isolates.

The forward primer of each primer set was end-labelled with [γ-^{32}P]-ATP (O'Ryan *et al*. 1998). PCR was then performed in 10-μL reaction volumes at the following reaction conditions: 25 pmoles of each primer, 5 mM dNTPs, 1.5 mM $MgCl_2$ and 0.5 U *Taq* polymerase (Bioline). Cycling parameters for PCR amplifications were as follows: a 1-min denaturing step at 94 °C, 1 min at the annealing temperature (50 °C to 62 °C) and a 45-s extension step at 72 °C. The amplified products were then electrophoresed on a 6% denaturing polyacrylamide gel. Genotypes were scored from the autoradiographs and allele lengths (in base pairs) determined using a sequenced size ladder of M13 ssDNA. Complete data sets for all nine loci were obtained for all 19 *Diceros bicornis michaeli* individuals, for 51 out of the 53 *Diceros bicornis bicornis* individuals and for 42 out of the 49 *Diceros bicornis minor* individuals, where some loci remained untyped. Calculations were performed on the complete data set and a data set omitting those individuals with missing data. The results showed minimal differences and the values reported below correspond to those from the complete data set.

Linkage measures

Linkage equilibrium was assessed using GENEPOP (Raymond & Rousset 2000) with critical significance levels corrected using the sequential Bonferroni test. Locus DB52 was found to be in linkage disequilibrium with both locus DB49 and locus DB66 in all populations ($P < 0.0001$). Loci DB49 and DB66 remained in linkage equilibrium. Data from locus DB52 were therefore omitted when determining measures of genetic variability and population differentiation.

Genetic variability measures

Genotype distributions were tested for deviations from Hardy–Weinberg expectations using the exact Hardy–Weinberg test in GENEPOP (Raymond & Rousset 2000). Genetic diversity within each population was quantified using measures of the number of polymorphic loci, allele numbers and heterozygosity. The total number of alleles was summed across all loci, and the mean number of alleles per locus was calculated using jackknifing (sampling without replacement with the sample size set at that of the smallest population sample size, *D. b. michaeli*) implemented in $AGAR_{ST}$ (Harley 2002) to correct for variation in sample

Table 3 Estimates of genetic variation and the population reduction statistic, '*M*', in three black rhinoceros subspecies

Population	*N*	Total alleles	Alleles/locus (absolute)	Alleles/locus (corrected*)	H_O	H_E	Estimated N_e (SMM)	Estimated N_e (IAM)	*M* (variance)
D. b. Minor	46	49	5.44	4.10	0.436	0.459	1472	1034	0.739 (0.025)
D. b. bicornis	53	36	4.00	3.44	0.523	0.505	1876	1243	0.691 (0.081)
D. b. michaeli	19	50	5.56	5.56	0.731	0.675	5173	2536	0.726 (0.033)

N, number of individuals analysed. Alleles per locus values were corrected for differing population numbers by 1000 jackknife iterations with sampling replicates of 19 (this being the size of the smallest of the three populations). H_O, observed heterozygosity; H_E, heterozygosity expected; N_e, effective population size assuming either the stepwise-mutation model (SMM) or the infinite allele model (IAM).

sizes. Heterozygosity was measured as the mean observed heterozygosity (H_O) and the mean expected heterozygosity (H_E) based on Hardy–Weinberg assumptions.

A qualitative descriptor of allele frequency distribution (the mode-shift indicator), which is reported to discriminate between bottlenecked and stable populations (Luikart *et al*. 1998), was obtained using the program BOTTLENECK. The mean ratio of the number of alleles to total range in allele size '*M*' (Garza & Williamson 2001) was implemented in AGAR$_{ST}$ (Harley 2002).

Population differentiation and effective population size

To assess population genetic differentiation Fisher's exact test (Raymond & Rousset 1995), implemented in GENEPOP, was initially performed to test whether differences in allele frequencies could be attributed to sampling error. Population differentiation was then quantified from genotypic (as opposed to allelic) data by calculation of Weir and Cockerham's estimator of F_{ST} (Weir & Cockerham 1984) using the program FSTAT, as well as R_{ST} (Chakraborty & Nei 1982; Slatkin 1995) computed using the program R_{ST} CALC (Goodman 1997), averaging across variance components using centred normalized allele sizes.

The effective population size, assuming mutation–drift equilibrium, was calculated as $N_e = [1/(1 - H_E)^2 - 1]/8\mu$ using the assumptions of the stepwise-mutation model (SMM) (Ohta & Kimura 1973) or as $N_e = H_E/[4\mu(1 - H_E)]$ using the infinite allele model (IAM), and assuming a mutation rate μ of 2.05×10^{-4} estimated from an average of three studies (Rooney *et al*. 1999).

Assignment testing of individuals

Assignment tests were performed in AGAR$_{ST}$ as described by Paetkau *et al*. (1997), with the individual's alleles being subtracted from the population under comparison, and a frequency value in that population of 0.01 assumed for alleles present in the individual being tested but absent from the population being compared.

Results

Genetic diversity

It was observed that two *Diceros bicornis minor* individuals shared identical genotypes at all loci. Since these represented two individuals sampled at the same time, the possibility that the samples represented the same individual was assumed and data from only one of the individuals was used in the subsequent analyses.

Measures of genetic diversity show that *Diceros bicornis michaeli* retained the most diversity in terms of both heterozygosity and relative number of alleles (Table 3). However, appreciable levels of genetic variation still persisted within both *D. b. minor* and *Diceros bicornis bicornis*. Only the *D. b. minor* population showed significant departure from Hardy–Weinberg equilibrium, with slight overall homozygous excess ($P = 0.0004$). This is likely to reflect a degree of within-population substructure, since the individuals came from several game reserves in South Africa and, in the case of eight individuals, from Zimbabwe (Table 2). Although the sampling of *D. b. bicornis* appears to be more scattered, most of these individuals represented animals recently translocated from Etosha National Park in Namibia, allowing insufficient time for genetic substructuring to have developed.

The effective population size (N_e) that would be expected from the data under the assumption of mutation–drift equilibrium was also calculated from each population under two models of microsatellite evolution: SMM and IMM. N_e values (Table 3) calculated under SMM were somewhat greater than those estimated under IMM.

Two statistical methods can be applied to microsatellite data to test which of the two processes, a bottleneck followed by expansion, or prolonged low population size in mutation–drift equilibrium, are more consistent with the data. In the first method, implemented in the program BOTTLENECK (Cornuet & Luikart 1996), the allele frequency distribution is established in order to see whether there is a mode shift from an approximately L-shaped distribution, as expected under mutation–drift equilibrium, or not (recent bottlenecks

Table 4 Pairwise population comparisons, averaged across loci, using Fisher's exact test for allelic differentiation as a qualitative measure of significance, and *F* and related statistics for quantification

Populations	Exact test	F_{IS}	F_{ST}	R_{ST}
D. b. minor vs. *D. b. bicornis*	< 0.00001	0.024	0.250	0.231
D. b. minor vs. *D. b. michaeli*	< 0.00001	0.020	0.280	0.231
D. b. bicornis vs. *D. b. michaeli*	< 0.00001	–0.040	0.294	0.242

provoke a mode shift). In none of the three populations was such a mode shift detected.

The second method (Garza & Williamson 2001) measures the ratio of the allele size range in repeat units to the number of alleles (*M* statistic). This method is manifested as a decrease in ratio from a value of > 0.8 typical of outbred populations to values typically < 0.7 in severely bottlenecked populations, and has the advantage that the effect persists, unlike BOTTLENECK, for very many generations. This *M* statistic gave values (Table 3) consistent with a population reduction in all three subspecies. Heterozygote deficiencies within the three populations, as measured by F_{IS} (Table 4), were close to zero.

Population differentiation

Population differentiation was assessed initially using Fisher's exact test for allelic distributions implemented in the program GENEPOP. Results are summarized in Table 4. The exact test showed highly significant differentiation between each of the three subspecies. The degree of differentiation was quantified using two measures of population differentiation, F_{ST} and R_{ST}. Values showed approximately the same degree of differentiation between all three populations, with these values proving to be greater than 0.2 in all cases.

An additional means of analysing population differentiation is by the use of assignment tests, which provide a measure of whether an individual is more likely to belong to one population rather than to another. It also provides a means of measuring the affinities or differences of the single samples of *Diceros bicornis longipes* and *Diceros bicornis chobiensis* relative to the other three subspecies. All individuals of all three quantitatively predominant subspecies were assigned correctly to their own subspecies group and the median values and range of the likelihood values are given in Table 5. The distribution of values is detailed in Fig. 1, which provides a more insightful way of appreciating the differences between both individuals and groups. Likelihood values for individuals of the *D. b. michaeli* subspecies are lower than those of the other two populations and this is a function of the greater genetic diversity of this population. The overall high values for the likelihood ratios express an additional measure of the population differentiation between the three subspecies.

The plots in Fig. 1 also provide some indication as to whether there is significant migration between the populations. A migrant would be assigned to a population other than the one from which it is sampled, and the first generation offspring of a migrant would plot close to the diagonal. No misassignments are observed in the data, although one individual from the *D. b. minor* population and a few from the *D. b. michaeli* population plot near the diagonal.

Individuals from outlying populations

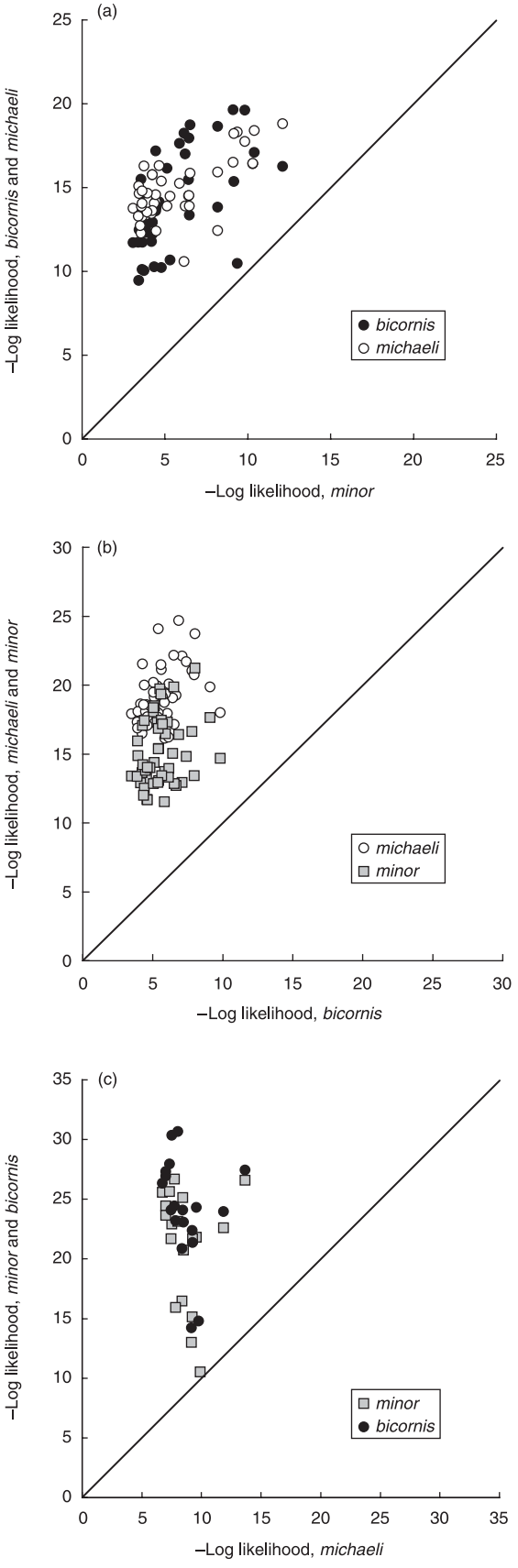
The single individual from Angola attributed to the *D. b. chobiensis* subspecies showed a greater affinity to the *D. b. minor* subspecies than to either of the other two subspecies, by a factor of 10^4, and the likelihood value fell within the range of assignments of individuals of the *D. b. minor* subspecies to that population. The single individual from the *D. b. longipes* population showed likelihood values for assignment to the other three populations that were markedly lower than the modal values for assignments of individuals within those populations to their own population (Table 6). No individual from any of the three main subspecies had a lower likelihood of belonging to its correct group than did that of *D. b. longipes* to that group. This individual was homozygous at eight out of the nine loci examined. In contrast the corresponding (mean) value for homozygous loci for *D. b. michaeli* was 2.6, for *D. b. minor* was 5.0, and for *D. b. bicornis* was 4.3.

Discussion

The East African *Diceros bicornis michaeli* was historically distributed from southern Sudan, Ethiopia and Somalia, through Uganda, Rwanda and Kenya into northern

Table 5 The likelihood values of assignment of individuals to their own population (left-hand columns) and likelihood ratios of assignment of individuals to their own as compared to the next closest population (right-hand columns). Median values and the range are given

Population	Median value of likelihood (own population)	Range	Median value of likelihood ratio (to next closest population)	Range
D. b. minor	3.6×10^{-5}	8.5×10^{-4} to 8.0×10^{-13}	1.2×10^{8}	1.2×10^{1} to 2.8×10^{10}
D. b. bicornis	4.0×10^{-6}	3.4×10^{-4} to 1.6×10^{-10}	2.1×10^{9}	7.3×10^{4} to 7.8×10^{13}
D. b. michaeli	4.2×10^{-10}	1.9×10^{-7} to 2.1×10^{-14}	8.3×10^{12}	7.0×10^{0} to 7.0×10^{18}



Tanzania. By 1980 these rhinoceros numbered a little over 2000 individuals with a current estimate of 490 (P. M. Brooks, personal communication). The Addo National Park sample representing this population has retained the most diversity in terms of both heterozygosity and relative number of alleles despite this sample being a sanctuary population seeded from only six animals in the 1960s from an East African *D. b. michaeli* population. This diversity adds to the value of this small out-of-range population that had grown to a population of about 25 animals in the late 1990s.

The southwestern *Diceros bicornis bicornis* population was largely restricted to Namibia, northern Angola and the mid-southwestern area of South Africa. Little historical demographic information exists except for anecdotal evidence for a large-scale population decline reaching its nadir early in the last century. About 600 individuals remained in 1980 (P. M. Brooks, personal communication), which have more than doubled to a figure of 1310 at the present time.

The south-central *Diceros bicornis minor* population was originally widespread from southern Tanzania through South Africa. The population in 1980 was about 8000 individuals, compared to about 1775 in 2001 and with a nadir of about 110 individuals in the 1930s.

The difference in allelic diversity and heterozygosity among the three populations is reflected in the N_e values. The values estimated using SMM, which may be the more appropriate model for microsatellite evolution (but see Chakraborty & Nei 1977; Balloux & Goudet 2002 for more detailed discussion), reflect an effective population size for *D. b. michaeli* that is more than twice that for the other two subspecies. One value of this measure is that it describes a mutation–drift equilibrium population number for the subspecies, which once attained in practice should not result in further loss of genetic diversity. This of course assumes panmixia for the subspecies and would be an underestimate if conservation measures resulted in within-population substructuring. Until numbers of each subspecies expands to approximately these N_e values, continuing loss of genetic diversity is expected to occur.

The relative low genetic diversity in *D. b. minor* and *D. b. bicornis* could either be the consequence of a recent population bottleneck with subsequent population expansion, or low population numbers persisting for many generations. *D. b. michaeli* populations in eastern Africa have never reached and passed through a bottleneck, but are instead on a continually declining trajectory, a situation similar to that of the greater one-horned rhinoceros in Nepal (Dinerstein

Fig. 1 Plots from assignment tests comparing the likelihood of an individual belonging to its source population (ordinate) to the likelihood of it belonging to either of the other two populations (abscissa). A low likelihood corresponds to a high negative log likelihood. Plotted in (a) are values for *Diceros bicornis minor* individuals, in (b) values for *Diceros bicornis bicornis* individuals and in (c) values for *Diceros bicornis michaeli* individuals.

Individual	Population	Likelihood	Likelihood ratios
D. b. chobiensis	*D. b. minor*	4.6×10^{-12}	*D. b. minor* to *D. b. michaeli* 10 700
	D. b. michaeli	4.2×10^{-16}	*D. b. michaeli* to *D. b. bicornis* 13 800
	D. b. bicornis	3.1×10^{-20}	
D. b. longipes	*D. b. michaeli*	2.4×10^{-17}	*D. b. michaeli* to *D. b. minor* 19 200
	D. b. minor	1.2×10^{-21}	*D. b. minor* to *D. b. bicornis* 1.5×10^{7}
	D. b. bicornis	8.0×10^{-29}	

Table 6 The likelihood of assignment of the single individuals of the subspecies *D. b. chobiensis* and *D. b. longipes* to the other major populations. The values in the right-hand column depict how many times more likely the individual is to be assigned to the first specified population than to the second

& McCracken 1990) and it is of note that both show relatively high measures of genetic diversity. *D. b. minor*, on the other hand, underwent a population bottleneck in the last century but has recovered substantially since, with *D. b. bicornis* following a similar pattern. One question is whether the bottlenecks for either of these two subspecies in the last century were of sufficient degree or duration to explain their relatively low genetic diversity, or whether a bottleneck or prolonged period of low population numbers further back in time would be necessary to explain the data. It is interesting that of the two methods reported to identify population bottlenecks, only Garza and Williamson's *M* statistic showed some evidence for this from the allele distributions. This limitation of these methods on populations with relatively well-known demographic history has been noted and discussed previously for bottlenecked large mammal populations (Whitehouse & Harley 2001), but the lack of any mode shift demonstrated by the BOTTLENECK program would be less consistent with a very recent bottleneck.

Genetic and demographic data are available on four southern African populations of large mammals that are known to have passed through, and mostly recovered from, bottlenecks which reached their nadir about 100 years ago. Outbreaks of foot-and-mouth disease in 1894 and rinderpest in 1896 brought about a 95% reduction in population numbers of the Cape African buffalo (*Syncerus caffer*) in South Africa (Smithers 1983). The total southern white rhinoceros (*Ceratotherium simum simum*) population fell to about 20 animals a century ago, but has recovered to over 9000 today (Emslie & Brooks 1999). The African elephant (*Loxodonta africana*) population in the Kruger National Park was reduced to only about 10 animals in 1905, increasing rapidly thereafter to more than 6000 by 1967 (Hall-Martin 1992), with growth by recruitment boosted by immigration of elephants from Mozambique. Once common Cape mountain zebra (*Equus zebra zebra*) populations were decimated to the extent that by 1935 only six isolated populations remained, three of which became extinct, leaving three relict populations which at their lowest points numbered 19, 6 and 5 individuals, respectively (Lloyd 1984). The maximum mean heterozygosities measured in populations of these species (buffalo, rhinoceros, elephant and zebra) from microsatellite analyses were 0.73, 0.27, 0.42 and 0.38, respectively (O'Ryan *et al*. 1998; Whitehouse & Harley 2001; Moodley & Harley 2005; O'Ryan, unpublished). However, care needs to be taken in comparing different microsatellite studies because some studies select microsatellite loci on the basis of high polymorphism which could bias such comparisons; for example, greater diversity than that observed here was observed in East African elephant populations which had not experienced such severe bottlenecks at that time (Nyakaana & Arctander 1999), and in buffalo populations north of the Limpopo (Van Hooft *et al*. 2000). In the above examples, the same loci were used in some comparisons of South and North African populations to justify the observed increased heterozygosity levels in northern over southern African elephant and buffalo populations. Other species are not in the position of recovering from bottlenecks but are in continuing decline; for example, the Roan antelope, *Hippotragus equinus*, with average expected heterozygosities across 18 populations across Africa being only 0.46 (Alpers *et al*. 2004).

Since most large mammal populations in Africa and elsewhere have reduced population sizes relative to those of several centuries ago, an interesting question is what levels of heterozygosity existed before the declines set in? A possible way to gain insight into this question is to examine small mammal species that have usually not been so severely affected by bottlenecks. In this context the African wild cat, *Felis lybica*, which is still widespread and relatively common in southern Africa, was found to have a mean expected heterozygosity across nine microsatellite loci of 0.795 (Wiseman *et al*. 2000), a value which is not much more than that in the East African *D. b. michaeli* population or in the African buffalo. A more direct way of addressing the question is to use material such as dried skin or teeth from individuals who died prior to the population decline; for example, analysis of microsatellite DNA from museum skin samples of elephants from Addo National Park in South Africa, home to a highly inbred elephant population, suggested genetic diversities prior to the bottleneck similar to those of more outbred neighbouring populations (Whitehouse & Harley 2001).

It might be supposed that R_{ST} should perform better than F_{ST} with microsatellite data because R_{ST} assumes SMM whereas F_{ST} assumes IAM, and also because R_{ST} is not so

significantly influenced by the introduction of new mutations since population separation. There is, however, still much debate as to the relative value of these and other measures of differentiation, and the limitations of SMM with regard to microsatellite evolution (Balloux & Goudet 2002), and all measures suffer to various extents from large bias and variance. In addition, measures of differentiation using these estimators cannot exceed the level of homozygosity, which will tend to be low for multiallelic loci such as microsatellites (Hedrick 1999). The relatively high values for both measures in Table 4 nevertheless show a significant degree of population differentiation between each of the three main groups. These values either reflect the rapid genetic drift taking place in small, recently separated populations, especially after a bottleneck event, or a long period of slow drift in large populations. The similarity of the R_{ST} and F_{ST} values is more consistent with relatively recent separation times since if there had been sufficient time for new mutations to have contributed to the differentiation, then F_{ST} values would tend to be lower than the values for R_{ST} (Slatkin 1995).

To summarize the analyses of population differentiation and bottleneck measures, it seems likely that the differentiation observed is not the result of the recent drastic population declines but rather the result of a period of separation sufficiently long for significant genetic drift to have occurred, but not long enough for new mutations in the microsatellite sequences to have made any noticeable contribution. Although there are a number of statistical methods to estimate the number of migrants between populations, either by calculation from F_{ST} values (Cockerham & Weir 1993) or using likelihood or Bayesian-based methods (e.g. Beerli & Felsenstein 1999), these usually require assumptions, such as constancy of N_e and negligible mutational effects, which are not realistic for these rhinoceros populations. Apart from the known recent population declines in all the rhinoceros populations, previous demographic fluctuations in population size are not known, and these can also affect estimates of population differentiation (e.g. Chakraborty & Nei 1977; Hedrick 1999). However, the assignment tests provide some insight into this question and imply that there is little migration between the populations, with *D. b. bicornis* especially showing no evidence of recent admixture in the individuals sampled.

This amount of differentiation has significant management implications; if differentiation had arisen from genetic drift acting over a long period of time, it would give more reason for maintaining populations separately, since new mutations would have established the populations on different evolutionary trajectories. Consequently, although outbreeding depression is unlikely without much greater degrees of differentiation, there might be some dilution of desirable attributes if populations were mixed. In the case of a bottleneck, rapid genetic drift causes random allele loss to predominate with minimal contribution from new mutations, and the populations would then be more appropriately managed by mixing to regenerate much of the original genetic diversity. A feature of the rapid genetic drift scenario is that populations may become fixed not only for neutral microsatellite alleles but also for morphological features that may appear to carry more evolutionary significance than they deserve. As a consequence such features can influence management to keep populations separate when a more appropriate strategy might be to allow interbreeding between them. The amount of diversity remaining, together with the degree of differentiation observed in the three populations, suggests that an intermediate conservation policy between these two extremes is appropriate. While the degree of diversity as reflected by heterozygosity and mean allele numbers remains near current levels it would seem appropriate to continue with current management policies of keeping the populations separate. However, were the effective population size or the genetic diversity of one of the groups to fall to much lower levels (as seems to be the case with the *Diceros bicornis longipes* population, see below) then it would become appropriate to consider interbreeding at least some individuals of that population with individuals from one of the others to maintain that population's genetic potential in the species as a whole.

The single individual which crossed over from Angola into the Caprivi in Namibia, attributed at the time of its capture there to the *Diceros bicornis chobiensis* subspecies, after Groves (1967), showed a greater affinity to the *D. b. minor* subspecies than to the others. This is consistent with a previous observation, using restriction mapping of mtDNA (O'Ryan *et al*. 1994) that the mtDNA haplotypes were identical between *D. b. chobiensis* and *D. b. minor*. The range of *D. b. bicornis* had been described as extending to the arid southern Angola, and *D. b. minor* to northern Angola. However, the microsatellite and mitochondrial results imply that the Caprivi/southern Angolan region where this individual was found is a component of the south-central geographical area. They also confirm that *D. b. chobiensis* does not warrant separate subspecific status.

It would be inappropriate to read too much into the data obtained from only a single individual of the northwestern ecological grouping, corresponding to *D. b. longipes*. Nevertheless, the high degree of homozygosity across the nine loci would be consistent with a major loss of genetic diversity in this bottlenecked population, and the assignment test suggests that this individual at least shows a high degree of divergence from the other populations, implying a high conservation status for the subspecies. The cell line derived from this species could have additional value: if *D. b. longipes* were to become extinct *in situ*, the cell line would provide nuclei for generation of an adult animal using cloning technology. This also emphasizes the need to at least

establish more cell lines from the population, and the need for attempts to establish cloning procedures in black and other rhinoceros species to prepare for such an eventuality, since a collection of cell lines from such individuals could constitute a 'virtual population' from which an effective 'copy' of the original population might eventually be recovered.

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Eric Harley and Collen O'Ryan are using molecular and computational approaches to address problems in population genetics and systematics with a conservation emphasis on endangered or vulnerable species in Southern Africa. Jessica Cunningham's PhD thesis was on rhinoceros and tortoise genetics. Ingrid Baumgarten's expertees is in data collection and organisation of the University of Cape Town Wild Life Cell Bank.
