Mitochondrial DNA Variation in Black Rhinoceros (*Diceros bicornis*): Conservation Management Implications

C. O'RYAN

Department of Chemical Pathology
Medical School
University of Cape Town
Cape Town, South Africa

J. R. B. FLAMAND

Natal Parks Board
P.O. Box 456
Mtubatuba 5939
Natal, South Africa

E. H. HARLEY

Department of Chemical Pathology
Medical School, University of Cape Town
Observatory 7925
South Africa

Abstract: *Cell cultures have been established from 33 individual black rhinoceroses. These were from wild populations from various localities in southern Africa and include representatives from three geographical regions (southwestern, south-central, and eastern) corresponding to currently accepted conservation units, and include individuals previously attributed to one of the four subspecies,* Diceros b. minor, D. b. bicornis, D. b. michaeli, *and* D. b. chobiensis *(du Toit et al. 1987). Comparative mitochondrial DNA restriction maps were constructed using 16 restriction enzymes. These showed in each case two site differences between representative individuals from any two of the above geographical regions. Maps were monomorphic within geographical regions and, therefore, have the potential to provide diagnostic markers. The map from a single individual attributed to the* D. b. chobiensis *subspecies was identical to other individuals (attributed to* D. b. minor*) in the south-central geographical region. The low amount of genetic diversity implied by these few differences renders it unlikely that problems with outbreeding depression will arise if, given the*

Address all correspondence to E. H. Harley. Paper submitted February 2, 1993; revised manuscript accepted July 29, 1993.

Variación de ADN mitrocondrial en rinocerontes negros (Diceros bicornis): Implicaciones para el manejo de conservación

Resumen: *Se establecieron cultivos de células de 33 rinocerontes negros. Estos provinieron de poblaciones salvajes de varias localidades del sur de Africa e incluyen representantes de tres regiones geográficas (Sudoccidental, Sud-central y Oriental) que corresponden a las unidades de conservación corrientemente aceptadas e incluyen individuos que previamente fueron atribuidos a otras cuatro subespecies,* Diceros b. minor, D. b. bicornis, D. b. michaeli *y* D. b. chobiensis *(du Toit, Foose & Cummings, 1987). Se construyeron mapas de restricción de ADN mitocondrial comparativos usando 16 enzimas de restricción. En cada caso estos mostraron diferencias en dos sitios entre individuos representantes de un par cualquiera de las regiones geográficas antes mencionadas. Los mapas fueron monomórficos dentro de cada región geográfica y por lo tanto tienen el potencial para proveer marcadores diagnósticos. El mapa de un único individuo que se atribuía a la subespecie* D. b. chobiensis *fue idéntico a otros individuos (atribuidos a* D. b. minor*), en la región geográfica Sud-central. El bajo nivel de diversidad genética*

continuing decline in numbers of black rhinoceroses, it becomes necessary to supplement wild or captive populations with individuals from a different conservation unit in order to avoid inbreeding depression.

que implican estas pocas differencias hace poco probable que surjan problemas de depresión de exogamia si, dada la declinación continua en el número de rinocerontes negros, se hace necesario suplementar las poblaciones salvajes o cautivas con individuos de unidades de conservación diferentes a los efectos de evitar la depresión de endogamia.

Introduction

There are five extant species of rhinoceros (Ryder 1991; Groves 1967), all of which are listed as endangered in the 1986 IUCN Red Data Book. The two African species, *Diceros bicornis* and *Ceratotherium simum* were once widely distributed in the sub-Saharian region, but with the increasing human settlement of Africa, both species have suffered severe declines in abundance and range (Smithers 1983).

Largely as a result of poaching, black rhinoceros numbers have decreased rapidly over the past 20 years. Numbers have fallen from 65,000 in 1970 to fewer than 4000 in recent years, which represents a decrease of over 90% (du Toit et al. 1987). As a consequence of this decline in numbers, black rhinoceros populations have become increasingly fragmented and isolated. Management of the species is complicated by the assignment of each population to one of several subspecies. The current management policy is to maintain these subspecies separately in order to conserve their genetic integrity. This is done on the basis that interbreeding might compromise any specific ecological adaptation possessed by a subspecies, or that outbreeding depression might arise from interbreeding if the populations were sufficiently divergent. But small fragmented populations, perhaps containing useful components of the overall genetic composition of the species, are susceptible to local extinction due to the effects of demographic fluctuations, local ecological perturbation, or loss of genetic diversity (Gilpin & Soulé 1986; de Toit et al. 1987).

The original subspecific designations defined seven subspecies (Table 1) based on morphological characteristics. But the sample sizes were small (only two of these subspecies were based on measurements of more than 10 adults), and the criteria used were limited to minor cranial and pelage differences (Groves 1967). Therefore it was agreed at the African Rhino Workshop in Cincinnati in 1986 (du Toit et al. 1987) that four basic ecological groups or conservation units should be recognized for practical management purposes and be maintained as separate breeding entities. These units are as follows:

Table 1. Subspecies of black rhinoceros: *Diceros bicornis* (Groves 1967).

Subspecies	*Distribution*
D. b. minor	Kenya to South Africa (Natal)
D. b. michaeli	Kenya and Tanzania
D. b. bicornis	South Africa (Cape Province) and Namibia
D. b. chobiensis	Angola
D. b. longipes	Central African Republic
D. b. ladoensis	Northern Kenya and Sudan
D. b. brucii	Ethiopia and Somalia

(1) the southwestern populations in Namibia, corresponding by general agreement of those in the field to *D. b. bicornis,* although this designation was originally applied by Groves to the Cape rhinoceros, now extinct;
(2) the south-central populations, extending from Natal in the Republic of South Africa through Zimbabwe and Zambia into southern Tanzania, and corresponding essentially to *D. b. minor;*
(3) the Eastern populations in Kenya and northern Tanzania, corresponding to *D. b. michaeli,* and
(4) the northwestern populations, extending from the horn of Africa to the Central African Republic and Cameroon. This would include most of the remaining subspecies. For the purposes of this study we will use the geographic designations instead of the trinomial subspecific designations.

MtDNA is a small circular molecule of about 16,400 base pairs (bp) in mammals (Anderson et al. 1981). It is easily purified, maternally inherited, undergoes no recombination, and evolves 5–10 times more rapidly than single-copy nuclear DNA (Brown et al. 1979). At the intraspecific level, analyses of mtDNA differences have been useful in defining phylogeographic partitioning (Avise et al. 1979; Avise & Lansman 1983; Wilson et al. 1985; Harrison 1989). Within-species differences in large mammal populations are typically less than 2%, although in some cases—for example, in cervids (Cronin 1991)—higher values have been reported.

Analysis of mtDNA has been used here to assess genetic differentiation among black rhinoceros subspecies and populations in Southern Africa, in order to assist in conservation decisions on how best to manage the remaining populations.

Methods

We examined a total of 33 individuals from a number of localities in southern Africa (Table 2). Cell culture lines were established from skin biopsies taken from animals that had been immobilized for veterinary or translocation purposes. Total DNA was extracted from confluent cell cultures, digested with the appropriate restriction enzymes, Southern blotted, and probed with purified mtDNA (Southern 1975). The purified mtDNA was prepared from heart tissue (Lansman 1981) obtained from an individual from Hluhluwe that died naturally in the field. DNA was digested with 16 restriction enzymes having six base-pair recognition sites, electrophoresed on 1–1.5% agarose gels, and transferred onto Hybond-N^+ membranes (Amersham, U.K.) for hybridization to the probe DNA. The latter was labeled by the random-priming method, using ^{32}P-dCTP (Amersham). Labeled mtDNA fragments were visualized by autoradiography. The restriction-site map was constructed from the purified mtDNA by the double-digestion method. The changes found in individuals from the other geographical areas were mapped using this map as a reference.

Results

The mtDNA restriction enzyme profiles are summarized in Table 3. Between one and seven restriction fragments were obtained with the 16 enzymes used. The most frequent fragment patterns observed were assigned the letter *A*, and subsequent patterns used *B*. For 13 of the 16 enzymes used, no mtDNA variability among the different geographical areas was observed. Only three enzymes gave differing patterns. They were Bcl I, Dra I, and Stu I.

Of the 26 south-central and five southwestern individuals screened, all were monomorphic for these respective mtDNA genotypes with the 16 enzymes tested. Although only one individual from the Eastern geographical region was tested, Ashley screened 11 individuals from this area in a mtDNA fragment-size analysis using a similar panel of restriction enzymes (Ashley et al. 1990). The documented fragment patterns of these 11 individuals tested were also found to be monomorphic and are consistent with our mapped restriction sites for the Eastern individual.

Table 2. Source of cell cultures

Subspecies	*Number*	*Location*
South-central	6	Mkuzi (Natal, S.A.)
	17	Umfolozi/Hluhluwe (Natal, S.A.)
	3	Andries Vosloo Reserve (Eastern Cape Province, S.A.)
	1	Caprivi (Namibia)*
Southwestern	4	Etosha (Namibia)
	1	Damaraland (Namibia)
Eastern	1	Addo (Eastern Cape Province, South Africa)**

* *This individual was found four kilometers from the unfenced Angolan/Namibian border, east of the Kavango river barrier.*
** *The Addo population of* D. b. michaeli *was derived from a founder population of two cows and two bulls translocated from the Kiboko region in southeast Kenya in 1961–1962.*

Table 3. Summary of Restriction Enzyme Digestions.

Enzyme	*Sites*	*Chobiensis* n = 1	*Minor* n = 26	*Michaeli* n = 1	*Bicornis* n = 5
Bcl I	6	A	A	B	B
Dra I	5	A	A	A	B
Stu I	6	A	A	B	A
Bam HI	2	A	A	A	A
Bgl I	1	A	A	A	A
Eco RI	2	A	A	A	A
Eco RV	2	A	A	A	A
Hind III	4	A	A	A	A
Hpa I	2	A	A	A	A
Nco I	1	A	A	A	A
Pvu II	4	A	A	A	A
Sac I	1	A	A	A	A
Sac II	3	A	A	A	A
Sal I	2	A	A	A	A
Sca I	7	A	A	A	A
Xba I	4	A	A	A	A

These fragment patterns are shown in Fig. 1, and the site differences giving rise to the variant fragment patterns in the three geographical regions are shown in Fig. 2. Two site changes differentiate south-central from eastern, two differentiate south-central from southwestern, and two differentiate eastern from southwestern. In each case this corresponds to an estimated sequence divergence of 0.4% between any two of the subspecies (Nei & Li 1979).

Discussion

The number of individuals sampled from the southwestern geographical region is clearly lower than ideal for a population genetic study, but the logistical problems of sampling endangered megavertebrates surviving in only small populations in the wild requires sampling to be largely an opportunistic exercise. Conclusions can be made with confidence for the south-central geographical region, where 26 individuals have been studied from a number of localities. Only five individuals from two separate localities of the southwestern population were studied, so it would be desirable to study more animals to establish whether the patterns are fixed for these populations. We have somewhat more confidence in our understanding of the monomorphic nature of the mtDNA pattern found in the eastern geographic area. Although only one individual was studied here, our mtDNA map is consistent with the fragment-size analysis

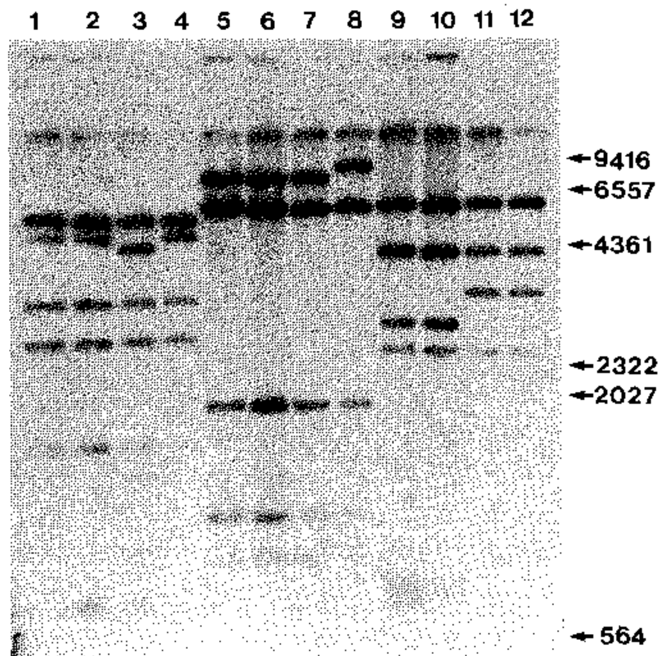

Figure 1. Southern blot analysis of DNA extracted from cell cultures from lanes 1, 5, and 9, D. b. chobiensis; *lanes 2, 6, and 10,* D. b. minor; *lanes 3, 7, and 11;* D. b. michaeli; *and lanes 4, 8, and 12,* D. b. bicornis. *DNA was digested with lanes 1–4, StuI; lanes 5–8, DraI; lanes 9–12, BclI. The sizes, in base pairs, of lambda Hind III molecular weight markers are indicated on the right-hand side.*

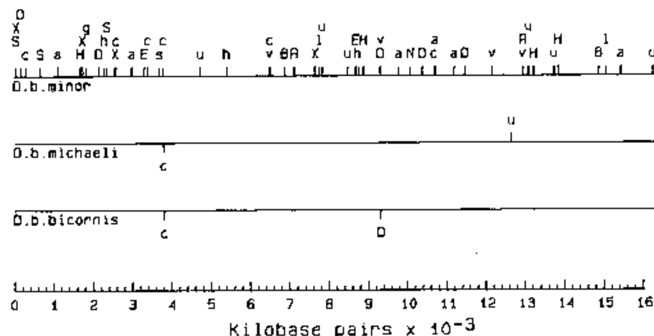

Figure 2. Black rhinoceros mitochondrial DNA restriction maps, aligned on the invariant Sac II sites at positions 676 and 2364 and oriented with the invariant Hpa I site at position 5480, both in the bovine sequence (Anderson et al. 1982). For D. b. michaeli *and* D. b. bicornis, *only the variant sites are shown, with site losses below the line and site gains above the line. a, ScaI; B, BamHI; c, BclI; D, DraI; E, EcoRI; g, BglII; h, HpaI; H, HindIII; l, SaI; N, NcoI; R, EcoRV; s, SacI; S, SacII; u, StuI; v, PvuII; X, Xba I.*

of Ashley et al. (1990), who found no variation among 11 individuals from the eastern geographical region originating from Kenya but held in zoological collections in the U.S.A. These patterns, or morphs, provide the basis for a useful set of diagnostic markers for each of these conservation units.

Populations with different mtDNA restriction patterns or morphs could arise as a consequence of either longstanding geographical separation or from demographic stochasticity, with genetic drift tending to fix morphs in small, fragmented populations. But although severe fragmentation of wild rhinoceros populations, coupled with a drastic decline in numbers of individuals within these populations, has occurred over the last few decades, there would have been insufficient time, given the long generation time of these animals (about 15 years), for this to explain the distribution of the morphs that we have observed. In addition, we have sampled individuals from separate localities in each of the three geographical areas studied, and the morphs are consistent in type within each area.

The identity of the restriction pattern of the individual from Caprivi, previously attributed to *D. b. chobiensis* with the restriction pattern of the south-central group provides a confirmation that the geographical conservation unit grouping is appropriate and indicates that the Caprivi/southern Angolan region is a component of the south-central geographical area. This is also consistent with the bioclimatic aspects of the region, the southwestern area consisting of an arid, semidesert habitat as compared to the savanna characteristic of the south-central region.

This result would be consistent with the abolition of *D. b. chobiensis* as a recognized subspecies. There are only a very few individuals of this population remaining in the wild. If translocations are required as a result of habitat loss or for protection from poachers, and if the numbers of the translocated individuals are considered to be too low for their establishment as a separate breeding population, it would seem more prudent to allow them to interbreed with populations in the south-central region than with the geographically closer southwestern populations.

Another result of practical value is the small amount of mtDNA diversity between the conservation units. This value (0.4%) is only approximate, because it is based on only two restriction site differences in each case—with a consequent large stochastic error—but it is no more than that typically found among members of any large panmictic mammalian population (Avise et al. 1986; Cann et al. 1987; Essop et al. 1991; Wayne et al. 1991). On this basis alone it would seem unlikely that interbreeding between these conservation units would result in any decrease in fitness or fecundity in the offspring (outbreeding depression). On the other hand, it is necessary to confirm that no significant chromosomal differences are found among the conservation units, because such differences could result in decreased fecundity among offspring of such crosses (O'Brien et al.

1985; Laikre & Ryman 1990; Packer et al. 1990). In this context it is relevant that a preliminary study (Ryder, personal communication) has indicated that there may be some chromosomal differences between and within southwestern and eastern populations.

In the absence of major chromosomal differences, the justification for managing a number of separate conservation units should be made on the basis of preserving some desirable features of morphology or adaptive specialization. These justifications will need to be well defined, because keeping small populations separate—at least on a regional basis—requires significant management planning and could contribute to increasing loss of genetic diversity and the resulting possibility of inbreeding depression.

This study was performed on individuals from wild populations. Given the constraints of space and financial resources in ex situ captive breeding programs, the above arguments apply even more strongly. But if the intention of ex situ captive breeding programs is to restock depleted wild populations in Africa, then problems might be encountered if local managers wish to avoid mixing their dwindling populations with cross-bred animals. The counter argument to this would be that if in situ populations have dwindled to the extent that inbreeding depression is either imminent or established, then introduced cross-bred animals would maximize genetic diversity most effectively. Captive breeding programs provide an excellent opportunity to resolve this problem: studies on fecundity and fitness of interpopulation crosses, especially if the individuals concerned show chromosomal differences, would be invaluable in helping to demonstrate the lack or otherwise of outbreeding depression. Until this is done, the most conservative approach would be to maintain separate captive breeding stocks of the different geographical conservation units. Attempts at population viability analysis suggest, however, that it is undesirable to initiate a captive breeding population with an effective founder number of fewer than 25 individuals (du Toit et al. 1987). This may be significantly lower than the actual number, given that many founders never breed. The effective population size needs to be maintained at 100 to 200 animals to ensure the maintenance of reasonable levels of heterozygosity over the foreseeable future.

Therefore, although it seems best at present for wild populations to be maintained as separate conservation units, the question that will need to be addressed by the captive breeding program will be whether the expense and increased management complexity of maintaining equivalent conservation units ex situ, given the number of individuals required in each conservation unit and the inability to benefit from exchange of individuals between such units, is justified by the advantages of keeping morphologically or genetically distinct entities separate.

Acknowledgments

We thank the FRD for financial assistance; I. Espie, A. Hall-Martin, M. Lindeque, P. Morkel, C. Raath, P. Rogers, and T. Sandwith for the collection of rhinoceros samples; and the Natal Parks Board, the National Parks Board, and the Namibian Ministry of National Conservation and Tourism for logistical assistance. Contructive comments were provided by an anonymous reviewer. I. Baumgarten assisted with the maintenance and curation of the rhinoceros cell cultures.

Literature Cited

Anderson, S., M. H. L. De Bruijn, A. R. Coulson, I. C. Eperon, F. Sanger, and I. G. Young. 1982. Complete sequence of bovine mitochondrial DNA. Journal of Molecular Biology **156**:683–717.

Ashley, M. V., D. J. Melnick, and D. Western. 1990. Conservation genetics of the black rhinoceros (*Diceros bicornis*). I. Evidence from the mitochondrial DNA from three populations. Conservation Biology 4:71–77.

Avise, J. C., and R. A. Lansman. 1983. Polymorphisms of mitochondrial DNA in populations of higher animals. Pages 147–164 in M. Nei and R. K. Koehn, editors. Evolution of genes and proteins. Sinauer Associates, Sunderland, Massachusetts.

Avise, J. C., C. Giblin-Davidson, J. Laerm, J. C. Patton, and R. A. Lansman. 1979. Mitochondrial DNA clones and matriarchal phylogeny within and among geographical populations of the pocket gopher, *Geomys pinetis*. Proceedings of the National Academy of Science, U.S.A. **76**:6694–6698.

Avise, J. C., G. S. Helfman, N. C. Saunders, and L. S. Hales. 1986. Mitochondrial DNA differentiation in North Atlantic eels: Population genetic consequences of an unusual life history pattern. Proceedings of the National Academy of Science, U.S.A. **83**:4350–4354.

Brown, W. M., M. George, and A. C. Wilson. 1979. Rapid evolution of animal DNA. Proceedings of the National Academy of Science, U.S.A. 76:1967–1971.

Cann, R. L., M. Stoneking, and A. C. Wilson. 1987. Mitochondrial DNA and human evolution. Nature **325**:31–36.

Cronin, M. A. 1991. Mitochondrial DNA phylogeny of deer (Cervidae). Journal of Mammalogy **72(3)**:533–566.

du Toit, R., T. J. Foose, and D. H. M. Cummings. 1987. Pachyderm Special Issue No. 9. Proceedings of African Rhino Workshop, Cincinnati, October 1986.

Essop, M. F., E. H. Harley, P. H. Lloyd, and H. J. van Hensbergen. 1991. Estimation of the genetic distance between bontebok and blesbok using mitochondrial DNA. South African Journal of Science **87**:271–273.

Gilpin, M. E., and M. E. Soulé. 1986. Minimum viable populations: The process of species extinction. Pages 13–34 in M. E. Soulé, editor. Conservation: The science of scarcity and diversity. Sinauer Associates, Sunderland, Massachusetts.

Groves, C. P. 1967. Geographic variation in the black rhinoceros. Zeitschrift fur Saugetierkunde **32**:267–276.

Harrison, R. G. 1989. Animal Mitochondrial DNA as a genetic marker in population and evolutionary biology. Trends in Ecology and Evolution **4(1)**:6–11.

Laikre, L., and N. Ryman. 1990. Inbreeding depression in a captive wolf (*Canis lupus*) population. Conservation Biology **5(1)**:33–40.

Lansman, R. A., R. O. Shade, J. F. Shapira, and J. Avise. 1981. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. Journal of Molecular Evolution **17**:214–226.

Nei, M., and W. Li. 1979. A mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences, U.S.A. **76**:5269–5273.

O'Brien, S. J., M. E. Roelke, L. Mourker, A. Neuman, C. A. Winkler, D. Meltzer, L. Colly, J. F. Evermann, M. Bush, and D. E. Wildt. 1985. Genetic basis for species vulnerability in the cheetah. Science **227**:1428–1434.

Packer, C., A. E. Pussey, H. Rowley, D. A. Gilbert, J. Martenson, and S. J. O'Brien. 1990. Case study of a population bottleneck: Lions of the Ngorongoro Crater. Conservation Biology **5(2)**:219–230.

Ryder, O. 1991. Abstract. International Symposium on Rhinoceros Biology and Conservation, San Diego, California.

Smithers, R. H. N. 1983. Order Perissodactyla. Pages 558–562 in The mammals of the southern Africa Subregion. University of Pretoria, South Africa.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology **98**:503–517.

Wayne, R. K., N. Lehman, D. Girman, P. J. P. Gogan, D. A. Gilbert, K. Hansen, R. O. Peterson, U. S. Seal, A. Eisenhawer, L. D. Mech, and R. J. Krumenaker. 1991. Conservation genetics of the endangered Isle Royale grey wolf. Conservation Biology **5(1)**:41–51.

Wilson, A. C., R. L. Cann, S. M. Carr, M. George, U. B. Gyllensten, K. M. Helm-Bychowski, R. G. Higuchi, S. R. Palumbi, E. M. Prager, R. D. Sage, and M. Stoneking. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. Biological Journal of Linnean Society **26**:375–400.