MOLECULAR GENETIC STUDIES OF SOUTHERN AFRICAN RHINOCEROS

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Our investigations have the goal of developing and applying DNA-based molecular genetic techniques to address aspects of rhinoceros biology which have both academic interest and practical value to conservation management. We therefore have utilized three approaches:

1) Systematics: restriction endonuclease site mapping of mitochondrial DNA to estimate the time of divergence of black and white rhinoceros from their common ancestor.

2) Population genetics: defining mitochondrial DNA haplotypes in subspecies of black rhinceros.

3) Developmental: exploring the practicability and usefulness of some relevant new techniques emerging from molecular biology.

SYSTEMATICS RESULTS

Restriction maps of mitochondrial DNA were constructed by the double digestion technique, with the help of a restriction mapping management computer program (E.H. Harley, 1991) for 18 restriction endonucleases recognizing six base sequences. Heart tissue from opportunistic deaths in the field of D. bicornis minor and C. simum simum was used to prepare highly purified mitochondrial DNA suitable for end labelling with ^{32}P, an approach which is straightforward, robust, and highly sensitive. Maps are shown in Fig. 1 aligned and oriented on two Sac II sites and a Hpa 1 site which are invarient throughout the vertebrata. Sites on the two maps aligned to within 1% of the total map length were assumed to be homologous. From the proportion of shared sites (0.667) the sequence divergence was calculated, using equation 9 of Nei and Li (1979) to be 6.79 (+/- 1.6)%. Assuming the calibration of sequence divergence against time for mammalian DNA reported by Brown, George, and Wilson (1979, this translates to a time of divergence of the two species of about 3.4 (+/- 0.8) million years ago, a value only slightly greater than that reported by George and Ryder (this proceedings) using a restriction fragment comparison method.

POPULATION GENETICS RESULTS

There is controversy about the validity of the various subspecific designations currently or recently applied to the Black Rhinoceros, therefore we gathered specimens from four of these subspecies for comparative mitochondrial DNA haplotype analysis. Since it is desirable to gather as many individuals as possible from each subspecies, we established skin fibroblast cell cultures from ear nicks taken when animals were immobilized for translocation or veterinary purposes. These ear nicks provided viable cultures even after five days in transit to our processing laboratory in Cape Town, provided they were kept cold and damp in sealed plastic bags on ice. Total DNA was extracted from the cultures by standard methods and restriction fragments were

separated by agarose gel electrophoresis. The mitochondrial DNA bands were visualized after Southern blotting using ^{32}P labelled black or white rhinoceros DNA, prepared as above, as a probe, and autoradiography. Table 1 summarizes the source and number of the cell lines established. All the 23 specimens of D.b. minor were monomorphic for each of the 12 restriction endonucleases used, as were the five specimens of D.b. bicornis.

Three enzymes identified sites polymorphic between subspecies and these are summarized in Table 2. D.b. minor and D.b. chobiensis gave identical results for all enzymes. Two site differences differentiated minor from michaeli, minor from bicornis, and michaeli from bicornis. In each case this implied a sequence divergence of no more than about 0.4% between the subspecies .

DEVLOPMENTAL RESULTS

DNA fingerprinting is a technique which has value at the within population level for identifying first degree relationships and for giving indications of the amount of genetic diversity in a population. Southern blots of both white and black rhinoceros total DNA failed to give consistent, reproducible, and easily interpretable results from a number of probes currently used to display polymorphic areas of the human genome (e.g. M13, Jeffrey's probes, and $(CAC)_5$).

On the other hand, more success was obtained using the polymerase chain reaction method (PCR) to amplify segments of the mitochondrial genome, which was used for direct DNA sequencing using the amplification primers. Clean sequences from the cytochrome B region have been obtained so far from D.b. minor which can be readily aligned with the corresponding sequence in the bovine DNA to give a sequence divergence of about 31%.

DISCUSSION

Molecular techniques give results of both academic interest and of value in practical management. The results of our systematics investigation are relevant to the definition of the timing of evolutionary events in the family Rhinocerotidae. This in turn contributes, together with other phylogenetic studies on larger mammals such as the Bovidae, to deeper understanding of biogeographic and climatic events from the Miocene to the present day on the African continent. The development of direct rapid sequencing methods will also be most relevant in this academic context.

On the other hand, the population genetic results have practical value for rhinoceros conservation. The haplotype analysis provides markers which can be used to identify the subspecific designation of an animal whose origin is uncertain. One especially useful feature of the three diagnostic enzymes illustrated in Table 2 is that they appear to be monomorphic for each subspecies . This conclusion can be made with near certainity for D.b. minor, where 23 individuals from a number of locations were studied, with moderate confidence for D.b bicornis and with moderate confience even for D.b. michaeli, since although only one individual of this subspecies was studied here, Ashley et al. (1990)

obtained similar patterns for Dra 1 in 11 michaeli individuals, and for Bcl 1 in the only michaeli individual they studied with this enzyme.

It is of interest that the chobiensis subspecies gives an identical pattern to D.b. minor. Although geographically it is closer to D.b. bicornis, the habitat of chobiensis is more similar to that of D.b. minor. The results would be consistent with the abolition of chobiensis as a recognized subspecies.

The second result of practical value to emerge from the haplotype studies is the small amount of mitochondrial DNA genetic diversity between the subspecies which these few differences demonstrate. The amount of diversity found is no more than that typically found between individual members of any large panmictic mammalian population. It is therefore very unlikely that interbreeding between these subspecies would result in any decrease in fitness or fecundity in the offspring (out-breeding depression); on the other hand, any recently evolved adaptive features might be compromised. In other words, if subspecies are to be managed as separately breeding entities, the justification will need to be on the basis of preserving some desirable feature of morphology or adaptive specialization in a subspecies. These justifications will need to be rigorously defined, since keeping the subspecies separate requires more expense, greater management complexity, and contributes, if numbers of rhinoceros populations remain small, to increasing loss of genetic diversity than if all the D. bicornis populations were allowed to interbreed.

Our development studies have suggested that DNA fingerprinting as currently performed is not a practical method for studying populations on a short term basis, and will be of little help to conservation management. On the other hand, techniques are being evolved which may change this rather negative conclusion. Techniques which may provide the same useful information at the intra-population level include isolation of species-specific single-locus hypervariable probes, and random primed PCR methods (G. Amato, this proceedings).

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LEGEND

Fig. 1 Restriction endonuclease maps of mitochondrial DNA aligned on the invariant Sac II site at position 676 in the bovine sequence. a, Sca 1; B, Bam H1; c, Bcl 1; D, Dra 1; E, Eco R1; g, Bgl II; h, Hpa 1; H, Hind III; l, Sal 1; N, Nco 1; o, Xho 1; P, Pst 1; R, Eco RV; s, Sac 1; S, Sac II; u, Stu 1, v, Pvu II; X, Xba 1.

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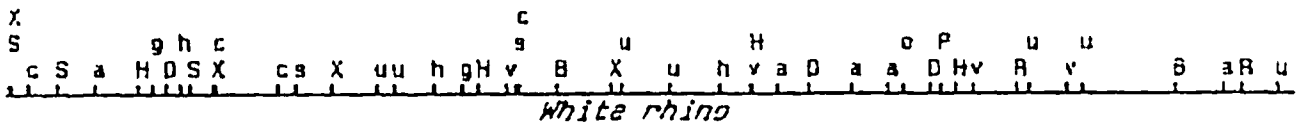
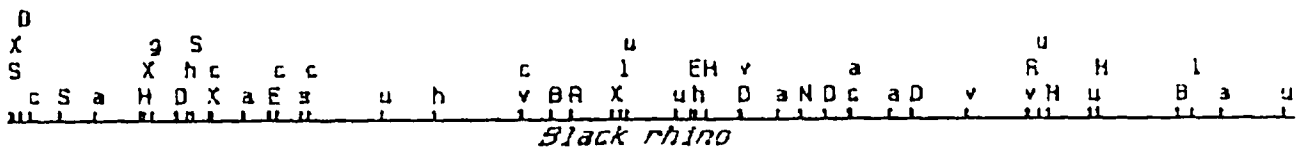

White rhino


Black rhino

CELL CULTURES ESTABLISHED

Diceros bicornis

Subspecies	Number	Source
D.b.minor	15	Umfolozi/Hluhlue
	6	Mkuzi
	2	Zimbabwe
D.b.chobiensis	1	Caprivi
D.b.michaeli	1	Addo
D.b.bicornis	4	Etosha/Vaalbos
	1	Damaraland
Total	30	

Ceratotherium simum

C.s.simum	3	Umfolozi/Hluhlue

Mitochondrial haplotypes in subspecies of Diceros bicornis

Enzyme	No.of sites	minor	chobiensis	michaeli	bicornis
Hind III	4	A	A	A	A
Sca I	7	A	A	A	A
Eco RI	2	A	A	A	A
Eco RV	2	A	A	A	A
Pvu II	4	A	A	A	A
Xba I	4	A	A	A	A
Bam HI	2	A	A	A	A
Sal I	2	A	A	A	A
Hpa I	3	A	A	A	A
Dra I	5	A	A	A	B
Bcl I	6	A	A	B	B
Stu I	6	A	A	B	A
Total	47				

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MITOCHONDRIAL DNA COMPARISONS IN BLACK AND WHITE RHINOCEROS

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The black rhinoceros, Diceros bicornis, and white rhinoceros, Cerototherium simum, are the two African representatives of the family Rhinocerotidae. As with the three other Asian members of this family they comprise dwindling populations in imminent danger of extinction. Defining inter- and intra-specific genetic relationships of these endangered megavertebrates provides a database contributing both to a fuller understanding of their phylogenetic relationships, and to the problem of maintaining viable populations. We report here restriction endonuclease maps of mitochondrial DNA prepared from heart tissue obtained after natural deaths in the field of Diceros bicornis subspecies minor, and Ceratotherium simum subspecies simum, both from Hluhluwe game reserve, Natal.

Mitochondrial DNA was extracted and purified by centrifugation in CsCl/Ethidium bromide gradients (Ausubel et al., 1989). Restricted DNA was end-labelled with ^{32}P using the Klenow fragment of DNA polymerase I and ^{32}P-deoxycytidine triphosphate (Amersham, U.K.). Restriction fragments were separated by agarose or polyacrylamide gel electrophoresis and visualised by autoradiography of the dried gel,

and sized by reference to appropriate end-labelled molecular weight markers. Maps were constructed for each animal independently by the double digestion method using a total of 19 restriction endonucleases recognising 6 base pair sequences. Maps were aligned with each other and with the known bovine sequence using the two Sac II sites and a Hpa II site, at positions 676, 2364, and 5480, respectively in the published bovine sequence (Anderson et al., 1982), which are invariant throughout the vertebrata. Sites which were aligned to within 1% of the total map length, estimated to be 16417 $\pm$ 298 and 16411 $\pm$ 225 for Black and White rhinoceros respectively, were taken to represent shared sites.

It is desirable to have an estimate of intra-specific genetic variation, since the significance of the inter-specific variation increases as the former decreases; for example, in the extreme case where the two values are the same then there would be no genetic basis for differentiation of the species. Since post-mortem material was available for very few individuals, cell cultures were established from the ear-nicks taken while marking 3 white rhinos from Hluhluwe (all C. simum simum) and 23 black rhino, 15 from Hluhluwe, 6 from Mkuzi (Natal), and 2 from Zimbabwe (all D. bicornis minor). Total DNA was extracted at an early passage number from cell cultures, propagated in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. The restriction patterns given by each of the enzymes used to construct the maps were compared for all the individuals after agarose gel electrophoresis and visualisation of mitochondrial DNA bands by hybridisation of Southern blots to a random primed rhinoceros mitochondrial DNA probe made from the purified heart preparation. No polymorphic sites were found for any enzyme for the black rhinoceros

and only one Sca I polymorphic site was found for the white rhinoceros population. This is consistent with the extremely small amounts of intraspecific variation observed in allozyme studies on these two species (Merenlender et al., 1989). Our studies are at present limited to the subspecies minor in D. bicornis and to simum in C. Simum, but will be extended to other subspecies when sufficient material has been collected.

The proportion of shared sites between Black and White rhinoceros is estimated by $2N_{xy}/(N_x + N_y)$ where N_x is the number of sites in Black Rhinoceros, N_y is the number of sites in white Rhinoceros and N_{xy} is the number of sites shared. With $N_x = 52$, $N_y = 45$, and $N_{xy} = 31$, the proportion of shared sites was estimated to be 0.667. Sequence divergence was calculated from this value using formula 9 of Nei and Li (1979) and gave a value of 6.79% with a standard deviation of 1.62%. The initial rate of sequence divergence between two mammalian mitochondrial DNA lineages has been calculated by Brown et al. (1979) to be about 2% per 10^6 years. If this holds true for the Rhinocerotidae it would give a time for the divergence of these two mitochondrial DNA lineages of 3.4 ± 0.8 million years before the present. This agrees well with a value of 3.5 million years suggested by George (1987) using restriction fragment size comparisons and with fossil evidence. The fossil record of the recent Rhinocerotidae is fragmentary, but the description of Ceratotherium praecox from deposits of about 4 million years before present (Hooijer, 1972), and its similarity to both C. simum and D. bicornis, was used to support the proposal that Ceratotherium split off from the Diceros lineage somewhere in the Pliocene. George and Ryder (1986) used restriction

site comparisons of mitochondrial DNA in another family in the Perissodactyla to estimate that the common ancestor of the Equidae was present about 3.9 million years before the present. This similarity to the figure of 3.7 in the African Rhinocerotidae may be coincidental but contributes to the gradual accumulation of a data set which may define major radiation episodes of African mammals in the Pliocene and Pleistocene.

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