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 detected 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 confidence 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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Table 1

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
	30	

Ceratotherium simum

C.s. simum	3	Umfolozi/Hluhlue

Table 2

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				

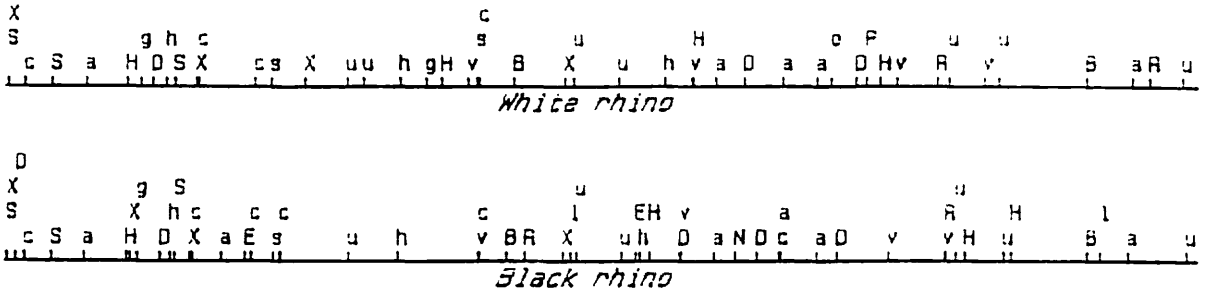

Fig. 1 Restriction endonuclease maps of mitochondrial DNA aligned on the invariant Sac II site at position 676 in the bovine sequence. a, Sca 1; B, Bam H1; c, Bcl 1; D, Dra 1; E, Eco R1; g, Bgl II; h, Hpa 1; H, Hind III; l, Sal 1; N, Nco 1; o, Xho 1; P, Pst 1; R, Eco RV; s, Sac 1; S, Sac II; u, Stu 1,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