Allozyme Variation and Differentiation in African and Indian Rhinoceroses

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We studied variation at 25 to 31 allozymic loci in African and Asian rhinoceroses. Four taxa in three genera were examined: African *Ceratotherium simum simum* (northern white rhinoceros), *C. s. cottoni* (southern white rhinoceros), *Diceros bicornis* (black rhinoceros), and *Rhinoceros unicornis* (Indian rhinoceros). Extremely small amounts of intraspecific variation were observed in sample sizes of 2 to 10 presumably unrelated individuals per taxon: $\bar{P}$ = .00–.10, $\bar{H}$ = 0.00–0.02. We examined demographic bottlenecks and sampling errors as possible reasons for the low levels of detectable variation. The very small intraspecific genetic distance ($\bar{D}$ = 0.005) between the two living white rhinoceros subspecies is far less than the distance that has been reported for other mammal subspecies. The mean *D* value of 0.32 ± 0.11 between the two African genera was also less than expected given the divergence time of greater than 7 million years suggested by the fossil record. Rhinoceroses may be evolving more slowly at the structural gene loci than are some other mammal groups. The estimate of $\bar{D}$ = 1.05 ± 0.24 for the African–Indian split supports this idea, as the lineage diverged at least 26 million years ago. Our results contribute to the currently available scientific information on which management decisions aimed toward saving endangered rhinoceroses should be based.

Rhinoceros populations have been decimated in the last 100 years. In light of these historical declines and the small numbers of animals remaining, there is an urgent need to manage the survivors more intensively. Information on genetic variation, breeding systems, and population structure in the various taxa is applicable to the problems of maintaining viable populations.[17,52,62,63] We report the results of a preliminary electrophoretic survey of genetic variation at protein and allozyme loci and address the following questions: 1) How much genetic variation resides in each of the recognized taxa? 2) What level of genetic differentiation exists between the two named subspecies of African white rhinoceros and among African white, African black, and Indian rhinoceroses? and 3) To what degree does this observed interspecific genetic differentiation conform to phylogenetic hypotheses based on data from morphology and the fossil record? We thus used multilocus genetic distances to construct phenetic trees, define extant evolutionary significant units, and elucidate their phylogenetic relationships.[13,61]

Rhinoceroses, which today are confined to parts of Asia and Africa, were once more widely distributed in Eurasia and North America. On the basis of paleontological evidence, their phylogeny can be traced back 30 to 35 million years to the Oligocene[26] (Figure 1A). The fossil record indicates that living African and Asian rhinoceroses arose separately from the Old World *Caenopus* group, a group of genera that included small, hornless, long-skulled animals of Oligocene age.[33] African rhinoceroses belong to two genera: *Diceros*, the black or hook-lipped rhinoceros, and *Ceratotherium*, the white or square-lipped rhinoceros. Both genera co-occur in 7-million-year old Kenyan deposits,[6,15] and the living species, *D. bicornis* and *C. simum*, are unusually old for mammals, having diverged from their congeneric ancestors approximately 4 million years ago.[26] For comparison, the mean species duration of European Plio-Pleistocene mammals was only about 1.5 million years.[53]

The two extant species of African rhinoceroses have been subdivided into several subspecies. In the case of the white rhinoceros, two subspecies are recognized: *C. s. simum*, the southern white rhinoceros, and *C. s. cottoni*, the northern white rhinoceros. These taxa are very poorly defined; Lydekker's[31] original description was based on only three skulls.

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Table 1. The origin of rhinoceroses examined

Sample[a]	Subspecies	Origin	Source[b]
1	*C. s. cottoni*	Sudan	SD Zoo (WC)
2 (28)	*C. s. cottoni*	Sudan	SD WAP (WC)
3	*C. s. cottoni*	Sudan	Dvur Kralove (WC)
4	*C. s. cottoni*	Sudan	Dvur Kralove (WC)
5	*C. s. cottoni*	Sudan	Dvur Kralove (WC)
6	*C. s. cottoni*	Sudan/Uganda[c]	Dvur Kralove
7	*C. s. cottoni*	Sudan/South Africa[d]	Dvur Kralove
8	*C. s. simum*	South Africa	SD WAP (52/159)
9	*C. s. simum*	South Africa	SD Zoo (?/156)
10 (203)	*C. s. simum*	South Africa	SD WAP (52/151)
11 (238)	*C. s. simum*	South Africa	SD WAP (52/150)
12 (53)	*C. s. simum*	South Africa	SD Zoo (WC)
13 (333)	*C s. simum*	South Africa	SD WAP (52/150)
14 (286)	*C. s. simum*	South Africa	SD WAP (52/147)
15 (287)	*C. s. simum*	South Africa	SD WAP (52/155)
16 (289)	*C. s. simum*	South Africa	SD WAP (52/?)
17 (142)	*C. s. simum*	South Africa	SD WAP (WC)
18	*C s. simum*	South Africa	SD WAP (?/155)
19 (688)	*C. s. simum*	South Africa	SD WAP (52/155)
20	*C. s. simum*	South Africa	SD Zoo (?/157)
21 (773)	*C. s. simum*	South Africa	SD WAP (52/159)
22 (774)	*C. s. simum*	South Africa	SD WAP (?/?)
23 (284)	*C. s. simum*	South Africa	SD WAP (52/150)
24 (52)	*C s. simum*	South Africa	SD Zoo (WC)
25 (819)	*C. s. simum*	South Africa	SD WAP (52/155)
26 (823)	*C. s. simum*	South Africa	SD WAP (268/271)
27 (820)	*C s. simum*	South Africa	SD WAP (52/159)
28 (821)	*C. s. simum*	South Africa	SD WAP (52/147)
29 (545)	*C. s. simum*	South Africa	SD WAP (52/?)
30	*C s. simum*	South Africa	SD WAP (52/150)
31 (78)	*D. bicornis*	Kenya	SD Zoo (WC)
32 (146)	*D. bicornis*	Kenya	SD Zoo (WC)
33 (239)	*D. bicornis*	East Africa	SD WAP (188/110)
34 (179)	*D. bicornis*	East Africa	SL Zoo (120/121)
35 (110)	*D. bicornis*	East Africa	SD WAP (46/47)
36 (104)	*D. bicornis*	East Africa	Germany
37 (188)	*D. bicornis*	Kenya	SD Zoo (WC)
38	*D. bicornis*	East Africa	Detroit Zoo (54/55)
39 (233)	*D. bicornis*	Kenya	Brookfield Zoo (WC)
40 (85)	*R. unicornis*	Assam, India	SD WAP (26/29)
41 (116)	*R. unicornis*	Assam, India	SD WAP (26/29)
42 (111)	*R. unicornis*	Assam, India	LA Zoo (7/8)

[a] Stud book number in parentheses if available.

[b] Sire/dam stud book numbers if available; ? = not recorded; WC = wild-caught; SD Zoo = San Diego Zoo; SD WAP = San Diego Wild Animal Park; SL Zoo = St. Louis Zoo; LA Zoo = Los Angeles Zoo.

[c] Zoo-born from Sudanese sire and Ugandan dam.

[d] Hybrid from South African sire and Sudanese dam.

Groves[21] argued that subspecific status was not warranted, and more recent studies by Hillman-Smith[23,24] and du Toit (unpublished observations) have failed to resolve this question. If in fact these two are subspecies, one can expect to find some fixed differences between the two at the protein level.

In the black rhinoceros, Groves[20] described seven subspecies on the basis of measurements on 79 skulls and some photographs: *D. b. minor* (ranges from Kenya to South Africa and Namibia), *D. b. michaeli* (Kenya and Tanzania), *D. b. bicornis* (South Africa), *D. b. longipes* (Central African Republic), *D. b. ladoensis* (northern Kenya and Sudan), *D. b. chobiensis* (Angola), and *D. b. brucii* (Ethiopia and Somalia). However, Groves[20] noted that in many cases the skull measurements were not diagnostic, and he was forced to make a more subjective assessment of skull photographs to distinguish the various subspecies. Recently, the African Rhino Workshop[1] ignored the many subspecific designations and simply recommended that populations in three geographic regions be targeted for conservation efforts. They based their recommendation on recent unpublished studies of skull morphology that revealed geographical clines in characters previously used to distinguish subspecies. Consistent with this reinterpretation are the results of a mitochondrial DNA study completed on the remaining black rhinoceros subspecies. Ashley et al.[4] found no significant differences in restriction fragment length polymorphisms between these subspecies. As our samples of black rhinoceros were all originally from east Africa, we were not able to measure the extent of allozymic divergence between subspecies or further evaluate their validity. Thus, for both the black rhinoceros and the white rhinoceros, it appears that the named subspecies are of questionable utility in defining evolutionarily significant units for purposes of conservation management.

Materials and Methods

We collected rhinoceros tissues opportunistically over a 10-year period at the Research Department of the San Diego Zoological Society, mostly from animals at the San Diego Wild Animal Park and the San Diego Zoo. Blood samples of *C. s. cottoni* were collected from the herd at Dvur Kralove, Czechoslovakia, in 1986. We examined the following numbers of individuals of each species, with the numbers of presumably unrelated individuals shown in parentheses: *C. s. simum* (South Africa), 23 (4); *C. s. cottoni* (north Africa), 7 (6); *D. bicornis* (east Africa), 9 (8); and *R. unicornis* (Assam, India), 3 (2). Available information on the origin and ancestry of these animals[35] is presented in Table 1.

Organ tissues were frozen after necropsy. Blood samples were collected in heparinized tubes; plasma and buffy coat were separated from the red blood cells by centrifugation. Tissues were held at −70°C until used. Prior to electrophoresis, 2 g of tissue was homogenized with a glass rod in 0.5 ml of distilled water. We then centrifuged the homogenate for 2 min to obtain an aqueous protein extract. Red blood cells were lysed with distilled water (1:1 dilution), and plasma was used without dilution.

We used standard horizontal starch gel electrophoresis to resolve allozyme patterns.[45,48] Gels were made with 12.5% Sigma starch (Sigma Chemical, St. Louis, Missouri). Samples were absorbed onto 10 × 3 mm tabs of chromatography paper and inserted into the gel. The specific enzymes examined and the buffer systems used to resolve them (Table 2) were generally those of Harris and Hopkinson.[22] Isozymes in multilocus systems were numbered in order of decreasing anodal mobility. Using all the available data, we calculated an average number of alleles per locus (A), percentage of polymorphic loci with no limiting criterion (P), average heterozygosity by direct count (H), and Nei's[36] unbiased genetic distance (D) with one standard error (66% confidence interval) for each pairwise comparison.[39] Intersample D values were clustered using the UPGMA algorithm. Most of these statistical analyses were performed with the BIOSYS-1 computer program.[54]

Table 2. Loci examined and electrophoretic conditions

Protein (EC no.)	Locus	Source[a]	Conditions[b]
Adenylate kinase (2.7.4.3)	*Ak*	All exc p	AP 6; 80/15/17
Asparate aminotransferase (2.6.1.1)	*Aat*	All exc p and RBC	TC 7; 60/15/18
Esterase (3.1.1.1)	*Est-1*	All exc p and RBC	TRIS-HCl 8.3/8.6; 15/80/20
Esterase (3.1.1.1)	*Est-2*	All	TRIS-HCl 8.3/8.6; 15/80/20
Esterase (3.1.1.1)	*Est-3*	All exc RBC	TRIS-HCl 8.3/8.6; 15/80/20
Esterase (3.1.1.1)	*Est-4*	p	TRIS-HCl 8.3/8.6; 15/80/20
Esterase (3.1.1.1)	*Est-5*	All	TRIS-HCl 8.3/8.6; 15/80/20
Fumarase (4.2.1.2)	*Fum*	All exc p	TRIS-HCl 8.3/8.6; 15/80/20
General protein (Amido Black)	*AB-1*	All	TRIS-HCl 8.3/8.6; 15/80/20
General protein (Amido Black)	*AB-2*	p	TRIS-HCl 8.3/8.6; 15/80/20
General protein (Amido Black)	*AB-3*	p	TRIS-HCl 8.3/8.6; 15/80/20
General protein (Amido Black)	*AB-4*	All exc p and RBC	TRIS-HCl 8.3/8.6; 15/80/20
General protein (Amido Black)	*AB-5*	All	TRIS-HCl 8.3/8.6; 15/80/20
Glucose phosphate isomerase (5.3.1.9)	*Gpi*	All	AP 6; 80/15/17
Hemoglobin	*Hb*	All exc p	All
Hexokinase (2.7.1.1)	*Hk*	All exc p	TBE 9; 20/175/18
Isocitrate dehydrogenase (1.1.1.42)	*Idh-1*	All exc p and RBC	AP 6; 80/15/17
Isocitrate dehydrogenase (1.1.1.42)	*Idh-1*	All exc p and RBC	AP 6; 80/15/17
Lactate dehydrogenase (1.1.1.27)	*Ldh*	All	TC 7; 60/15/18
Leucine aminopeptidase (3.4.1.1)	*Lap-1*	All exc p	AP 6; 80/15/17
Leucine aminopeptidase (3.4.1.1)	*Lap-2*	p	AP 6; 80/15/17
Malate dehydrogenase (1.1.1.37)	*Mdh-1*	All exc p and RBC	AP 6; 80/15/17
Malate dehydrogenase (1.1.1.37)	*Mdh-2*	All	AP 6; 80/15/17
Malic enzyme (1.1.1.40)	*Me*	All	TRIS-HCl 8.3/8.6; 15/80/20
Peptidase (leucyl-glycyl-glycine) (3.4.11)	*Pep-lgg*	All	TBE 9; 20/175/18
Phosphoglucomutase (2.7.5.1)	*Pgm-1*	RBC	TC 7; 60/15/18
Phosphoglucomutase (2.7.5.1)	*Pgm-2*	RBC	TC 7; 60/15/18
6-Phosphogluconate dehydrogenase (1.1.1.44)	*Pgd*	All exc p	AP 6; 80/15/17
Purine nucleoside phosphorylase (2.4.2.1)	*Np*	p	TBE 9; 20/175/18
Superoxidase dimutase (1.15.1.1)	*Sod-1*	p	TC 7; 60/15/18
Superoxidase dimutase (1.15.1.1)	*Sod-2*	RBC	TC 7; 60/15/18

[a] Proteins were detected in all tissues or in plasma (p) or red blood cells (RBC) except (exc) as noted.
[b] Electrophoretic conditions: buffer (pH); voltage/amps/time in h.

Table 3. Allele frequencies for polymorphic loci

Locus/ allele	*D. bicornis*	*C. s. simum*	*C. s. cottoni*	*R. unicornis*
Aat				
No.	4	8	*	2
A	1.000	0.938		0.000
B	0.000	0.063		1.000
AB-3				
No.	4	9	7	1
A	0.125	0.389	0.571	1.000
B	0.875	0.611	0.429	0.000
Pgm-2				
No.	6	11	5	1
A	0.000	0.136	0.000	0.000
B	0.583	0.636	0.500	0.000
C	0.417	0.227	0.000	1.000
D	0.000	0.000	0.500	0.000

[a] Missing cell because of lack of organ tissue. The remaining loci in Table 2 are monomorphic.

Table 4. Estimates of the average number of alleles per locus, (*A*), percentage polymorphic loci (*P*), and mean individual heterozygosity (*H*)

	D. bicornis	*C. s. simum*	*C. s. cottoni*	*R. unicornis*
A	1.1	1.1	1.1	1.0
P	0.065	0.097	0.080	0.000
H	0.013	0.013	0.019	0.000

Results

Eighteen enzyme and protein systems were examined, and genetically interpretable results were obtained for 31 presumptive loci for the three genera (Table 2). We suspect that AB-1 is albumin and that AB-2 or AB-3 is transferrin. Other proteins were examined, but as a result of poor resolution they were not genetically interpretable; these include ADA (EC 3.5.4.4), CAT (EC 1.11.1.6), and DIA (EC 1.6.2.2).[35]

Low amounts of genetic variation were observed in African rhinoceroses; *Pgm-2, Aat,* and one general protein locus (*AB-3*) revealed the only detectable variation in the surveyed black and white rhinoceroses (Table 4). No variation was detected in the three Indian rhinoceroses. Table 3 shows the allele frequencies for the polymorphic loci we examined.

Figure 1B shows the phenogram based on multilocus genetic distances among the four taxa, using all available data. The genetic distance between Indian *R. unicornis* and African black *D. bicornis* was $\bar{D} = 0.89 \pm 0.21$; between *R. unicornis* and African white (*C. s. simum* and *C. s. cottoni*), $\bar{D} = 1.05 \pm 0.24$; and between the two African genera, $\bar{D} = 0.32 \pm 0.11$. The genetic comparison of the two subspecies of the white rhinoceros (25 loci in the absence of organ tissue for *C. s. cottoni*) showed an insignificantly small genetic distance ($\bar{D} = 0.005$).

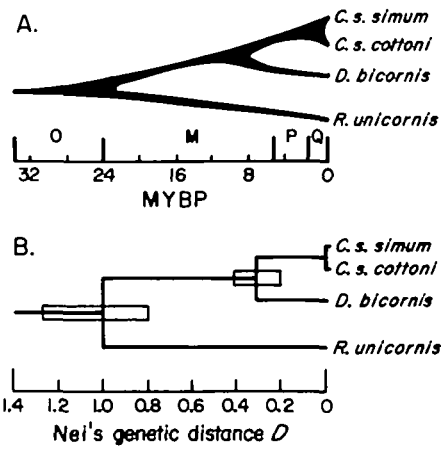

Figure 1. Relationships among rhinoceros taxa studied. (A) Schematic tree based on fossil evidence (see text). (B) UPGMA tree based on allozyme data and calibrated around a *Ceratotherium-Diceros* split at 7 million years before the present.

Discussion

Genetic Variation

Typically, mammals are genetically variable. In a review[40] of previous studies of allozyme variation in 184 species, Nevo et al. found $\bar{P} = 0.191$ and $\bar{H} = 0.0416$. Large mammals are generally less variable than are smaller mammals; studies of variation in 138 mammals revealed a positive correlation between increased body size and decreased genetic variation.[65] Large mammals with little detectable genetic variability include the northern elephant seal (*Mirounga angustirostris*),[7] polar bear (*Thalarctos maritimus*),[3] Atlantic walrus (*Odobenus r. rosmarus*),[50] cheetah (*Acinoyx jubatus*),[41,42] British fallow deer (*Dama dama*),[44] Arabian oryx (*Oryx leucoryx*),[64] Père David's deer (*Elaphurus davidianus*),[64] and Weddell seal (*Leptonychotes weddelli*).[57] There are, however, exceptions: White-tailed deer (*Odocoileus virginianus*),[51] Przewalski's horse (*Equus przewalskii*),[8] African and Asiatic lions (*Panthera l. leo* and *P. l. persica*),[43] and Florida manatee (*Trichechus mamatus*)[34] have high levels, and the small red fox (*Vulpes vulpes*)[49] a low level, of variability.

The lower levels of allozyme variation in some large mammals, including rhinoceroses, could be a result of sampling errors. One problem is that our samples of only 2 to 10 unrelated individuals might have constrained the amount of detectable variation. From simulations run with 32 sets of published data, Gorman and Renzi[19] found that reducing larger sample sizes (mean of 24) to two individuals yielded

estimates of heterozygosity that differed from the estimate based on the larger sample by an average of only 1.72%. Therefore, our small sample sizes should provide adequate preliminary estimates of intraspecific variability. The second sampling problem involves bias in the types of loci examined, as certain loci mutate more rapidly than do others. However, we examined a large number of loci representing different classes of enzymes, including the typically variable peptidases and esterases. Although our samples were lamentably small, the low genetic variabilities observed probably represent an accurate picture of the level of genetic variability remaining in the white rhinoceros, because our study included a reasonable number of white rhinoceroses from two different locations. On the other hand, recent results from a more thorough study of the Indian rhinoceros[16] revealed higher levels of variability (H = 9.9%) than we found in our extremely limited sample of Indian rhinoceroses. Our limited samples were from Assam, India, from a different population than those examined by Dinerstein and McCracken;[16] this could have contributed to the observed differences.

Two suggested hypotheses may account for low levels of genetic variation. An early selectionist hypothesis states that levels of genetic variability are related to the grain of the environment. Selander and Kaufman[47] argued that large, highly mobile animals tend to encounter environmental conditions that are fine-grained and are selected for a single general-purpose genotype that is adapted to the conditions most frequently encountered. Perhaps African rhinoceroses are such generalists; they occupy a wide range of habitats and show very little morphological or genetic differentiation. This hypothesis has been difficult to test, however, and is usually refuted by counterexamples of generalists that exhibit large amounts of genetic variation.

More commonly, demographic bottlenecks have been hypothesized as the cause of decreased genetic variability in the northern elephant seal, British fallow deer, and cheetah.[7,41,42,44] Many different variables influence the effects of a bottleneck on a population, including 1) the size of the initial population, 2) the size to which the population is reduced, 3) the duration of the bottleneck, and 4) the rate of recovery of the population after a crash.[17,38] There is evidence of recent severe population reductions in rhinoceroses. The African southern white rhinoceros was decimated in the 19th century but has recovered from about 100 individuals (probably not as few as 10)[28] in 1920 to over 4,600 today.[12,59] Similarly, the African northern white rhinoceros, *C. s. cottoni*, has lost 95% of its population since 1980.[60] There are now 40 animals in captivity and the wild.[25] Although the fossil record suggests that rhinoceroses were widespread across Africa, leading us to believe the population sizes were fairly large, the fact that the southern and northern white subspecies show no genetic differences suggests that the level of polymorphism was low before the recent bottlenecks in the two groups. This could be due to earlier bottlenecks or constant low population sizes.

The Indian rhinoceros, once widespread and abundant, has recovered from an estimated 12 individuals in 1908 in the Kaziranga area of Assam plus a few scattered in other areas[29] to a fairly stable population of 1,500 today.[16,32] African black rhinoceros numbers have fallen from 60,000 to 3,800 in the last 17 years alone; the remaining 70 to 100 isolated populations are highly vulnerable.[59] These recent demographic bottlenecks coupled with possible historical bottlenecks may be sufficient to explain the lack of observed genetic variability today.

Genetic Differentiation

Small sample sizes probably did not significantly affect our preliminary estimates of genetic differentiation, because statistics such as Nei's genetic distance are relatively independent of sample sizes when large numbers of loci are studied.[19,37] The population bottlenecks, on the other hand, could lead to overestimates of D, as they have a temporary accelerating effect on apparent differentiation.[37]

The estimated genetic distances among the various taxa of rhinoceroses are all less than expected based on morphological and paleontological evidence and on allozymic studies of other mammals.[5,37,58] The genetic distance between the two African genera ($\bar{D}$ = 0.32) is actually typical of the values seen for congeneric species comparisons[37] in other mammals. Similarly, the distance between the two subspecies *C. s. simum* and *C. s. cottoni* ($\bar{D}$ = 0.005) is less than expected for mammalian intrapopulation comparisons and far less than the average distances among subspecies ($\bar{D}$ = 0.23)[58] of mammals. Furthermore, these unexpectedly low genetic distances may be inflated, as the populations compared have gone through bottlenecks and are almost monomorphic.[14] Even lower values might have been obtained a century ago.

Rhinoceros taxonomy is currently based on morphology and paleontological records, and it is not surprising that the allozyme data suggest that taxonomic oversplitting has occurred at the subspecific level. Matthew[31] concluded that if population variability is considered, the majority of the then-described species would have been synonymized. Furthermore, he argued that the unnatural splitting of species, genera, and higher taxa resulted in a taxonomy without phylogenetic merit. His criticisms remain valid, as no comparative multivariate analysis of rhinoceros morphology has been published. Rhinoceros taxonomy was established long before isolation and recognition species concepts were applied to mammals and still longer before the development of the cohesion species concept.[55,63] Current evolutionary and genetic concepts must be applied to these animals if we are to define evolutionarily significant units in time to manage them effectively.[46,56] Clearly, electrophoresis can contribute significant information relevant to decisions regarding species and their conservation.

Rhinoceros Electrophoretic Clock

Published electrophoretic protein clock calibrations employed for mammals vary within a narrow range, such that a Nei's D of 1.0 = 0.8 to 6.7 million years.[37] This clock is based on congeneric species differences because of the wealth of data available at this taxonomic level. It is difficult to extrapolate to higher taxonomic levels, but to provide some comparison with previous data, we examined whether rhinoceros genera divergence times coincide with predicted divergence times using these predetermined estimates. As it turns out, such calibrations underestimate the age of rhinoceros species.

Calibrations of the electrophoretic clock against absolute time and comparisons of these calibrations across different organisms are possible, provided that independent paleontological estimates are available on times of speciation. To calibrate the rhinoceros allozyme clock, we used fossils the ages of which are generally agreed on by paleontologists. The first record of coexistence of *Ceratotherium* and *Diceros* came from a radiometrically dated 7-million-year-old deposit in Baringo, Kenya (J. Berry, personal communication,

1986).[6,26] Thus, the two taxa had already diverged from their common ancestor 7 million years ago. Nevertheless, if we let a genetic distance of 0.32 (the *D* value between *C. s. simum* and *D. bicornis*) represent 7 million years for a rhinoceros molecular clock, a *D* value of 1.0 equals 22 million years. This calibration is much larger than those reported for other mammals but is concordant with observed rates of evolution in some fish and reptiles.[5,37]

The mean rate of amino acid substitution as detected by electrophoresis is estimated to be about 10^{-7} per locus per year.[37] It is possible that the genetic distances among the African taxa are less than expected because the rate of allelic substitutions is lower in rhinoceroses than it is in other families of mammals. In fact, the black and white rhinoceros divergence as measured by genetic distance ($\bar{D} = 0.32$) is the same as reported for human-chimpanzee and human-orangutan ($\bar{D}$ = 0.3–0.4).[11] Humans are known to be evolving at a slower rate than are other well-studied mammals.[66] This supports the hypothesis that the rhinoceros clock is slower than expected. This slower rate may be due to a generation time effect, as rhinoceroses have a generation time of approximately 8 to 10 years, which is longer than that of most mammals.

Far less is known about the divergence time of the African and Indian lines. The Sumatran rhinoceros, *Dicerorhinus sumatrensis,* the oldest extant Asian species, occurred as early as the middle Miocene, 16 million years ago, and the African-Asian divergence probably occurred in the late Oligocene.[26] The *Ceratotherium-Diceros* molecular clock calibration can now be used to estimate when the African and Asian lines diverged. Assuming neutrality, we find that a Nei's *D* of 1.05 is equivalent to 23 million years (early Miocene). This is only slightly younger than we would have predicted from the sketchy fossil record. However, as estimated divergence times do not increase linearly when *D* values are greater than 1.0, the "infinite allele" model may be unrealistic for events that occurred 20 to 30 million years ago.[37] Consequently, we interpret the Indian-African *D* value as suggesting that cladogenesis occurred toward the more recent end of the time range based on the fossil record.

As estimates of time since divergence based on paleontological evidence are probably conservative, we interpret the observed slow electrophoretic clock in rhinoceroses as a real phenomeon. It is not clear, however, whether the long duration of rhinoceros species is due to a fundamental slowing of their rates of evolution (at least as monitored by the allozyme clock) or to an artifact of the technique employed.

Conclusions

This preliminary study reveals a marked lack of genetic variability in all four taxa. One possible consequence of such low genetic variability is that despite their broad ecological tolerance as species, each local population of rhinoceroses may not be able to adapt to environmental changes as well as do populations of more variable species (see Allendorf and Leary[2] and Ledig[30] for reviews of the relationship between heterozygosity and fitness). This lack of variability may not be significant in regard to short-term conservation, when ecological factors are often more important,[27] but its long-term effects have not been studied. The actual risks of monomorphism at electrophoretically detectable enzyme loci are still unknown. O'Brien et al.[42] found lower juvenile survivorship, spermatozoal abnormalities, and lower resistance to disease in cheetahs. Similarly, the Torrey pine (*Pinus torreyana*) may have lost reproductive capacity[30] as a result of the loss of genetic variability. As such possible effects on evolutionary fitness are important to conservationists, our observations should be confirmed by additional studies involving wild animals and the use of different molecular genetic techniques.

Our results do not permit us to use allozymes to distinguish the two named subspecies of white rhinoceros, *C. s. simum* and *C. s. cottoni.* This conclusion is in apparent opposition to observations derived from a preliminary study[18] of mitochondrial DNA, in which a 4% difference between the two white rhinoceros subspecies suggested that they have been isolated for 2 million years. A greater degree of divergence in mtDNA than in nuclear DNA is to be expected (as vertebrate mtDNA evolves at a rate five times that of vertebrate single copy nDNA),[10] but the 4% difference was unexpected, because in primates the average rate of divergence is 0.5 to 1.0% per million years. The limited sample sizes used in the study by George et al.[18] did not allow an assessment of intraspecific mtDNA variation in each white rhinoceros subspecies; in other mammals, this ranges from 1 to 7%.[9] Consequently, discussion of this apparent discrepancy is premature, and a second investigation based on more individuals is under way (M. George, personal communication).

Data on genetic variability may be used, together with ecological and behavioral data, to define and manage viable populations of endangered species. If the named subspecies are truly genetically distinct from one another, their conservation requires the preservation of viable populations of each taxon. If they are not, further research should be conducted to determine whether the geographic races of the species could be pooled to maintain the existing variability of each species as a whole. If the two subspecies of *Ceratotherium* are simply remnant populations from two extremes of a once-continuous geographic range, relocation of southern white rhinoceroses to areas formerly occupied in the north might be considered as a means of alleviating the ecological effects of the recent extirpation of northern white rhinoceros populations.

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