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COMPARISONS OF MITOCHONDRIAL DNA IN BLACK AND WHITE RHINOCEROSES

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Mitochondrial DNA restriction maps of *Diceros bicornis*, the black rhinoceros, and *Ceratotherium simum*, the white rhinoceros, were constructed to provide a basis for population genetic and systematic studies. The sequence divergence between DNA of the two species was calculated to be 6.79% from which it could be estimated that the time of divergence from a common ancestor was ca. 3.4 × 10^6 years ago. Little intraspecific variation was found in the 24 black rhinoceroses or the 4 white rhinoceroses studied.

Key words: mitochondrial DNA, sequence divergence, rhinoceros

The black rhinoceros, *Diceros bicornis*, and white rhinoceros, *Ceratotherium simum*, are the two African representatives of the family Rhinocerotidae. As with the three Asian members of this family, they comprise dwindling populations in imminent danger of extinction. We report here restriction-endonuclease maps of mitochondrial DNA (mtDNA) prepared from heart tissue obtained after natural deaths in the field of *Diceros bicornis minor* and *Ceratotherium simum simum*, both from Hluhluwe Game Reserve, Natal, South Africa. The restriction-site data were used to provide a measure of the sequence divergence between mtDNAs of the two species and hence, an estimate of the time of their divergence from a common ancestor. As more members of the family become available for study, they also will contribute to a more detailed biogeographic and phylogenetic study of extant species and populations of rhinoceroses.

METHODS

Mitochondrial DNA was extracted from heart tissue frozen shortly after death and purified by centrifugation in CsCl/ethidium bromide gradients (Ausubel et al., 1989; Lansman et al., 1981). Restricted DNA was end-labelled with ^{32}P by using the Klenow fragment of DNA polymerase I and ^{32}P-deoxycytidine triphosphate (Amersham, UK). Restriction fragments were separated by agarose or polyacrylamide-gel electrophoresis and visualized 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 by using a total of 18 restriction endonucleases recognizing six base-pair sequences. Maps were aligned with each other and with the known bovine sequence (Anderson et al., 1982) using the two *Sac* II sites and a *Hpa* I site at positions 676, 2364, and 5480, respectively. These sites are invariant throughout most of the Vertebrata (Carr et al., 1987). Sites that were aligned to within 1% of the total map length, estimated to be 16,417 ± 298 and 16,411 ± 225 for black and white rhinoceroses, respectively, were interpreted to represent shared sites.

Since postmortem material was available for only one black and one white rhinoceros, both from Hluhluwe (Natal), cell cultures were established from the ear nicks taken while marking three additional white rhinoceroses from Hluhluwe (all *C. s. simum*) and 23 black rhinoceroses. The latter come from three populations of *D. b. minor*, which consist of 15 individuals from Hluhluwe, 6 from Mkuzi (Natal), and 2 from Zimbabwe. Total DNA was extracted at an early passage number from cell cultures propagated in Dulbecco's modified Eagle's medium (Gibco, UK) containing 5% fetal-calf serum (Highveld, South Africa). The restriction

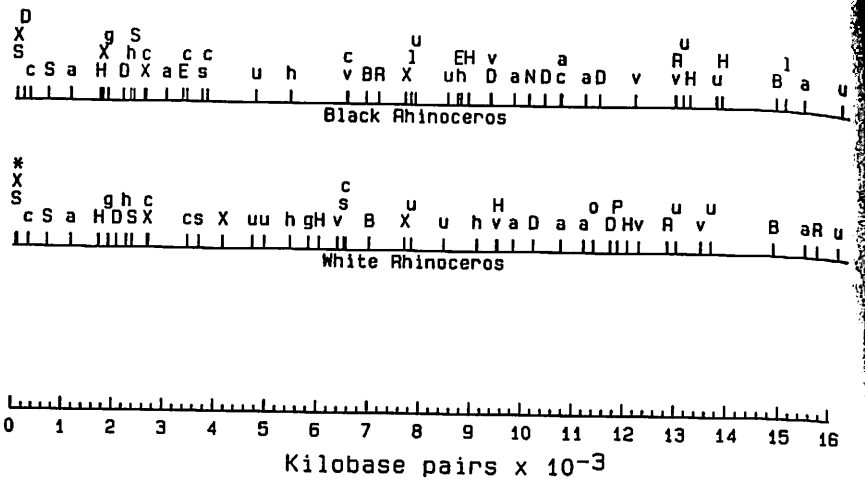

FIG. 1.—Restriction-site maps of mtDNA from *Diceros bicornis minor* and *Ceratotherium simum simum*. B, *Bam* H1; c, *Bcl* I; g, *Bgl* II; D, *Dra* I; E, *Eco* RI; R, *Eco* RV; H, *Hind* III; h, *Hpa* I; N, *Nco* I; P, *Pst* I; v, *Pvu* II; s, *Sac* I; S, *Sac* II; l, *Sal* I; a, *Sca* I; u, *Stu* I; X, *Xba* 1; o, *Xho* I. *Kpn* I did not cut either DNA. Asterisk indicates *Sca* I site present in one *C. s. simum*.

patterns given by each of the enzymes were compared for all the individuals after Southern blotting and hybridization to random primed-mtDNA purified from the rhinoceros heart tissue.

Results and Discussion

The fragments produced by 18 restriction endonucleases were used to construct restriction-site maps (Fig. 1). The restriction-site positions and fragment sizes are listed in Table 1. No polymorphic sites were found for any enzyme among the 24 black rhinoceroses sampled and only one *Sca* I polymorphic site was found among the four white rhinoceroses sampled. This is consistent with the small amounts of intraspecific variation observed in allozyme studies on these two species (Merenlender et al., 1989). Ashley et al. (1990), using a restriction-fragment size-comparison, in contrast to the restriction-site comparison used here, found only a small degree of intraspecific variation between the subspecies *D. b. minor* and *D. b. michaeli*, and found no variations within 12 *D. b. minor* (one of which was of South African origin) and within 11 *D. b. michaeli*. Their fragment sizes for *D. b. minor* are compatible with our restriction maps.

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 (Nei and Li, 1979). 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% (*SD* = 1.63%). The initial rate of sequence divergence between two mammalian-mtDNA lineages was calculated by Brown et al. (1979) to be ca. 2%/1 × 10^6 years. If this holds true for the Rhinocerotidae, it would give a time for the divergence of these two mtDNA lineages of 3.4 ± 0.8 × 10^6 years before present. It must be made clear, however, that absolute time estimates for divergence events calculated in this way are

TABLE 1.—*Restriction-site positions and fragment sizes in mitochondrial DNA of* Ceratotherium simum simum *and* Diceros bicornis minor. *Site positions are estimated to the nearest 10 base pairs. In one of the four white rhinoceroses, an additional* Sca I *site cuts the 2,060 base-pair fragment (marked by asterisk) into two equal-sized fragments.*

	Ceratotherium simum simum			*Diceros bicornis minor*		
Enzyme	Number of sites	Site position	Fragment sizes (base pairs)	Number of sites	Site position	Fragment sizes (base pairs)
Bam HI	2	6990, 14870	8520, 7890	2	6890, 14890	8420, 7990
Bcl I	4	300, 2680, 3540, 6520	10190, 3070, 2380, 770	6	300, 2600, 3400, 3800, 6520, 10700	6020, 4170, 2720, 2300, 800, 400
				1	1850	16420
Bgl II	2	1890, 5800	12500, 3910	5	170, 2170, 9330, 10400, 11470	7160, 5120, 1990, 1070, 1070
Dra I	3	2060, 10210, 11720	8160, 6750, 1510	2	3320, 8690	11040, 5370
Eco RI	0	Nil	Nil	2	7140, 12960	10600, 5820
Eco RV	2	12830, 15750	13490, 2920	4	1700, 8900, 13230, 13850	7200, 4330, 4270, 620
Hind III	4	1700, 6010, 9500, 12050	6060, 4310, 3490, 2550	3	2300, 5430, 8750	9970, 3320, 3130
Hpa I	3	2250, 5450, 9100	9560, 3650, 3200	1	10100	16420
Nco I	0	Nil	Nil	0	Nil	Nil
Pst I	1	11850	16410	4	6510, 9340, 12170, 12940	9980, 2830, 2830, 770
Pvu II	4	6370, 9480, 12270, 13480	9300, 3110, 2800, 1210	1	3700	16420
Sac I	2	3670, 6480	13600, 2810	3	40, 680, 2360	14100, 1690, 630
Sac II	3	50, 680, 2360	14100, 1680, 630	2	7770, 15070	9110, 7310
Sal I	0	Nil	Nil	6	1120, 3000, 9800, 10720, 11200, 15450	6800, 4250, 2090, 1880, 920, 480
Sca I	5	1150, 9820, 10750, 11200, 15500	8670, 4300, 2060,* 930, 450	6	4750, 7850, 8550, 13100, 13750, 16260	4910, 4600, 3100, 2510, 650, 650
Stu I	7	4720, 4950, 7810, 8450, 13000, 13680, 16200	4930, 4550, 2860, 2420, 680, 640, 230	4	50, 1740, 2570, 7660	8810, 5090, 1680, 830
Xba I	4	60, 2650, 4140, 7680	8790, 3540, 2590, 1480	0	Nil	Nil
Xho I	1	11390	16410			

only approximate. Factors contributing to the uncertainty are not only error due to the stochastic nature of the mutational process (the value given above as ±0.8 million years), but also the applicability of the calibration of rate of sequence divergence against time to the group under study, and the amount of within-species divergence. Although the latter would appear to be low, at least within the population of black rhinoceros sampled here, and in Ashley et al.'s study (1990), levels of intraspecific divergence as high as 6.8% have been reported for other mammalian species (Carr et al., 1987; Cronin, 1991). Our value of 3.4 ± 0.8 × 10^6 years is, therefore, a measure of the divergence of the mtDNA of these two species and only a qualified estimate of the species' actual divergence time. Nevertheless, this agrees well with the value of 3.5 × 10^6 years suggested by George (1987), who used comparisons of restriction-fragment sizes, as well as with fossil evidence. The fossil record of the Rhinocerotidae is fragmentary, but the description of *Ceratotherium praecox* from deposits of ca. 4 × 10^6 years before present (Hooijer and Singer, 1972), and its similarity to *C. simum* and *D. bicornis*, was used to support the proposal that *Ceratotherium* split from the *Diceros* lineage sometime during the Pliocene. George and Ryder (1986) used restriction-site comparisons of mtDNA in another family of Perissodactyla to estimate that the common ancestor of the Equidae was present ca. 3.9 × 10^6 years before present. This similarity to the figure of 3.7 × 10^6 years before present in the African Rhinocerotidae may be coincidental, but contributes to the gradual accumulation of a dataset that may define major episodes of radiation of African mammals in the Pliocene and Pleistocene.

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THE ZYGOMATIC ARCH OF *HYAENODON* (HYAENODONTIDAE: CREODONTA)

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The horizontally oriented zygomatic arch of *Hyaenodon* traditionally has been viewed as thin and structurally weak. This condition has been correlated with atrophy of the masseteric musculature, itself reduce because of stresses imposed along with increased gape. Physical reconstruction of the adductor-masticatory musculature of *Hyaenodon* suggests that the mass of the masseteric musculature was proportionally smaller in this genus as compared to other carnivores, but not to the degree suggested by Mellett (1977, Contrib. Vert. Evol., 1:1-134). The condition of the arch primarily is not a result of atrophy of the masseteric musculature because such atrophy is not a solution to the problem of stress; indeed, it would aggravate the problem. Rather, the condition of the arch probably reflects the function of reducing the distance between the origins and insertions of the masseteric musculature, while the length of the fibers remained largely unaltered.

Key words: ***Hyaenodon*, Creodonta, morphology, evolution**

The skull of *Hyaenodon* is highly specialized for carnivory, but possesses a suite of characters that distinguishes it from the skulls of creodonts and carnivorans of a comparable grade of carnassiality. Some of these modifications have been described by various authors (e.g., Mellett, 1977; Scott and Jepsen, 1937).

Mellett (1977) proposed a sequence of factors that could give rise to the skull morphology of *Hyaenodon*. He suggested that the masseteric musculature was reduced because of the increased stress imposed by selection for increased gape, and that the horizontal and weak zygomatic arch resulted from this muscular atrophy. This opinion commonly is accepted by paleomammalogists and has become so pervasive that it is expressed consistently by researchers otherwise only vaguely familiar with *Hyaenodon*. However, Mellett's (1977) assertion that the condition of the zygomatic arch is due solely and simply to masseteric reduction is questionable based on muscle physiology. A more probable explanation is that the morphology of the arch may in part be a product of selective pressures to decrease the distance between the origin and insertion of the masseteric musculature, assuming that selection for increased gape imposed an initial stress on the musculature.

Materials and Methods

Hyaenodontids were obtained from a number of institutions (American Museum of Natural History, AMNH: Frick Collection, American Museum of Natural History, FAM; Natural History Museum of Los Angeles County, LACM [CIT]) and included four taxa: *Hyaenodon crucians* (AMNH 647), *H. horridus* (AMNH 394390, FAM 756920, LACM [CIT] 83/102, LACM [CIT] 143/1660), *H. vetus* (LACM [CIT] 150/1359), and *H. ?vetus* (LACM [CIT] 150/1381). Extant taxa examined, from the Royal Ontario Museum, Department of Mammalogy (ROM), and Grant Hurlburt Private Collection (GHPC), include: *Alopex lagopus* (ROM 21591), *Bassariscus astutus* (ROM 91-10-1-3), *Canis adustus* (ROM 28188), *C. familiaris* (GHPC 7, GHPC 24), *C. latrans* (ROM 19940), *C. lupus* (ROM 18669), *Crocuta crocuta* (ROM 16754), *Dusicyon* (ROM 14214), *Felis concolor* (ROM 33-9-25-1), *F. catus* (GHPC 39), *Hyaena hyaena* (ROM 80312), *Genetta trigrina* (ROM 65103) *Urocyon*