KENYA BLACK RHINOCEROS

METAPOPULATION WORKSHOP

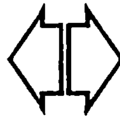
BRIEFING BOOK

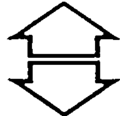
SECTION 5
SYSTEMATICS

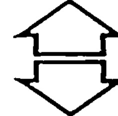
PACHYDERM

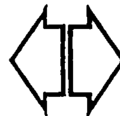
NEWSLETTER OF THE AFRICAN ELEPHANT AND RHINO SPECIALIST GROUP

CAPTIVITY **WILD**









SPECIAL ISSUE

PROCEEDINGS OF AFRICAN RHINO WORKSHOP, CINCINNATTI, OCTOBER 1986

Edited by R.F. du Toit, T.J. Foose and D.H.M. Cumming

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INTERNATIONAL UNION FOR CONSERVATION
OF NATURE AND NATURAL RESOURCES
SPECIES SURVIVAL COMMISSION

WCI
Wildlife Conservation International

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(iv) the resources that have already been expended on its conservation, and the interest and willingness of Zaire to conserve the species;

(v) the flagship nature of the species for conservation in this region of Africa.

2. The Workshop recommends integration of the conservation programs for the wild and captive populations. Ultimately, these programs are expected to entail exchange of genetic material between the wild and captive populations. Fewer than 15 founder animals are known to exist for both the small wild and captive populations. These founders are evenly divided between the wild and captive populations. However, over the short term it is recommended that no animals be exchanged between the wild and captive populations; at this time it is recommended that every effort be exerted to expand the wild and captive populations as rapidly as possible from their small founder bases.

3. The Workshop endorses continued support for the *in situ* conservation programs in Garamba National Park. In particular, the Workshop believes that, in addition to the activity currently occurring, funds should be provided for a field biologist who can be deployed continuously in the Park with the rhinos. Further, the Workshop also strongly recommends that there be an intensive effort to train Zairois biologists to continue with these conservation programs into the future.

4. With respect to expansion of the captive population, the Workshop acknowledges and commends the considerable efforts of Dvur Kralove, in collaboration with the IUCN/SSC CBSG, to enhance the captive breeding program, as reflected in the report and recommendations by CBSG chairman Dr. U.S. Seal and CBSG member Dr. D. Jones, issued after their visit to Dvur Kralove in February 1986. Many of these recommendations have been implemented, including some reproductive examination of females, the movement of a lone male rhino from London to Dvur Kralove, the initiation of a facility enlargement at Dvur Kralove, and collection of samples for genetic analysis.

However, further analysis and evaluation of both the captive and wild population emphasizes the urgent need to expand the captive nucleus as soon as possible. Concerns over the demographic risks of maintaining the entire captive nucleus in one facility have intensified.

Therefore, the Workshop recommends that Dvur Kralove consider movement of 1/2 adult animals to another facility with experience in breeding the southern white rhino. Further, the Workshop recommends that Dvur Kralove be requested to suggest a timetable by which, if further reproduction does not occur there, other relocations will be undertaken. The reasons for these recommendations relate to enhancement of reproduction and reduction of demographic risks, as will be explained more fully in a white paper to be prepared over the next few months by Dr. Jones and Dr. Seal.

5. The Workshop encourages the use of the southern white rhino for development of reproductive technology to help the northern white rhino.

6. The Workshop also encourages continued investigation of the genetic and ecological differences between the northern and southern forms. With respect to the genetic studies, both field and zoo programs are encouraged to provide sample materials as requested and where practical to Dr. O. Ryder and colleagues.

AFRICAN RHINO SYSTEMATICS

Session Chairman RAOUL DU TOIT

RATIONALE FOR INVESTIGATIONS OF AFRICAN RHINO SYSTEMATICS

Comments by David Western (New York Zoological Society)

To ensure that efforts to conserve rhinos in the wild as well as in captivity are maintaining the existing genetic diversity of the species, it is necessary to establish the "evolutionarily significant units" within the different species. In the case of the northern white rhino, there has been much debate over whether this "subspecies" is sufficiently different from the southern white rhino to merit the expense and effort required to maintain the last remaining population in the Garamba National Park, Zaire. Funds allocated to conservation of these northern white rhinos might be better spent on initiatives to conserve black rhinos, which have dwindled from about 15 000 at the time when this issue was first debated to a present level of under 4 000. The importance of subspecies designations thus requires critical review in order to assign priorities for rhino conservation action in Africa, but conservation initiatives need not be delayed while the necessary research is undertaken.

In debating the significance of genetic differences between allopatric groups of rhinos, it is necessary to consider not only the need to maintain the evolutionary potential of the species by preserving overall genetic diversity, but also the need to maintain genetic traits that constitute specific ecological adaptations, allowing some of the rhinos to thrive in habitats which may be unfavourable for other members of the species. Altitudinal zonation of habitats in East Africa may be one important factor influencing ecological adaptations of rhinos.

A further aspect to consider in strategies for conservation in Africa is the likelihood that the recognition of a certain group of a spectacular "flagship species" as being different to other groups of the same species elsewhere gives impetus to national and international efforts to save those animals and their habitats — the effort to protect the mountain gorilla in Rwanda has been a case of this "political" aspect of systematics.

THE EXISTING BASIS FOR SUBSPECIES CLASSIFICATION OF BLACK AND WHITE RHINOS

Summary of presentation by Raoul du Toit (IUCN African Elephant and Rhino Specialist Group)

The efforts of Hopwood (1939) and Zukowsky (1965) in revising black rhino systematics did not greatly improve the classification since these authorities erected subspecies on the basis of very small numbers of representative skulls, and in some instances the skulls representing their subspecies were those of immature animals (notably the subspecies *holmwoodi*). In view of these deficiencies, Groves (1967) produced a revision which identified 7 subspecies, but

sample sizes were still very low (only 2 of these subspecies were based on measurements of more than 10 adult skulls). Groves' breakdown was as follows (with sample sizes indicated in brackets):

Diceros bicornis bicornis (5)	South Africa — Cape area;
D.b. chobiensis (4)	Southern Angola, Chobe area;
D.b. minor (23)	South Africa to Kenya;
D.b. michaeli (22)	Kenya and Tanzania;
D.b. ladoensis (6)	Northern Kenya and Sudan;
D.b. longipes (4)	Central Africa;
D.b. brucii (10)	Somalia and Ethiopia.

Confusion was introduced since Groves did not indicate in this paper that he believed his subspecies *bicornis* to be extinct. This was only made clear in a paper he co-authored with Rookmaker in 1978. Here they stated that *bicornis* was a very large rhino that was exterminated in Namibia and the Cape in about 1850.

Several zoologists continued to refer to *bicornis* as one of the surviving species in southern Africa. Ansell (1978), in his Mammals of Zambia, excluded *bicornis* but had previously stated (1974) that some living rhinos of southern Africa were of this subspecies, and in his recent work Smithers (1983) apparently follows Ansell's original classification; he states that *bicornis* occurred widely in the subcontinent and now has a restricted distribution (presumably meaning this to be Zululand), while he thought *minor* may occur in northern Namibia/Angola (he does not clarify how this fits in with *chobiensis*).

Joubert (1970) compared some Namibian rhino skulls with a sample from Natal. He may not have checked that all skulls were of fully-grown animals, but found that all the Namibian skulls were significantly greater than those from Natal. However, he calculated that the differences between the populations were below the level conventionally accepted for subspecies differences (i.e. the ranges of dimensions had more than 10% overlap) and said all the skulls were of the *bicornis* subspecies.

Rookmaker and Groves (1978) commented that *bicornis* (as described by them from Cape specimens) was similar to *chobiensis* in that both had large skulls, and postulated that this was due to independent adaption to similar (wet) environments. This is clearly fallacious, since the climates of southern Angola/Chobe and the Cape/Namibia are dissimilar, and are not wet.

Thus, the published literature contains rather confusing statements on black rhino taxonomy, and sample sizes are small. Dr. C. Groves recently sent the African Elephant and Rhino Specialist Group (AERSG) an outline of his current ideas on the topic, including data from a few more skulls. His new, interim classification is similar to that he published in 1967, but excludes *bicornis* as an extant subspecies, and has the following criteria for the taxonomic divisions: presence or absence of crista (a tooth feature), greatest length of skull, zygomatic breadth, toothrow length and occipital breadth. Three of the subspecies still have less than 10 representative skulls (*chobiensis, ladoensis* and *longipes*).

In view of the poor state of black rhino systematics, AERSG initiated a survey of black rhino skulls in African wildlife areas and in some museums. This survey is not complete, but initial results can be presented. The data indicate that there is statistically significant variation between certain dimensions of female skulls and the equivalent dimensions of male skulls from the same population (notably in toothrow, basilar length and zygomatic breadth). Groves' latest classification is not supported by the data; for instance, all the skulls that were measured in Etosha National Park have occipital breadths greater than the maximum range indicated by Groves (which was for *chobiensis).* The range in toothrow length which Groves gives for *brucii* totally covers the range he gives for *minor* (and thus would be a poor distinguishing feature anyway), but there are a number of fully-grown skulls measured recently from supposed *minor* populations which have even shorter toothrow lengths.

The 300 skulls measured so far in the AERSG survey are mainly from southern Africa and thus only a very tentative conclusion can be reached on the clinal variation in black rhinos. This conclusion is that there may be possibly a trend of decreasing skull size towards the north of the continent, with the largest skulls being from the Namibia animals, a range of intermediate sized skulls extending up to Kenya and possibly west from there to the Central African Republic, and small skulls from the population to the horn of Africa (Somalia and Ethiopia; where in fact the animals may be effectively exterminated by now). If there is a large-skulled rhino group in Namibia, this may well have been linked with the supposed *bicornis* population as well as with the *chobiensis* population; based on collection localities of skulls designated as *bicornis,* and on ecological similarities between the postulated range of *bicornis,* and that of the extant Namibian rhino, Hall-Martin (1985) has also suggested that these may be the same race.

Thus, in general, it would appear that taxonomic distinctions between black rhinos have been exaggerated and a concerted effort to measure more skulls is justified (the AERSG survey will now build up data from East Africa, but it is expected that few data will be forthcoming from Central Africa). The working premise of AERSG that efforts to conserve rhinos and to create captive breeding groups should concentrate on rhinos from either end of their current range in Africa and from the middle of the distribution is supported. It is also clearly important to undertake further investigations of the ecological adaptions (physiological and behavioural) which suit rhinos to particular environments (notably the Namibian desert and Kenyan highlands) — adaptions to blood parasites may be particularly important, and would not be revealed by the classical taxonomic approach of measuring skulls.

There has been consensus between taxonomists in the identification of the two subspecies of white rhinos: *Ceratotherium simum cottoni* and *C.s. simum*. However, these subspecies have been nominated largely on the basis of geographical separation — several taxonomists have noted that on the basis of skull characteristics the two are not well differentiated. Groves (1972;1975) feels that the major difference is that *simum* has a much deeper dorsal concavity (the occipital crest is raised higher). There is an overlap of only 5% in the ranges of this dimension for the two groups thus the difference, taken in isolation, could be said to constitute a valid subspecies distinction (but, as with the black rhinos, the sample sizes were small — less than 10 *simum* skulls were measured). On the basis of the less indented skull of *cottoni,* Groves (1975) postulates that this subspecies has evolved further than *simum;* he believes that the fossil record indicates an advance from *Diceros* via *C. praecox* to *C. simum* with the dorsal outline of the skull becoming flatter.

The other major skull difference between the subspecies is in toothrow length, with *simum* having a longer toothrow, but the coefficient of difference is too small for taxonomic

separation on this character (there is an overlap in the ranges of 20%). Alexander and Player (1965) have also stated that the southern race, *simum*, has sparse body hair while the northern has no hairs, only follicles. Groves (1975) suggests that the northern may be longer-legged and shorter-bodied than the southern, but this is not based on any data.

A BRIEF PALAEONTOLOGICAL HISTORY AND COMPARATIVE ANATOMICAL STUDY OF THE RECENT RHINOS OF AFRICA

Summary of presentation by Claude Guerin
(Universite Claude Bernard — Lyon)

Information on this subject has been published by Guerin (1980).

The black rhino *(Diceros bicornis)*

The lineage begins in the upper part of the middle Miocene, about 12 million years ago, with *Paradiceros mukirii* known from Fort Ternan (Kenya) and Beni Mellal (Morocco). The genus *Diceros* appears later in the upper Miocene and is known at that time in Spain, Greece and Turkey with *D. pachygnathus*, in Turkey with *D. neumayri*, and in Tunisia and Italy with *D. douariensis*. The first of these three very large Miocene species may be the ancestor of the white rhino, *Ceratotherium*.

The species *D. bicornis* appears during the Pliocene about 4 to 5 million years ago, and is known in more than 20 sites of Pliocene up to middle Pleistocene age, especially Hadar (Afar) in Ethiopia, Omo (Mursi, Usno and Shungura formations) in Ethiopia, East Turkana in Kenya, Laetolil and Olduvai in Tanzania. More sites of upper Pleistocene and Holocene age are recorded. However, the material is always rare and the fossil form has not yet received any precise taxonomic status. Anatomical differences between the fossil and extant forms are minimal. Thus the fossil form warrants no more than a subspecific status.

I have studied about 60 adult skulls and more than 30 postcranial skeletons of *D. bicornis*, most of these being of Groves' (1967) medium-sized East African forms: subspecies *ladoensis*, *michaeli* and *brucii*. It is not easy to distinguish between these subspecies, whereas *minor* appears to be smaller-skulled and *bicornis* exceptionally large-skulled. I have not been able to study *chobiensis* and *longipes*. Statistical anayses show that, from the data I collected, *D. bicornis* is homogeneous, with rather normal variability (see Guerin, 1980). The various subspecies appear to constitute a complicated cline.

The white rhino *(Ceratotherium simum)*

The lineage of the white rhino is much more recent than that of the black. The genus *Ceratotherium* appears during the Pliocene with *C. praecox*, a species defined in 1972 by Hooijer and Patterson with material from Kanopòi and Ekora in East Africa. The same year Hooijer described abundant material of the same species from Langebaanweg in South Africa. I have studied the material from Chemeron formation (Lake Baringo) and a good deal of material from Hadar (Ethiopia) and from Laetolil (Tanzania). The species is now known in 11 localities of East and South Africa.

The recent species *C. simum* appears about 3 million years ago. It is classically held that there are two fossil subspecies, *C.s. germanoafricanum* from East Africa and *C.s. mauritanicum* from North Africa. I have studied material of *germanoafricanum* from Afar, East Turkana, Olduvai, Omo, Rawi and sever minor locations, and *mauritanicum* material from Ternifine (0.8 million years), Ain Hanech (1.5 million years) and other minor localities. The postcranial material shows clear differences between the fossil and the recent subspecies.

For the two recent forms, *simum* and *cottoni*, I have been able to find only about 30 skulls and 12 postcranials, and many were without specified origin. In fact, only 16 skulls and 8 postcranial skeletons were certainly from *cottoni*, and 8 skulls with 2 postcranial skeletons from *simum*. Hence the results are little more than an indication of differences. On average, *simum* has a skull slightly larger than that of *cottoni*, with a lower and broader skull roof, and a differently-shaped occipital surface (confirming observations of Groves, 1975).

Comparison of fossil forms with the complete sample of recent species shows that the skull of *C. praecox* is shorter, broader and lower, while the skull of *C.s. germanoafricanum* seems like that of a gigantic white rhino with comparatively narrower occipital surfaces, broader cheek teeth and correspondingly narrower palate widths. A comparison of limb elements again shows *germanofricanum* to be like a giant white rhino, while *mauritanicum* has similar (or exaggerated) proportions to *C. praecox*, being dissimilar to recent white rhinos and *germanoafricanum*.

Since the two Pleistocene subspecies seem to be very different to each other and from the recent ones, *germanoafricanum* probably deserves full species rank and may be the ancestor of the two recent forms; *mauritanicum*, which has no descendants, seems closer to their common ancestor, *C. praecox*, and probably also deserves species rank. The two recent subspecies are clearly distinct from each other and seem to be in the course of a speciation process. More postcranial material, particularly from southern Africa, is required to help verify this.

BIOCHEMICAL INVESTIGATIONS OF RHINO SYSTEMATICS

Summary of presentation by Matthew George
(Howard University)

A comparative study was undertaken of genetic differences between individual northern and southern white rhinos, and a black rhino. This study was based on comparisons of mitochondrial DNA (mtDNA), which is a useful means of investigating closely related species since 1.) the molecule is maternally inherited, thus complications arising from paternal contributions and recombination events (which affect nuclear DNA) are avoided; 2.) the molecule evolves very rapidly (5-10 times faster than nuclear DNA) so that if differences exist between races they are more likely to be detected than through other methods.

After purification of mtDNA molecules extracted from liver and spleen tissue of the three animals, these were subjected to digestion by 21 different restriction enzymes (which cut the mtDNA at specific sequences of nucleotide units). The cleaved fragments were separated electrophoretically. With most of the restriction enzymes, the migration patterns of mtDNA of the black rhino were different to those of the two white rhinos, while comparison of the two white rhinos showed 13 patterns to be identical and the remaining 8 different.

Analysis of these data indicate that the white rhinos differ by 4% in their nucleotide sequence and they both differ by 7% from the black rhino. If rhinoceros mtDNA changes at a rate of 2% per million years as has been shown in primate mtDNA, the divergence time between the white rhinos is 2 million

CONFERENCE RESOLUTION

The Conference encourages the international donor community to consider the five species of rhino in Africa and Asia as flagship and umbrella species for conserving biodiversity and critical ecosystems. The critically endangered status of these species emphasizes the urgent need for immediate conservation action and funding. The Conference urges the donor community to use the Global Environmental Facility of The World Bank to fund conservation projects in Africa and Asia that protect rhinos and their habitats, involve local community participation in rhino management and conservation education and awareness programs.
(This resolution was unanimously approved at the final plenary session)

Keynote Addresses

Esmond Bradley Martin: *The present-day trade routes and markets for rhinoceros products*
Rolf Benirschke: *The spiritual value of habitats with rhinos in an increasingly urban world*
Mark R. Stanley Price: *What will it take to save the rhino?*
Michael Werickhe: *The rhino will live or die because of us*

Plenary I - Summary
Rhinoceros Evolution and Systematics: Conservation Implications

R. Aman, chair: *Genetic analysis of rhino populations in Kenya*
G. Amato: *Molecular evolution in rhinos*
M. George: *Mitochondrial DNA analysis of rhinoceros subspecies*
E. Harley: *Molecular Genetic studies of Southern African black rhinoceros*
D. Prothero: *Fifty million years of rhinoceros evolution*
O. Ryder: *Rhinoceros chromosomal studies: Application to gene pool conservation*
N. van der Merwe & A. Hall-Martin: *The determination of species and geographic origin of rhinoceros horn by isotopic analysis*

Fossil records indicate that the evolutionary history of rhinoceroses dates back to 50 million years ago. The newer molecular genetic techniques are quite useful in tracing the relatively recent evolutionary history of the five extant species of rhinos. What implications do those new techniques have from the conservation point of view? Here the concerns require a practical application of information gathered from such techniques. The questions may be: Is there a genetic basis for the species divisions at the sub-species level? Are such genetic differences between the sub-species large enough to contraindicate managing them at the sub-species level or are they small enough so that strategy of management could be dictated by factors other than genetic? Can these techniques determine with certainty the identity and relationships of individuals within a population? And from the point of view of regulation in trade of rhino products and forensics, can these techniques lead to identification of such products and trace their points of origin? It is clear from the proceedings that these new approaches hold great promise in providing answers to such questions.

Summary Report

This session opened with a brief description by Dr. Aman of efforts underway in Kenya in setting up a molecular genetics lab to conduct research on assessing genetic variation within large wildlife mammals within the region using a DNA-based approach. A project, in collaboration with Dr. Ryder and CRES, to examine genetic variation in black rhinos in Kenya has already been initiated. An interesting introduction on the subject of rhinos was provided by Dr. Prothero who traced back the evolutionary history of this family over the last 50 million years since its origin. Rhinoceroses were at one time dominant large land mammals on all the northern continents and in Africa and comprised over 65 genera that occupied diverse ecological niches. Today only five species in four genera survive.

Dr. Ryder described his work on analysis of chromosomes of the African rhino. Karyotypes of the northern and southern black rhino were found to be similar in terms of chromosome numbers but dissimilar when the proportion of biarmed chromosomes was examined. In a chromosomal survey of 7 black rhinos from Zimbabwe and 22 black rhinos from Kenya, the distribution of chromosome arm lengths was found to follow a bimodal pattern grouped according to origin. This may be reflecting changes in heterochromatin as a result of a recent lack of gene flow between populations in the two regions.

At the DNA level, low levels of intraspecifc variation are observed in the two African species of rhino. By analysis of mitochondrial DNA restriction fragment length polymorphisms, Dr. George found that the level of genetic variation ranged between 0 and 0.07% among northern white rhinos and between 0 and 0.04% among southern white rhinos. The differences between northern and southern white rhinos ranged between 1 and 1.4%. Much larger levels of variations ranging from 4.2 to 5% were observed between the white and the black rhino species. Using the same technique, Dr. Harley reported a similar level of sequence divergence of 6.8 ± 1.6% between the two species of African rhino which translated to a divergence time of 3.4 ± 0.8 million years. Harley also found that mitochondrial haplotypes that were unique to *D.b. minor*, *D.b. bicornis*, and *D.b. michaeli* could be defined and could serve as useful markers for those subspecies. He noted that the amount of divergence between the black rhino sub-species represented by these changes was small. Thus, there was no indication to maintain these subspecies separately based on mitochondrial DNA distance estimates. Any outbreeding depression as a result of subspecies interbreeding would be quite unlikely and the choice, therefore, of management strategy would have to be dictated by other factors such as the preservation of some desirable morphological, behavioral, or adaptive specializations.

Data on genetic variation as assessed by the finest level of resolution, that is DNA sequencing, was presented by Dr. Amato. He described the merits of the powerful and versatile polymerase chain reations (PCR) and its application to rhino genetics. DNA sequence information generated by analysis of PCR amplified

products from the 12S and 16S ribosomal genes of the mitochondrial genome was used to construct phylogenetic trees for four species of the rhino using the cow or zebra as outgroups. The phylogenies strongly supported the monophyly of the African species. Both the Sumatran and Indian rhinos separated as another branch with a distant lineage split. Dr. Amato also described a new technique called RAPD (random amplified polymorphic DNA markers) that seems to hold better promise in paternity and pedigree analysis in rhinos than the conventional DNA fingerprinting techniques. The technique also has the advantage that there is no need for Southern blotting and hybridization with radioactive probes in generating data.

That techniques developed in other scientific disciplines can be applied to resolve zoogeographical separation in genetics was demonstrated by Dr. van der Merwe. He presented data on the measurements of light stable isotopes such as S, C, H, O, and N in specimens of rhino horn to determine the species of rhino that the sample originated from and its geographic origin. Carbon isotope ratios ($^{13}C/^{12}C$) are sufficient for species identification because of the natural differences in this ratio in the vegetation types that the two species of African rhino feed on, i.e. browse vs. grasses. Analysis of $^{13}C/^{12}C$ ratios in horn material from the two African rhino species resulted in an unequivocal identification of the species. Identification of the geographic origin, however, is more complex and requires multiple analysis of isotope ratios including $^{15}N/^{14}N$ (which correlates with rainfall) and heavy isotope ratios of Sr, Pb, and Nd (which register the age of geological substrate). Multivariate cluster analysis of these isotopes ratios was found to separate the geographic refuges with very little overlap, thereby identifying the geographic origin of the sample.

Plenary II - Summary
Biology and Conservation of the Greater One-Horned Rhinoceros

E. Dinerstein, chair: *Demographic characteristics of greater one-horned rhinoceros populations*
Sunder P. Shrestha: *The role of translocation of greater one-horned rhinos in species conserva-tion: The Bardia Park example*
G. McCracken: Genetic variat*ion in the greater one-horned rhino and implications for population structure*
Satya Priya Sinha: *Management of the reintroduced great one horned rhinoceros (Rhinoceros unicornis)in Dudwa National Park, Uttar Pradesh, India*

This session took a conservation biology approach to the management of this species. We first looked at some of the demographic considerations of this population which have been under study over the last few years and found that the Chitwan and Kaziranga populations (essentially the only viable population at the moment) had made significant recovery since the early 1900s, when they were down to very low levels. In the case of the Kaziranga population, its numbers decreased to less than a hundred individuals; for the Chitwan population, the low point was between 60 and 80 individuals in 1962. These rhino populations are an example of how adequate protection and sufficient habitat can lead to recovery.

In looking at the genetics of this population a rather startling and interesting discovery was made. The average heterozygosity in greater one-horned rhinoceroses approaches the highest levels recorded for free-ranging mammals. Gary McCracken explained how, through the historical demography of this species, genetic diversity might have been maintained in spite of the population bottleneck. The speakers in this session also recognize that the remnants of a population almost going extinct may still carry high levels of genetic variability, and that the next step in any effort to conserve the species is to reestablis h these populations within the historic range of the species, particularly in areas that are now well protected and where there is adequate habitat.

Dr. Shrestha from Nepal and Dr. Sinha from Uttar Pradesh gave examples of where translocations have begun. In the very successful reintroduction and translocation of rhinoceros from Chitwan the population at Bardia is now up to 38 individuals. There have been five births. Of the first installment of rhinos that were sent there in 1986, all those born were from females that were bred in national parks, rather than females that arrived pregnant. Dr. Sinha reported on a different, opposing scenario involving the Dudwa Sanctuary population for which it appears unlikely at the moment there is enough habitat within the Dudwa Sanctuary to support a viable population.

There are a number of other areas within the historic range of the greater one-horned rhinoceros that are available for future translocation efforts. From Dr. Shrestha's work it is clear that the technology is available for the translocation of animals and a high success rate may be anticipated, unlike some of the problems we heard about with black rhinos. Thus, the translocations should become a very important part of the conservation activities for *Rhinoceros unicornis*.

Plenary III - Summary
African Rhino Status and Conservation Plans

C. Gakahu, chair: *African rhinos: Current numbers and distribution*
R. Brett: *The management of rhinos in sanctuaries in Kenya*
P.M. Brooks: *Conservation plan for the black rhinoceros in South Africa, the TBVC states and Namibia*
K.H. Smith: *Conserving rhinos in Garamba National Park*
M. Atalia: *Strategies for the conservation of rhino in Zaire*
N. Steele: *Development and management of rhino sanctuaries in South Africa: The effects of socio economic and political changes in Southern Africa on developments*

1. Numbers, distribution, and whether the trend of population(s) is decreasing, stable, or increasing in sanctuaries, nations, or regions are the basis of assessing status and therefore, vital data for management and conservation of rhinos. The databases for rhino populations should progress toward continuous monitoring of births and deaths, including, when possible, the identification of individuals. A permanent and centralized database should be established.

2. The required field surveys and monitoring are expensive and require finance, personnel, and equipment. These requirements must therefore be used rationally for max output. Finance and equipment are a major problem and assistance is required. Efforts should therefore be concentrated in areas with significant (viable) numbers of rhinos.

3. Sanctuaries offer great hope and future for rhinos, but they must be actively managed and supported by long term intensive monitoring of all aspects including vegetation, food and nutrition requirements, genetics, and disease together with physiology and veterinary needs especially for capture and translocation.

NEW YORK ZOOLOGICAL SOCIETY

August 6, 1991

Robert W. Reece
Kings Island Wild Animal Habitat
Kings Island, Ohio 45034

Dear Bob,

Enclosed are copies of relevant papers. If there are any questions please give me a call.

In regards to our phone conversation, I would like to offer my services for organizing a workshop to address black rhino ESU's. There is a great deal of data now and a few of us (Ollie, E. Harley, and myself) are continuing to expand various data sets.

A good place to hold such a workshop would be in one of the two new molecular systematics labs (the Smithsonian's or the American Museum of Natural History). There would be a number of experts already present at either facility and we could invite a few outside researchers in this field (e.g. John Avise, Wes Brown, Joel Cracraft, Alan Templeton). Of course, I would be happy to discuss this with you further. I strongly feel that this workshop could serve as an example of how we should handle ESU questions in the future.

Sincerely,

George Amato

cc: Ulysses S. Seal

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CONSERVATION EDUCATION RESEARCH

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; I, 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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X
S c
g h c s u H o P u u
c S a H D S X c s X u u h g H v B X u h v a D a a D H v R v B a R u

White rhino

D
X g S u u
S X h c c c c 1 E H v a R H 1
c S a H D X a E s u h v B R X u h D a N D c a D v v H u B a u

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 ± 298 and 16411 ± 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

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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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Conservation Genetics of the Black Rhinoceros (*Diceros bicornis*), I: Evidence from the Mitochondrial DNA of Three Populations

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Abstract: *A drastic decline in the number of black rhinoceroses (*Diceros bicornis*), primarily as a result of poaching, places this species in imminent danger of extinction. The remaining black rhinos are divided into small, isolated populations that are vulnerable to demographic extinction, disease epidemics, genetic drift, and inbreeding. Some conservationists have suggested minimizing these threats by moving as many animals as possible from different isolated populations to a few safe "rhino sanctuaries." To examine the possible long-term genetic consequences of such a strategy, we focused our efforts on determining the level of genetic differences among the remaining black rhino populations by examining restriction fragment length polymorphisms of the rapidly evolving mitochondrial DNA molecule. The 23 black rhinos in our survey, including animals from*

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Resumen: *Una disminución drástica en el número de rinocerontes negros (*Diceros bicornis*), principalmente debido a la caza ilegal, pone a dicha especie en peligro de extinción inminente. Los rinocerontes negros restantes estan divididos en poblaciones pequeñas y aisladas, que son vulnerables a la extinción demográfica, las epidemias, la deriva genética y la endogamia. Algunos conservacionistas han sugerido disminuir estas amenazas, trasladando tantos animales como sea posible, de diferentes poblaciones aisladas, a un par de "santuarios seguros" para rinocerentes. Para examinar las posibles consecuencias genéticas a largo plazo de dicha estratégia, enfocamos nuestros esfuerzos en determinar el nivel de diferencia genética entre las poblaciones restantes de rinocerontes negros. Examinamos polimorfismos de fragmentos de longitud restringida de la molécula ADN, de evolución mitocondrial rápida. Los 23 ejemplares de rinocerontes negros de nuestro estudio, que incluyen a animales provenientes de tres regiones geográficas y de dos subespecies descritas, denotaron muy poca diferenciación del ADN mitocondrial. Unicamente 4 de 18 enzimas re-*

three geographic regions and two named subspecies, showed very little mitochondrial DNA differentiation. Only 4 out of 18 restriction enzymes revealed any mtDNA polymorphisms, and the average estimated percent sequence divergence between the four mtDNA genotypes observed as 0.17%. Mitochondrial DNA divergence between the two named subspecies, D. b. minor *and* D. b. michaeli, *was estimated to be only 0.29%. These results indicate a very close genetic relationship among the black rhinos in our survey. Thus, the mitochondrial DNA data suggest that within national boundaries, the black rhino populations we sampled may be considered single populations for breeding purposes, which might increase the species' probability of survival.*

strictivas, denotaron poliformismo mitocondrial del ADN (ADNmt) y, el porcentaje promedio estimado de divergencia secuencial entre los cuatro genotipos ADNmt observados, fue de 0.17%. La divergencia de ADN mitocondrial entre las dos subespecies descritas, D. b. minor *y* D. b. michaeli, *se estima que fue solo de 0.29%. Estos resultados indican una relación genética muy cercana entre los rinocerontes negros de nuestro estudio. Por lo tanto, los datos de ADN mitocondrial sugieren que, dentro de las fronteras nacionales, las poblaciones de rinocerontes negros que estudiamos pueden considerarse una sola población para finalidades de reproducción, lo cual puede incrementar la probabilidad de supervivencia de dicha especie.*

Introduction

Despite its reputation as a powerful and invincible beast, the rhinoceros has suffered precipitous declines in number and is threatened with extinction. Fewer than 11,000 individuals of all five species survive in small scattered populations throughout Africa, India, and Southeast Asia. The black rhino (*Diceros bicornis*), the focus of this study, has suffered the most dramatic decline, disappearing faster than any other large mammal. The species once occupied most of sub-Saharan Africa and numbered in the hundreds of thousands (Fig. 1). Even by the turn of the century, large, nearly contiguous populations of black rhino were spread across much of central, eastern, and southern Africa. However, by 1970 their numbers had declined to 65,000 and over the past 18 years poaching has reduced this number by 95%. The remaining 3,800 animals are split into some 75 populations, only ten of which have more than 50 animals (Western & Vigne 1985; Du Toit et al. 1987; Wildlife Conservation International News 1988).

Although the loss of any species is tragic, the plight of the rhinoceros is particularly apalling because the overwhelming cause of their demise is not destruction of their preferred habitat but continued slaughter by poachers to supply two major markets. Horns are fashioned into ornamental dagger handles costing up to $30,000 in the Near East, particularly in North Yemen. In many parts of East Asia, rhino horn is valued at $8,000 to $15,000 per kilogram for various medicinal purposes (Martin 1983; Penny 1988). Increasing affluence in the Far East and oil wealth in the Near East, coupled with declining availability, have drastically increased the value of rhino horn in recent years, creating devastating repercussions for rhinos in Africa. The market value of rhino horn is currently so high that all age and size classes are susceptible to poachers (Leader-Williams 1988).

Assuming for the moment that the governments of countries still harboring sizeable numbers of black rhino (e.g., Kenya, Tanzania, Zimbabwe, and South Africa) are successful in controlling poachers, another major problem exists for the species. The small, isolated populations of black rhino that remain are vulnerable to the effects of demographic fluctuations, local ecological perturbations and disease epidemics, and loss of genetic variability due to drift and inbreeding (e.g., Crow & Kimura 1970; Gilpin & Soulé 1986; Wilcove et al. 1986). Additionally, low population density reduces the probability that a male will find a female during the 1–2-day period in her estrous cycle when she is sexually

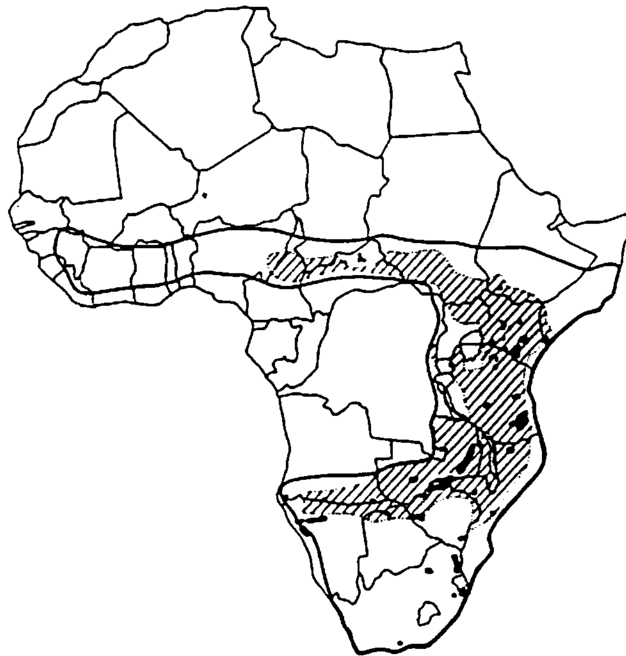

Figure 1. Map of Africa showing black rhino distributions during historical times (heavy black line), 1900 (hatched area), and 1987 (black area). Redrawn from Western & Vigne (1985), incorporating data from WCI for 1987.

receptive (Hitchins & Anderson 1983). Hence, what were once thought to be minimum viable populations on the basis of genetic parameters (i.e., inbreeding depression and loss of genetic heterogeneity) are now in many instances considered underestimates (Soulé 1987).

The shortage of manpower and resources within national conservation departments across Africa is a primary factor in the decline of the rhino (Leader-Williams & Albon 1988). Consolidating groups is an easier and less expensive way to reestablish former densities. The idea of creating more sanctuaries, which are effectively "species parks," has received widespread interest following the increase in Kenya's rhino populations in such sanctuaries. However, management strategies such as this are complicated by the fact that each remaining population has been assigned to one of several subspecies, based upon aspects of external morphology such as horn shape and body size. The genetic and evolutionary relationships of these morphologically defined subspecies are unknown. The most widely accepted classification (Groves 1967) recognizes seven subspecies of *Diceros bicornis*, one of which *D. b. ladoensis*, is probably extinct. Three other subspecies — *D. b. brucii*, found in Ethiopia and Somalia; *D. b. longipes*, which remains only in Cameroon and perhaps Chad; and *D. b. chobiensis*, found in Angola — are down to a few dozen animals, if they are not already extinct (Western & Vigne 1985). *D. b. bicornis*, if it can be considered a distinct taxon at all, is found in Namibia and probably numbers less than 100 (Hall-Martin 1985; Du Toit et al. 1987). The remaining two subspecies, *D. b. michaeli* and *D. b. minor*, or populations designed as such, will figure most importantly in the return of the black rhino, should this be accomplished. *D. b. michaeli*, found in Kenya and Tanzania, has declined drastically as well, but still numbers between 500 and 1,000 and has increasingly received more protection in Kenya. The vast majority of the approximately 150 black rhinos in North American and European zoos are of this subspecies (Du Toit et al. 1987) and so will be important for future captive breeding efforts. *D. b. minor* is the most common remaining race, ranging from Kenya to South Africa, and with numbers at about 2,500, it is relatively the most secure.

Should all the remaining black rhinos be considered as a single population for breeding purposes? This tactic might increase their chances of survival by increasing effective population sizes and thus forestalling stochastic demographic extinctions, inbreeding depression, and loss of the species' existing genetic variability (Soulé 1983; Gilpin & Soulé 1986; Ralls et al. 1986; Goodman 1987). Alternatively, do different populations (which may or may not coincide with subspecies designations) merit separate conservation as genetically and possibly ecologically distinct units? The latter strategy might prevent outbreeding depression or the production of animals with genetic makeups inappropriate for a given environment (Templeton 1986). These questions should, ideally, be tackled from both an ecological and genetic standpoint. Ecologically, it might be possible to distinguish locally adapted traits. Statistically significant differences in serum vitamin E levels, for example, have been found between Kenyan and southern African samples, which may reflect substantial differences in diet (Dierenfield, personal communication). Ecological differences also distinguish the desert rhinos of Namibia from the highland forest rhinos of Kenya (Du Toit 1987).

In the absence of any clear morphometric differences, IUCN's African Elephant and Rhino Specialist Group has placed a high priority on genetic studies of black rhinos to resolve whether discrete populations could be identified (Du Toit et al. 1987). As a first step in applying molecular genetic techniques to questions of black rhino conservation, we have examined the mitochondrial DNA (mtDNA) of 23 black rhinos representing two morphologically defined subspecies and three geographic populations. We chose mtDNA because its rapid evolutionary rate has shown it to be a useful molecule for determining intraspecific relationships of many animals (e.g., Wilson et al. 1985; Avise & Lansman 1983). If the rhino populations surveyed here have had separate evolutionary histories for a considerable length of time, it should be reflected in the divergence of mtDNA's from animals in different populations.

The mitochondrial genome consists of a closed circular DNA molecule which codes for 13 proteins and a complete set of transfer RNAs. It is extremely conserved in size (about 16,000 base pairs in all mammals that have been examined) and gene arrangement (Brown 1983). It lacks the complicating features of repetitive DNA or introns; therefore, a relatively simple restriction enzyme analysis of the molecule can be undertaken to yield good estimates of genetic relationships among fairly large numbers of individuals. It is maternally inherited without recombination and thus represents an unambiguous marker of maternal phylogeny. Because it evolves 5–10 times more rapidly than single-copy nuclear DNA (Brown et al. 1979) and intraspecific mtDNA variability has been widely demonstrated, this approach seemed the most likely to uncover genetic differentiation among black rhinos, should it exist.

Materials and Methods

With the cooperation of field biologists, wildlife managers, and zoo personnel in both Africa and the United States, we were able to obtain whole blood from both

captive and wild-caught black rhinos (Table 1). Our sample included 11 *D. b. michaeli* of Kenyan origin now kept in U.S. zoos, 11 *D. b. minor* taken from wild populations in Zimbabwe, and one captive (U.S.) *D. b. minor* of South African origin. While blood was separated into plasma, red blood cells, platelets, and white blood cells or buffy coats, the latter two components being our primary source of DNA. Total DNA was extracted from white blood cells or buffy coats by standard procedures. We also obtained frozen organ tissue from three animals that died during the period of our study (Table 1). This frozen tissue served as a source of purified mtDNA, which was isolated by the method of differential centrifugation (Lansman et al. 1981).

We have used restriction enzymes to survey the black rhinos for mtDNA polymorphisms. Restriction enzymes recognize specific oligonucleotide sequences, usually 4 to 6 base pairs in length, and cleave double-stranded DNA wherever these sequences occur. By surveying mtDNAs with a set of restriction enzymes, we can obtain an accurate estimation of similarity by determining the proportion of restriction fragments and/or restriction sites they share.

Samples of total DNA were digested with 14 restriction enzymes (Bethesda Research Laboratories) having 5 or 6 base pair (b.p.) recognition sites, according to manufacturer's instructions. These enzymes typically cleave mtDNA into 1–7 fragments. The DNA fragments once obtained were separated electrophoretically in 1% agarose gels along with a radioactively labeled (α-32-P) one-kilobase ladder (Bethesda Research Laboratories), then transferred to GeneScreen-plus membranes (New England Nuclear) by an alkaline blotting procedure (Southern 1975; Reed & Mann 1985). Purified mtDNA obtained from tissue was then nick-translated with α-32-P labeled nucleotides and was used to "probe" the southern blots. Membranes were then washed under high-stringency conditions and exposed to Kodak XAR film.

Additionally, to increase our resolution, we digested the three purified mtDNA samples (one from each population) with four enzymes having 4 b.p. recognition sites. These enzymes cleave the mtDNA into 20–30 fragments and thus have a greater likelihood of revealing differences between individuals. Because each sample contained only purified mtDNA, the resulting restriction fragments could be directly labeled with α-32-P (Brown 1980) before being separated electrophoretically on 3.5% polyacrylamide gels. Again, an appropriate radioactively labeled molecular weight/size standard was included in the gel. Gels were subsequently dried under vacuum and exposed to Kodak XAR film.

The proportion of shared restriction fragments was calculated between the observed mtDNA genotypes. The percent sequence divergence between the mitochondrial genotypes was estimated using equation 6b of Upholt (1977). Calculations for restriction enzymes having 6, 5, and 4 b.p. restriction sites were calculated separately, then weighted according to the total number of base pairs recognized by each type of restriction enzyme. This procedure allowed an overall estimate or weighted average of nucleotide sequence divergence, based on the differences revealed by all the restriction enzymes used, between the mtDNA of different individuals.

Results

Each restriction fragment pattern produced by a given enzyme was arbitrarily assigned a letter. The results of the enzymes having 5 or 6 base-pair recognition sites for all 23 animals are listed in Table 2. The results of enzymes for a smaller set of three animals, including en-

Table 1. Black rhino samples.

Sample	*Origin**	*Subspecies***	*Tissue*	*Source*
1–8	Zimbabwe	*D. b. minor*	Buffy coat	Department of Parks and Wildlife Management Zimbabwe
9	Zimbabwe	*D. b. minor*	Frozen liver	Department of Parks and Wildlife Management Zimbabwe
10, 11	Zimbabwe	*D. b. minor*	W.B.C.***	Los Angeles Zoo
12	South Africa	*D. b. minor*	Frozen brain	Calvin Bentsen Ranch Brownsville, Texas
13–16	Kenya	*D. b. michaeli*	W.B.C.	Denver Zoo
17–19	Kenya	*D. b. michaeli*	W.B.C.	St. Louis Zoo
20	Kenya	*D. b. michaeli*	W.B.C.	Zoo Atlanta
21	Kenya	*D. b. michaeli*	W.B.C.	Busch Gardens
22	Kenya	*D. b. michaeli*	Frozen liver	Kansas City Zoo
23	Kenya	*D. b. michaeli*	W.B.C.	Detroit Zoo

* *For animals born in captivity, the origin of the individual's mother is given.*
** *Subspecific designation according to range distributions given by Groves, 1967.*
*** *White blood cells.*

Table 2. mtDNA Patterns for enzymes with 5 and 6 b.p. sites.

		D. b. minor		*D. b. michaeli*
Enzyme	*# Sites**	*South Africa n = 1*	*Zimbabwe n = 11*	*Kenya n = 11*
AvaI	2	A	A	A
BamHI	2	A	A	A
BglII	1	A	A	A
ClaI	1	A	A	A
DraI	5	A	A	A
EcoRI	2(1)	A	A(B)	A
EcoRV	2	A	A	A
HaeII	4	A	A	A
HindIII	4	A	A	A
ScaI	7	A	A	A
XbaI	4	A	A	A

* *The number in parentheses represents the number of restriction sites for haplotype B.*

Table 3. mtDNA Patterns for additional enzymes.

		D. b. minor		*D. b. michaeli*
Enzyme	*# Sites**	*South Africa n = 1*	*Zimbabwe n = 1*	*Kenya n = 1*
AvaII	4	A	A	A
BclI	6(5)	A	A	B
HincII	7	A	A	A
HinfI	30(29)	A	A	B
HpaII	16	A	A	A
MboI	23	A	A	A
TaqI	24(25)	A	A	B

* *The number in parentheses represents the number of restriction sites for haplotype B.*

zymes having 4 b.p. recognition sites (i.e., HinfI, HpaII, MboI, TaqI), are presented in Table 3. The restriction enzymes used in our survey yielded an average of 140 restriction sites per mitochondrial genome. This corresponds to a recognized total of over 630 b.p., or 3.9% of the mitochondrial genome. For 14 out of a total of 18 restriction enzymes, absolutely no mtDNA variability was observed. That is, all rhinos surveyed had the identical restriction fragment pattern (designated as "A" in Tables 2 and 3) for any one of these 14 restriction enzymes. One enzyme, EcoRI, was found to be polymorphic among the Zimbabwe rhino, with 3 of 11 animals possessing only one EcoRI restriction site instead of the two sites found in the other 8 members of this population. Three enzymes, BclI, HinfI, and TaqI, revealed a difference between the Kenyan population and the Zimbabwe and South African populations. In each case, the result could be interpreted as a single loss or gain of a restriction site.

In total, then, for our sample of 23 animals, only three mtDNA haplotypes could be distinguished: (1) the Kenyan haplotype with fragment pattern "B" for BclI, HinfI, and TaqI; (2) the Zimbabwe haplotype with fragment pattern "B" for EcoRI; and (3) the Zimbabwe and South African haplotype with fragment pattern "A" for all 18 restriction enzymes. These three mtDNA haplotypes are extremely similar to one another (Table 4), with an average estimated percent sequence difference between any pair of haplotypes and/or populations of 0.17%. The average difference between subspecies was only slightly higher, 0.29%.

Discussion

The results of the mtDNA analysis strongly suggest a very close genetic relationship among all the black rhinos in our survey. Because of the generally rapid rate of mtDNA evolution in mammals, differences observed among rhino populations appear to indicate a very recent common ancestry. If mtDNA evolves at a rate of 2% per million years as suggested (Brown et al. 1979; Wilson et al. 1985), this common ancestry probably dates back no farther than 100,000 years. Indeed, the level of differentiation between the so-called subspecies is well within the range (0–4%) observed among members of other mammalian species (e.g., Avise & Lansman 1983), and even within the range (0–2%) that has been observed among members of the same local population (Ashley & Wills 1987). Thus, there is no evidence from these data that the black rhinos we sampled represent "evolutionarily distinct units."

These findings for the black rhino stand in sharp contrast to the level and distribution of mtDNA differences reported for the white rhino. The southern white rhino, *Ceratotherium simum simum,* has recovered quite well from a population bottleneck that occurred at the turn of the century, and now more than 3,000 members of this population can be found in South Africa and other African countries (Penny 1988). The status of the northern white rhino, *Ceratotherium simum cottoni,* is much bleaker, with only 22 individuals known to exist in the wild (Western 1987). Management plans originally proposed supplementing the northern race with members from the southern population. Based on a survey of one individual from each of the two races, however, George et al. (1983) reported a very high level of mtDNA divergence (approximately 4.0%). Partially as a result of this study, managers decided against interbreeding the two races. Unlike the black rhino, the white rhino subspecies have existed in nonoverlapping

Table 4. Estimated percent sequence divergence between mtDNA types, based on the proportion of shared restriction fragments (Upholt, 1977).

	Zimbabwe 1	*Zimbabwe 2*	*S. Africa*
Kenya	0.24	0.39	0.24
Zimbabwe 1	—	0.08	0.00
Zimbabwe 2		—	0.08
S. Africa			—

ranges, at least during historical times. George et al. suggest, on the basis of their molecular data, that the two white rhino subspecies have been isolated from each other for at least two million years. However, recent research on variability of nuclear-coded allozymes found little differentiation between the northern and southern subspecies (Merenlender et al. 1989), suggesting a more recent isolation.

We chose mtDNA analysis because we thought it would be most likely to uncover genetic differences between the black rhino populations, should they exist. It seems unlikely from our results that significant barriers to successful interbreeding would exist, given what we estimate to be a brief history of separation between the populations in question. However, more information should be obtained before final management decisions are made. The mitochondrial genome represents only a tiny fraction of an organism's genetic makeup, and problems that might arise from interbreeding might not necessarily be reflected in mtDNA differentiation. For this reason, we are conducting an allozyme survey in our laboratory to determine if the findings regarding the mtDNA hold for nuclear-coded genes as well. The allozyme survey will also be more informative for determining if the black rhinos suffer from reduced levels of genetic variability, as has been reported for some species that have passed through recent population bottlenecks (Bonnell & Selander 1974; O'Brien et al. 1983). Thus far, we have found no allozyme polymorphisms within or between populations, despite the fact that (1) we have included in our analysis animals from Kenya, Zimbabwe, and three different populations in South Africa (Etosha, Ado, and Zululand) and (2) our initial surveys have included all three allozyme loci identified by Merenlender et al. (1989) as polymorphic in African rhinos (Amato & Melnick, unpublished data).

Karyotype analysis is also recommended, because chromosomal differences reducing the fertility of hybrids could conceivably exist in the absence of either allozyme or mtDNA differentiation. There have been no known crosses of black rhinos from different subspecies in captivity, which might indicate reduced viability or fertility.

The application of genetics to conservation issues is a practical endeavor and should yield concrete recommendations for management strategies. Black rhinos from the populations included in our study will probably be the ancestors of all future black rhinos, as their successful breeding is the only hope for the survival of the species. Our results provide strong evidence for a very close genetic relationship among these populations. At the national level, the level at which management decisions are currently made, the pooling of black rhinos carries with it little risk of mixing distinct genetic adaptations worthy of separate conservation efforts. This finding should allow managers to aggregate individuals to create larger local populations or demes. Preserving the black rhino in relatively large local populations would have several beneficial effects. These include retarding the rate of loss of genetic variability, buffering each aggregate against the possibility of demographic extinction, restoring previous population densities, and allowing the wildlife managers with limited resources to provide better production against poachers. Taken together, these effects should, in the long run, increase the probability of survival of this critically endangered species.

Acknowledgments

This research grew out of the participation by two of us (DJM and DW) in the October 1986 African Rhino Workshop organized by the AAZPA SSP, the Cincinnati Zoo, and the King's Island Wild Animals Habitat. It represents one part of a larger effort to understand the genetic structure of the black rhinoceros and help preserve this highly endangered species. We are grateful to many institutions and individuals who helped put together the research materials and provided financial support for the laboratory analysis. In particular we wish to thank David Cumming, Raoul Du Toit, and the Wildlife Department of Zimbabwe, and Perez Olindo and the Department of National Parks and Wildlife Management of Kenya, for supplying us with blood and organ tissue samples critical to this study. We would also like to thank the administration and veterinary personnel of Busch Gardens, Tampa, Florida; the Calvin Bentsen Ranch, Brownsville, Texas; Denver Zoo; Detroit Zoo; Kansas City Zoo; Los Angeles Zoo; St. Louis Zoo; and Zoo Atlanta for supplying samples from captive black rhino of Kenyan and South African origin. We appreciate the comments of two anonymous reviewers and Robert Vrijenhoek on an earlier version of this article. Finally, we would like to single out Mary Pearl, Tom Foose, Ed Maruska, and Eric Miller for their logistical support and encouragement throughout this project. Financial support for this research was supplied by Wildlife Conservation International, a division of the New York Zoological Society, the Cincinnati Zoo, the AAZPA, and those other U.S. zoos that contributed to the Black Rhino SSP's fund-raising effort.

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July 9, 1991

Dr. George Amato
New York Zoological Society
Bronx Zoo
Bronx, NY 10460

Dear George:

I've completely run out of time before I have to go to Mongolia. Consequently, the only help I can offer at this moment is the enclosed figure. I will be back in San Diego on 5 August.

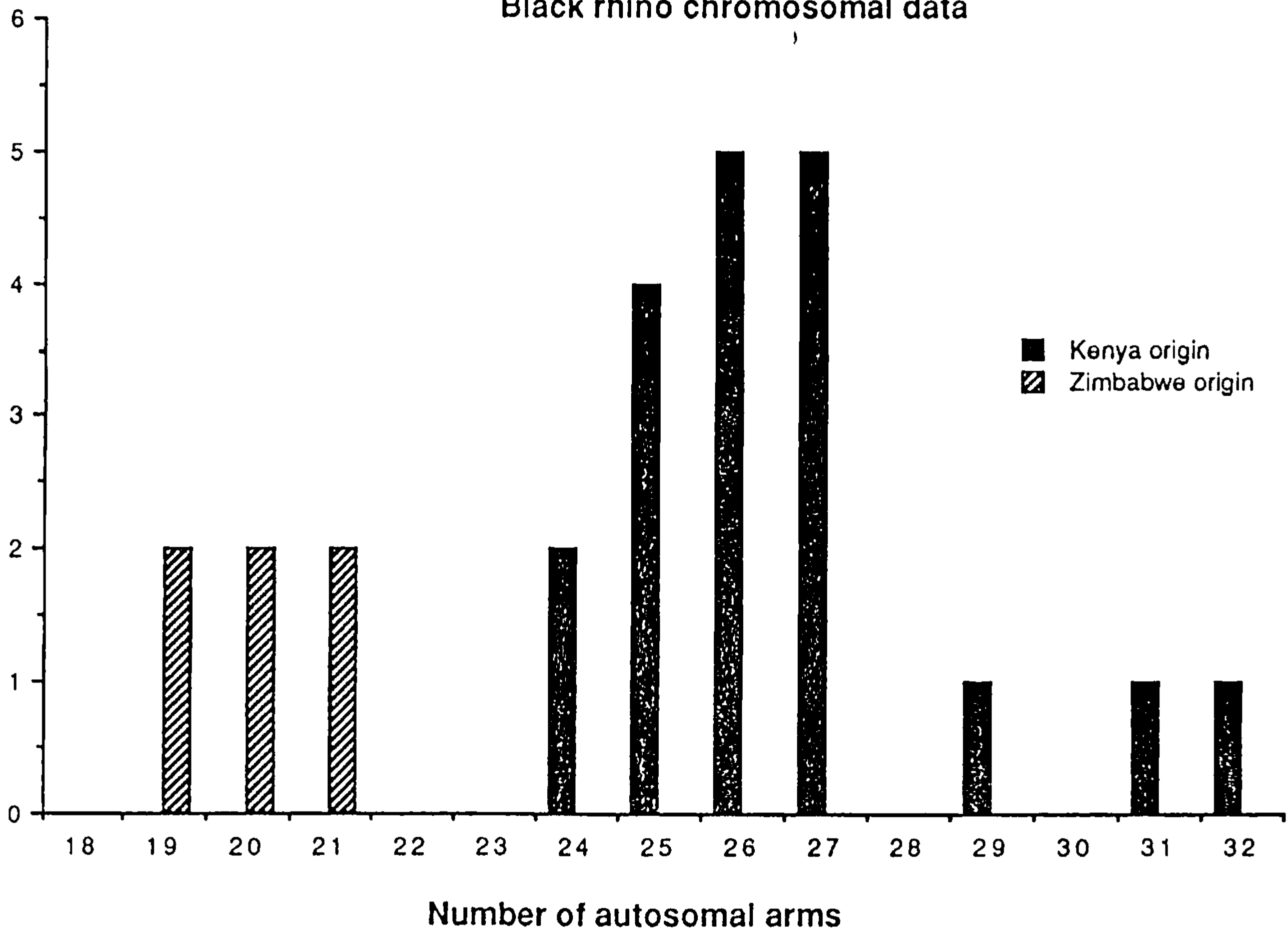
Best wishes.

Sincerely yours,

Ollie

Oliver A. Ryder, Ph.D.
Kleberg Genetics Chair
Center for Reproduction of Endangered Species

OAR/gm


Black rhino chromosomal data
6
5
4
3
2
1
0
Kenya origin
Zimbabwe origin
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
Number of autosomal arms

RHINOCEROS GENETICS: THE STATE-OF-THE-ART AND APPLICATION TO CONSERVATION MEASURES

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The mammalian family Rhinocerotidae, is one of three families that comprise the Order Perissodactyla. There are five extant species of rhinoceros (Table 1). All five species are listed as endangered in the 1986 IUCN Red List of Threatened Animals (IUCN, 1986). Estimated numbers of African rhinos have been declining rapidly, due mainly to their selective removal from available habitat by poaching (Bradley Martin, 1982). Of the three Asian species, population numbers are stabilized only for the Indian rhinoceros. Some 1987 estimates for population numbers of extant rhino species are also listed in Table 1.

Decline in population numbers and increasing isolation and fragmentation of rhino populations raises concern for the long-term survival of this unique group of mammals, even if population numbers are stabilized at current levels, because small isolated populations are vulnerable to extinction from random demographic events, inbreeding, and genetic drift.

Current strategy for conservation of rhinoceroses is in the context of national plans with oversight by the IUCN/SSC African Elephant and Rhino Specialist Group (AERSG), IUCN/SSC Rhino Specialist Group, United Nations Environmental Program, UNESCO, and other international and national agencies. In some cases populations of rhinos within a single nation-state are so low that their long-term viability is seriously in question. Under these circumstances, current conservation strategies for rhinos involve proposals for genetic mixing of some of the named subspecies. Three of the extant rhino species have named subspecies (Table 1).

The use of subspecies designations in the zoological nomenclature was established long before modern studies in population genetics revealed spatial and temporal patterns of genetic diversity within species of mammals. Often, subspecies status is conferred assuming that it reflects genetic and/or ecological differences. However, the results of modern genetic studies employing chromosomal analysis, protein electrophoresis, and other biochemical-genetic methods have not always been consistently correlated with recognized subspecies designations. Subspecies were traditionally designated by morphological criteria including minor cranial and pelage differences. Often these were not subjected to the types of statistical analysis that are available today. Consequently, the subspecific distinctions among mammals are somewhat arbitrary and inconclusive, particularly among neighboring subspecies with contiguous distribution or those showing continuous variation.

Alternately, populations designated as only being distinct at the subspecies level have been shown to be reproductively isolated. In some instances, chromosomal differences between subspecies have been shown to be of sufficient magnitude that progeny of first-generation crosses between subspecies are sterile.

Comparative genetic studies may be useful in providing data that will help in the evaluation of the degree of evolutionary differentiation of rhino populations, subspecies and species. Previous genetic studies of rhinoceroses have been limited to investigations of chromosome numbers for relatively few individuals of a limited number of populations of a few named subspecies.

Thus, it is recognized that additional genetic studies of rhinoceroses are urgently needed. The purpose of this paper is to provide a review of the data gathered in our laboratory in San Diego or in collaboration with investigators elsewhere.

BLACK RHINOCEROS

Limited chromosomal data has been published on black rhinoceroses. An adult female specimen from Kenya was studied by Hungerford and Snyder (1967). Heinchen (1969) reported that an animal from Krueger Park had 84 chromosomes. To our knowledge, no other geographic forms of black rhinoceros have been subjected to chromosomal investigations.

We have studied the chromosomes of 16 individual black rhinos for which we are reasonably certain of the subspecies status of 13. Of these, with the help of the black rhino SSP species coordinator, Ed Maruska, we have been able to determine that 12 are *Diceros bicornis michaeli*. All of these individuals possess 84 chromosomes. However, we have found a variation in the number of chromosome arms in individuals of this subspecies. C-banding reveals that this variation is due to the presence or absence of heterochromatic small arms on chromosomes exhibiting G-banding homology.

To date, we have studied one male individual held in Los Angeles that belongs to the *D. b. minor* subspecies. Remarkably, this individual has a smaller number of chromosome arms than the *michaeli* individuals we have studied. C-banding analysis of this single male animal reveals only four chromosomes that have heterochromatic small arms of appreciable size. In this regard, the pattern of heterochromatin in this single *michaeli* individual is more similar to that of white rhinos, *Ceratotherium simum*.

Major karyotypic variation in the context of variable numbers of acrocentric chromosomes has been observed in other mammals, e.g., *Peromyscus* (Pathak *et al*., 1973).

Further chromosomal studies of black rhino subspecies should be conducted in order to learn more about the chromosomal differentiation of the geographically distributed remnant populations of this endangered species.

SUMATRAN RHINOCEROS

The Sumatran rhinoceros, *Dicerorhinus sumatrensis*, is an endangered species for which efforts are underway to establish captive populations derived from animals captured in habitats designated for deforestation and agricultural purposes.

As a result of this effort, a total of ten Sumatran rhinos are now in

captivity in Indonesia, Malaysia, Thailand, and Great Britain. At the time of writing, potential breeding pairs exist in the Jakarta Zoo and at a capture site in Sumatra. In order to constitute additional breeding pairs, animal translocations will be made producing pairs of animals from different subspecific backgrounds. While the establishment of breeding groups is of the highest priority, some concern does exist as to whether the pairing for reproduction of individuals from different geographic regions is appropriate. The potential consequences of inappropriate pairing for reproduction of these animals include a reduced rate of population growth, the production of offspring with reduced fertility, and the production of individuals with genetic backgrounds that do not accurately reflect the situation found in wild populations. In recent times the species has occurred on Borneo (*D. s. harrissoni*), Sumatra and Malaysia (*D. s. sumatrensis*), and on the Asiatic mainland as far north as Assam (*D. s. lasiotis*) (Groves and Kurt, 1972). In the Mammalian Species account for the Sumatran rhino, Groves and Kurt summarized the genetic knowledge of this species in the following way: "Nothing whatever is known of the genetics of this species."

When a female Sumatran rhinoceros died unexpectedly at the Port Lympne Estate in Kent, England, zoo director Dr. Tom Begg collected skin biopsy specimens that were forwarded to our laboratory in San Diego. Cell cultures were successfully established and chromosomal preparations made. The female, "Subur," possessed 82 chromosomes. With the exception of the sex chromosomes, we believe the chromosomal complement consists entirely of acrocentric chromosomes. The sex chromosomes are submetacentric with prominent distal blocks of heterochromatin. This individual was captured on Sumatra and, accordingly, would belong to the *sumatrensis* subspecies. It is anticipated that opportunities for sample collection will arise during the process of translocating animals in order to create breeding groups. Samples will be collected by individuals involved in the field activities of the AAZPA Sumatran Rhino Trust and forwarded to San Diego for analysis.

WHITE RHINOCEROS

Two subspecies of white rhino, *Ceratotherium simum*, are recognized. Unlike the black rhino that, until recently, consisted of contiguously-distributed populations, the white rhino is thought to have been discretely distributed for thousands of years (Groves, 1972), although this is not a unanimous opinion (D. Western, pers. comm.). The Southern form, *C. s. simum*, went through a population reduction and bottleneck estimated to be approximately 30 animals within the last 100 years. The previously more numerous Northern form, *C. s. cottoni*, survives now as a single population estimated at 17 - 20 animals in Garamba National Park in Zaire. A captive population of Northern white rhinos is held in the Dvur Kralove Zoo in Czechoslovakia.

Chromosomal studies of Southern white rhinos in Kruger National Park involved direct preparations from bone marrow. These studies were successful on only a few numbers of individuals, but, when successful, a diploid chromosome number of 82 was obtained. More recent studies involving cell culture obtained diploid chromosome numbers of 84 utilizing statistical analysis of a large number of well-prepared metaphase plates.

We have studied the chromosomes of nine Southern white rhinos, three Northern white rhinos and one first-generation hybrid between parents belonging to the two different subspecies. Successful blood cultures always revealed a diploid chromosome number of 82. Early passaged fibroblasts revealed a diploid number of 82 as well. However, upon extended culturing, diploid chromosome numbers of 84 and higher have been obtained. We currently believe that, with extended time in culture, artifactual cell transformation occurs resulting in chromosome counts of varying numbers including tetraploidy.

The availability of a first-generation captive-born individual, one of whose parents was a Northern white rhino and one a Southern white rhino, provides the opportunity for detailed comparisons of the chromosomes of the two subspecies in a single individual. We can conclude at this time that the diploid chromosome number for both C. s. simum and C. s. cottoni is 82 and that, in broad perspective, the G-banding patterns of their chromosomes are highly similar if not identical.

An electrophoretic comparison of enzymes and other blood proteins of the two white rhino subspecies, involving an analysis of 31 electrophoretic loci resulted in a very small intraspecific distance between the two living white rhino subspecies (Merenlender, A., Woodruff, D. and Ryder, O.A., in preparation). A study involving comparison of mitochondrial DNA from one Northern white rhino and two Southern white rhino individuals suggested that the mitochondrial DNAs of the two rhinos differ by approximately 4% in their nucleotide sequences (George, M., Puentes, L.A. and Ryder, O.A., 1982). By comparison to calibrations made for primate species, these results indicated that the white rhino subspecies last shared a common ancestor at least two million years ago (George, M., Puentes, L.A. and Ryder, O.A., 1982). These results, while not necessarily in conflict, indicate that further analyses are necessary in order to provide a more complete picture about the genetic differentiation of the two named subspecies of Ceratotherium simum.

INDIAN RHINO

The greater Indian rhinoceros has one named subspecies. Currently, two populations exist in the wild, one in India in Assam and the other in Nepal. We have studied the chromosomes of a single male Indian rhino and have determined a chromosome number of 82. This is consistent with a previous report in the literature (Wurster, D.H. and Benirschke, K., 1968). We have obtained G- and C-banded preparations from the single individual and hope to analyze additional samples.

CONCLUSIONS

Additional genetic studies of all extant rhino taxa are clearly indicated with priority allocated to investigations of of black and Sumatran rhinos. Chromosomal analysis has been shown to be an important aspect of the genetic comparisons following the findings derived from the single animal of Zimbabwe origin held in the Los Angeles Zoo. Additional samples urgently need to be collected for chromosomal, electrophoretic and mitochondrial DNA analyses. At the SSP/AERSG workshop held October, 1986 in Cincinnati, OH, protocols were

developed for sample collection by Dr. Eric Miller of the St. Louis Zoo. Genetic studies of rhinos are currently being undertaken by our group at the San Diego Zoo and in the laboratory of Dr. Don Melnick, Department of Anthropology, Columbia University, NY. The findings of these continuing investigations may significantly impact conservation management plans for the endangered rhinoceroses and, for this reason alone should be expedited.

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Molecular Evolution in Living Species of Rhinoceros:

Implications for Conservation

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Introduction

Molecular genetic techniques provide valuable new tools for understanding patterns of biodiversity and elucidation of evolutionary events. These patterns have the potential to aid in the design of effective management programs for endangered species. The accurate identification of distinct taxa is crucial for conservation, both *in situ* and through captive management (Avise 1989). Groups that represent observable evolutionary events, recently termed "evolutionarily significant units" (ESUs) (Ryder 1986), comprise logical entities on which to focus our conservation efforts. Molecular genetic data, combined with more traditional information on morphology, ecology, and behavior, should be used to establish ESUs within the *Rhinocerotidae*.

Identifying ESUs is essentially a systematics question at a fine level of resolution. However, in order to adequately define these units, it is frequently neccessary to address higher level relationships with similar genetic data to that which will be used to resolve this lower level. A higher level phylogeny provides an important context in which to view lower level data sets and suggests which molecular techniques and genome regions are appropriate for subspecific analysis.

We present here genetic data that address the relationships among populations and species of living rhinos. Our goal was to apply the most powerful techniques currently available to resolve both the higher order phylogenetics of the family *Rhinocerotidae*, and the relationships among subspecies and populations within individual rhino species. With regard to the latter goal, we were especially interested in determining whether the named subspecies of the black rhino, *Diceros bicornis*, are genetically distinct. Finally, we present a preliminary report of a

new technique currently being applied to address similar issues concerning the Sumatran rhino.

Higher level phylogenetics

The family *Rhinocerotidae* is comprised of four living genera (*Ceratotherium, Diceros, Dicerorhinus,* and *Rhinoceros*). Three genera are represented by a single species while the genus *Rhinoceros* is represented by two species. The relationships of these genera have proven controversial (Groves 1983). In order to construct a phylogeny for living rhinos, we sequenced genes encoded in the mitochondrial genome. Such DNA sequence data provides a large number of characters for phylogenetic reconstruction, and the rapidly evolving mitochondrial genome is especially useful for resolving relationships among closely related species and genera (Brown 1985).

In this study, 445 bases of 12S ribosomal and 16S ribosomal mitochondrial genes were sequenced for five taxa, Sumatran rhino *Dicerorhinus sumatrensis*, white rhino *Ceratotherium simum*, black rhino *Diceros bicornis*, Indian rhino *Rhinoceros unicornis*, and Malayan tapir *Tapirus indicus* (Table 1). Sequences of templates constructed by unbalanced polymerase chain reaction (PCR) with universal vertebrate primers (Kocher et al., 1989, Palumbi pers. com.) were obtained by the dideoxy method of sequencing (Gyllensten and Erlich 1988). Samples were sequenced at least twice from independent amplifications, and all taxa were represented by more than one individual. Sequences were aligned by eye, and were analyzed using PAUP version V.3 (Swofford 1990) for the Macintosh. All trees were rooted with the domestic cow mitochondrial sequence (Anderson et al., 1987).

A single most parsimonious tree was recovered (Figure 1). (An identical tree was recovered when transversions were weighted 9:1 to test the effect of transition/transversion bias (Hixson and Brown, 1986)). A bootstrap analysis (Swofford 1990) with branch-and-bound search was performed yielding 81% and 89% replicates for the Sumatran rhino/Indian rhino node and the black rhino/white rhino node respectively (Figure 1).

Our tree based on sequence data is identical to that generated from DNA/DNA hybridization data (Ryder, George, Benviniste, unpublished) as well as to that of Groves based on morphological characters (Groves 1983). Agreement with these two independent data sets increases our confidence in the topology of Figure 1. Percent sequence divergence between taxa was calculated to provide an estimate of genetic distance (Table 2).

Genetic Distinctness of Black Rhino Subspecies

Higher order phylogenetics provide an important framework for interpreting surveys of intraspecific genetic variability and identification of ESUs. Our next goal was to assess the relationships among populations of a single species. If subspecies or populations have been isolated from each other for substantial periods of time, the best management strategy would likely be to conserve such groups as separate ESUs. Such populations may have important adaptations to local environments which would be lost through interbreeding. Genetically distinct populations or subspecies might also be vulnerable to outbreeding depression if managed as a single unit in captivity.

As recently as 100 years ago, the black rhino (*Diceros bicornis*) ranged through much of sub-Saharan Africa, and populations numbered in the hundreds of thousands. Animals from different regions were observed to have slight variations in morphological characters such as size of horn. Whether these polytypic characters represented population subdivision or simply intrapopulation variation was not clear. The most widely accepted classification, that of Groves (1967), recognized seven distinct subspecies. Today, with fewer than 4,000 animals remaining in the wild, four of the seven named subspecies are extinct or nearly extinct. Several questions arise from this situation. Do the three remaining subspecies, *D. b. bicornis* (found in Namibia), *D. b. michaeli*, (found in Kenya and Tanzania), and *D. b. minor* (found in Zimbabwe and South Africa) represent distinct ESUs? Is there evidence of genetic or ecological distinctions that would support separate conservation of the remaining populations? Should those in captive management avoid breeding animals that originated from different regions?

To address such issues of intraspecific population structure of black rhinos, we surveyed restriction fragment length polymorphisms (RFLPs) of the mitochondrial genome of animals from three countries, Kenya, Zimbabwe, and South Africa (Ashley et al., 1990). These animals represented two of the remaining named subspecies, *D. b. michaeli* and *D. b. minor*. Although encoding just a tiny fraction of an organism's genetic material, there are several reasons why the mitochondrial genome should reveal population subdivision, if it exists. As mentioned above, it has a rapid rate of evolution, reportedly 5-10 times that of single-copy nuclear genes (Brown 1985). Therefore, if genetic differences did exist between the subspecies, they would likely be seen in the mitochondrial genome to a greater extent than in the nuclear genome. Furthermore, a growing number of empirical

studies report that populations are often subdivided for mitochondrial genes in the absence of nuclear genetic subdivision (Avise 1987). This pattern is most likely determined by the transmission genetics of mitochondrial genes as well as the dispersal patterns in many species. Since mitochondrial DNA is clonally transmitted through maternal lineages, the effective population size for the mitochondrial genome will differ from that of the nuclear genome, and the distribution of variability may also differ. In general, intrademic variation will be lower and interdemic divergence will be higher for mitochondiral genes compared to nuclear genes (Avise 1987). These differences will be magnified if there is preferential dispersal by males, because migrating males will transmit nuclear genes but not mitochondrial genes. Because of these considerations, if representatives of black rhino subspecies had divergent mtDNAs, this would warrant further studies of genetic differentiation. If the black rhino mtDNA showed little or no genetic differentiation, this would be strong evidence that genetic exchanges had occurred recently among the populations, and that the named subspecies did not warrant status as separate ESUs for conservation purposes.

We collected blood samples from 11 *D. b. michaeli* from Kenya, 11 *D. b. minor* from Zimbabwe, and 1 *D. b. minor* from South Africa. Total DNA was extracted from either white blood cells or buffy coats by standard procedures (Ashley et al., 1990). DNA samples were digested with 11 restriction enzymes having 5 or 6 base pair recognition sites. Restriction fragments were separated by electrophoresis in 1% agarose gels and transferred to nylon membranes by alkaline blotting (Reed and Mann 1985). Membranes were probed with ^{32}P-labelled mtDNA purified from frozen organ tissue of three black rhinos. After high-stringency washes, membranes were exposed to Kodak XAR film. The purified mtDNA from three animals,

one from each population sampled, were digested with an additional seven restriction enzymes. Restriction fragments were directly labelled with ^{32}P and separated on 1% agarose or 3.5% polyacrylamide gels. For these three samples, approximately 630 base pairs were surveyed per individual.

Our results indicate that intraspecific mtDNA variation is extremely low in black rhinos. Only three out of eighteen restriction enzymes, *Bcl,I Hinf*I and *Taq*I, revealed RFLP patterns that differed between the named subspecies. For each variable enzyme, differences appeared to be due to a single loss or gain of a restriction site. Average mtDNA differentiation was 0.29% (Upholt 1977) between subspecies. When comparing *D. b. minor* from different regions, the most common mitochondrial genotype in Zimbabwe was indistinguishable from that found in the animal of South African origin. We feel that these results indicate that the populations surveyed are genetically very similar and that present populations of black rhino shared a common ancestor quite recently. In addition, mitochondrial RFLP data on *D. b. bicornis* showed similar results (Harley, this volume). There is no indication from the mitochondrial genome that the designated subspecies warrant consideration as separate ESUs for conservation and management. However, cytogenetic data may indicate further investigation into heterochromatin variation (Ryder unpublished).

A New Technique and its Application to Sumatran Rhino Conservation

Subspecific taxonomy may be of even greater importance in designing a conservation program for the Sumatran rhino (*Dicerorhinus sumatresis*). Extant populations of Sumatran rhinos currently are distributed in three disjunct populations (Borneo, Sumatra and peninsular Malaysia) that are isolated from each

other by open ocean. There likely has been no opportunity for genetic exchange between these populations for thousands of years, at least since the glacial episodes of the Pleistocene caused a lowering of sea levels and exposure of the Sunda Shelf. There exists a distinct possibility that this isolation has resulted in genetic differences that should be preserved by managing the populations separately. However, if the separate populations are found to be genetically similar, it would be much easier to manage the small number of individuals in captivity as a single population in order to maintain maximum genetic variability and effective population size.

A similar approach to that used for the black rhino, a survey of variability in the mitochondrial genome, would be a valid strategy for the Sumatran rhino. Unfortunately, it has been difficult to obtain samples such as blood and organ tissue that can be analyzed by these methods. We have, however, obtained hair samples from 14 animals, and have begun an analysis of Sumatran rhino genetics from this non-traditional sample material. The ability to conduct genetic studies on such material would greatly enhance the applicability of these approaches not only to rhino conservation, but to the conservation of many other highly endangered species where sampling is problematic.

DNA was isolated from these samples by first grinding the hair in liquid nitrogen with mortar and pestle and then isolating DNA by standard techniques (Caccone et al, 1987). Sequences were amplified by PCR technology and compared with known rhino sequences to determine that this technique had resulted in the isolation and amplification of DNA from the animal and not a contaminant. Having demonstrated that we could amplify Sumatran rhino DNA from hair samples, we

proceeded to attempt a new procedure.

A new technique, RAPD, (ramdomly amplified polymorphic DNA markers) (Williams et al., 1990) can potentially provide an additional set of molecular characters with which to identify evolutionarily significant units. Since it relies on amplification by PCR, minute quantities of DNA such as that obtained from hair follicle cells can provide an appropriate sample. Genomic sequences are amplified with randomly chosen 10 base oligonucleotides and the resultant DNA fragments are compared between individual animals. This technique has revealed polymorphisms that are inherited in a Mendelian fashion (Williams et al., 1990).

Aside from the opportunity to utilize nontraditional material for genetic studies, there are several other advantages of the RAPD technique over other approaches such as mtDNA RFLP surveys and DNA fingerprinting. Because amplified sequences are directly visualized on agarose gels with ethidium bromide, the use of radio-labeled probes and Southern blotting are unnecessary. Additionally, arbitrarily chosen primers survey for sequences/characters throughout the entire genome.

Rhino samples were amplified in a Perkin Elmer Cetus DNA thermal cycler. Reaction volumes were a total of 25 µl containing 100 ng DNA, 0.2 mM primer, 2.5 µl Cetus Gene Amp buffer, 0.1 mM dNTPs, and 1 unit Taq polymerase. Each cycle of the polymerase chain reaction consisted of denaturation for 1 minute at 94°, hybridization for 1 minute at 35°, and extension for 2 minutes at 72°. This cycle was repeated 40 times. The entire amplified product was run on 2% agarose gels and stained with ethidium bromide. Species specific markers and polymorphic bands

were identified (Figure 2). Currently, additional oligonucleotide primers are being sampled to identify additional polymorphisms which will be tested for covariance. These data, as well as mtDNA data will be used to make future recommendations about Sumatran rhino ESUs.

Discussion

Presented here are molecular data that provide new information on the relationships of species and subspecies of rhinos. We are taking advantage of current molecular biology to address issues in conservation that have been very difficult to answer in the past. While the role of molecular genetics in conservation is still being defined, we strongly feel that the large number of genetic characters generated by these new techniques, along with the application of new methods of data analysis, provide an increasingly accurate picture of the patterns of biodiversity that we are committed to preserving.

As we continue to add data and make strong arguments for particular relationships, it will be up to managers to implement policies that will reflect our new understanding of ESUs. Political and economic issues surely will affect these decisions. In addition, biological concerns other than genetics will need to be addressed in specific circumstances. For example, managers of black rhino populations are faced with different concerns regarding *in situ* conservation versus captive management. The genetic data support the notion that subspecific designations in black rhinos do not reflect genetically distinct taxonomic groups. Concerns about outbreeding depression and loss of unique adaptive gene complexes are therefore probably unfounded. However, in managing wild populations, environmental factors such as unique social interaction and exposure to different

parasites and diseases must be considered if translocation of animals between distinct environments is planned (R. DuToit pers. comm.). Fortunately, preliminary information suggests that moving highland black rhinos to lowland areas in Kenya has not yet revealed any such problems (R. Brett, pers. comm.). Also, research on nutritional requirements has shown black rhinos to be generalists that should do well in a translocation situation (E. Dierenfeld pers. comm.). At this stage, *in situ* conservation can proceed by affording greater protection to designated populations while avoiding the problems associated with translocation. Moving animals is most likely to be employed for reintroducing rhinos to areas where they have been extirpated and is more likely to be successful than population reinforcement (W. Conway, pers. comm.).

While it is necessary to consider many factors beside population genetic structure in the field, our captive management strategy may be different. In captivity environmental factors are controlled so we can consider captive black rhinos as a single population designed to maximize founder contribution and maintenance of genetic variation. Since we will never have truly large populations in captivity, it will greatly increase our chances of success if we avoid unnecessary splitting. Reintroductions are more likely to be successful from "genetically healthy" animals than by attempting to maintain groups from specific localities in numbers that will likely be affected by loss of genetic variation.

We are currently continuing work on the molecular genetics of rhinos, and in the near future we will make further recommendations. Our primary goal is for this information to contribute to the preservation of this highly endangered

group.

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Table 1. Aligned sequences with the reference cow *Bos taurus* sequence (Anderson et al., 1982). Periods signify nucleotide identity with reference sequence. Dashes represent positions where gaps were introduced. (sr=*Dicerorhinus sumatrensis*, wr=*Ceratotherium simum*, br=*Diceros bicornis*, ir=*Rhinoceros unicornis*, ta=*Tapirus indicus*)

```
16S                                                  [50]
cow  ctgtctctta cttccaatca gtgaaattga ccttcccgtg aagaggcggg
sr   .......... .c.t...... .......... ...c...... ..........
wr   .......... ...t...... .......... .......... ..........
br   .......... ...t...... .......... .......... ..........
ir   ....t..... ...t...... .......... ...c...... ..........
tap  .......... ...t...... .......... .......... ..........

                                                     [102]
cow  aat-gcacaaa- taagacgaga agaccctatg gagctttaac taaccaaccc
sr   g..-.a..g..a c......... .......... ......c..t ....t..tt.
wr   ...a......-- .......... .......... .........t ....t..tt.
br   ...ga..c.c.a .......... .......... .........t ..g.t..tt.
ir   g..-.a------ .......... .......... .........t c...t..tt.
tap  ...-.a.....a .......... .......... .........t ....t..tt.

                                                     [153]
cow  aaagagaa--- tagatttaa cca----ttaagg aataacaac aatctccat
sr   .c.a.a.-caa a.cc..c.. ..tatatc..... ........a .t.tcgat.
wr   .c.a.a..taa a.tc.c... ..cacatcc.g.. g.......a .c.t.gac.
```

```
br   .c.a.a.-taa a.tc.c... ..tacatcc.... g.......a .c.t.gac.

ir   .c.a.a--taa a.tc..c.. ..tacata..... g.......a .t...aac.

tap  .cta.a..taa actt.c-.. ..tacc----... t......ga .c.t.aac.


                                                     [202]

cow  gagttggta     gtttcggttg gggtgacctc ggagaataaa aaatcctccg

sr   ..a..a.c.     a......... .......... ......c... .c.a......

wr   ..a..a...     a......... .......... ......c..g cc.a......

br   ..a.(c,t)a.c. a......... .......... ......c--- cc.a......

ir   ..a..aacg     a......... .......... ......c.c. .c.a......

tap  ..a...ac.     a......... .......... .......... .c.a..c...


12S                                                  [253]

cow  tagcaacagc ttaaaactca aaggacttgg- cggtgcttta tatccttcta

sr   ......t... c......... ........... .......... .....cc...

wr   .......... c......... ..........a .a........ .....cc...

br   .......... .......... ..a........ .......... .....cc...

ir   .......... .......... ........... .......... .....cc...

tap  .......... c...----.. ........... .......... ..c.......


                                                     [303]

cow  gaggagcctg ttctataatc gataaacccc gataaacctc accaattctt

sr   .......... ...c....c. .......... .........t .....cc...

wr   .......... ...c....c. .......... ........c. .....cc...

br   .......... ...c...... .......... ........ct ....gcc...

ir   .......... ...c....c. .......... .........t ....gcc...
```

```
tap  .......... ...cg..... .......... .........t ....ccc...

                                                    [353]
cow  gctaatacag tctatatacc gccatcttca gcaaacccta a-aaaggaaa
sr   ......t... c......... .......... .......... .a.......c
wr   ......t... c......... .......... .......... .-.......c
br   ......t... c......... .......... .......... .-c......c
ir   ......t... c......... .......... ..c....... .-.......c
tap  ..c....... c......... .......... .......... .-........

                                                    [403]
cow  aaaagtaagc gtaattatga tacataaaaa cgttaggtca aggtgtaacc
sr   t......... ac..g.---. g......... .......... .......g.t
wr   t......... ac..g...a. a......... .......... .......g.t
br   t......... ac..g...a. g......... .......... .......g.t
ir   c......... ac..g...a. g......... .......... .......g.t
tap  c......... ac..gc---- --........ .......... .......g.t

                                         [445]
cow  tatgaaatgg gaagaaatgg gctacattct ctacaccaag ag
sr   ....gg.... ag........ ........t. ....ta.... .a
wr   ....gg.... ag........ ........t. ...tttt... .a
br   ....gg.... ag........ ........t. ....t.t... .a
ir   ....gg.... ag........ ........t. ....tt.... .a
tap  .....gg... ag........ ........t. ...-...... .a
```

Table 2. Percent sequence divergence of 445 bases of 12S and 16S ribosomal mitochodrial genes. (cow=*Bos taurus*, sr=*Dicerorhinus sumatrensis*, wr=*Ceratotherium simum*, br=*Diceros bicornis*, ir=*Rhinoceros unicornis*, ta=*Tapirus indicus*)

	sr	wr	br	ir	ta
Cow	15.9	15.7	16.4	15.7	13.7
sr		6.9	6.9	4.7	8.5
wr			4.3	6.3	8.3
br				5.8	8.1
ir					7.9

FIGURE 1

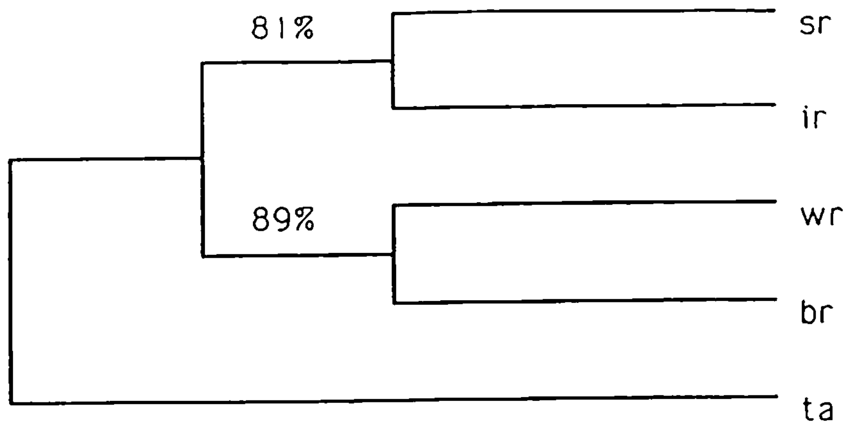

Figure 1. A single most parsimonious tree was derived from the sequence data when analyzed using PAUP V.3 (Swofford 1990). A bootstrap analysis with branch and bound search yielded 81% and 89% replicates for the sr/ir node and the wr/br node respectively. (sr=*Dicerorhinus sumatrensis*, wr=*Ceratotherium simum*, br=*Diceros bicornis*, ir=*Rhinoceros unicornis*, ta=*Tapirus indicus*).

ALLOZYME VARIATION AND DIFFERENTIATION IN AFRICAN AND INDIAN RHINOCEROSES

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Merenlender *et al*.: Allozyme variation in rhinoceroses

ABSTRACT

This preliminary study focuses on variation at 25-31 allozymic loci in African and Asian rhinoceroses. Four taxa in three genera are examined: African *Ceratotherium simum simum* (northern white rhinoceros), *C. s. cottoni* (southern white rhinoceros), *Diceros bicornis* (black rhinoceros), and Indian *Rhinoceros unicornis*. Extremely small amounts of intraspecific variation were observed in sample sizes of 2-10 presumably unrelated individuals per taxon: $\bar{P} = 0.00$-0.10, $\bar{H} = 0.00$-0.02. Demographic bottlenecks and sampling errors are discussed 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 reported for other mammal subspecies. The mean D value between the two African genera of 0.32 ± 0.11 is also less than expected given the >7 million year divergence time suggested by the fossil record. It may be that rhinoceroses are evolving more slowly at the structural gene loci than some other mammal groups: the estimate of $\bar{D} = 1.05 \pm 0.24$ for the African-Indian split supports this idea as the lineage diverged at least 26 m.y.a. These results contribute to the currently available scientific information on which management decisions should be based to save the endangered rhinoceroses.

INTRODUCTION

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 here report 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 between 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? Multilocus genetic distances will thus be used to construct phenetic trees, define extant evolutionary significant units, and elucidate their phylogenetic relationships[13,61].

Rhinoceroses, today 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 30-35 m.y. to the Oligocene[26] (Figure 1A). The fossil record indicates living African and Asian rhinoceroses arose separately from the Old World Caenopus group, a group of genera which 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 m.y. 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 m.y.a.[26] For comparison, the mean species duration of European Palio-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 also very poorly defined; Lydekker's[31] original description was based on only 3 skulls. Groves[21] argued that subspecific status was not warranted and more recent studies by Hillman-Smith[23,24] and du Toit (unpublished) fail to resolve this question. If in fact these two are subspecies we 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 Republic of Africa), D.b. ladoensis (northern Kenya and Sudan), D. b. chobiensis (Angola), and D. b. brucii (Ethiopia and Somalia). However, Groves 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 based on recent unpublished studies of skull morphology using a larger sample size, which revealed a geographical cline not worthy of

subspecies distinctions. Consistent with this recent fossil data are the results of a mitochondrial DNA study completed on the remaining black rhinoceros species. Ashley et al.[4] showed no significant differences for 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 electrophoretic divergence, which is necessary to evaluate the subspecies status genetically. For both black rhinoceros and white rhinoceros, it appears that the named subspecies are of questionable utility in defining evolutionary significant units for conservation management.

MATERIALS AND METHODS

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

Organ tissues were frozen following necropsy. Blood sample 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 were homogenized with a glass rod in 0.5 ml of distilled water. The homogenate was then centrifuged for 2

minutes to obtain an aqueous protein extract. Red blood cells were lysed with distilled water (1:1 dilution) and plasma was used without dilution.

Standard horizontal starch gel electrophoresis was used to resolve allozyme patterns[45,48]. Gels were made with 12.5% Sigma starch (Sigma Chemical Co., St. Louis). Samples were absorbed onto 10 x 3 mm tabs of chromatography paper and inserted into the gel. The specific enzymes examined and the buffer systems used to resolve them (Table II) generally follow Harris and Hopkinson[22]. Isozymes in multilocus systems were numbered in order of decreasing anodal mobility. Using all of the available data we calculated an average number of alleles per locus (A), percent 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 the above statistical analyses were performed with the BIOSYS-1 computer program[54].

RESULTS

Eighteen enzyme and protein systems were examined and genetically interpretable results were obtained for 31 presumptive loci for the three genera (Table III). We suspect that AB-1 is albumin and AB-2 or AB-3 is transferrin. Other proteins were examined but due to poor resolution they were not genetically interpretable; these include ADA (E.C.3.5.4.4), CAT (1.11.1.6), and DIA (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 out of all the loci surveyed in the black and

white rhinoceroses. No variation was detected in the three Indian rhinoceroses. Table IV includes the allele frequencies for the polymorphic loci examined.

Figure 1B shows the phenogram based on multi-locus genetic distances between the four taxa, using all available data. The genetic distance between Indian R. unicornis and African black D. bicornis is $\bar{D} = 0.89 \pm 0.21$; between R. unicornis and African white (C. s. simum and C. s. cottoni) the $\bar{D} = 1.05 \pm 0.24$; 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) yielded an insignificantly small genetic distance ($\bar{D} = 0.005$).

DISCUSSION

Genetic Variation

Typically, mammals are genetically variable. A review[40] of previous studies of allozyme variation in 184 species found $\bar{P} = 0.191$ and $\bar{H} = 0.0416$. Large mammals are generally less variable than 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 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 leocooryx, Pere David's deer, Elaphurus davidianus[64], and Weddell seals, 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 higher, and the small red fox, Vulpes vulpes[49], lower levels 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 2-10 unrelated individuals may constrain the amount of detectable variation. From simulations run with 32 sets of published data, Gorman and Renzi[19] found reducing larger sample sizes (mean of 24) to 2 individuals yielded estimates of heterozygosity that differed from that based on the larger sample by an average of only 1.72%. Therefore, this small sample size can provide a an adequate preliminary estimate of intraspecific variability. The second sampling problem involves bias in the type of loci examined as certain loci mutate more rapidly than others. However, we examined a large number of loci representing different classes of enzymes, including the typically variable peptidases and esterases. Although our samples are lamentably small the low genetic variabilities observed probably represent an accurate picture of the level of genetic variability remaining in the white rhinoceros, because this study includes a substantial number of white rhinoceroses from three different locations. On the other hand, recent results from a more thorough study of the Indian rhinoceros reveals higher levels of variability (H - 9.9%) than we found in our extremely limited sample of Indian rhinoceroses[16]. Our limited samples are from Assam, India which is a different population than those examined by Dinerstein and McCracken[16]; this could contribute to the observed differences.

Two suggested hypotheses may account for low levels of genetic variation. An early selectionist hypothesis predicts levels of genetic variability will be related to the grain of the environment. Selander and Kaufman[47] argued that large, highly mobile animals tend to encounter environmental conditions which are fine-grained and will be selected for a single general purpose genotype which 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 counter examples of generalists which 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 the population is reduced to, 3) 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 the rhinoceroses. The African southern white rhinoceros was decimated in the nineteenth century but has recovered from about 100 individuals (probably not as few as ten[28]) in 1920 to over 4,600 today[12,59]. Similarly, the African northern white rhinoceros, C. s. cottoni , lost 95% of its population since 1980[60]; there are now 40 animals in captivity and the wild[25]. Although, the fossil record suggests rhinoceroses were wide spread 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 the level of polymorphism was low before the recent bottlenecks in the two groups. This could be do 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[32,16]. African black rhinoceros numbers fell from 60,000 to 3,800 in the last seventeen years alone; the remaining 70 to 100 isolated populations are highly vulnerable[59]. These recent demographic bottlenecks coupled with a possible historical bottlenecks may be sufficient to explain the lack of observed genetic variability today.

Genetic Differentiation

Small sample sizes are probably not significantly affecting 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 between 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 distance between

subspecies (D = 0.23[58]) of mammals. Furthermore, these unexpectedly low genetic distances may be inflated as the populations being 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 taxonomic oversplitting has occurred at the subspecific level. Matthew[33] concluded that if population variability was considered the majority of the then described species would be synonymized. Furthermore, he argued that the unnatural splitting of species, genera and higher taxa resulted in a taxonomy without phylogenetic merit. His criticisms are still valid today as no comparative multivariate analysis of the rhinoceros morphology has yet been published. Rhinoceros taxonomy was established long before the isolation and recognition species concepts were applied to mammals and longer still before the development of the cohesion species concept[55,63]. Current evolutionary and genetic concepts must now be applied to these animals if we are to define evolutionary significant units in time to manage them effectively[46,56]. Clearly electrophoresis is just one component of the information necessary to make decisions regarding species 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 is equivalent to 0.8-6.7 million years[37]. This clock is based on congeneric species differences because of the multitude of available data at this taxonomic level. It is difficult to extrapolate to higher taxonomic levels,

but to provide some comparison with previous data, we will examine whether or not the rhinoceros genera divergence times coincide with the predicted divergence times using these predetermined estimates. As it turns out, such calibrations underestimate the age of the rhinoceros species.

Calibrations of the electrophoretic clock against absolute time and the comparisons of these calibrations across different organisms is possible provided independent paleontological estimates are available on times of speciation. To calibrate the rhinoceros allozyme clock we used fossils, the age of which are generally agreed upon by paleontologists. The first record of coexistance of Ceratotherium and Diceros is from a radiometrically dated 7 m.y. old deposit in Baringo, Kenya (John Berry, Harvard, pers. comm., 1986[6,26]). The two taxa had thus already diverged from their common ancestor 7 m.y.a. Nevertheless, if we let a genetic distance of 0.32 (the D value between C. s. simum and D. bicornis) represent seven m.y. for a rhinoceros molecular clock, a D value of 1.0 will equal 22 m.y. 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 between the African taxa are less than expected because the rate of allelic substitutions is lower in rhinoceroses than in other families of mammals. In fact, the black and white divergence ($\bar{D} = 0.32$) is the same as was found for human-chimpanzee and human-orangoutang ($\bar{D} = 0.3$-0.4)[11]. Humans are known to be evolving at a slower rate than other well studied mammals[66]. This supports the hypothesis that the rhinoceros clock is slower than expected. This slower rate may be do to

a generation time effect as the rhinoceroses have a generation time of approximately 8-10 years, which is longer than 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 m.y.a., 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 m.y. (early Miocene). This is only slightly younger than we would have predicted from the sketchy fossil record but 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 20-30 m.y.a.[37]. Consequently, we will interpret the Indian-African D value as suggesting cladogenesis occurred towards 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 phenomenon. It is not clear, however, if 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 an artifact of the technique employed.

CONCLUSTIONS

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 tolerances as species, each local

population of rhinoceros may not be able to adapt to environmental changes as well as 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 their short-term conservation, when ecological factors are often more important[27], but its long-term effects have yet to be studied. The actual risks of being monomorphic 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 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.

The results reported here 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 of mitochondrial DNA in which a 4% difference between the two white rhinoceros subspecies suggested that they have been isolated for 2 million years[18]. 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 in primates was unexpected because the average rate of divergence is 0.5-1.0% per million years. The limited sample sizes utilized in the study by George et al.[18] did not allow an assessment of intraspecific mtDNA variation in each white rhinoceros subspecies and in other mammals this

ranges from 1-7%[9]. Consequently, discussion of this apparent discrepancy is premature and a second investigation based on more individuals is underway (George, pers. comm., 1987).

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 really genetically distinct from one another then their conservation requires the preservation of viable populations of each taxon. If not, then further research should be conducted to determine whether the geographic races of the species could be pooled in order 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, then relocation of southern white rhinoceroses to areas formerly occupied in the north might be considered to alleviate the ecological effects of the recent extirpation of northern white rhinoceros populations.

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Table I.

Sample[1]		Subspecies	Origin	Source[2]
1		_C. s. cottoni_	Sudan	SD Zoo (WC)[3]
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[4]	Dvur Kralove
7		_C. s. cottoni_	Sudan/S.A.[5]	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	[illegible]
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[6] (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)

[1]Stud book number in parentheses if available
[2]sire/dam stud book numbers if available, ? indicates not recorded, WC = wild caught
[3]SD Zoo = San Diego Zoo, SD WAP = San Diego Wild Animal Park
[4]Zoo born from Sudan sire and Uganda dam
[5]Hybrid from South African sire and Sudan dam
[6]SL Zoo = St. Louis Zoo, LA Zoo = Los Angeles Zoo

Table I I. Loci examined and electrophoretic conditions.

Protein (EC no.)	Locus	Source*	Conditions†
Adenylate kinase (2.7.4.3)	*Ak*	All ex p	AP 6; 80/15/17
Asparate aminotransferase (2.6.1.1)	*Aat*	All ex p & RBC	TC 7; 60/15/18
Esterase (3.1.1.1)	*Est-1*	All ex p & RBC	TrisHCl 8.3/8.6; 15/80/20
Esterase (3.1.1.1)	*Est-2*	All	" "
Esterase (3.1.1.1)	*Est-3*	All ex RBC	" "
Esterase (3.1.1.1)	*Est-4*	p	" "
Esterase (3.1.1.1)	*Est-5*	All	" "
Fumarase (4.2.1.2)	*Fum*	All ex p	" "
General protein (Amido Black)	*AB-1*	All	TrisHCl 8.3/8.6; 15/80/20
General protein (Amido Black)	*AB-2*	p	" "
General protein (Amido Black)	*AB-3*	p	" "
General protein (Amido Black)	*AB-4*	All ex p & RBC	" "
General protein (Amido Black)	*AB-5*	All	" "
Glucose phosphate isomerase (5.3.1.9)	*Gpi*	All	AP 6; 80/15/17
Hemoglobin	*Hb*	All ex p	All
Hexokinase (2.7.1.1)	*Hk*	All ex p	TBE 9; 20/175/18
Isocitrate dehydrogenase (1.1.1.42)	*Idh-1*	All ex p & RBC	AP 6; 80/15/17
Isocitrate dehydrogenase (1.1.1.42)	*Idh-1*	All ex p & 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 ex 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 ex p & 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	TrisHCl 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 ex 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

* Proteins were detected in all tissues or in plasma (p) or red blood cells (RBC) except (ex) as noted.
† Electrophoretic conditions: buffer (pH); voltage/amps/time in hours.

Table III. Allele frequencies for polymorphic loci.

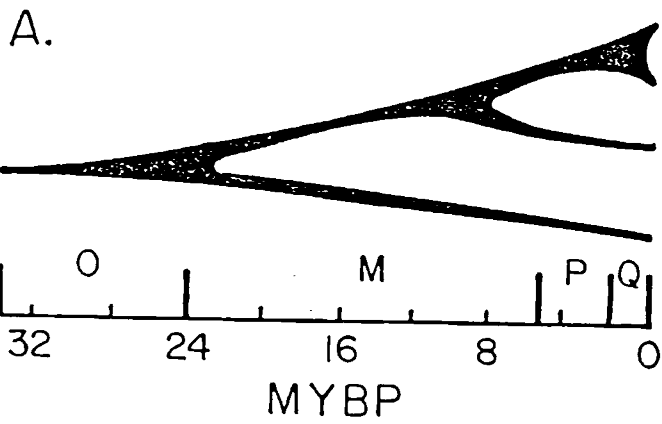
Locus/allele	*D. bicornis*	*C.s. simum*	*C.s. cottoni*	*R. unicornis*
AAT				
(N)	4	8	*	2
A	1.000	0.938		0.000
B	0.000	0.063		1.000
AB-3				
(N)	4	9	7	1
A	0.125	0.389	0.571	1.000
B	0.875	0.611	0.429	0.000
Pgm-2				
(N)	6	11	5	1
A	0.000	0.136	0.000	0.000
B	0.583	0.636	0.500	0.000
C	0.417	0.227	0.000	1.000
D	0.000	0.000	0.500	0.000

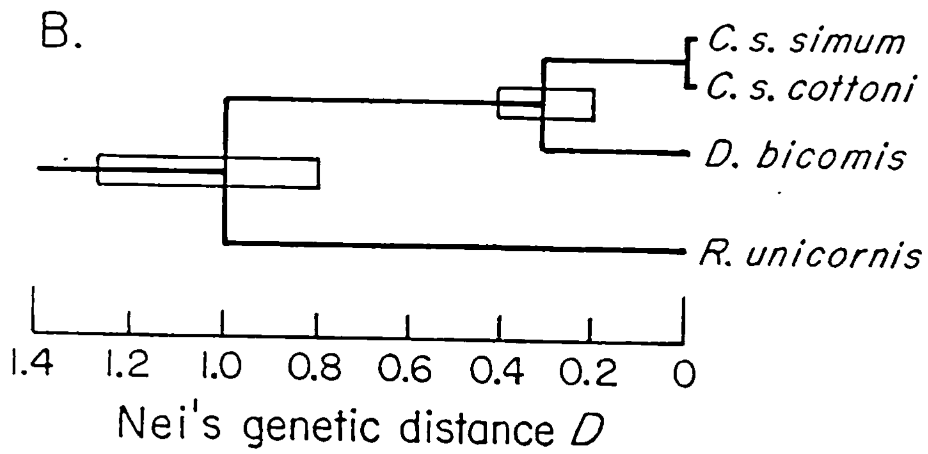
*Missing cell due to lack of organ tissue. The remaining loci found in Table II are monomorphic.

Table IV. Estimates of the average number of alleles per locus, A, percent polymorphic loci, P,* and mean individual heterozygosity, $\bar{H}$.

	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
$\bar{H}$	0.013	0.013	0.019	0.000

FIGURE 1. Relationships among rhinoceros taxa studied. A. Schematic tree based on fossil evidence (refs. in text). B. UPGMA tree based on allozyme data and calibrated around a Ceratotherium-Diceros split at 7 MYBP.


A.
O
M
P
Q
32
24
16
8
0
MYBP


B.
C. s. simum
C. s. cottoni
D. bicomis
R. unicornis
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Nei's genetic distance D