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 HinfI* and *TaqI*, 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

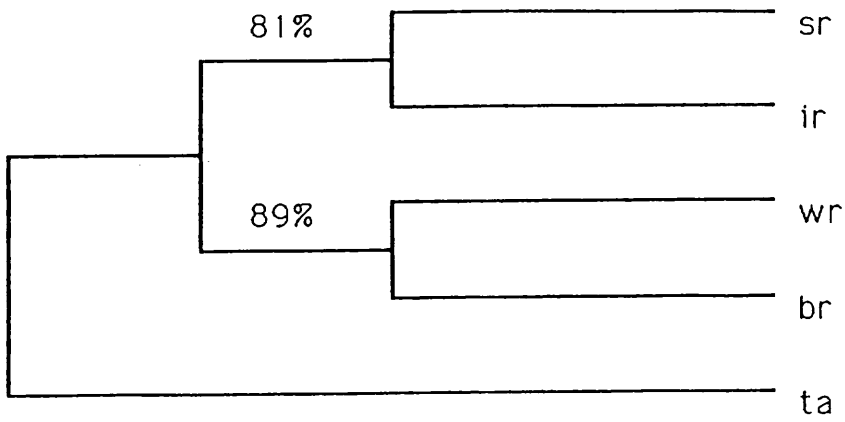

Figure 1. A single most parsimonious tree was derived from the sequence data when analyzed using PAUP V.3 (Swofford 1990). A bootstrap analysis with branch and bound search yielded 81% and 89% replicates for the sr/ir node and the wr/br node respectively. (sr=*Dicerorhinus sumatrensis*, wr=*Ceratotherium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