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Conservation genetics of the black rhinoceros (*Diceros bicornis*)

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Abstract

We analysed genetic variation in mitochondrial DNA (mtDNA) control region in the *Diceros bicornis minor* and *D. b. michaeli* animals at the Western Plains Zoo, which form part of an international *ex-situ* breeding program. Six of the nine *D. b. minor* animals were wild-caught from Zimbabwe during the 1990s, and our study revealed five distinct mtDNA haplotypes, and a haplotype diversity of 0.86 in the colony. Phylogenetic relationships between mtDNA haplotypes analyzed using the neighbor joining method reveal that for the small sample available, *D. b. minor* and *D. b. michaeli* are reciprocally monophyletic and represent separate ancestral lineages. Nucleotide divergence between the black and and white rhinoceros (*Ceratotherium simum*) was 14.0%, and nucleotide divergence between the *D. b. minor* and the *D. b. michaeli* subspecies was 2.6%. This suggests a divergence time for the two black rhinoceros subspecies of between 0.93 MY and 1.3 MY.

Introduction

The black rhinoceros *Diceros bicornis* once ranged throughout most of sub-Saharan Africa. Only one hundred years ago, hundreds of thousands of black rhinoceros were widely distributed in central, eastern and southern Africa (Western and Vigne 1985). However, rhinoceros populations have been decimated by poaching (Leader-Williams 1988), and although 65,000 animals existed in 1970 (du Toit et al. 1987), only a few thousand animals currently remain in the wild.

A key component of black rhinoceros conservation both *in-situ* and *ex-situ* is the identification and management of evolutionarily significant units (Ryder et al. 1986; Moritz 1994). The majority of extant rhinoceroses belong to two of the seven recognised *D. bicornis* subspecies: *D. b. michaeli*, found in Kenya and Tanzania, and *D. b. minor*, which ranges from Kenya to South Africa (Natal) (Groves 1967). In captivity, *D. b. minor* and *D. b. michaeli* are maintained as separate breeding entities known as the southern and eastern rhinoceros populations respectively (du Toit 1987; Ryder 1993). The subspecies are maintained separately to preserve potentially locally adapted traits and to avoid outbreeding depression, which could arise if they were sufficiently genetically divergent (Templeton 1986).

However, mtDNA RFLP analysis has suggested a common ancestry between the *D. b. minor* and *D. b. michaeli* subspecies dating back no more than 100,000 years (Ashley et al. 1990). Furthermore, managing remaining black rhinoceros populations as a single evolutionarily significant unit may facilitate conservation of genetic biodiversity better, since small isolated populations face an increased threat of extinction due to genetic factors (Frankham 1995, 1998). Nevertheless, estimates of divergence time between the black rhinoceros subspecies should be interpreted cautiously, particularly given the limited power of restriction enzymes to detect intraspecific variation in mtDNA (Ashley et al. 1990; O'Ryan and Harley 1993; O'Ryan et al. 1994). To address this issue further, we reanalysed the evolutionary relationship between *D. b.*

minor and *D. b. michaeli* subspecies based on DNA sequence of the hypervariable domain of the mtDNA control region from animals at the Western Plains Zoo colony in Australia. The mtDNA control region was chosen due to its high rate of evolution and proven utility in phylogenetic studies (e.g. Hoelzel et al. 1991; Luikart and Allendorf 1996; Pope et al. 1996; Taberlet 1996; O'Corry-Crowe et al. 1997; Wooding and Ward 1997).

We also quantified mtDNA diversity in the colony at the Western Plains Zoo, which received animals threatened by poaching from a small population in Chete National Park, Zimbabwe during the 1990s (Kelly et al. 1995). While allozyme studies have demonstrated that at least some wild black rhinoceros populations have maintained high levels of gene diversity to date (Swart et al. 1994; Swart and Ferguson 1996), mtDNA variation is reported to be limited (Ashley et al. 1990; O'Ryan and Harley 1993; O'Ryan et al. 1994). In contrast to the latter reports, we detect high levels of variation in mtDNA control region, which will be useful for monitoring genetic biodiversity and for tracing maternal gene flow in rhinoceroses.

Material and methods

Samples of blood, faeces, or biopsy material were obtained from the animals listed in Table 1. DNA was extracted from blood and tissue biopsy following proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation (Sambrook et al. 1989). Faecal material was extracted according to method 2 of Hopwood et al. (1996). Alternatively, 50–100 mg of frozen faecal material was added to 500 μl of TRIZOL^{TM} (Gibco BRL), mixed and incubated at room temperature for 30 min. The mixture was centrifuged at 5,000 g for two minutes and the supernatant was purified by phenol/chloroform extraction and ethanol precipitation.

The 450 bp 5′ end of the control region (d-loop) was amplified from total genomic DNA and sequenced using the primers mt15996L (5′-TCCA-CCATCAGCACCCAAAGC-3′) (Campbell et al. 1995), located in the tRNA^{Pro} gene flanking the control region, and mt16502H (5′-TTTGATGGCCC-TGAAGTAAGAACCA-3′), located in the central conserved domain of the control region (Moro et al. 1998) as described (Houlden et al. 1999). Cycle sequencing was performed using the ABI PRISM^{TM} BigDye Kit (Perkin-Elmer) with AmpliTaq DNA polymerase, FS (Perkin-Elmer). Products were electrophoresed on an Applied Biosystems Model 310 DNA sequencer (Foster City, California). Sequence was evaluated using Factura^{TM} 1.2.0 (Applied Biosystems) and a consensus derived in AutoAssembler^{TM} 1.3.0 (Applied Biosystems). Sequences have been deposited in GenBank/EBML (accession numbers AF187825-AF187839). Sequence data were compared to published reports for mtDNA from *D. b. michaeli* (Jama et al. 1993) and *Rhinoceros unicornis* (greater Indian rhinoceros) (Xu et al. 1996). Distance values between pair-wise comparisons of haplotype sequences were calculated by the method of Tamura (1992), using MEGA (Kumar et al. 1993). Estimates of haplotype and nucleotide diversity within populations (excluding the offspring Kusomona), and nucleotide divergence were calculated as described by Nei (1987) using REAP 4.0. (McElroy et al. 1991).

Results

Mitochondrial DNA variation within the D. b. minor *and* D. b. michaeli *subspecies*

DNA sequence of a 488 bp segment of the mtDNA control region revealed five distinct mtDNA haplotypes present in nine *D. b. minor* animals. *Kwanzaa*, *Ibala*, and *Utahwedande* each had a unique haplotype (Table 2). *Kalungwizi* (and her captive-born offspring *Kusomona*), *Musina Ponga* and *Chitundumuseremus* shared a common mtDNA haplotype, as did *Pepe Kalle* and *Dongajumu* (Table 2). Thus, the *D. b. minor* colony contains at least five distinct maternal lineages, and has a haplotype diversity of 0.86 (n = 8). Each of the two *D. b. michaeli* individuals at Western Plains zoo had unique haplotypes, which were distinct from previously published sequence of *D. b. michaeli* (Jama et al. 1993) (Table 2). However the three white rhinoceroses at Western Plains Zoo, which originated from Umfolozi Game Reserve, all shared a common haploytpe (Table 2).

D. b. minor control region sequences varied by not more than four substitutions between individuals, giving 99% sequence identity (Table 2). Sequence variation was detected as single base pair substitutions at positions 112, 203, 205, 251, 256, and 290 (Table 2). Nucleotide diversity (π, Nei 1987) was 0.43% for *D. b. minor* (n = 8).

Table 1. Rhinoceros species and individuals analysed for DNA variation

Species	Individual	Sex	Studbook no.	Origin
D. b. minor	Kwanzaa	Male	463	Captive born, Milwaukee Zoo
	Ibala	Male	485	Captive born, San Diego Zoo
	Musina Pongo	Female	494	Chete National Park, Zimbabwe
	Chitundumuseremus	Female	496	Chete National Park, Zimbabwe
	Pepe Kalle	Female	498	Chete National Park, Zimbabwe
	Kalungwizi	Female	499	Chete National Park, Zimbabwe
	Kusomona	Male	609	Captive born, Western Plains Zoo
	Utahwedande	Female	500	Chete National Park, Zimbabwe
	Dongajumu	Female	502	Chete National Park, Zimbabwe
D. b. michaeli	Mwaniki	Male	302	Captive born, Cincinnati Zoo
	Taronga	Female	197	Captive born, Taronga Zoo
Ceratotherium simum simum	Thomas	Male	195	Umfolozi Game Reserve, South Africa
	Alexander	Female	614	Umfolozi Game Reserve, South Africa
	Nicol	Female	196	Umfolozi Game Reserve, South Africa
	Unknown	Unknown	Unknown	London Zoological Gardens

The sample identified only as *C. s. simum* was donated by Kelly Ewen and Helen McPartlan

Table 2. MtDNA d-loop variation in Rhinoceros

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Individual          Nucleotide position
                                  1111111111111111111111111222222222222222222222222222223333333344444
                    1122333344556778999000111122233456888889999990000001112233333444445566778901245592235 57
                    5969356967255586046045024601212242134692456892356893695912689012471612457025243621707 81

UTAHWEDANDE         TC-CT--TATCTTGGTGGTTTCAGTTGTGCCTAGAGTCCC---TAGTGTTGTCGAAACGTTTCTGTGATCATTGTTCCCATCCCGG-
MUSINA PONGO        ..........................................................................AG.....A.............
KALUNGWIZI          ..........................................................................AG.....A.............
KUSOMONA            ..........................................................................AG.....A.............
CHITUNDUMUSEREMUS   ..........................................................................AG.....A.............
PEPE KALLE          ..............................................C...........................AG.....A.............
DONGAJUMU           ..............................................C...........................AG.....A.............
IBALA               ........................A.......................A.....................A.....................
KWANZAA             ..........................................................................A.....................
MWANIKI             ........G.......................................A.......GGT..C.T.A..G.T...A...T...T.T...
TARONGA             ........G..............C...A...................A.C.....GGT..C.T.A..G.TG..A...T...T.....
D. b. michaeli      ........G.......A.......C...A.-..........TAGAT..A.C.....GGT....T.A..G.AG..A..GT.....TT-.
C. s. simum         CTAGCTACGCT-CAAC.AACCTT.CCACAT.CCAGACTTT.....A.AC.ACTAGGG.ACAC.CACAGC.CCC.CG..TGATA...C
NICHOL              CTAGCTACGCT-CAAC.AACCTT.CCACAT.CCAGACTTT.....A.AC.ACTAGGG.ACAC.CACAGC.CCC.CG..TGATA...C
ALEX                CTAGCTACGCT-CAAC.AACCTT.CCACAT.CCAGACTTT.....A.AC.ACTAGGG.ACAC.CACAGC.CCC.CG..TGATA...C
THOMAS              CTAGCTACGCT-CAAC.AACCTT.CCACAT.CCAGACTTT.....A.AC.ACTAGGG.ACAC.CACAGC.CCC.CG..TGATA...C
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Only variable positions are shown . Dots indicate identity with the sequence from Utahwedande, and gaps were introduced for alignment. Sequence of *D. b. michaeli* (Jama et al. 1993) was obtained from GenBank (L22010). Other sequences have GenBank/EMBL accession nos. AF187825-AF187839.

Mitochondrial DNA variation between D. bicornis *subspecies*

Sequence information was used to quantify the extent of genetic differentiation between these *D. b. minor* and *D. b. michaeli* animals, and revealed a divergence of 2.6%. Nucleotide divergence between the *D. b. minor* and *D. b. michaeli* subspecies and the white rhinoceros was 14.7% and 14.2% respectively.

Phylogenetic relationships between rhinoceroses based on neighbour joining analysis showed strong bootstrap support for three lineages (Figure 1). Within the black rhinoceros branch, *D. b. minor* and *D. b. michaeli* each form a monophyletic group (Figure 1). This suggests that the subspecies have been evolving separately for a significant period of time. Based on a rate of nucleotide substitution of $\leq$0.02 per MY, and 7MY divergence between white and black rhinoceros (Cook 1972), the divergence time between the two black rhinoceros subspecies is estimated to be $\sim$1.3 MY. Employing an alternative rate of mtDNA nucleotide substitution of 0.028 per MY calculated for black bears (Wooding and Ward 1997) yields a more recent divergence time of approximately 0.93 MY.

Discussion

Intensive conservation efforts following the precipitous decline in the numbers of all rhinoceros species this century have prompted quantification of levels of genetic biodiversity in extant populations. Despite recent population bottlenecks, significant amounts of genetic diversity have been retained by both the white rhinoceros (Stratil et al. 1990), and the Indian rhinoceros (Dinerstein and McCracken 1990). We conclude that genetic variation has also been conserved in the black rhinoceros, given the high levels of mtDNA haplotype diversity (0.86) and nucleotide diversity (0.43%) in the *D. b. minor* colony at Western Plains Zoo, which is comprised principally of wild-caught animals from Chete National Park, Zimbabwe. These findings probably reflect an historically large effective population size for rhinoceroses in Chete, given that Zimbabwe has one of the few remaining large populations of black rhinoceros in Africa, and Chete National Park had a stable population until approximately ten years ago (Kelly et al. 1995).

Our findings are consistent with the high levels of allozyme variation (H = 0.018 to 0.046) detected in isolated populations of *D. b. minor* from South Africa and Zimbabwe (Swart et al. 1994). However, many previous studies have reported low levels of genetic variation in black rhinoceros populations. For example, little or no mtDNA RFLP variation was detected in populations of *D. b. minor* in Zimbabwe and South Africa (Ashley et al. 1990; O'Ryan and Harley 1993; O'Ryan et al. 1994). In addition, no genetic variation was detected at 3 allozyme loci in 10 *D. b. minor* individuals from South Africa (Osterhoff and Keep 1970). A more extensive analysis of allozyme variation in nine captive *D. b. michaeli* animals also revealed low heterozygosity levels (H = 0.013) (Merenlender et al. 1989). The low levels of genetic variation detected in these studies could be attributed to the effects of demographic bottlenecks rhinoceros populations had undergone. However, this interpretation is inconsistent with our findings, which most likely reflect both the more variable nature of the control region, and the sensitivity of DNA sequencing to detect polymorphism.

The level of mtDNA control region differentiation between *D. b. minor* and *D. b. michaeli* (2.6%) supports the subdivision of rhinoceros into eastern and southern conservation units defined by the IRF (du Toit et al. 1987; Kelly et al. 1995) and the subspecies status based on morphological analyses (Groves 1967). Our findings conflict with the previous mtDNA studies of Ashley et al. (1990) and O'Ryan et al. (1994), which found little or no genetic differentiation between the subspecies. However, a subsequent allozyme study clearly revealed a pattern of differentiation among three *D. b. minor* populations and a *D. b. bicornis* population (from Etosha) which was consistent with an east-west cline (Swart and Ferguson 1997). We cannot exclude the possibility that the mtDNA haplotypes we characterised are discrete representatives that form part of a genetic continuum, since the black rhinoceros was more or less continuously distributed from Zululand to Somalia historically (Cooke 1972). Resolution of this issue would require an extensive genetic analysis of the black rhinoceros across its continental range, which may be obtained from forensic samples where populations are locally extinct.

Fossil evidence dates the divergence between the black rhinoceros and the white rhinoceros genera of Africa at $\geq$7 MY (Cooke 1972). We calculated a rate of nucleotide substitution of 0.02/MY for the rhinoceros mtDNA control region, based on corrected nucleotide divergence between the black (represented by *D. bicornis*) and white (represented by *C. s. simum*)

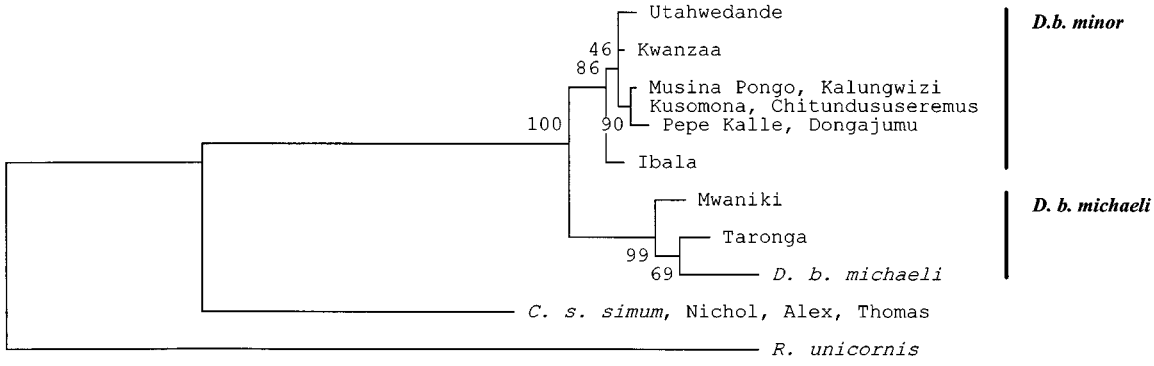

Figure 1. Neighbour-joining tree of Tamura's distance between mtDNA control region haplotypes in the rhinoceros. Numbers indicate the percent confidence level of each node estimated by 5000 bootstrap samplings of the data. Tree includes sequence from *D. b. michaeli* individual (Jama et al. 1993).

rhinoceroses of 14%. This is consistent with rates of about 0.02/MY reported for larger mammals (Slade et al. 1994; Árnason et al. 1996). Applying the conservative rate of 0.028 for bears (Wooding and Ward 1997) leads to estimates of divergence times of 0.93 MY for black rhinoceros subspecies. These divergence estimates are significantly greater than suggestions of a common ancestry dating no more than 100,000 years (Ashley et al. 1990). However, the rate of sequence divergence in mammalian mtDNA control region varies substantially (e.g. see Hoelzel et al. 1991; Stewart and Baker 1994). Because the application of a molecular clock can only estimate divergence times approximately, our results need to be interpreted with caution. However, given the level of differentiation suggested by this study, we advise that the two subspecies be managed as separate genetic entities for as long as is practical in captivity.

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