GENETIC DIVERSITY OF BLACK AND WHITE RHINOCEROS USING RAPD AND MICROSATELLITE GENETIC MARKERS: CAN FECAL SAMPLES SERVE AS A NON-INVASIVE TOOL?

by

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ABSTRACT

The endangered black and white rhinoceroses of Africa have suffered extensive population decreases in the last several decades due primarily to poaching. In an effort to save the remaining populations of these incredible animals, conservationists have been forced to commence with some drastic efforts to save these species. An area of interest that has not been extensively explored for these efforts is conservation genetics. Monitoring genetic diversity of the remaining populations and making management decisions based on that information would help increase the success of current management programs. Additionally, less invasive approaches to this monitoring, such as the use of fecal samples for DNA extraction, would be even more advantageous for this type of research. This study set out to first address the possibility that fecal samples from black and white rhinoceroses could be used to extract good quality DNA. This project also wanted to take the quick "snap-shot" of the current diversity of Hluhluwe-Umfolozi Reserve in South Africa rhinoceros population used in the study. Using both RAPD and microsatellite analyses, fecal samples were inadequate in providing us with comparable quality DNA found in blood samples. Additionally, a microsatellite analysis of the genetic diversity of this population confirmed research on other populations that both the black and white rhinoceros have relatively low genetics variation.

INTRODUCTION

The black rhinoceros (*Diceros bicornis*) is one of the most endangered species in Africa, with population estimates ranging from 3,600 to 2,400 individuals remaining (International Rhino Foundation 2006). The white rhinoceros (*Ceratotherium simum*), while not as critically endangered, is estimated to have a remaining population size around 11,000 individuals (International Rhino Foundation 2006). The primary threat to rhinoceroses at this point is poaching (Leader-Williams 2002). To fight the threat of poaching conservation managers have been forced to take extreme measures in protecting and preserving the remaining rhinoceros populations. There are currently multiple areas where help is needed to conserve the rhinoceros species of Africa. Security needs to be provided for the remaining populations to reduce poacher access to the animals. Researchers are needed to further investigate the behavior and ecology of the rhinoceroses. Medical professionals are needed to ensure the safety and health of the animals that might be translocated or immobilized for various reasons. Lastly, the use of genetic diversity information to aid conservation decisions is starting to be explored in the conservation of the rhinoceros species as well as other endangered species.

The establishment of a genetic diversity database within these species will help conservation efforts with regards to translocation of individuals, population viability assessments that are in the best interest of the rhinoceroses (Harley et al 2005, Florescu et al 2003) and will help decisions in the future with regards to increasing or decreasing genetic diversity of the rhinoceroses. Overall this will help conservation managers make critical decisions that will ultimately lessen the species' risk for extinction. This project set out to contribute to the establishment of the diversity of a group of white and black rhinoceroses from the Hluhluwe-Umfolozi Reserve in South Africa by using microsatellites, which are hypermutable, fast-evolving repetitive sequences which are co-dominantly inherited and can serve as highly variable genetic markers. They were chosen for this group of rhinoceros samples because of their sensitivity in detecting variation among individuals found in a reduced geographical area (Estoup et al 1998). Based on the limited studies of rhinoceros genetic diversity that is currently available (Brown and Houlden 1999, Florescu et al 2003, Harley et al 2005), we anticipated the diversity of these animals to be relatively poor.

Additionally, it has been found recently that immobilization of back rhinoceroses can decrease the females' fertility rates (Alibhai et al 2001). Since blood samples from wild rhinoceroses requires immobilization, this project also set out to determine whether or not fecal samples from rhinoceroses could provide researchers with comparable DNA for genetics research. Based on previous research with other wild mammal species ranging from brown bears, to big cats, and even chimpanzees (Hoss et al 1992, Whittier et al 1999, Goldberg et al 1995), we hypothesized that we would be able to obtain good quality DNA from the rhinoceros fecal samples.

The specific objectives of this study are to: 1) address whether or not fecal samples could provide good quality DNA to be used as a substitute for blood sample DNA in white and black rhinoceroses, and 2) establish baseline data on genetic diversity of the black and white rhinoceros from the Hluhluwe-Umfolozi Reserve in South Africa. Both objectives were completed with RAPD and microsatellite techniques.

MATERIALS AND METHODS

ANIMALS

While at Tufts School of Veterinary Medicine Dr. Annelisa Kilbourn collected blood, fecal, and fur samples from 22 white rhinoceroses and 6 black rhinoceroses within the Hluhluwe-Umfolozi Reserve in South Africa. DNA was isolated from the samples by Dr. Kilbourn following published procedures (Garcia *et al* 1994). For this study, we used all of the blood samples (white rhinoceros n=22, and black rhinoceros n=6), as well as 6 fecal samples yielding the highest quality DNA of all the fecal samples from individuals within the larger study (white rhinoceros n=3, black rhinoceros n=3). Information on the samples included in this study can be found in Table 1.

RAPD ANALYSIS

Originally we tested 20 primers (Operon Inc.) of which we selected the six (B3, B4, B5, A7, A8, and Z3) that yielded the best results for this portion of the study. Every DNA sample was diluted to 100 ng and used with each of the six primers in separate reaction mixtures. Genetic diversity tests were run using the RAPD technique on all 28 samples collected by Annelisa. The six fecal samples yielding the highest quality DNA of all the fecal samples and their paired blood samples from the same animals was tested using a DNA quality control gel. The PCR reaction mixtures were run following PCR cycle conditions: 92°C for 1 minute, 35°C for 1.5 minutes, 72°C for 1 minute, repeat cycle for 40 cycles. DNA quality control gels were performed using 1% agarose gel electrophoresis for both the RAPD and microsatellite analyzes (Figure 1). From the QC results dilutions were established to bring all the samples to 100ng. Amplified fragments were loaded onto a 2%ethidium bromide containing agarose TAE gel and electrophoresed at 50volts for 16-20 hours. The RAPD gel photographic results were analyzed by scoring based on band presence or absence in the photographs.

MICROSATELLITE ANALYSIS

Microsatellite loci were obtained from GenBank sequences and published papers for both the black and white rhinoceros and primers were designed using the Primers3 program (Rozen and Skaletsky 1996, 1997). Primers were selected based on the number of simple sequence repeats and GC content using sequences from previous papers (Florescu et al 2003, Brown and Houlden 1999), and primers from rhinoceros sequences on GenBank. Initially a total of 21 original primers were

designed (black rhinoceros n=16, white rhinoceros n=5). Only one (AY138544) of the five primers for white rhinoceroses published by Florescu *et al* (2003) could be confirmed from the sequences available on GenBank.

All DNA used was diluted to 20ng. The primer sequences were synthesized at Integrated DNA Technologies, Inc. and all the reverse primers were fluorescently labeled with one of the three Well-Read dyes used in the CEQ8000 Beckmann Coulter Genotyper®. The PCR mixtures (15ul) contained the following: 1.0ul of 20ng DNA, 5.88ul molecular biology grade H2O, 1.5ul of 1.25mM dNTPs, 3.0ul of 5X GoTaq Flexi Buffer, 1.5ul of 25mM MgCl2, 1.0ul forward primer, 1.0ul reverse primer, and 0.12ul of 5U/ul GoTaq Polymerase (Promega®). PCR was run on a PTC-100 thermal cycler (MJ Research Inc.) using the following profile: 95°C for 12 minutes, 94°C for 1 minute, 52°C or 56°C for 1 minute, 72°C for 2 minutes, for 30 total cycles, then 72°C for 30 minutes. PCR products were diluted into a sample mix plate using the following ratios: 1ul (D4) blue-labeled; 4ul (D2) black labeled; and 5ul (D3) green labeled. From each sample in the mix plate 1ul of the dilution was added to 28ul of Standard Loading Solution with Beckman's 400 or 600 bp size standard.
All results were analyzed and binned with the CEQ8000 fragment analysis software. The results were entered into Cervus Version 2.0 software to perform allele frequency analysis including [allele number, H(O), H(E), and PIC] (Marshall et al 1998). A SSRs locus was regarded as polymorphic when the frequency of the most common allele is equal to or less than 0.99 (Nei, 1987). Unfortunately, we are unable to determine if the animal is homozygous or if they have a null allele.

RESULTS

RAPD ANALYSIS

The gel from the fecal and blood samples showed that fecal samples did not reveal comparable DNA to the blood samples from the same individuals. Specifically, DNA from fecal samples had low molecular weight, poor quality DNA patterns suggesting highly degraded DNA using RAPD analysis (Figure 2). On the other hand, the blood samples from the same individuals had high molecular weight, good quality DNA with distinct band patterns using RAPD analysis.

Additionally, the RAPD analysis using only blood DNA to look at genetic variation was not extremely successful. Specifically, not all of the white rhino samples amplified PCR products with the primers tested. The only generalizations that could be made were that black rhinos were less polymorphic then white rhinos and that some of the RAPD bands were species specific.

MICROSATELLITE ANALYSIS

Using 20ng DNA the same six paired fecal and blood samples used in the RAPD analysis were used with nine of the microsatellite primers. Similar results were found using this analysis, specifically that fecal samples did not reveal comparable patterns to blood samples (Figure 3). More particularly, fecal samples showed a low peak size with a lot of background peaks, suggesting degraded or contaminated DNA samples. The blood samples from the same animals showed high quality DNA with large peaks at each of the alleles.

In addition to the fecal and blood sample comparisons, each of the 21 primer sets was tested against all of the blood samples for a diversity analysis. Five of the primers did not amplify even after attempting to use adjusted annealing temperatures. The remaining 16 primers amplified results in both or one of the rhinoceros species tested (Table 2). Thirteen primers proved to be polymorphic in white rhinoceroses and thirteen primers were polymorphic in black rhinoceroses. With the exception of one of these primers, (AF129727, which did not amplify in white rhinoceroses), all the

primers amplified in both African rhinoceros species. The mean number of alleles was 4.94 and 6.50 and the expected heterozygosity was 0.661 and 0.632 for black and white rhinoceroses, respectively.

DISCUSSION

The quality of DNA from the fecal samples was mostly fair or poor and, thus, it was expected that the RAPD technique and microsatellite results would also be compromised. The RAPD technique is not recommended for fecal and blood DNA comparison in future studies because of its poor results, (but this could be due to technician or collection technique problems). Since the results using the RAPD technique were not as conclusive as desired, the next step was to confirm these results using the more sophisticated and powerful technique with the co-dominant marker microsatellites.

Despite the fact that microsatellites are a more sensitive technique for analyzing DNA genetic variation, fecal and blood DNA samples from the same individual did not reveal comparable patterns either. The fecal samples all instead amplified a high background with no man product peak, suggesting contamination or poor quality DNA.

Unfortunately, we have concluded that fecal DNA is not an adequate source of DNA for genetic variation analyses with either white or black rhinoceroses. Since fecal DNA has been used successfully in other wild animal species, we will strongly encourage researchers working with other species to pursue the use of fecal DNA for genetic studies in other wild animals. However, after completing this research we suggest that the attempt to use fecal DNA for genetic variation studies in white and black rhinoceroses be abandoned until a more refined technique is designed for extracting rhinoceros DNA from fecal samples.

The high number of homozygous loci among individuals supports previous findings that the black and white rhinoceros species may have low genetic variation (Harley et al 2005, Florescu et al 2003). Interestingly, 7 of the primers produced different allele sizes and variations making it relatively easy to distinguish black from white rhinoceroses, and one of our samples labeled as a white rhinoceros matched the black rhinoceros alleles on every primer, possibly changing the sample size for each species (white rhinoceros n=21, black rhinoceros n=7). This means that many of these primers are adequate in distinguishing between the black and white rhinoceros species. Further genetic analyses with larger wild population sample sizes are recommended to obtain a better understanding of the genetic structure of the black and white rhinoceros populations.

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REFERENCES

Alibhai, S. K., Jewell, Z. C., Towindo, S. S. 2001. Effects on immobilization on fertility in female black rhino. *Journal of Zoology* 253: 333-345.

Brown, S. M., Houlden, A. 1999. Isolation and characterization of microsatellite markers in the black rhinoceros (*Diceros bicornis*). *Molecular Ecology* 8: 1551-1561.

Estoup A, Rousset F, Michalakis Y, et al (1998) Comparative analysis of microsatellite and allozyme markers: a case study investigating microgeographic differentiation in brown trout (*Salmo trutta*). *Molecular Ecology,* 3, 339-353.

Florescu, A, Davila, J. A., Scout, C., Fernando, P., Kellner, K., Morales, J. C., Melnick, D., Boag, P.T., Van Coeverden De Groot, P. 2003. Polymorphic microsatellites in white rhinoceros. *Molecular Ecology Notes* 3:344.

Garcia DK, Faggart MA, Rhoades L, et al (1994) Genetic diversity of cultured *Penaeus vannamei* shrimp using three molecular genetic techniques. *Molecular Marine Biology Biotechnology,* 3, 270-280.

Goldberg T., Yoder A., Ruvolo M. 1995. Isolation and amplification of chimpanzee DNA from feces: dealing with plant polysaccharides**.** In press.

Harley, E. H., Baumgarten, I., Cunningham, J., O'Ryan, P. 2005. Genetic variation and population structure in remnant populations of Black Rhinoceros, *Diceros bicornis*, in Africa. *Molecular Ecology* 14:2981-90.

Hoss, M., Khon, M., Paabo, S., Knauer, F. Schroder, W. 1992. Excrement analysis by PCR. *Nature* 359:199.

International Rhino Foundation. (2006) IRF Program Office, Yullee, Florida.
http://www.rhinos-irf.org/ Cited 1 May 2006.

Leader-Williams, N. 2002. Regulation and protection: successes and failures in rhinoceros conservation. In: *The Trade in Wildlife: Regulation for Conservation* (ed. Oldfield S), pp. 89-99. Earthscan, London.

Rozen, S., and Skaletsky, H.J. 1996, 1997. Primer 3. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html

Whittier, C. A., Dhar, A. K., Stem, C., Goodall, J., Alcivar-Warren, A. 1999. Comparison of DNA extraction methods for PCR amplification of mitochondrial cytochrome c oxidase subunit II (COII) DNA from primate fecal samples. *Biotechnology Techniques* 13:771-779.

Table 1. Rhinoceros information for all rhinoceroses included in study.							
	Species	Blood ID#	Date Collected	Age[a]	Sex	Capture Date	Ear Tag
1	White Rhinoceros	WR002	29-Jun	A	M	APRIL2	18BLU
2	White Rhinoceros	WR004	6-Jul	SA	F	APRIL1	4BLUE
3	White Rhinoceros	WR005	6-Jul	SA	F	APRIL1	16RD
4	White Rhinoceros	WR007	6-Jul	A	F	APRIL1	5RD
5	White Rhinoceros	WR008	8-Jul	A	M	APRIL2	17BLU
6	White Rhinoceros	WR22(9/94)	12-Jul	A	F	MAY12	26RD
7	White Rhinoceros	WR1/50/50	28-Jul	A	F	JUL29	
8	White Rhinoceros	WR12/60/60[b]	1-Aug	A	F	MAY12	27NCN
9	White Rhinoceros	WR13/54/54[c]	31-Jul	C	?	MAY12	28NCN
10	White Rhinoceros	WR14/53/53	31-Jul	C	?	AUG1	5GRN
11	White Rhinoceros	WR010/30/30	7-Jul	A	M	MAY5	23BLU
12	White Rhinoceros	WR2/35/35	28-Jul	A	M	JUL29	
13	White Rhinoceros	WR3/36/36	28-Jul	A	M	JUL29	
14	White Rhinoceros	WR 1/50/50	29-Jul	A	F	JULY29	
15	White Rhinoceros	WR 16/45/45	1-Aug	C	F	AUG1	17
16	White Rhinoceros	WR 18/34/34	2-Aug	A	F	AUG1	15RD
17	White Rhinoceros	WR 21/5/5	2-Aug	C	?	AUG2	18
18	White Rhinoceros	WR22/17/17[d]	3-Aug	C	M	AUG3	20
19	White Rhinoceros	WR 23/20/20[e]	3-Aug	A	F	AUG3	19
20	White Rhinoceros	WR 24/58/58[f]	3-Aug	A	F	APRIL4	3BL
21	White Rhinoceros	WR 25 [g]	3-Aug	C	M	APRIL4	2RD
22	White Rhinoceros	WR 26/28/28	15-Aug	A	M	APRIL1	10BL
23	White Rhinoceros	WR 27/23/23	15-Aug	A	F	APRIL1	25
24	Black Rhinoceros	BR 4/32/32	29-Jul	A	M	MAY10	
25	Black Rhinoceros	BR 5/8/8	29-Jul	A	M	MAY10	
26	Black Rhinoceros	BR 7/40/40	29-Jul	SA	F	MAY8	
27	Black Rhinoceros	BR 8/49/49	29-Jul	A	F	JUN9	
28	Black Rhinoceros	BR 9/52/52	29-Jul	A	M	JUL30	
29	Black Rhinoceros	BR 10/59/59	30-Jul	SA	M	JUL30	

[a]A=adult, C=calf, SA= small adult
[b] mother to WR13/54/54
[c] calf to WR12/60/60
[d] calf to WR23/20/20
[e] mother to WR22/17/17
[f] mother to WR25
[g] calf to WR 24/58/58

Table 1. Microsatellite Loci Tested in 28 Black and White Rhinoceroses from the Hluhluwe-Umfolozi Reserve in South Africa.[a]

Accession Number (GenBank)	Forward Primer Sequence	Reverse Primer Sequence	Microsatellite Motif	Ta °C	Allele Size Range (bp)	# of Alleles (White, Black Rhinoceros)	Expected Heterozygosity H(E) (White, Black rhinoceros)	Observed Heterozygosity H(O) (White, Black rhinoceros)	PIC value[c] (White, Black rhinoceros)
AF129724	taagtcacagggactaatctg	gagggtttattgtgaatgag	$(ac)_{15}$	52	155-160	4, 3	0.654, 0.868	0.350, 0.714	0.616, 0.786
AF129726	aacaccctaaatgtccatc	tagcataatgccctcaag	$(ac)_{13}$	52	137-138	2, 3	0.524, 0.533	0.267, 0	0.462, 0.365
AF129727	cttgctggataatactgctc	cttctcacatctctccaaag	$(tg)_{13}$	52	176-177	0[b], 2	0, 0.758	0, 0.143	0, 0.657
AF129729	agatggtcacaccatttg	cttcctcagcaaaaacag	$(gt)_3$…$(tc)_4$	52	87-188	3, 2	0.796, 0.714	0.619, 0.714	0.745, 0.754
AF129730	agggtggaatgtcaagtag	cttctagagggagactaggag	$(tg)_4c(gt)_{16}$	52	200-208	2, 1	0.566, 0.484	0.381, 0	0.523, 0.406
AF129734	atcttcctcagcaataagg	atcatcagagtttccagttc	$(ca)_{12}$	52	237-251	1, 2	0.264, 0.769	0.143, 0.857	0.246, 0.666
AY606078	gatcagtaacaccaaagtcc	agtgaagacagaaggatcac	$(gt)_{13}gca(tg)_3$	56	237-243	2, 2	0.557, 0.747	0.381, 0.571	0.508, 0.641
AY606079	agattcttggaaaggtcact	aacattgggtttcacctc	$(ac)_{17}g(ca)_4$	52	118-139	2, 3	0.688, 0.742	0.474, 0.167	0.639, 0.622
AY606080	agtcccctatcaacataaac	cttgtgggaagtaatcaaac	$(ca)_{14}ga(ca)_4$	52	237-250	2, 2	0.580, 0.868	0.238, 0.714	0.548, 0.782
AY606082	caacaaagtgggtatagagg	cttcagtaaaactggcatct	$(ca)_{14}$	52	211-228	2, 2	0.496, 0.813	0.150, 0.286	0.458, 0.730
AY606083	acatgtgtaaacttgggaac	tggttcattgatctcttctc	$(tg)_6(ag)_{11}ga(ag)_5$	52	200-241	1, 4	0.605, 0.864	0.200, 0.333	0.553, 0.763
AY138542	ggcaaaactaagagaacttg	gataccaaactggaaatgg	$(ac)_{18}$	52	171-186	3, 1	0.658, 0	0.818, 0	0.563, 0
AY138543	gtcagttcaagtttttgctc	ctcatccatgcttcttctac	$(ct)_{14}(at)_{11}$	52	138-158	3, 2	0.669, 0.758	0.476, 0.429	0.623, 0.657
AY138544	aaccaacttgtaatgagagg	aatgaacaggaaggaagac	$(tg)_{16}a(gt)_5$	52	214-220	4, 2	0.712, 0.593	0.619, 0.286	0.657, 0.521
AY138545	acagctagaatcaccaaaac	tcctgctgcataaatctc	$(ta)_8(ca)_4$	52	223-239	5, 2	0.793, 0.681	0.762, 0.286	0.743, 0.605
AY138541	ctagcaaaatctcaaagagg	ttactaagggaatcaccaag	$(ac)_6$…$(ac)_{15}$	52	199-203	3, 1	0.762, 0	0.727, 0	.672, 0

[a]Black rhinoceros n=6, White rhinoceros n=22
[b]unsuccessful cross-species amplification.
[c]PIC, polymorphic information content

Figure 1. Quality Control RAPD Gel

Figure 2. Fecal VS. Blood RAPD Analysis Gel

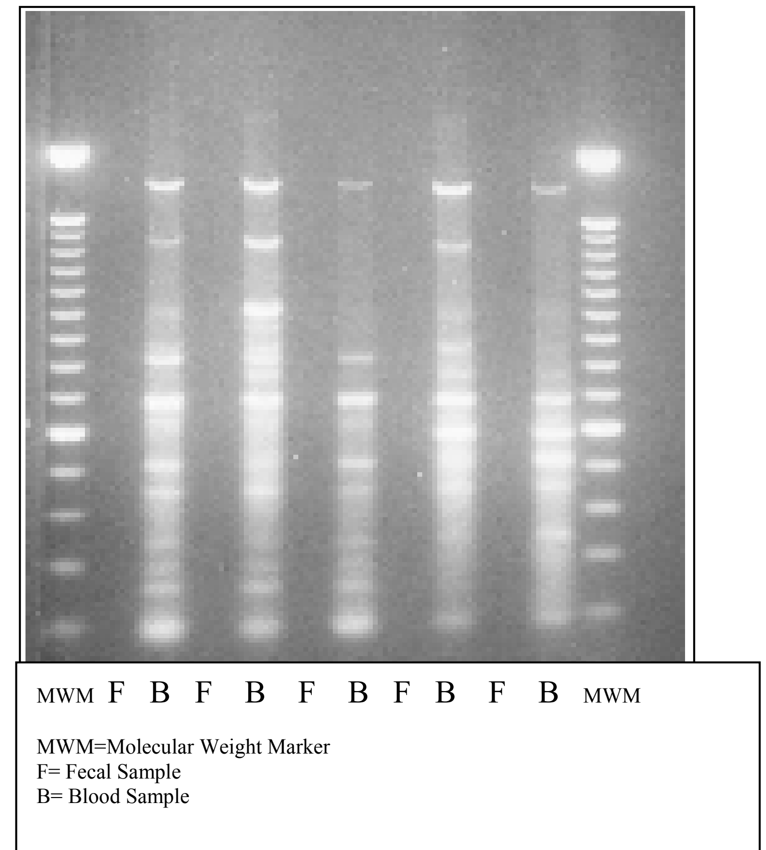

Figure 3. Microsatellite variation with blood and fecal samples from the same animal.