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TECHNICAL NOTE

Characterization of microsatellite loci in the black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*): their use for cross-species amplification and differentiation between the two species

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Abstract As the population sizes of the black and white rhinoceroses continues to decline, more efforts are needed in multiple areas to help with the conservation efforts. One area being explored is the use of genetic diversity information to aid conservation decisions. In this study, we designed 21 microsatellite primers for white and black rhinoceroses, 16 and 17 of which amplified bands in the white and black rhinoceros, respectively. Out of these primers all 16 were polymorphic in the white rhinoceros and 12 of the 17 were polymorphic in the black rhinoceros. The mean number of alleles was 3.31 and 2.12, the expected heterozygosities were 0.420 and 0.372, and the observed heterozygosities were 0.436 and 0.322 for the white and black rhinoceroses, respectively. Seven of the primers produced different allele sizes and variations that distinguished between black and white rhinoceroses. Further genetic analyses with larger wild population sample sizes and markers are recommended to obtain a better understanding of the genetic structure of the black and white rhinoceros populations in order to be useful in the conservation efforts of these critically endangered species.

Keywords Black rhinoceros (*Diceros bicornis*) · White rhinoceros (*Ceratotherium simum*) · Microsatellites · Cross-species amplification · Heterozygosity

A. Kilbourn—In memoriam.

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There are five species of rhinoceroses in the world. 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), and the white rhinoceros (*Ceratotherium simum*), is estimated to have a remaining population size around 11,000 individuals (International Rhino Foundation 2006). 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. To assist with this effort, microsatellite genetic markers can be used. Microsatellites 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).

The samples used in this study were collected in 1994 and included blood and fecal samples from 22 white rhinoceroses and 6 black rhinoceroses within the Hluhluwe-Umfolozi Reserve in South Africa. DNA was isolated using an SDS lysis buffer following published procedures (Garcia et al. 1994; Whittier et al. 1999). The fecal and blood DNA from each individual was used previously for an analysis using Randomly Amplified Polymorphic DNA (RAPD) and microsatellites to address the feasibility of using fecal samples for non-invasive population genetics work. It was found that fecal samples were not an ideal

alternative to blood for genetic analysis in the study of Kilbourn et al. (2006), and this may be due to the technique used to preserve the fecal samples and isolate the DNA. Differences in sample storage and DNA isolation protocols have resulted in variable estimates of DNA quality for primate fecal samples (Whittier et al. 1999). Alternative DNA isolation techniques have successfully purified DNA from black rhinoceros feces for genetic analysis using microsatellites (Garnier et al. 2001). For this study, only the blood samples with good quality DNA were used.

Microsatellite primer sets were obtained from GenBank sequences and published papers for both the black and white rhinoceros (Florescu et al. 2003; Brown and Houlden 1999). 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. Due to the confusion between GenBank and published primers, new primers were designed using the Primers3 program (Rozen and Skaletsky 1996, 1997) and were selected based on the number of simple sequence repeats and GC content. Initially a total of 21 primers were designed (black rhinoceros $n = 16$, white rhinoceros $n = 5$) and synthesized at Integrated DNA Technologies, Inc. Reverse primers were fluorescently labeled with one of the three Well-Read dyes (D2-black label, D3-green, D4-blue) used in the CEQ8000 Beckmann Coulter Genotyper®. PCR mixtures (15 µl) contained the following: 1.0 µl of 20 ng DNA, 5.88 µl molecular biology grade H_2O, 1.5 µl of 1.25 mM dNTPs, 3.0 µl of 5× GoTaq Flexi Buffer, 1.5 µl of 25 mM $MgCl_2$, 1.0 µl forward primer, 1.0 µl reverse primer, and 0.12 µl of 5 U/µl GoTaq Polymerase (Promega®). PCR was run on a PTC-100 thermal cycler (MJ Research Inc.) using the following profile: 95°C for 12 min, 94°C for 1 min, 52°C or 56°C for 1 min, 72°C for 2 min, for 30 total cycles, then 72°C for 30 min. PCR products were diluted into a sample mix plate using the following ratios: 1 µl (D4), 4 µl (D2), and 5 µl (D3). From each sample in the mix plate 1 µl of the dilution was added to 28 µl of the mixture of 960 µl Standard Loading Solution and 11 µl 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 obtain population statistics (Marshall et al. 1998). PopGene version 1.31 software was also used to analyze for linkage disequilibrium (Yeh et al. 1999).

Out of the 21 primers tested, 16 amplified in the white rhinoceroses and 17 amplified in the black rhinoceroses. Four of the 21 primers did not amplify even after attempting to use different annealing temperatures. The remaining 17 primers provided allele data in both or one of the rhinoceros species tested (Table 1). One primer (AF129727) did not amplify in the white rhinoceros. With the exception of one primer (AY606078), 16 primers amplified at an annealing temperature of 52°C, which is lower than the annealing temperatures of 58–62°C used for white rhinoceroses by Florescu et al. (2003) and 55–64°C used for black rhinoceroses (Brown and Houlden 1999; Garnier et al. 2001).

All 16 primers proved to be polymorphic in white rhinoceroses with a mean number of alleles of 3.31 (range 2–6), an observed heterozygosity (H_o) of 0.436 (range 0.0–0.889), and an expected heterozygosity (H_e) of 0.420 (range 0.094–0.741) (Table 1). The number of alleles observed with the white rhinoceroses is similar to the 2.8 observed with 5 microsatellites by Florescu et al. (2003), which is not surprising since the animals used in this study were from the same Hluhluwe-Umfolozi Reserve in South Africa. The expected heterozygosity is slightly lower than the 0.578 reported by Florescu et al. (2003). The mean PIC for the 16 primers was 0.368. Whether or not these results suggest relatively low genetic variation for white rhinoceroses (Florescu et al. 2003) or average genetic variation is subjective and should perhaps be based on variability estimates across microsatellite loci cloned from all species using similar selection criteria, as suggested by Florescu et al. (2003), or a genome-wide microsatellite assessment without regards to species (i.e., one marker per chromosome), which awaits the development of a rhinoceros linkage map (Nielsen et al. 2006). Data analysis using PopGene software found that two of the loci in the white rhinos had significant linkage disequilibrium (AF129724 and AY606083, $P < 0.0001$), but the remaining loci were not in linkage disequilibrium.

Twelve out of 17 primers were polymorphic in black rhinoceroses with a mean number of alleles of 2.12 (range 1–4), observed heterozygosity of 0.322 (range 0.0–0.833), and expected heterozygosity of 0.372 (range 0.0–0.712). The number of alleles is lower than the 4.2 identified in captive black rhinos in Africa (Brown and Houlden 1999) and 4.0 in wild black rhinos in Zimbabwe (Garnier et al. 2001). The mean observed heterozygosity was lower than the 0.660 reported by Brown and Houlden (1999) using 11 microsatellites, six of which were sequences also used in this study. This number is also lower then the 0.726 reported by Garnier et al. (2001) using 10 microsatellites and 35 wild black rhinoceros. The heterozygosity levels reported here are lower than the 0.675 reported by Harley et al. (2005), which may in part be due to the small sample size in this study. Even though our sample size was small, it showed that the wild black rhinoceros population in South Africa still retains a moderate degree of allele diversity. Allele size comparisons could not be completed with the results from 121 black rhinoceroses genotyped with 9 microsatellites because neither allele number nor H_e was available for each locus (Harley et al. 2005). Data analysis using PopGene found no linkage disequilibrium for any of the loci in the black rhinos.

Table 1 Population statistics of 17 microsatellite loci tested in 6 black and 22 white rhinoceroses from the Hluhluwe-Umfolozi Reserve in South Africa

Accession number (GenBank)	Forward primer sequence	Reverse primer sequence	Microsatellite motif	T_a (°C)	Allele size range (bp)	# of alleles (white, black rhinoceros)	Expected heterozygosity (white, black rhinoceros)	Observed heterozygosity (white, black rhinoceros)	PIC value (white, black rhinoceros)
White rhinoceros primers									
AY138542[a, d]	ggcaaaactaagagaacttg	gataccaaactggaaatgg	$(ac)_{18}$	52	171–186	4, 1	0.646, 0	0.727, 0	0.558, 0
AY138543[a, d]	gtcagttcaagtttttgctc	ctcatccatgcttcttctac	$(ct)_{14}(at)_{11}$	52	138–158	4, 2	0.525, 0.545	0.571, 0.333	0.473, 0.375
AY138544[a]	aaccaacttgtaatgagagg	aatgaacaggaaggaagac	$(tg)_{16}a(gt)_5$	52	214–220	3, 2	0.570, 0.200	0.667, 0.200	0.480, 0.164
AY138545[a]	acagctagaatcaccaaaac	tcctgctgcataaatctc	$(ta)_8(ca)_4$	52	223–238	4, 2	0.677, 0.303	0.857, 0	0.601, 0.239
AY138541[a, d]	ctagcaaaatctcaaagagg	ttactaagggaatcaccaag	$(ac)_6 \ldots (ac)_{15}$	52	199–203	5, 1	0.735, 0	0.550, 0	0.669, 0
Black rhinoceros primers									
AF129724[b]	taagtcacagggactaatctg	gagggtttattgtgaatgag	$(ac)_{15}$	52	156–160	4,2	0.391, 0.467	0.350, 0.600	0.359, 0.332
AF129726[b]	aacaccctaaatgtccatc	tagcataatgccctcaag	$(ac)_{13}$	52	137–138	2, 1	0.285, 0	0.333, 0	0.239, 0
AF129727[b]	cttgctggataatactgctc	cttctcacatctctccaaag	$(tg)_{13}$	52	176–177	NA, 2	NA, 0.485	NA, 0	NA, 0.346
AF129729[b]	agatggtcacaccatttg	cttcctcagcaaaaacag	$(gt)_3 \ldots (tc)_4$	52	87–188	3, 3	0.627, 0.682	0.889, 0.833	0.527, 0.545
AF129730[b]	agggtggaatgtcaagtag	cttctagagggagactaggag	$(tg)_4c(gt)_{16}$	52	200–208	2, 1	0.333, 0	0.227, 0	0.272, 0
AF129734[b, d]	atcttcctcagcaataagg	atcatcagagtttccagttc	$(ca)_{12}$	52	237–251	3, 2	0.094, 0.545	0.048, 1.00	0.090, 0.375
AF129732[b]	catgtgaaatggaccgtcagg	atttctgggaaggggcagg	$(ca)_{21}$	52	215–220	6,4	0.741, 0.712	0.727, 0.167	0.676, 0.599
AY606078[c, d]	gatcagtaacaccaaagtcc	agtgaagacagaaggatcac	$(gt)_{13}gca(tg)_3$	56	237–243	3, 2	0.427, 0.530	0.455, 0.833	0.360, 0.368
AY606079[c, d]	agattcttggaaaggtcact	aacattgggtttcacctc	$(ac)_{17}g(ca)_4$	52	118–139	2, 3	0.371, 0.591	0.474, 0.667	0.296, 0.460
AY606080[c]	agtcccctatcaacataaac	cttgtgggaagtaatcaaac	$(ca)_{14}ga(ca)_4$	52	238–250	3,3	0.099, 0.621	0.050, 0.500	0.094, 0.477
AY606082[c]	caacaaagtgggtatagagg	cttcagtaaaactggcatct	$(ca)_{14}$	52	211–212	2,1	0.093, 0	0, 0	0.087, 0
AY606083[c, d]	acatgtgtaaacttgggaac	tggttcattgatctcttctc	$(tg)_6(ag)_{11}ga(ag)_5$	52	200–241	3, 4	0.110, 0.636	0.056, 0.333	0.104, 0.530

[a] From sequences in GenBank deposited by Florescu et al. (2003). All primer sequences are new, designed using Primer3

[b] From sequences in GenBank deposited by Brown and Houlden (1999). All primer sequences are new, designed using Primer3, except primer AF129732

[c] From sequences in GenBank for which primers have not been published. Submitted by Van Coeverden de Groot PJ, Scott CA and Boag PT

[d] These primers could be useful in distinguishing between the black and white rhinoceros species

NA = did not amplify in the white rhinoceros

Interestingly, 7 of 17 primers produced different allele sizes and variations making it possible to distinguish black from white rhinoceroses. These 7 primers are AY138541, AF129734, AY138543, AY606080, AY606083, AY606079 and AY138542. 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 these primers may be useful in distinguishing between the black and white rhinoceros species. Considering there are 41 and 42 chromosomes for the white and black rhinoceroses, respectively, the number of markers needed for a proper population wide genetic analysis should be increased. The development of a genetic map for these species is recommended to obtain a better understanding of the genetic structure and genome organization of rhinoceros populations.

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