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Counting rhinos from dung: estimating the number of animals in a reserve using microsatellite DNA

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WE REPORT A DNA-BASED METHOD TO estimate the minimum number of individuals in a rhinoceros population in a reserve in southern Tanzania. The size of this population could not be determined by conventional means. In this pilot study, total genomic DNA was extracted from dung samples collected in the reserve and polymorphic microsatellite DNA loci were amplified using the polymerase chain reaction. Although very small amounts of DNA were extracted and inhibitors of plant origin were co-extracted, positive amplification products were obtained from 60% of the dung samples. Eight unique genotypes were observed using a black rhinoceros-specific polymorphic microsatellite locus. Preliminary data suggest that these eight genotypes represent the minimum number of individuals in this population.

African rhinoceros have suffered a precipitous decline in numbers over the past 25 years. This decline, though partly a result of habitat destruction, is predominantly the result of widespread hunting of these animals for their horns. Black rhinoceros (*Diceros bicornis*) populations have decreased by more than 96% and have become extinct in most parts of their previous range.[1] Tanzania has two taxonomic units of black rhinoceros, *Diceros bicornis michaeli* and *D. b. minor*. Recent investigations in the Selous Game Reserve in southern Tanzania have reaffirmed at least four small breeding populations of *D. b. minor*,[2] with the Lukuliro area showing the greatest potential for conservation in terms of the maintenance of a stable rhinoceros population.[3] In order to formulate a more objective foundation on which to decide the extent and form of management intervention that should be given to these breeding populations, the Wildlife Division of the Ministry of Natural Resources and Tourism requires that their minimum numbers, distribution and demographic structure be determined. The nocturnal and secretive nature of these animals and the vast areas of thick, evergreen coastal thicket vegetation of the reserve do not permit a comprehensive and protracted ground or aerial survey of these populations. This problem is compounded by time and funding constraints. Consequently, the proposed Selous rhino survey is being augmented by the use of dung DNA analysis to determine the minimum number of individuals present.

Microsatellite DNA consists of units of di-, tri, or tetra-nucleotide tandem repeats, and are found randomly distributed throughout all eukaryotic genomes studied to date.[4] They are highly polymorphic, with alleles varying in the number of repeat units.[5] Microsatellites are being used increasingly to examine and quantify genetic variation in animal populations on account of their accuracy, reproducibility, and amenability to a wide range of statistical analyses.[6,7] The hypervariability at microsatellite loci allows one to calculate the minimum number of individuals in a population, provided there are sufficient polymorphic loci and enough discrete genotypes at these loci[8] This method makes use of the polymerase chain reaction (PCR), so that only small amounts of starting material are required, for example, a small skin biopsy, a few drops of blood, single hairs, and faecal samples.[9,10] Microsatellite DNA is becoming particularly useful in the study of endangered species and especially where a limited amount of ecological data is available.[11]

The primers used to amplify microsatellite loci are directed at unique DNA regions flanking the microsatellite repeat units, which generally means that a new microsatellite library has to be compiled for each new group of animals studied. A black rhinoceros library was constructed in our laboratory and a number of primer sets were designed and tested.[12]

Dung DNA extraction

DNA was extracted from rhino dung samples, collected in Selous Game Reserve, using a published method.[13,14] Approximately 100–200 mg of the outer part of the dung bolus was placed into 1 ml of extraction buffer L6 (containing 5 M GuSCN, 0.1 M Tris-HCl, pH 6.4, 0.02 M EDTA, pH 8 and 1.3% TritonX-100); vortexed and incubated with constant agitation at room temperature overnight. Extractions were then centrifuged at 5000 rpm in an Eppendorf desktop microfuge for 10 minutes and the supernatants transferred to a tube containing 300 µl of fresh L6 buffer and 50 µl of diatomaceous earth, vortexed and incubated for 10 minutes at room temperature. The diatomaceous earth was washed twice with 1 ml of buffer L2 (containing 5 M GuSCN, 0.1 M Tris-HCl pH 6.4, 0.02 M EDTA) and once with 70% ethanol. The pellets were dried in a heating block at 56°C and the DNA eluted by incubation at 56°C with 120 µl water. Extraction blanks were included in each extraction.

Microsatellite genotyping

The primers used to amplify microsatellite loci in the experimental animals were first described in *Diceros bicornis*.[12] The forward primer of each primer set was end-labelled with [^{32}P]dATP.[11] PCR was then performed in 10-µl reaction volumes under the following reaction conditions: 25 pM of reverse primer, 5 mM dNTPs, 1.5 mM $MgCl_2$ and 0.5 U *Taq* polymerase (Bioline). Cycling parameters for PCR amplifications were as follows: a one-minute denaturing step at 94°C, one minute at the annealing temperature (50–62°C to maximize the specificity of the hybridization) and a 45-second extension step at 72°C. The amplified product was then electrophoresed on a 6% denaturing polyacrylamide gel. Genotypes were scored from the autoradiographs and allele lengths (in base pairs) determined using a sequenced size ladder of M13 ssDNA.

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Table 1. DNA extracted from dung of black rhinoceros in the Selous Game Reserve and subsequent amplification at BR6.

Sample ID	Amplification	Genotype
LU1	Yes	N/S*
LU2	Yes	N/S
LU3	Yes	N/S
LU4	No	-
LU5	Yes	N/S
LU6	No	-
LU7	No	-
LU8	No	-
LU9	Yes	A/A
LU10	Yes	N/S
LU11	Yes	N/S
LU12	Yes	N/S
LU13	No	-
LU14	No	-
LU15	No	-
LU16	No	-
LU17	Yes	A/B
LU18	Yes	A/C
LU19	Yes	A/D
LU20	Yes	D/E
LU21	Yes	A/F
LU22	No	-
LU23	Yes	C/F
LU24	Yes	G/B
LU25	Yes	A/A

*N/S, although there was amplification, the product was not scorable.

Analysis of dung samples

Twenty-five of the dung samples from the game reserve were tested for DNA quality and concentration by PCR amplification. A standard agarose gel was not used to check the extraction owing to the large amounts of plant, bacterial and fungal DNA that would have been co-extracted with the faecal DNA. The first polyacrylamide gel resulted in positive amplification of 60% of these initial extractions with primer BR6 (Table 1), although not all the PCR products scored gave clear genotypes. These amplifications resulted in microsatellite products in the appropriate size ranges as found in the original testing of the primers with *D. b. bicornis* and *D. b. minor* samples (126–158 bp).[12] We observed eight distinct genotypes within the sample set with primer BR6, which was represented by a sub-set of seven of the original alleles seen in the South African black rhinoceros population. We were unable to repeat this amplification to the same specificity in subsequent experiments at the same locus. Instead, we obtained amplification product, but the gels displayed multiple non-specific banding patterns, which could not be scored with a high degree of confidence. This same non-specific, variable pattern was obtained when testing primers at the two other loci. Allelic dropout, the non-amplification of one of the two alleles at a locus, was also observed on duplicate experiments and has been reported elsewhere.[8] This allelic dropout could lead to a misrepresentation, or over-representation of the number of homozygotes in a population. Apparent homozygotes are also referred to as null alleles and could be a result of poorly designed primers for amplification.[15] In this case, however, it was as a result too little, poor quality DNA in the starting sample.[8]

Tests were performed to test why our PCR reactions were not reproducible. The results suggest that we co-extracted an inhibitor of DNA amplification. This inhibitor was of plant origin, as the addition of 1% PVP to the DNA extracts (a known plant-inhibitor chelator) allowed for more efficient amplification of the extracted samples. Another reason for the irreproducibility of our PCR reaction is the low DNA concentration, which causes allelic dropout. Our current experiments are aimed at minimizing or removing the plant inhibitors from the dung extractions, as well as optimizing the quality and quantity of rhinoceros DNA extracted.

Conclusions

We have demonstrated that DNA can be amplified, although not reproducibly, from black rhinoceros dung samples. We observed a minimum of eight unique genotypes (8 of the 25 samples tested) from preliminary data from our initial extractions and genotyping at one locus. The potential for the efficient characterization of rhinoceros in natural populations from faecal material cannot be underestimated because it allows for the inexpensive and rapid collection of these samples as opposed to the classic tissue collection, which is labour-intensive and requires time and considerable financial resources. Additionally, we plan to identify more polymorphic repeat loci and develop additional primers for the black rhinoceros, as well as testing recently published primers.[16] The results of optimizing our experimental protocols and successfully determining the minimum number of animals in a population will be invaluable because the black rhinoceros is a highly endangered land animal that is in urgent need of conservation management.

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