Electrophoresis 1999, *20*, 1778–1780

Jessica Cunningham
Eric H. Harley[1]
Colleen O'Ryan

Department of Biochemistry, University of Cape Town, Rondebosch, Cape Town, South Africa
[1]Department of Chemical Pathology, University of Cape Town, Observatory, Cape Town, South Africa

Isolation and characterization of microsatellite loci in black rhinoceros (*Diceros bicornis*)

The black rhinoceros (*Diceros bicornis*) is currently one of the most endangered African mammals as a direct result o habitat destruction and intensive poaching. A microsatellite library was constructed to address a number of population genetic questions. This paper describes the isolation of five black rhinoceros microsatellite loci. Three of these loci were found to be polymorphic. In addition, the paper demonstrates the utility for cross-hybridization of these primers in other species of rhinoceros.

Keywords: Microsatellites / Black rhinoceros / *Diceros bicornis* EL 3473

African rhinoceros have suffered a precipitous decline in population numbers over the past 25 years. This decline, though partially a result of habitat destruction, is predominantly the result of widespread hunting of these species 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]. In 1995 there were about 2400 black rhinoceros remaining worldwide [2], of which 35% of the population occurred in South Africa. Although it is debatable whether there were as many as seven different subspecies of black rhinoceros [3], it is irrefutable that more than half of these populations have become extinct. Currently, the three major "evolutionary significant units" of black rhinoceros that are recognized are: (i) the south-central populations extending from Natal in South Africa through to Zimbabwe and Zambia into southern Tanzania, corresponding to *D. b. minor*, (ii) the south-western populations in Namibia, corresponding to *D. b. bicornis*, and (iii) the eastern African populations in Kenya and northern Tanzania, corresponding to *D. b. michaeli* [4].

There are two subspecies of white rhino (*Ceratotherium simum*) with the northern subspecies, *C. s. cottoni*, thought to exist as a single, small population of 25 individuals in Garamba National Park in Zaire [5]. The southern subspecies, *C. s. simum*, was hunted to the brink of extinction in southern Africa by the early 1900s, except for a single population in Umfolozi-Hluhluwe Reserve, South Africa [6]. This reserve now has a relatively stable population of 7000 animals and there are several seeded populations in Kenya, Namibia, Swaziland, and Botswana [7]. African rhinoceros now occur as small, highly fragmented populations confined to game reserves. In order to ensure the long-term survival of these remaining fragmented populations, not only poaching has to be halted, but these populations require interventive management to prevent loss of genetic diversity due to stochastic demographic events [8]. This requires a knowledge of the mating systems, paternity, population demographics, intra- and interpopulation diversity, ecology, and behavior as well as an understanding of the population genetics of these remaining populations. This will be crucial to the implementation of successful conservation management programs. In this paper, the isolation and characterization of microsatellite primers from black rhinoceros are described, and the utilization of these primers in white rhinoceros is investigated.

A microsatellite library was constructed and screened for CA repeat loci as previously described [9, 10]. Black rhinoceros DNA fragments were restricted with *Alu*I, *Hae*III and *Rsa*I, size-selected, and then the purified fragments were cloned into pUC 18. The library was screened with an α^{32}P-labeled poly CA/GT probe (Pharmacia, Piscataway, NJ, USA). Positive colonies were isolated and sequenced by cycle sequencing using an ABI 373 Sequencer. Primers were designed for amplification using the Primer Premier Lite program. Genomic DNA was extracted from cell cultures established from the ear nicks of black and white rhinoceros [11] using standard DNA extraction methods [12]. Individuals were genotyped for each microsatellite locus using the following protocol: 1.5 pM of the forward primer of each primer set was end-labeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase at 37°C for 90 min [12]. The PCR was performed in 10 µL reaction volumes using the following reaction conditions: 1.5 pM of the reverse primer, 0.2 mM of each deoxyribonucleotide triphosphate, 1.5 mM $MgCl_2$, 10% glycerol and 0.2 U *Taq* polymerase. DNA was amplified in a Stratagene thermocycler (La Jolla, CA, USA) using the following program: a 1 min denaturing step at 94°C, 1 min at the annealing temperature, and a 45 s extension step at 72°C. A final 10 min extension step was included. The

Correspondence: Dr. C. O'Ryan, Department of Biochemistry, Molecular Biology Building, University of Cape Town, Rondebosch, 7700, South Africa
E-mail: colleen@molbiol.uct.ac.za
Fax: +021-6855931

© WILEY-VCH Verlag GmbH, 69451 Weinheim, 1999
0173-0835/99/0808-1778 $17.50+.50/0

Table 1. Description of five microsatellite loci for *Diceros bicornis*

Locus	Repeat array	Heterozygozity (observed)	Number of alleles	Size range of alleles (bp)	Annealing temperature (°C)	Primer sequence (5′–3′)
BR3F	$(CA)_{10}CG(CA)_5$	0	1	143	60	TGA GCC TGG ATC ATC TTG TAG T
BR3R						GAA GTC TTG AGT CAT TGG GAA G
BR4F	$(CA)_{19}$	0.68	10	124–146	49	CCC CTA AAT TCT AGG AAC AC
BR4R						CCA AAG ACC ACC AGT AAT TC
BR6F	$(CA)_{15}$	0.59	10	126–158	51	TCA TTT CTT TGT TCC CCA TAG CAC
BR6R						AGC AAT ATC CAC GAT ATG TGA AGG
BR17F	$(AT)_6(GT)_{18}$	0.49	6	123–135	60	ACT AGC CCT CCT TTC ATC AG
BR17R						GCA TAT TGT AAG TGC CCC AG
BR20F	$(CA)_{20}$	0	1	149	49	ATA GGT ATT TCA AAG ATG CCC
BR20R						TGG ACA ACT TAC TTC CAC TTC

F, forward primer; R, reverse primer
Allele number, size ranges and heterozygosities were obtained by screening 72 black rhinoceros from a variety of localities

Table 2. Cross-species amplification of microsatellite loci for primers developed in the black rhinoceros (*Diceros bicornis*)

	P3	P4	P6	P17	P20
White rhinoceros	M	M	P	M	M
Indian rhinoceros	M	P	P	P	M
Black rhinoceros	M	P	P	P	M

P, polymorphic product
M, monomorphic product

amplified product was electrophoresed on a 6% denaturing polyacrylamide gel, and the gels were dried and exposed to autoradiographic film. The genotypes were scored from the autoradiographs and allele lengths were determined by comparison with a sequence size ladder of the M13 polycloning site.

After screening 27 000 colonies, 117 putative repeat loci were identified. Of these clones 42 were sequenced and 24 were found to contain $(CA)_n$ loci. Amplification primers were designed for ten loci since the remaining repeat loci were cloned in such a manner that there was insufficient flanking sequences on either side of the repeat locus to design PCR primers, or the clones did not contain a long enough repeat locus to be informative, *vig.*, $(CA)_{<6}$. Three of the microsatellite loci yielded positive polymorphic bands, two a monomorphic pattern, and the primers for the remaining five loci yielded multiple amplification bands. The three polymorphic microsatellite loci tested in black rhinoceros ($n = 72$) demonstrated high allelic variation, with the number of alleles ranging from 6–10 alleles per locus, and displayed relatively high levels of heterozygosity (Table 1). The heterozygosity values ranged from 0.49 to 0.68, with a mean heterozygosity of 0.58. All three populations were found to conform to Hardy-Weinberg expectations using the chi-squared test [13]. Additionally, our unpublished data suggests that there is significant population differentiation between the different subspecies of black rhinoceros [14].

The primers isolated in black rhinoceros hybridized and successfully amplified loci in the white rhinoceros. These loci were less polymorphic in white rhinoceros with four of the five loci demonstrating monomorphic patterns. Three of five isolated loci amplified a polymorphic pattern in the Indian rhinoceros (Table 2). This demonstrates the successful isolation of microsatellite loci in black rhinoceros, which will be useful in population genetics studies, thus benefiting the management of this endangered species.

We thank I. Baumgarten for establishing and maintaining the rhinoceros cell cultures; Rachel Wiseman for assistance with genotyping, and Mike Bruford for assistance with library construction. This research was supported by grants from the Foundation for Research and Development (SA), Institute of Zoology, London (UK) and the International Rhinoceros Foundation (USA).

Received February 27, 1999

References

[1] Gakahu, C. K., in: Ryder, O. A. (Ed.), *African Rhinos: Current Numbers and Distribution in Proceedings of a Conference: Rhinoceros Biology and Conservation*, Zoological Society of San Diego 1993, pp. 160–165.

[2] Swaringen, K., Wiese, R. J., Willis, K., Hutchins, M. (Eds.), *Aza Annual Report Conservation and Science*, Bethesda, MD, American Association of Zoological Parks and Aquariums 1995.

[3] Groves, C. P., *Zeitschrift Säugetierkunde* 1967, *32*, 267–276.

[4] du Toit, R., Foose, T. J., Cummings, D. H. M., *Pachyderm Special Issue No. 9, Proceedings of the African Rhino Workshop*, Cincinnati 1987.

[5] Brooks, M., *Pachyderm* 1994, *18*, 16–18.

[6] Owen-Smith, R. N., *Ph.D. Dissertation*, University of Wisconsin 1974.

[7] Kemf, E., Jackson, P., *WWF* 1994, 20.

[8] Gilpin, M. E., Soule, M. E., in Soule, M. E. (Ed.), *Minimal Viable Populations: The Process of Species Extinction in: Conservation: The Science of Scarcity and Diversity*, Sinauer Associates, Sunderland, Massachusetts 1986.

[9] Rassman, K., Schlötterer, C., Tautz, D., *Electrophoresis* 1991, *12*, 113–118.

[10] Bruford, M., Cheesman, D., Coote, T., Green, H., Haines, S., O'Ryan, C., Williams, T., in: Smith, T. B., Wayne, R. K. (Eds.), *Microsatellites and their Application to Conservation Genetics in Molecular Genetic Approaches in Conservation*, Oxford University Press 1996, pp. 278–297

[11] O'Ryan, C., Flamand, J. R. B., Harley, E. H., *Conserv. Biol.* 1994, *8*, 494–500.

[12] Sambrook, J., Fritsch, E. F., Maniatis, T., *Molecular Cloning: a Laboratory Manual*, Habor Laboratory Press, Cold Spring Harbor 1989.

[13] Raymond, M., Rousset, F., *Genepop (V1.2)*, Montpellier, France 1995.

[14] Goodman, S., *Mol. Ecol.* 1997, *6*, 881–885.