Molecular Ecology Notes (2003) **3**, 233–235 doi: 10.1046/j.1471-8286.2003.00400.x

PRIMER NOTE

Polymorphic microsatellite loci in the endangered Indian rhinoceros, *Rhinoceros unicornis*

S. ZSCHOKKE,* B. GAUTSCHI† and B. BAUR*

**Department of Integrative Biology, Section of Conservation Biology (NLU), University of Basel, St. Johanns-Vorstadt 10, CH-4056 Basel, Switzerland, †ECOGENICS GmbH, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland*

Abstract

Eleven novel polymorphic microsatellite loci are presented for the highly endangered Indian (or greater one horned) rhinoceros *Rhinoceros unicornis* (Mammalia: Rhinocerotidae). These will be used to analyse the genetic variability within and between the two remaining large populations of the Indian rhinoceros and to manage captive breeding.

Keywords: captive breeding, microsatellite, population genetics, *Rhinoceros unicornis*

Received 18 October 2002; revision accepted 8 January 2003

The Indian rhinoceros (*Rhinoceros unicornis*) is highly endangered. Currently, only 2400 animals are living in the wild, mainly in two populations in Nepal and Assam, India (Foose *et al*. 2000). A further 137 animals are kept in zoos and wild parks (Wirz-Hlavacek *et al*. 2001). Protein electrophoresis in 23 individuals from the Nepal population revealed a high variability (Dinerstein & McCracken 1990), whereas another study in three individuals from the Assam population revealed a low variability (Merenlender *et al*. 1989). In addition, indirect evidence suggests that the two populations differ genetically from each other (Groves 1993; Zschokke & Baur 2002). However, the genetical variability between the two populations has not yet been analysed. Microsatellite primers have been developed for the Black rhinoceros *Diceros bicornis* but not for any other rhinoceros species (Brown & Houlden 1999; Cunningham *et al*. 1999).

Blood samples were collected from 14 captive *Rhinoceros unicornis* descending from the two populations (four from the Nepal population, nine from the Assam population and one animal with a parent from each population). Samples were buffered with K_3EDTA (Becton-Dickinson, USA) and stored at −80 °C. Total genomic DNA was isolated using a standard phenol–chloroform extraction protocol (Sambrook *et al*. 1989). An enriched library was made by ECOGENICS GmbH (Zurich, Switzerland) from size-selected genomic DNA ligated into TSPAD-linker (Tenzer *et al*. 1999) and enriched by magnetic bead selection with biotin-labelled $(CA)_{13}$ and $(GA)_{13}$ oligonucleotide repeats (Gautschi *et al*. 2000a,b). Of 288 recombinant colonies screened, 179 gave a positive signal after hybridization. Plasmids from 60 positive clones were sequenced and primers were designed for 19 microsatellite inserts. Of these, 16 were tested for polymorphism.

To assay variation among individuals, polymerase chain reaction (PCR) amplifications were performed in a 10 μL reaction volume containing 10 ng of genomic DNA, 3 μL double-distilled water, 0.5 μM of each forward and reverse primer, and 5 μL HotstarTaq master mix (Qiagen). The latter contains 400 μM dNTP each, 0.5 units of HotStar*Taq* DNA Polymerase (Qiagen), and 2× PCR buffer (Qiagen), consisting of Tris-Cl, KCl, and $(NH_4)_2SO_4$, with a final concentration of 1.5 mM $MgCl_2$. We used the following thermotreatment on a PTC-100™ Programmable Thermal Controller (MJ Research): 30 cycles with 95 °C for 30 s, locus-specific annealing temperature (Table 1) for 30 s, and 72 °C for 30 s. Before the first cycle, a prolonged denaturation step (95 °C for 15 min) was included and the last cycle was followed by an 8-min extension. The amplified products were separated on precast Spreadex®· EL-400 and EL-1200 gels (Elchrom Scientific AG, Switzerland), which consist of a novel and fully synthetic matrix of gel polymers (Elchrom Scientific AG). All electrophoresis was performed using the SEA 2000™ advanced submerged gel electrophoresis apparatus (Elchrom Scientific AG). Products were visualized using SYBR Gold (Molecular Probes) and scored against the M3 Marker (Elchrom Scientific AG). Expected and observed heterozygosity was determined, and exact tests for departure from Hardy–Weinberg equilibrium

Correspondence: S. Zschokke. Fax: + 41 61 267 08 32; E-mail: samuel.zschokke@alumni.ethz.ch

© 2003 Blackwell Publishing Ltd

Table 1 Microsatellite loci from *Rhinoceros unicornis*

Locus	Repeat motif based on sequenced clone	Primer sequence (5′–3′)	T_a (°C)	No. of alleles	Size (bp)	n	H_O	H_E	Accession no.
Rh1	$(TG)_{13}$	F: GTGCCATTATTATCCCAGGTC R: CGTAAGACCTCAAGGGATGC	60	3	148–152	13	0.462	0.640	AJ508894
Rh2	$(GT)_{36}$	F: GACTTCAAACTTCGCAGCAATC R: GCCCTAGACCTGGAAATAACC	60	7	212–228	14	0.643	0.709	AJ508895
Rh3	$(TC)_8TG(TC)_7CCTG(TC)_4TG(TC)_{16}$	F: TGTGTGGAGCACATCAGTCTTC R: CCAGGGACCCGTGAGGAT	62	3	114–146	14	0.571	0.611	AJ508896
Rh4	$(AC)_{22}$	F: CAAAATGTGGGTTTTGTGAGC R: GACGAGCTTTGTTTGAATGC	60	6	80–106	14	0.643	0.786	AJ508897
Rh5	$(TG)_{15}$	F: CCCATTAGAGGCTGTAGAGTAATATC R: GGACTCTAAACTCCAGGGTCAC	58	5	194–206	14	0.500	0.680	AJ508898
Rh6	$(CA)_4GT(AT)_2(GTAT)_4GCAT(GT)_2(AT)_2(GT)_{11}$	F: CCTTACTGTTGGGAAGATGTTATAGG R: CATCACCTGTGCGTAAGTGC	58	2	116–118	14	0.286	0.254	AJ508899
Rh7	$(TG)_{17}$	F: CCGTCACATATGACAGTGTGC R: GGGCAGCTTATGCTCAAGTC	62	2	200–202	14	0.357	0.389	AJ508900
Rh8	$(TG)_{22}(AG)_2ANACA(GA)_{28}CA(GA)_3CA(GA)_9CG(TA)_5$	F: ACACACCTTTATAAACAATATGGTCAC R: AGTCTCTAGTCAAAAGGGATTGC	60	6	218–230	14	0.143	0.738	AJ508901
Rh9	$(TG)_4TT(TG)_{17}TA(TG)_5$	F: TCTGGTACCACCAAATGTAGC R: ACGATTACGTCTTTCAGTTGC	60	2	146–168	14	0.214	0.304	AJ508902
Rh10	$(GT)_{24}(GC)_7$	F: TATGCCAGGGAAGAATCTGGTC R: TCCCTCACCAACTCTCGTAAAC	60	7	138–150	14	0.643	0.783	AJ508903
Rh11	$(CA)_{23}$	F: CTCGCATCCTCATCAATGC R: GCAGGTGTACCAGGCTGAG	64	4	147–159	14	0.643	0.659	AJ508904

F, forward primer; R, reverse primer; T_a, optimal annealing temperature; n, number of individuals genotyped; H_O, observed heterozygosity; H_E, expected heterozygosity (Nei 1973).

were performed using GENEPOP, web version 1.2 (Raymond & Rousset 1995).

Eleven primer pairs gave reproducible and interpretable PCR products (Table 1). Only one locus showed a significant discrepancy between observed and expected heterozygosity (Rh8, $P < 0.001$). These microsatellite markers will be used to examine the genetic variability within and between the populations of the Indian rhinoceros and they may also be useful to determine the origin of poached rhinoceros horns and skins from confiscated material.

Acknowledgements

We thank Jean Miller for helping us to obtain blood samples and Georg Armbruster for advice. Samples were kindly provided by the zoos of Antwerp B, Basel CH, Bronx NY, Buffalo NY, Cincinnati OH, Dvur Kralove CZ, Fort Worth TX, Lowry Park Tampa FL, Miami FL, Munich D, Rotterdam NL, Smithsonian Washington DC and Wilds Cumberland OH. This work was financially supported by Basel Zoo, Treubel Fonds Basel, Basler Stiftung für biologische Forschung and E. Guggenheim-Schnurr Stiftung.

References

Brown SM, Houlden BA (1999) Isolation and characterization of microsatellite markers in the black rhinoceros (*Diceros bicornis*). *Molecular Ecology*, **8**, 1559–1561.

Cunningham J, Harley EH, O'Ryan C (1999) Isolation and characterization of microsatellite loci in black rhinoceros (*Diceros bicornis*). *Electrophoresis*, **20**, 1778–1780.

Dinerstein E, McCracken GF (1990) Endangered greater one-horned rhinoceros carry high levels of genetic variation. *Conservation Biology*, **4**, 417–422.

Foose T, van Strien N, Rookmaaker K (2000) Asian rhino areas and estimates. *Asian Rhinos*, **3**, 20–21.

Gautschi B, Tenzer I, Müller JP, Schmid B (2000a) Isolation and characterisation of microsatellites in the bearded vulture (*Gypaetus barbatus*) and cross amplification in three Old World vulture species. *Molecular Ecology*, **9**, 2193–2195.

Gautschi B, Widmer A, Koella J (2000b) Isolation and characterization of microsatellite loci in the dice snake (*Natrix tessellata*). *Molecular Ecology*, **9**, 2191–2193.

Groves CP (1993) Testing rhinoceros subspecies by multivariate analysis. In: *Rhinoceros Biology and Conservation* (ed. Ryder OA), pp. 92–100. Zoological Society of San Diego, San Diego CA.

Merenlender AM, Woodruff DS, Ryder OA, Kock R, Váhala J (1989) Allozyme variation and differentiation in African and Indian rhinoceroses. *Journal of Heredity*, **80**, 377–382.

Nei N (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences of the USA*, **70**, 3321–3323.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Sambrook J, Fritsch EF, Maniatas T (1989) *Molecular Cloning: a*

Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Tenzer I, degli Ivanissevich S, Morgante M, Gessler C (1999) Identification of microsatellite markers and their application to population genetics of *Venturia inaequalis*. *Phytopathology*, **89**, 748–753.

Wirz-Hlavacek G, Zschokke S, Studer P (2001) *International Studbook for the Greater One-Horned Rhinoceros Rhinoceros Unicornis Linnè, 1758*, 11th edn. Basel Zoo, Basel, CH.

Zschokke S, Baur B (2002) Inbreeding, outbreeding, infant growth, and size dimorphism in captive Indian rhinoceros (*Rhinoceros unicornis*). *Canadian Journal of Zoology*, **80**, 2014–2023.