Molecular Ecology Notes (2003) **3**, 344–345 doi: 10.1046/j.1471-8286.2003.00440.x

PRIMER NOTE

Polymorphic microsatellites in white rhinoceros

A. FLORESCU,* J. A. DAVILA,*¶ C. SCOTT,* P. FERNANDO,† K. KELLNER,§ J. C. MORALES,† D. MELNICK,†‡ P. T. BOAG* and P. VAN COEVERDEN DE GROOT*

Queens University Molecular Ecology Laboratory (QUMEL), Queens University, Kingston, Ontario, Canada K7L-3N6, †Center for Environmental Research and Conservation (CERC), Columbia University, New York, NY, USA, ‡Department of Ecology, Evolution and Environmental Biology, Columbia University, New York, NY, USA, §Institut für Tierzucht der Ludwig-Maximilians, University of Munich, Munich, Germany, ¶Instituto de Investigacion en Recursos Cinegeticos, Ciudad Real, Spain

Abstract

The southern white rhinoceros (*Ceratotherium simum simum*) has suffered severe reductions in population size over the last 150 years as a result of overhunting. We optimized 10 southern white rhinoceros microsatellite loci and tested them on 30 individuals from the largest remaining population of this species. Five of the 10 loci were polymorphic with mean expected heterozygosity of 0.578, mean polymorphic information content of 0.481 and mean number of alleles 2.8. Although these data suggest low genetic variability in *C. s. simum*, an accurate comparison of variability awaits results of ongoing broad-scale microsatellite surveys in this family.

Keywords: genetic variability, microsatellites, polymorphism, primer design, Rhinocerotidae, white rhinoceros

Received 26 January 2003; revision accepted 10 March 2003

Microsatellites are fast-evolving noncoding repetitive sequences abundant in the nuclear genome of eukaryotes (Tautz & Renz 1984). These genetic markers have applications ranging from estimation of genetic differentiation among taxa (Paetkau *et al*. 1995) to assigning parentage within a group of individuals (Garnier *et al*. 2001). As part of ongoing studies of genetic differentiation and mating behaviour within the Rhinocerotidae we optimized five polymorphic microsatellite loci in southern white rhinoceros. Although microsatellites from other species have been tried with white rhinos, these are the first loci cloned from this species (Cunningham *et al*. 1999).

Since initial reports suggest that genetic variation in white rhinos is low (Merenlender *et al*. 1989; Cunningham *et al*. 1999; Kellner 2000) we adopted a twofold strategy to optimize variable microsatellites for this species. First, we screened a large number of microsatellite-containing clones isolated from a white rhino genomic library. The library was constructed by ligating white rhino genomic DNA fragments into pGen5Z plasmid grown in *Escherichia coli* (JM109 cells). After secondary screening of an initial 40 000 colonies with γ^{32}P-labelled double-stranded $(AC)_n$–$(TG)_n$ and $(AG)_n$–$(TC)_n$ probes (Pharmacia), 135 positive colonies were size-selected for sequencing. Sixty of these met the size requirements and were sequenced. Of these, 20 had sufficient flanking sequence for primer design.

Of the 20 primer sets designed, 10 have been optimized for white rhinos with the other 10 yielding either no product or nonspecific products under our optimization regime. Using a Gene Amp 9700® (Perkin Elmer) and 10 μL reaction volume, the polymerase chain reaction (PCR) conditions were: 2 min at 94 °C and 35 cycles consisting of 25 s at 94 °C, 25 s annealing (T_a; Table 1) and 20 s at 72 °C. The 35 cycles were followed by 5 min at 72 °C. Each primer set was optimized with respect to annealing temperature and $MgCl_2$ concentration (Table 1). All other components remained constant: 1 μL PCR reaction buffer (Gibco-BRL®), 0.1 μL 10 μM dNTP, 0.05 μL 10 mM forward primer, 0.1 μL 10 mM reverse primer, 0.25 μL 10 mM radioactively labelled forward primer cocktail (for 20 samples: 1.0 μL 10 mM forward primer, 0.6 μL PNK reaction buffer A (MBI Fermentas®), 0.4 μL dH_2O, 3.0 μL polynucleotide kinase, 1.0 μL [$^{33}\gamma$]-ATP; the end-labelling reaction was incubated at 37 °C for 30 min followed by 10 min at 68 °C) and 1.0 μL DNA. The PCR products were run on 6% polyacrylamide gels at 65 W for 3.5–4 h, dried for 2 h at 80 °C and exposed

Correspondence: A. Florescu. E-mail: toanaflorescu@yahoo.com

Table 1 Summary of five white rhinoceros microsatellite loci based on 30 adults from Umfolozi Game Reserve

Locus	Primer sequence (5′–3′)	$MgCl_2$ (mM)	T_a (°C)	Motif	PCR product (bp)	Allele no.	H_E	H_O	PIC	Null allele frequency
7B	F: CCTCTGTGATTAAGCAAGGC R: ATGAACAGGAAGGAAGACGC	1.75	62	$(TG)_{16}$	262–266	3	0.582	0.533	0.494	0.0376
7C	F: TGAACTCTGATGGAAATGAG R: AAACAGGTCTTGATTAGTGC	2.00	58	$(CT)_{14}(AT)_{11}$	253–257	3	0.542	0.462	0.450	0.0726
12F	F: AAGCAGCCAGCCTAGGACAC R: GGAGCTCTCCCATATGGTCG	1.75	62	$(CA)_{22}$	232–246	2	0.499	0.571	0.370	–0.0769
32A	F: CAGTCCTGCTGCATAAATCTC R: GCAGTACAGCTAGAATCACC	2.00	62	$(CA)_{14}$	162–168	3	0.662	0.739	0.573	–0.0806
32F	F: AATACTCAAGCTATGCATCC R: TGTCGTCTACATATAGGGTG	2.00	62	$(GT)_{20}$	226–236	3	0.607	0.679	0.519	–0.0699

Primer sequences, repeat motif, no. of alleles and annealing temperature (T_a) are included for each locus. Expected (H_E) and observed (H_O) mean heterozygosities, polymorphic information content (PIC) and null allele frequencies are also shown (calculated using CERVUS 2.0; Marshall *et al*. 1998). Locus sequences are deposited in GenBank with Accession nos AY138541–AY138545.
PCR, Polymerase chain reaction.

to BIOMAX® film (Kodak) for 24 h. Genotypes were assigned relative to a standard for each locus.

The second component of our strategy was determining polymorphism at these loci for a population likely to be the most variable for these markers. For this we compiled 30 tissue samples from the Umfolozi Game Reserve, currently part of the Hhluhwe-Umfoloze National Park, South Africa. This was the largest remaining population of white rhino during most of the 20th century (International Rhino Foundation 1999). These individuals formed part of the founding population for a World Conservation Union-sanctioned captive breeding programme and were moved to European zoos during the 1970s. DNA was extracted from tissue samples using phenol–chloroform methods (Sambrook *et al*. 1989) and from blood with a QIAGEN® DNA extraction kit.

Five of the 10 optimized primers were variable in this sample set and their details are summarized in Table 1. The mean number of alleles per locus was 2.8. The average observed heterozygosity was 0.597 ± 0.050 (± SE; range 0.462–0.739) and the average expected heterozygosity was 0.578 ± 0.028 (range 0.499–0.662). Expected heterozygosity under Hardy–Weinberg equilibrium was calculated using eqn 8.4 in Nei (1987). The average polymorphic information content for the five selected loci was 0.481.

The high number of monomorphic loci from our optimized primers (five of 10) and low number of alleles suggest that this species may be characterized by relatively low genetic variation. A valid assessment of relative microsatellite variation within the remaining five species of the Rhinocerotidae requires variability estimates across microsatellite loci cloned from all species using similar selection criteria. This work is ongoing in our laboratories.

Acknowledgements

Thanks to Sherry Hinchey, Erin Moody and Michael Kim for data collection, advice and technical expertise. Samples were provided by numerous European zoos. AF was funded by an NSERC summer internship (Canada). Molecular work was funded by an NSERC grant to PTB and an NSF grant to DM.

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