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PRIMER NOTE

Optimization of novel polymorphic microsatellites in the endangered Sumatran rhinoceros (*Dicerorhinus sumatrensis*)

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Abstract

Loss of habitat and poaching have led to a drastic reduction in numbers of the Sumatran rhinoceros (*Dicerorhinus sumatrensis*). To aid in the conservation management of this species, we isolated and optimized 10 polymorphic Sumatran rhinoceros microsatellite loci. A survey of six individuals yielded a mean number of alleles of 3.7, mean expected heterozygosity of 0.551 and probability of identity of 3.46×10^{-8}. Although this estimate is similar to estimates of microsatellite variability in the Black, Indian and White rhinoceroses, such a conclusion is premature as locus purity, sample size and number of loci surveyed vary significantly among studies.

Keywords: genetics, heterozygosity, microsatellite, Sumatran rhinoceros

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The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is one of the most critically endangered large mammal species. Among the five extant species of rhinoceros only the Javan rhinoceros (*Rhinoceros sondaicus*), with estimates of 60 individuals, numbers less than the Sumatran rhinoceros (n = 300). These animals once existed all across southeast Asia including Thailand and Vietnam but, due to a combination of loss of habitat and especially poaching for the horn, their numbers are drastically reduced with their current range restricted to Indonesia and Malaysia (International Rhino Foundation 2003). The rapid, ongoing decline in the population demands immediate intervention through well-informed conservation management strategies.

To assist conservation efforts we report 10 variable microsatellites cloned from the Sumatran rhinoceros. We used a standard cloning protocol (Sambrook *et al*. 1989) as well as an additional enrichment step (Hamilton *et al*. 1999). The *Escherichia* coli strain used for cloning of genomic DNA fragments was JM109 and the plasmid used as vector was pGEM5Zf(+) (Promega Corp.). An initial blue/white screening was followed by a secondary screening of 400 colonies performed by hybridizing filters with probes made by labelling synthetic polymer tracts of $(AC)_n(TG)_n$ and $(AG)_n(TC)_n$ (Pharmacia) with $\gamma^{32}P$. After sizing 298 putative positives on 1% agarose gels, clones of 300–500 bp were sequenced using a radiolabelled Thermosequenase sequencing kit. The $\alpha^{33}P$ dideoxy sequenced products were electrophoresed on a vertical gel rig and visualized with Kodak 100 Biomax MR film. Primers were designed from 18 clones that had both sufficient flanking regions and contained perfect microsatellite repeats of approximately 40 bases.

All primer pairs were optimized on a T-Gradient Thermocycler (Biometra) over a range of annealing temperatures (58–65 °C). The polymerase chain reaction (PCR) cocktail was comprised of 1 μL QIAgen 10× reaction buffer containing $MgCl_2$ (15 mM; QIAgen), 1 μL of dNTP mix (10 mM; 2.5 mM each dATP, dTTP, dCTP and dGTP), 1 μL each of forward and reverse primer (10 μM) (Table 1), 1 μL DNA and sterile ddH_20 up to a total volume of 10 μL. Cycling conditions were an initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 15 s, annealing

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Table 1 Details of 10 optimized polymorphic Sumatran rhinoceros microsatellites with polymorphism estimates from six Sumatran rhinoceros samples (see text for details)

Locus	Repeat motif	Sequence (5′–3′)	T_a (°C)	Alleles	Clone size (bp)	H_E	H_O	Accession no.
SR IIIA	$(CA)_{22}$	F: GGCGAAAGGTAAGAGCAGC R: GCTTCTTTCCGAGGATCTGG	62	6	132	0.806	0.857	AY427961
SR IIIB	$(GT)_{22}$	F: GCCAGCCACCTTCCTCAATG R: TTCATAGACGACGAATGCCTACATG	63	3	159	0.460	0.167	AY427962
SR 54	$(CA)_{26}$	F: CAATATCCAGGCTTCCAGG R: CTGTTTACTGTTATCGATGCTC	63	3	189	0.569	0.571	AY427964
SR 63	$(AC)_{19}$	F: CTTGAGCAGAGTAGAATTTGG R: CTCTGTATCCACCTCATTCC	63	2	201	0.219	0.200	AY427965
SR 71	$(CA)_{21}$	F: ATCATCTCTCTCACACAGACC R: CAACGCTGCACAGACTTCAC	63	7	122	0.806	0.429	AY427966
SR 74	$(CA)_{19}$	F: CAGCACAATGTTTGGCACTTG R: TTGGAGTCTTATGTCACCACC	63	6	176	0.800	0.833	AY427967
SR 191	$(CA)_{21}$	F: TGTAATGTAAAGCACAGTGAC R: GACGTGTATATTGCAAAGTG	63	2	199	0.444	0.429	AY427968
SR 261	$(CA)_{22}$	F: CTGCTGGCCTGTAGATTGC R: CTCCCTGAGCAGTAACTATCC	63	4	192	0.640	0.833	AY427970
SR 275	$(CA)_{25}$	F: GGACTTAGAACCAGGCAATC R: GTCTTGATGCCTGCATTCTG	62	2	149	0.278	0.429	AY427973
SR 281	$(GT)_{23}$	F: AGGTGATTAGGGAATTGCTGG R: TTCTTCTGTCCTGGCATTGC	62	2	234	0.486	0.857	AY427974

Primer sequences, annealing temperature (T_a), repeat motif, clone size of the repeat-containing fragment and number of alleles are indicated for each locus. H_E, mean expected heterozygosity (Nei 1987); H_O, mean observed heterozygosity. The combined probability of identity (Paetkau *et al*. 1995) value for all 10 loci was 3.46×10^{-8}. Accession nos were assigned to all sequences deposited in GenBank.

temperature (Table 1) for 30 s and 72 °C for 30 s. A terminal extension of 72 °C for 5 min completed the PCR. Optimum PCR conditions are summarized in Table 1. For genotyping, γ^{33}P dATP was attached to the forward primer in an end-labelling reaction and this primer was used in an optimized PCR reaction. The PCR products were electrophoresed on a vertical gel rig and visualized with Kodak 100 Biomax MR film.

Ten variable loci were optimized across six Sumatran rhinoceroses (studbook nos, 24, 25, 27, 28, 29 and 33) (Foose 2003). DNA was extracted using the DNEasy kit (QIAgen) and a locus was variable if two or more genotypes were observed in the six samples. Allele sizes for each locus were determined by comparison with an amplicon of the relevant clone. No evidence of null alleles was detected in this small data set as all samples amplified products at all loci. The absence of pedigree data and small sample size precluded further evaluation of the presence of null alleles. The mean number of alleles per locus was 3.7, mean observed heterozygosity 0.522 ± 0.081 (± SE) and mean expected heterozygosity (H_E) 0.551 ± 0.067 (eqn 4 in Nei 1987). The probability of identity was 3.46×10^{-8}. This value estimates the likelihood that two randomly drawn Sumatran rhinoceroses will have identical 10 locus genotypes (Paetkau *et al*. 1995).

Although our estimate of microsatellite variability in this species appears to be similar to three of the other four rhinoceros species, this conclusion is premature. Our estimate of $H_E = 0.551$ is similar to that of the black rhinoceros (*Diceros bicornis bicornis*), $H_E = 0.594$ (Brown & Houlden 1999) and $H_E = 0.500$ (Cunningham *et al*. 1999), the Indian rhinoceros (*R. unicornis*), $H_E = 0.593$ (from Table 1 in Zschokke *et al*. 2003) and the southern white rhinoceros (*Ceratotherium simum simum*), $H_E = 0.593$ (Florescu *et al*. 2003). However, these values are calculated from loci with very different repeat motifs, assays of different sample sizes and studies using different numbers of conspecific loci. For example, black rhinoceros estimates were from a sample size of $n = 7$ with 11 loci (Brown & Houlden 1999) and $n = 72$ with three loci (Cunningham *et al*. 1999), Indian rhinoceros estimates from $n = 14$ and 11 loci (Zschokke *et al*. 2003), Sumatran rhinoceros estimates from $n = 6$ and 10 loci (this study) and white rhinoceros estimates from $n = 30$ and five loci (Florescu *et al*. 2003). Locus purity and sample size are both important when estimating microsatellite variability in any taxon (Van Coeverden de Groot 2001). An accurate comparison of microsatellite variability among surviving rhinoceroses awaits the results of our ongoing study, which uses the same suite of microsatellite loci cloned from black, Indian, Sumatran and white rhinoceroses on samples of 20 or more for each of the above four rhinoceros species. Only then can the relative effects on genetic variability of recent severe bottlenecks be estimated for each species.

The primary goal of this work was to optimize loci necessary to determine relatedness among all captive Sumatran rhinoceroses. The data in this study were restricted to a few individuals from North American zoos, reducing the accuracy of their relatedness estimates. The assay of the combined set of these and Sumatran rhinoceroses at breeding facilities in Indonesia and Malaysia (n = 19) is currently underway.

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References

Brown SM, Houlden BA (1999) Isolation and characterization of microsatellite marker in the black rhinoceros (*Diceros bicornis*). *Molecular Ecology*, **8**, 1551–1561.

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

Florescu A, Davila JA, Scott C *et al*. (2003) Polymorphic microsatellites in white rhinoceros. *Molecular Ecology Notes*, **3**, 344–345.

Foose T (2003) *International Studbook for Sumatran Rhino (Dicerorhinus sumatrensis)*. International Rhino Foundation, Yulee, Florida.

Hamilton MB, Pincus EL, Di Fiore A, Fleischer RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *Biotechniques*, **27**, 500–502, 504–507.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Paetkau D, Calvert W, Sterling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology*, **4**, 347–354.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbour Laboratory Press, New York.

Van Coeverden de Groot PJ (2001) Conservation genetic implications of microsatellite variation in the mustox *Ovis moschatus*: The effect of refugial isolation and the Arctic Ocean on Genetic Structure. PhD thesis, Queens University, Ontario, Canada.

Zschokke S, Gautschi B, Baur B (2003) Polymorphic microsatellite loci in the endangered Indian rhinoceros (*Rhinoceros unicornis*). *Molecular Ecology Notes*, **3**, 233–235.