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PRIMER NOTES

Characterization of microsatellite DNA loci for a neotropical migrant songbird, the Swainson's thrush (*Catharus ustulatus*)

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Genetic analyses using highly variable DNA-based genetic markers can provide useful information for developing conservation plans for migrant birds. For example, analyses of population genetic structure can help define demographically distinct management units (cf. Moritz 1994) while identification of population-specific genetic variants enable the use of genetic 'tags' to study the movement patterns of individuals between breeding and wintering populations (Wenink & Baker 1996). Although a number of microsatellite loci have been isolated for some species of neotropical migrants (e.g. Dawson *et al*. 1997), the availability of loci for this group of birds remains limited. Here, we describe the isolation of variable microsatellite DNA loci which can be used for individual and population-level genetic analyses of a member of this group, the Swainson's thrush (*Catharus ustulatus*).

We studied thrushes in Prince Albert National Park, Saskatchewan from May to July 1994. Blood samples were collected from individually marked adults captured using song play-backs to lure individual birds into mist nets. DNA was then extracted from these samples using standard phenol–chloroform techniques and quantified using a fluorometer. We analysed DNA from 24 adults for the analyses described here.

To isolate microsatellite loci we digested 10 μg of thrush DNA (2 μg from each of five birds combined) with 10 U each of *Alu*I, *Hae*III, and *Rsa*I and then cloned genomic fragments 350–450 bp in length into the *Sma*I site of the pUC18 plasmid vector. The screening of colonies was then performed by hybridizing filters with probes made by labelling three dinucleotide polymer tracts $(TG)_n$ $(TC)_n$, and $(GC)_n$ (Pharmacia) with [α^{32}P]-dCTP using random priming. Primary and secondary screening of approximately 17 000 colonies yielded a total of 27 positive clones. These clones were then cycle sequenced (AmpliCycle; Perkin-Elmer) and primers were designed to amplify regions containing a microsatellite repeat for six of these clones (Table 1).

To assay variation among individuals, PCR reactions were performed on a Perkin-Elmer 480 DNA Cycler in 10 μL volumes, each containing 50 ng of genomic DNA, 0.25 pmoles of the forward primer end-labelled with [γ^{33}P]-ATP, 0.25 pmoles of unlabelled forward primer, 0.5 pmoles of unlabelled reverse primer, 300 μM dNTPs, 0.25 U AmpliTaq (Perkin-Elmer), 0.1 M Tris-HCl pH 8.3, 0.5 M KCl, and 2.5 mM $MgCl_2$. Following an initial denaturing step at 94 °C for 3 min, 30

Table 1 Genetic characteristics and primer sequences for six Swainson's Thrush microsatellite loci. Sizes are based on the sequenced allele. Data on numbers and frequencies of alleles are based on genotypes of 24 adults. Expected (H_E) and observed (H_O) levels of heterozygosity were calculated using GENEPOP. The clone sequences from which the primers were developed have GenBank accession numbers AF122890–AF122895

Locus	Repeat motif	Primers	T_m (°C)	Size (bp)	No. of alleles	Frequency of most common allele	H_E	H_O
Cuμ 02	$(TG)_{15}$	F-CCTTGGATTGCTTCCAAATG R-CCAATTTCCTGCAGACTCTTTC	60	146	10	0.21	0.89	0.96
Cuμ 04	$(GT)_{16}$	F-AATTGCATAAATGTGATCCAC R-AAATGAAATGTGGTAGAATTCC	55	132	20	0.15	0.94	0.96
Cuμ 05	$(GT)GC(GT)_2GC(GT)_8$	F-ACCTCTAAATACCTGTGAGTGC R-ACTGTGGTATTCTTTACCTAGCA	60	146	7	0.58	0.63	0.55
Cuμ 10	$(CA)_{10}$	F-AAAATGAGGAGAATACTAGGCA R-ACTTATTTCAGTCCTAAATTCACC	60	141	9	0.33	0.82	0.83
Cuμ 28	$(CA)_{12}$	F-GAGGCACAGAAATGTGAATT R-TAAGTAGAAGGACTTGATGGCT	60	157	15	0.15	0.90	0.83
Cuμ 32	$(GT)TT(GT)_9$	F-AGGAGAGTGAAAGAAAAGGG R-GAATTCTCAGCATGACAAATC	60	130	7	0.29	0.80	0.71

cycles of PCR were carried out, each cycle consisting of 45 s at 94 °C, 45 s at T_m in Table 1, and 45 s at 72 °C. Amplification products were resolved on 6% polyacrylamide denaturing gels containing 7.7 M urea. Gels were run at 55 W for 2 h. Dried gels were exposed to BIOMAX (Kodak) X-ray film overnight. Product sizes were determined by reference to a sequencing reaction of a known template, and a clone of known size for each locus run every 10 lanes.

All loci were variable with between 7 and 20 alleles among the adults surveyed; the frequency of the most common allele ranged from 0.15 to 0.58 (Table 1). Expected and observed heterozygosities are not significantly different for any locus (Table 1) with the exception that there is a significant ($P = 0.029$) deficiency of certain heterozygotes at Cuμ 05 as determined using exact tests in GENEPOP (Raymond & Rousset 1995), suggesting the possible presence of a null allele(s) at low frequency at this locus. The usefulness of these loci for parentage analyses was determined by calculating the probability of detecting the occurrence of an extra-pair fertilization by an additional male (assuming known maternity) (P[E]) using the formula in Chakravarti & Li (1983). The overall P(E) value is 0.999, indicating that when the six loci are used for parentage analyses there is a 99.9% chance of detecting extrapair paternity within nests of this species. Finally, four of these loci (Cuμ 02, 04, 10, and 28) appear to amplify polymorphic loci in some distantly related passerine species. Specifically, the following loci are variable in these birds: yellow warbler (*Dendroica petechia*), Cuμ 28; grasshopper sparrow (*Ammodramus savannarum*), Cuμ 02, 04, and 28; ovenbird (*Seiurus aurocapillus*), Cuμ 02, 04, and 28; brown-headed cowbird (*Molothrus ater*), Cuμ 04, 10, and 28.

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References

Chakravarti A, Li CC (1983) The effect of linkage on paternity calculations. In: *Inclusion Probabilities in Parentage Testing* (ed. Walker RH), pp. 411–422. American Association of Blood Banks, Arlington, Virginia.

Dawson RJG, Gibbs HL, Hobson KA, Yezerniac S (1997) Isolation of microsatellite markers from a passerine bird, the yellow warbler, *Dendroica petechia* and their use in population studies. *Heredity*, **79**, 506–514.

Moritz C (1994) Applications of mitochondrial DNA analysis in conservation: a critical review. *Molecular Ecology*, **3**, 401–411.

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

Wenink PW, Baker AJ (1996) Mitochondrial DNA lineages in composite flocks of migratory Dunlins (*Calidris alpina*). *Auk*, **113**, 744–756.

Primers for the detection of *Oryctes* virus from Scarabaeidae (Coleoptera)

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Oryctes virus (OrV), first described by Huger (1966), is an unusual virus which can infect some members of the insect family Scarabaeidae (Coleoptera). Presently designated as an 'unassigned virus' (Murphy *et al*. 1995), it is primarily a pathogen of the palm pest, *Oryctes rhinoceros* (rhinoceros beetle), against which it has been successfully used as a biocontrol agent throughout the Pacific and Indo-Asia. The virus is also under assessment as a control agent for *Papuana* spp. (Scarabaeidae), pests of taro, which it is not known to infect naturally. Detecting OrV infections can be difficult and usually involves examining for symptoms of a whitened, swollen gut (Zelazny 1978) with confirmation by electron microscopy. We have developed a set of PCR (polymerase chain reaction) primers which specifically detect OrV from insect host material and could be used for the routine detection of the disease. Such screening will greatly enhance knowledge of the epizootiology of OrV, both in *O. rhinoceros* and potential new hosts.

*Hin*dIII and *Eco*RI fragments of the 17.7 kb *Pst*I D fragment of OrV DNA (Fig. 1), originating from strain PV505 (Crawford *et al*. 1985), were cloned into the pUC19 vector and sequenced (GeneBank Accession no. AF126716). Primers were designed to amplify OrV fragments (Fig. 1; Table 1) and tested against larval and adult gut samples from *O. rhinoceros* and *Papuana* spp. collected in Papua New Guinea (PNG). The majority of guts showed no visual symptoms of OrV infection. Samples were stored at 4 °C in either 70% ethanol or buffer (0.05 M Tris pH 7.5, 1 mM EDTA). Tissue culture-grown OrV was included as a positive control and water as a negative control. Specificity of one primer pair, OrV15a/15b, was examined using gut extracts from a range of other Scarabaeidae, including the New Zealand species, *Costelytra zealandica*, *Odontria* sp., *Chlorochiton* sp. and *Adoryphorus couloni*, which had never been exposed to OrV.

Approximately 50 mg portions of insect guts were macerated in 500 μL of disruption buffer (100 mM Tris pH 8.0, 10 mM EDTA, 0.1% SDS, 0.1 mg/mL proteinase K) and incubated either overnight at 37 °C or for 3 h at 65 °C. Samples were twice phenol–chloroform extracted, ethanol precipitated and then resuspended in 200 μL of sdH_2O.

PCR reactions (25 μL) contained 0.4 μM of each primer (Gibco BRL), 200 μM dNTPs (Boehringer Mannheim), 2.5 μL of reaction buffer, and 2 μL of DNA. Three polymerases were compared: *Taq* (0.625 U/reaction) (Boehringer Mannheim), Expand™ High Fidelity *Taq* (0.7 U/reaction) (Boehringer Mannheim) and 'Red Hot' *Taq* (0.6 U/reaction) (Advanced

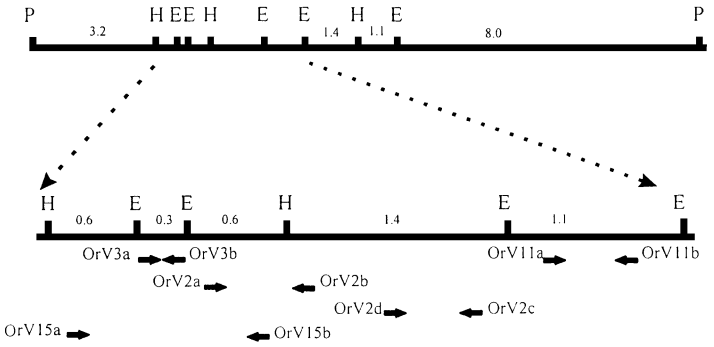

Fig. 1 Restriction map of OrV *Pst*I D fragment and location and direction of OrV primers (P = *Pst*I; H = *Hind*III; E = *Eco*RI).

Table 1 Primers designed for amplification of OrV DNA

Primer pair	Sequence (5′–3′)	Product size
OrV15a	ATTACGTCGTAGAGGCAATC	945 bp
OrV15b	CATGATCGATTCGTCTATGG	
OrV3a	GAACGCAAGTTTGCAGTATG	212 bp
OrV3b	ATACTATCGTCTCCGATGTC	
OrV2a	AGAGTCTACGAAATTCGAGC	481 bp
OrV2b	CATTTAAGCGTTTACGTTCG	
OrV2d	ACTGCTGTTTACGATCGAAG	504 bp
OrV2c	GTACAGTTGAGCGCTAGTAC	
OrV11a	GCAGTCCGTAGAGGATTCAC	409 bp
OrV11b	AAGCTCCTACCGCGACCACA	

Biotechnologies, UK). A $MgCl_2$ concentration of 2.5 mM per reaction was found to be optimal for both Expand and Red Hot *Taq* polymerases. Amplification was performed in a Perkin-Elmer 480 thermal cycler using 30 cycles of 1 min at 94 °C, 1 min at 45 °C and 2 min at 72 °C.

Replacement of Boehringer Mannheim *Taq* with Expand or Red Hot Taq significantly improved detection. Both phenol–chloroform extraction and proteinase K disruption were necessary steps as amplification from nonextracted insect tissue was poor. An extensive investigation of the optimal dilution of processed samples (10^0, 10^{-1} and 10^{-2}) demonstrated that most virus-positive results came from the 10-fold dilution. Some larval samples with high levels of gut organic matter could not be purified and never produced OrV-positive bands.

Positive PCR results were strongly correlated with visual disease symptoms in *O. rhinoceros* adults. In addition, a high proportion of the insects that appeared healthy but probed positive were collected from sites where the virus was known to be present. The OrV15a/15b primer pair detected OrV in > 40% of the *O. rhinoceros* larval samples, which are particularly difficult to visually assess for OrV infection. OrV was detected in *Papuana* spp. which had ingested large quantities of virus (exposed by swimming in virus stock), but no larval *Papuana* spp. collected from field sites or New Zealand scarab samples produced positive results, indicating that the primers are specific to the virus. In conclusion, the primer pair OrV15a/15b has proven useful for the routine detection of OrV. The virus was detected from tissue before visual symptoms were evident, suggesting that the primers will be useful in ecological studies on the virus and management of the pest.

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References

Crawford AM, Ashbridge K, Sheehan C, Faulkner P (1985) A physical map of the *Oryctes* baculovirus genome. *Journal of General Virology*, **66**, 2649–2658.

Huger AM (1966) A virus disease of the Indian rhinoceros beetle *Oryctes rhinoceros* (Linnaeus), caused by a new type of insect virus, *Rhabdionvirus oryctes* gen. n., sp. n. *Journal of Invertebrate Pathology*, **8**, 38–51.

Murphy FA, Fauquet CM, Bishop DHL *et al*. (1995) *Virus Taxonomy: the Classification and Nomenclature of Viruses*. The Sixth Report of the International Committee on Taxonomy of Viruses. Springer-Verlag, Wien.

Zelazny B (1978) Methods of inoculating and diagnosing the baculovirus disease of *Oryctes rhinoceros*. *FAO Plant Protection Bulletin*, **26**, 163–168.

Dinucleotide microsatellite loci in a migratory wood warbler (Parulidae: *Limnothlypis swainsonii*) and amplification among other songbirds

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Table 1 Characteristics of 10 microsatellite loci developed for Swainson's warbler (*Limnothlypis swainsonii*)

Locus	Primers (5′ – 3′)	T_a*	Amplification details†	Repeat	Fragment size (bp)‡	No. of alleles§	H_O	H_E	GenBank Accession no.
Lsw μ3	TATGGTTTGCTCTGGTTTTTC TTCTTTACACTGTGCCTTGCT	48	dNTPs, BSA	$(GT)_{15}$	229	7	0.80	0.76	AF129088
Lsw μ4	CTTTTTAACCCCTGTGTTTGT CTTTTGTCTGGATGTCTGTGG	48	dNTPs, BSA, 1.5 × buffer	$(GT)_{18}$	189	¶			AF129089
Lsw μ5B	CTTGCAGCATCAGTTTTTAGT GCTGGGTGCACACACACA	55	dUTPs	$(GT)_{12}$	227	4	0.50	0.69	AF129090
Lsw μ7	GATGTGACAAGTGTGCTCTCC TTTATATCTAGTGACGCTCTA	48	dNTPs	$(GT)_{14}$	165	7	1.00	0.87	AF129091
Lsw μ9	GAGCACATCCAAGAGGCAATA GATCCCTGTTCCACTCCAC	50	dUTPs, BSA	$(GT)_{10}$	143	2	0.40	0.34	AF129092
Lsw μ12	ATCCCATTGAGGACTTTCTTG TTCCCTGAAGGARATCAACATC	50	dUTPs	$(GT)_{11}$	≈400	8	0.88	0.89	AF129093
Lsw μ13	ATTTGAAAAAGAGGAGTCATTATAT AAAGGAAAGGAGCTTCATCAG	55	dUTPs	$(GT)_{11}$	≈450	¶			AF129094
Lsw μ14	GTTATGCTCCAACAAAATAGATA AGGTTTTRAAGGATAGATTTATA	50	dUTPs	$(AT)_6(GT)_{11}$	190	6	0.89	0.73	AF129095
Lsw μ18	TTGCTGAAAGAAGTACTAAGA CTGKTTGCAGGATATGTATAC	55	dUTPs	$(AT)_{14}$	240	7	1.00	0.84	AF129096
Lsw μ19	AGGTGACAATTATGAGGATAG ACTGGTGTTTATCTTCAATAG	48	dNTPs, BSA	$(AT)_{15}(GT)_9$	161	7	1.00	0.85	AF129097

*Annealing temperature.
†Variation from general amplification conditions (see text).
‡Size of cloned allele.
§Number of alleles found in screening 10 individuals from five populations.
¶Polymorphic, but not readily scoreable; two loci may be amplifying.

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Development of conservation and management plans can be aided by genetic data, and the utility of microsatellites for studies of genetic variability, population structure, gene flow, and relatedness makes these loci appropriate for studies of declining natural populations. Wood warblers (Parulidae), a New World family of 115 species of 25 genera, have played an important role in studies of avian ecology, evolution, hybridization, and behaviour. Also, 7% of the species are threatened or endangered (Collar *et al*. 1992), and populations of other species are locally threatened or declining.

We developed polymerase chain reaction (PCR) assays of microsatellite loci for Swainson's Warbler (*Limnothlypis swainsonii*), the only species in the genus *Limnothlypis* and among the rarest wood warblers in North America (Brown & Dickson 1994). Our PCR primers were tested for their ability to amplify orthologous loci in 19 other wood warblers and five other species of three different families.

Protocols used to obtain and score microsatellites (Glenn 1997) involved ligating 300–700 bp fragments of genomic DNA into a plasmid and enriching for dinucleotide microsatellites using $d(AG)_{12}$ and $d(AC)_{12}$ or $d(AG)_{12}$ and $d(TG)_{15}$ (Ostrander *et al*. 1992). Transformed bacterial colonies were screened by hybridization with radioactively labelled $d(AG)_{12}$ and $d(TG)_{15}$. Sixty positive clones were sequenced following Meeker *et al*. (1993) or Rouer (1994) using Sequenase 2.0 (US Biochemical). PCR primers were designed from sequences flanking the repetitive elements. Genomic DNA was extracted using a diatomaceous earth/guanidine thiocyanate extraction protocol (Carter & Milton 1993) and diluted to 20 ng/μL for use in PCR experiments.

Amplification protocols for each primer pair were optimized to amplify *Limnothlypis swainsonii* DNA using the highest possible annealing temperatures, the lowest Mg concentration, dUTP when possible, and BSA when necessary. dUTP was substituted for TTP to allow enzymatic degradation of carryover contaminants (Longo *et al*. 1990). Because dUTP is incorporated less efficiently by *Taq* DNA polymerase, TTP was used for loci not amplifying with dUTP. Following optimization, amplification experiments were conducted using Promega Thermo buffer (final concentrations 50 mM KCl, 10 mM Tris-HCl pH 9, 1% Triton X-100), 2 mM $MgCl_2$, 150 μM dNTPs (Table 1), 1 unit *Taq* DNA polymerase (Promega), 0.5 μM each primer, and 80 ng of DNA. For some primer pairs, optimization included addition of 125 ng/mL BSA (Table 1). A Perkin-Elmer Model 480 Thermal Cycler was used with Midwest Scientific thermal tubes under

Table 2 Amplification of 10 microsatellite loci in 24 species of oscine passerines (Passeriformes: suborder Passeres)

Taxon	*Lsw*μ3	*Lsw*μ4	*Lsw*μ5B	*Lsw*μ7	*Lsw*μ9	*Lsw*μ12	*Lsw*μ13	*Lsw*μ14	*Lsw*μ18	*Lsw*μ19
Ptilonorhynchidae										
Ptilonorhynchus violaceus	*	–	+/*	*	+	*	*	+	–	–
Amblyornis subalaris	*	–	+	–	+	–	–	*	–	–
Turdidae										
Catharus aurantiirostris	–	–	–	–	–	–	–	–	–	–
Hylocichla mustelina	–	–	+	–	–	–	–	–	–	–
Parulidae										
Vermivora pinus	±	+	+	+	+	+	+	–	+	–
Parula americana	–	–	+	–	+	–	+	+	+	–
Dendroica pinus	–	–	–	+	±	+	+	+	+	–
Dendroica kirtlandii	–	–	+	+	±	–	±	+	+	–
Mniotilta varia	*	+	+	+	+	–	+	+	+	+
Setophaga ruticilla	–	–	–	–	–	–	–	–	±	–
Protonotaria citrea	–	+	±	–	+	±	±	+	+	+
Helmitheros vermivorus	–	+	–	–	+	–	±	+	+	–
Seiurus aurocapillus	–	+	–	+	+	+	+	+	+	–
Seiurus noveboracensis	±	+	±	+	+	+	+	+	+	+
Oporornis philadelphia	–	+	–	+	+	–	–	+	+	–
Geothlypis trichas	*	+	+	+	+	+	–	+	–	+
Wilsonia citrina	*	+	–	+	+	+	+	+	+	+
Myioborus miniatus	–	±	–	+	+	+	+	+	+	±
Basileuterus culicivorus	–	±	–	+	±	–	+	+	+	–
Basileuterus rivularis	*	+	–	+	–	+	–	+	+	–
Zeledonia coronata	–	–	–	–	–	–	–	±	±	–
Icteria virens	–	–	+	–	+	–	–	+	+	–
Granatellus sallaei	*	–	–	–	–	–	–	+	+	–
Thraupidae										
Piranga flava	–	–	–	+	–	+	–	+	+	–

+indicates amplification of product similar in size to *Limnothlypis*.
±indicates less product, but of expected size.
*indicates amplification of nontarget product(s).
–indicates no amplification.

these conditions: 3 min 45 s at 94 °C; then 35 cycles of 94 °C for 1 min, primer-specific annealing temperature (see Table 1) for 30 s, and 72 °C for 30 s. Negative and positive controls were used in all amplification experiments. Twelve μL of completed PCR reaction were run in 1.5% agarose minigels containing ethidium bromide. GelMarker I (Research Genetics) was used to judge relative size and yield of PCR products. Results were examined under UV light and an Eagle Eye I was used to record fluorescence.

Following optimization, variation within *L. swainsonii* was assessed at each locus using two individuals each from five breeding populations (North Carolina, South Carolina, Florida, Louisiana, Arkansas). Cross-species amplification experiments were conducted by testing individuals for amplifiability using each primer pair separately in a 50 μL PCR reaction.

Five of the 15 microsatellite primer pairs amplified loci showing no variation within Swainson's Warbler (GenBank Accession nos: AF129098–AF129102). Two showed polymorphism, but were not scoreable (Table 1). In both cases two loci may have been amplifying. The eight remaining loci showed 2–8 alleles among the 10 individuals screened (mean of 6.4 alleles/locus; Table 1). Linear regression showed no correlation between maximum repeat length and the number of alleles occurring at each locus.

In cross-species amplification experiments only *Limnothlypis* amplified at every locus. Considering only perfect amplifications ('+' in Table 2), other Parulidae showed amplification at from 0 to 8 loci (mean of 4.9; Table 2). Species in the families Ptilonorhynchidae, Thraupidae, and Turdidae amplified at from 0 to 4 loci (Table 2), with a mean of 2.0 loci.

Loci developed in *Limnothlypis* amplified broadly, but with notable variability, among other species of songbirds. It is anticipated that these loci will prove useful for investigations of population genetics, geographic variation, speciation, hybridization, paternity, migration, and behaviour in this widely studied group of birds.

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References

Brown RE, Dickson JG (1994) Swainson's warbler (*Limnothlypis swainsonii*). *Birds of North America*, **126**, 1–19.

Carter MJ, Milton ID (1993) An inexpensive and simple method for DNA purifications on silica particles. *Nucleic Acids Research*, **21**, 1044.

Collar NJ, Gonzaga LP, Krabbe N *et al*. (1992) *Threatened Birds of the Americas: the ICBP/IUCN Red Data Book*, 3rd edn, *part 2*. International Council for Bird Preservation, Cambridge, U.K.

Glenn TC (1997) Genetic bottlenecks in long-lived vertebrates: mitochondrial and microsatellite DNA variation in American alligators and whooping cranes. PhD Dissertation, University of Maryland, College Park.

Longo MC, Berninger MS, Hartley JL (1990) Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene*, **93**, 125–128.

Meeker AK, Li YK, Shortle D, Stites WE (1993) A simplified protocol for isolation and characterization of ssM13 DNA templates for use in dideoxy sequencing. *Biotechniques*, **15**, 370–372.

Ostrander EA, Jong PM, Rine J, Duyk G (1992) Construction of small-insert genomic DNA libraries highly enriched for microsatellite repeat sequences. *Proceedings of the National Academy of Sciences of the USA*, **89**, 3419–3423.

Rouer E (1994) Direct neutralization of alkaline-denatured plasmid DNA in sequencing protocol by the sequencing reagent itself. *Nucleic Acids Research*, **22**, 4844.

Identification of polymorphic microsatellite loci in the gorilla (*Gorilla gorilla gorilla*) using human primers: application to noninvasively collected hair samples

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Thousands of microsatellite loci have been identified as part of the Human Genome Programme. Their evolutionary conservation allows for successful cross-species amplification in related species using the same primers and amplification conditions (Coote & Bruford 1996). Human polymerase chain reaction (PCR) primers have been applied to several primate species (e.g. Morin *et al*. 1994; Gerloff *et al*. 1995; Kayser *et al*. 1996). Similar degrees of polymorphism are found at the equivalent primate microsatellite loci, and they can be used to discriminate between individuals. As more than five alleles often segregate within a population (e.g. Morin *et al*. 1994), microsatellites are highly suited as Mendelian markers for pedigree and population analyses (e.g. Coote & Bruford 1996). Sequential typing of up to 12 such loci from 20 individuals can accurately characterize genetic variability within a population (Altmann *et al*. 1996). We report data about cross-species amplification in western lowland gorillas (*Gorilla gorilla gorilla*) for 32 human microsatellite markers. This work forms part of a population genetic study examining microsatellite variation throughout the lowland gorilla range using shed hairs as the DNA source material.

Genomic DNA was extracted from blood collected from 10 captive gorillas (Wickings & Dixson 1992). DNA was extracted from hair as follows: roots of 3–6 hairs from each individual were agitated in 250 μL of 5% chelex, 10 mM Tris pH 8.0 for approximately 24 h at 56 °C. Samples were vortexed vigorously, boiled for 15 min, vortexed and spun for 3 min. The supernatant was stored at 4 °C.

PCR amplification at 32 microsatellite loci was carried out using blood-derived DNA, in a 20 μL volume using a Hybaid Omnigene Thermal Cycler. Twenty pmoles 5′-primer was end-labelled using T4 polynucleotide kinase with [γ^{32}P]-ATP. The reaction mix contained 20–100 ng of DNA, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/mL BSA, 0.2 mM dNTP, 20 pmoles labelled primer, 20 pmoles 3′-primer and 1 U of *Taq* DNA polymerase. For dinucleotides initial denaturation was for 3 min at 93 °C followed by seven cycles of 30 s at 93 °C, 60 s at either 50 °C or 54 °C and 90 s at 72 °C, followed by 30 cycles of 45 s at 93 °C, 90 s at 55 °C or 59 °C and 120 s at 72 °C with 10 min at 72 °C after the last cycle. For tetranucleotides initial denaturation was 2 min at 93 °C followed by 35 cycles of 10 s at 94 °C, 15 s at 58 °C and 15 s at 72 °C, with 10 min at 72 °C after the last cycle. PCR products were electrophoresed on 6% denaturing polyacrylamide gels and visualized on autoradiographic film exposed for 12–72 h. Allele sizes were determined by comparison with previously sequenced human alleles or a M13 sequence ladder.

At the 32 loci tested, three or more alleles were detected at 21 loci (Table 1). As the aim of this study is to characterize a series of loci for use in noninvasively collected hair samples, these 21 loci were examined in hair-derived DNA from wild gorillas; only eight amplified consistently, i.e. gave identical amplification products in at least two PCRs (Table 2). Heterozygosity was reduced in this larger sample set, perhaps due to errors associated with using DNA derived from shed hair, as observed in other studies (Taberlet *et al*. 1996; Gagneaux *et al*. 1997). In this study marked differences in amplification success and accuracy were seen between DNA extracted from blood or from shed hairs. We are currently characterizing 16 different gorilla populations using these polymorphic microsatellite loci.

Table 1 Screening of 32 human microsatellite loci using blood-derived DNA from 10 gorillas

Locus	VNTR	No. of alleles	Allele sizes (bp)	GDB ID	Heterozygosity		Source of marker information
					Observed	Expected	
D1S161	$(CA)_n$	4	G00–182–195	141–153	0.833	0.875	Field *et al*. (1998)
D1S207	$(CA)_n$	6	G00–188–004	131–169	0.556	0.876	Coote & Bruford (1996)
D1S518	$(GATA)_n$	4	G00–686–838	196–284	0.625	0.867	CHLC
D1S533	$(GATA)_n$	6	G00–686–415	196–252	0.333	0.727	CHLC
D1S548	$(GATA)_n$	4	G00–689–691	165–177	0.738	0.750	CHLC
D1S550	$(GATA)_n$	5	G00–686–748	183–199	0.755	1.000	CHLC
D2S426	$(GATA)_n$	4	G00–686–484	184–204	0.659	0.857	CHLC
D2S434	$(GATA)_n$	5	G00–686–682	284–300	0.625	0.825	CHLC
D2S1326	$(GATA)_n$	7	G00–684–564	258–282	0.900	1.000	Morin *et al*. (1998)
D3S1768	$(GATA)_n$	5	G00–686–874	164–208	0.600	0.616	CHLC
D5S820	$(GATA)_n$	3	G00–686–796	194–202	0.769	0.857	Kayser *et al*. (1996)
D5S1457	$(GATA)_n$	6	G00–682–402	113–125	0.714	0.714	CHLC
D5S1470	$(GATA)_n$	4	G00–685–680	184–204	0.842	0.875	Kayser *et al*. (1996)
D6S271	$(CA)_n$	5	G00–188–051	169–203	0.867	1.000	Coote & Bruford (1996)
D6S311	$(CA)_n$	4	G00–188–611	246–264	0.667	0.727	Coote & Bruford (1996)
D6S501	$(GATA)_n$	2	G00–686–718	166–170	0.440	0.571	CHLC
D11S925	$(CA)_n$	2	G00–188–416	167–169	0.286	0.440	Coote & Bruford (1996)
D16S402	$(CA)_n$	4	G00–187–850	197–217	0.663	0.714	CHLC
D17S791	$(CA)_n$	3	G00–188–093	182–188	0.429	0.714	Coote & Bruford (1996)
D22S684	$(GATA)_n$	5	G00–685–080	240–256	0.714	0.835	Morin *et al*. (1998)
D22S685	$(GATA)_n$	5	G00–364–343	185–201	0.625	0.808	CHLC
DXS738	$(CA)_n$	5	G00–182–660	138–152	0.500	0.792	Field *et al*. (1998)
DXS6810	$(GATA)_n$	5	G00–364–494	213–229	0.375	0.725	CHLC

CHLC, Cooperative Human Linkage Center.

Table 2 Characterization of eight polymorphic microsatellite loci using hair-derived DNA from western lowland gorillas

Locus	VNTR	Annealing Temp (76 °C)	No. of individuals	No. of alleles	Heterozygosity		PCR primer sequences 5′–3′
					Observed	Expected	
DIS548	$(GATA)_n$	58	10	4	0.400	0.737	GAACTCATTGGCAAAAGGAA GCCTCTTTGTTGCAGTGATT
D1S550	$(GATA)_n$	58	22	10	0.409	0.868	CCTGTTGCCACCTACAAAAG TAAGTTAGTTCAAATTCATCAGTGC
D2S434	$(GATA)_n$	58	21	11	0.524	0.843	TAAATCACTAGCCTTTGCCG GCCATCTGTACTGTTCCCAG
D2S1326	$(GATA)_n$	58	21	12	0.571	0.808	AGACAGTCAAGAATAACTGCCC CTGTGGCTCAAAAGCTGAAT
D3S1768	$(GATA)_n$	58	31	11	0.523	0.601	GGTTGCTGCCAAAGATTAGA CACTGTGATTTGCTGTTGGA
D22S685	$(GATA)_n$	58	8	5	0.500	0.833	TTCTTAGTGGGGAAGGGATC TGAGTTTGATGTTTTTGATAGACA
DXS738	$(CA)_n$	54	10	5	0.273	0.723	AATGTGTTGTTGTATTCACCTTGC CCAGCAATAACCATAAGTAAAAC
DXS6810	$(GATA)_n$	58	24	7	0.063	0.678	ACAGAAAACCTTTTGGGACC CCCAGCCCTGAATATTATCA

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References

Altmann J, Alberts SC, Haines SA *et al*. (1996) Behavior predicts genetic structure in a wild primate group. *Proceedings of the National Academy of Sciences of the USA*, **93**, 5797–5801.

Coote T, Bruford MW (1996) Human microsatellites applicable for analysis of genetic variation in apes and Old World monkeys. *Journal of Heredity*, **87**, 406–410.

Field D, Chemnick L, Robbins M, Garner K, Ryder O (1998) Paternity determination in captive lowland gorillas and orangutans and wild mountain gorillas by microsatellite analysis. *Primates*, **39**, 199–209.

Gagneaux P, Boesch C, Woodruff DS (1997) Microsatellite scoring errors associated with non-invasive genotyping based on nuclear DNA amplified from shed hair. *Molecular Ecology*, **6**, 861–868.

Gerloff U, Schlötterer C, Rassmann K *et al*. (1995) Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild living bonobos (*Pan paniscus*). *Molecular Ecology*, **4**, 515–518.

Kayser M, Ritter H, Bercovitch F, Mrug M, Roewer L, Nurnberg P (1996) Identification of highly polymorphic microsatellites in the rhesus macaque *Macaca mulatta* by cross-species amplification. *Molecular Ecology*, **5**, 157–159.

Morin PA, Mahboubi P, Wedel S, Rogers J (1998) Rapid screening and comparison of human microsatellite markers in baboons: allele size is conserved, but allele number is not. *Genomics*, **53**, 12–20.

Morin PA, Moore JJ, Chakraborthy R, Jin L, Goodall J, Woodruff DS (1994) Kin selection, social structure, gene flow, and the evolution of chimpanzees. *Science*, **265**, 1193–1201.

Taberlet P, Griffin S, Goossens B *et al*. (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, **24**, 3189–3194.

Wickings EJ, Dixson AF (1992) Application of DNA fingerprinting to familial studies of Gabonese primates. In: *Paternity in Primates: Genetic Tests and Theories* (eds Martin RD, Dixson AF, Wickings EJ), pp. 113–130. Karger, Basel, Switzerland.

Isolation and characterization of microsatellite markers in the acorn barnacle *Semibalanus balanoides*

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The acorn barnacle *Semibalanus balanoides* is widely distributed in the intertidal rocky shores of both East and West Atlantic, north of Cape Atteras (USA) and northwestern Spain. The species also occurs on the Pacific coast of southern Alaska (Flowerdew 1983). *S. balanoides* has been traditionally used as a model organism in studies of factors shaping marine invertebrate communities (Menge & Sutherland 1976). Much less work has been devoted to the molecular ecology of this species. Of particular interest is a recent study of *S. balanoides* populations from the Gulf of St. Lawrence (Canada) that revealed a genetic discontinuity of allelic frequencies at two enzymatic loci (Mpi and Gpi) in the vicinity of the Miramichi estuary in the southern part of the Gulf (Holm & Bourget 1994). These authors proposed that selection imposed by thermal gradients was probably responsible for such genetic patterns in the absence of physical barriers to gene flow. In order to further elucidate the respective roles of selection and gene flow on the genetic structure of *S. balanoides* from that region, we developed primers for polymerase chain reaction (PCR) amplifications of five microsatellite loci. DNA was extracted from whole barnacles collected in Le Goulet, New-Brunswick, using standard phenol–chloroform techniques (Sambrook *et al*. 1989). Fifty μg of genomic DNA was digested using Sau3AI restriction enzyme. DNA fragments ranging from 400 to 900 bp were selected (as suggested in Estoup & Turgeon 1996), excised from a 1.7% agarose gel and purified using a QIAGEN extraction kit. The purified DNA was ligated into the pUC18 *Bam*HI/BAP vector ('Ready to go' kit, Pharmacia). The ligation products were transformed into XL-1 Blue competent cells following Estoup & Turgeon (1996). Approximately 3000 recombinant clones were obtained and screened with the following probes: $(CT)_{10}(GT)_{10}$ $(CAC)_5(CA)$, $CT(CCT)_5$, $CT(ATCT)_6$ and $(TGTA)_6$, using the nonradioactive DIG nucleic acid detection kit (Boehringer Mannheim). A total of 24 putative positive clones were extracted using the QIAprep plasmid DNA prep kit (Qiagen). Sequencing reactions were obtained using ABI prism BigDye terminator cycle sequencing ready reaction kits (Perkin-Elmer, Applied Biosystems) and run on a 373 stretch XL automated sequencer. Only seven of the 24 clones contained sequences with more than six repeats. Primers could be designed for five loci using both OSP (Hillier & Green 1991) and OLIGO™ version 4.0 (National Biosciences) software programs (Table 1). PCR amplifications were carried out using a Perkin-Elmer 480 DNA thermal cycler using $\alpha^{35}S$ in 20-μL reactions containing template DNA (50–100 ng), 10 pmol of each primer, 75 μM each of dCTP, dGTP, dTTP, 5 μM dATP, 1.5 μCi of [$\alpha^{35}S$]-dATP, 1.2 mM $MgCl_2$, 1× Taq buffer (10 mM Tris-HCl (pH 9), 50 mM (KCl) and 0.25 units of *Taq* polymerase. The PCR conditions were: denaturation for 3 min at 95 °C, followed by 30 cycles of 1 min at 94 °C, 30 s of annealing at 59 °C, extension for 1 min at 72 °C followed by a final elongation step of 5 min at 72 °C. Electrophoresis, fixation, drying, and autoradiography were performed following standard procedures (Sambrook *et al*. 1989). The M13 sequence was used as a size control.

Table 1 Microsatellite loci for the barnacle, *Semibalanus balanoides*. T_A is the annealing temperature, H_E and H_O are expected and observed heterozygosity, respectively

Loci	Primer sequences (5′ to 3′)	Repeat sequence	T_A (°C)	No. of alleles	Size range (bp)	H_E	H_O	Accession numbers
Sebal13	F: GCACGGTGTTTTTTATGGAAG R: CTTTCATCCTGATACATTTC	$(GT)_{10}$	59	4	171–177	0.37	0.40	AF135375
Sebal14	F: GATCGTCGAGTTGGTCAG R: GACATGGGCGGTGCTTCTTAG	$(CA)_{10}$	59	8	144–160	0.57	0.67	AF135376
Sebal98	F: CTGACTATGACCTTGACC R: CGATTTCTCGAACGTGTC	$(GT)_{26}$	59	16	106–144	0.83	0.73	AF135377
Sebal916	F: GTATTATCGGTGGAAGTTG R: GGATCGCATGACAAGATG	$(CA)_8GA(CA)_6$	59	3	157–163	0.53	0.37	AF135378
Sebal935	F: GATCAGGAATAGTACAAACTAC R: GATCTCACGGTGATGCAG	$(CA)_{16}$	59	8	185–241	0.6	0.57	AF135379

To assess polymorphism at each locus, we screened 30 individual barnacles from Anse Bleue, New-Brunswick. Observed and expected heterozygosity at each locus were calculated using version 3.0 of the GENEPOP software package (Raymond & Rousset 1995). All five microsatellite markers revealed high polymorphism, with numbers of alleles per locus ranging from 3 to 16 for the population studied (Table 1). Gene diversity at each locus varied between 0.37 and 0.83. Significant deficits in heterozygotes were observed for locus Sebal98, and particularly for Sebal916. This may indicate the presence of null alleles at those loci. Alternatively, departures from Hardy–Weinberg equilibrium for other reasons, such as fine-scale structuring and/or selection, cannot be ruled out at this stage.

To our knowledge, these loci represent the first published microsatellites for barnacles. The level of polymorphism detected with microsatellites is much greater than what has been found for allozymes (Flowerdew 1983) and will be adequate to address population structure questions. In addition, the most polymorphic loci (Sebal14, Sebal35 and Sebal98) should also be of interest for paternity analysis.

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References

Estoup R, Turgeon J (1996) Microsatellite markers: isolation with non-radioactive probes and amplification. HTTP://www.inapg.inra.fr/dsa/microsat/microsat.html

Flowerdew MW (1983) The circumboreal barnacle Semibalanus balanoides (L.) and its subpopulations. In: *Protein Polymorphism Adaptive and Taxonomic Significance* (eds Oxford GS, Robinson D), pp. 75–84. Systematics association Spec, Vol. 24. Academic Press, London.

Hillier L, Green P (1991) OSP: a computer program for choosing PCR and DNA sequencing primers. *PCR Methods and Applications*, **1**, 124–128.

Holm ER, Bourget E (1994) Selection and population genetic structure of the barnacle *Semibalanus balanoides* (L) in the northwest Atlantic and Gulf of St. Lawrence. *Marine Ecology Progress Series*, **113**, 247–256.

Menge BA, Sutherland JP (1976) Species diversity gradients: synthesis on the roles of predation, competition, and temporal heterogeneity. *American Naturalist*, **110**, 351–369.

Raymond M, Rousset F (1995) GENEPOP (Version 1.2): Population genetic software for exact tests and ecumenicisms. *Journal of Heredity*, **86**, 248–249.

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

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

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The black rhinoceros (*Diceros bicornis*) was once widely distributed across Africa but is now listed as endangered (Baillie & Groombridge 1996). Since the 1970s, poaching has reduced the total population by 96%, and at the present time less than 2400 individuals remain in highly fragmented populations spanning 9–12 countries (International Rhino Foundation 1998). Two *D.b. michaeli* individuals from Kenya and a group of *D.b. minor* from Chete National Park in Zimbabwe form an *ex situ* breeding conservation programme at Western Plains Zoo, Australia. Here we describe 11 $(CA)_n$ microsatellite

Table 1 Characteristics of microsatellite markers isolated from *Diceros bicornis minor*. The clone sequences from which the primers were designed have GenBank Accession nos AF129724–34

Locus	Primer pair sequence 5′–3′	Repeat Size	Size (bp)	T_a (°C)	No. of alleles	H_O	H_E
DB1	AGATAATAATAGGACCCTGCTCCC GAGGGTTTATTGTGAATGAGGC	$(CA)_{14}$	121–127	60	4	0.714	0.780
DB4	CCTAAGCCCCCTTTACCTTG GACCAATAAACTCTTAGCAAAATGG	$(CA)_{15}$	185–204	60	2	0.667	0.545
DB5	GACCCCCATGTTCACTGC AGGTCCATCCATTTTGTCCC	$(CA)_{13}$	185–204	60	7	0.857	0.868
DB14	CTTCTGGATTAATACTGCTCACC TCTCCCACAACATTCTCATCC	$(CA)_{13}$	282–288	60	2	0.571	0.527
DB23	CCTCAGCAATAAGGGGAGGATTAGC GTTGATTCTCTGCCCCTGAGTTTGGG	$(CA)_{12}$	179–185	55	3	0.286	0.385
DB30	GCGACTATGACATACAACTATCTAC GGTCAAGGATTATTCTGACTAGC	$(CA)_{21}$	201–205	64	4	0.714	0.670
DB42	CCTGTTAGTGTAACTTCTATGCTCCC CATGGATGTTAGCTCAGGGCTGATC	$(CA)_{21}$	60–80	58–64	4	0.333	0.712
DB44	GGTGGAATGTCAAGTAGCGG CTTGTTGCCCCATCCCTG	$(CA)_4G(CA)_{16}$	170–184	64	6	0.572	0.813
DB49	GTCAGGCATTGGCAGGAAG CAGGGTAAGTGGGGGTGC	$(CA)_{14}$	152–162	64	4	0.833	0.742
DB52	CATGTGAAATGGACCGTCAGG ATTTCTGGGAAGGGGCAGG	$(CA)_{21}$	210–220	64	4	0.857	0.758
DB66	CCAGGTGAAGGGTCTTATTATTAGC GGATTGGCATGGATGTTACC	$(CA)_7TA(CA)_{16}$	187–205	58	6	0.857	0.747

T_a = optimal PCR annealing temperature. Observed heterozygosity (H_O) and unbiased estimates of expected heterozygosity (H_E) were determined using BIOSYS (Swofford & Selander 1981).

loci amplified from *D.b. minor* and *D.b. michaeli* that were developed to assess genetic diversity in this captive colony.

Microsatellites were isolated from a partial genomic library, generated from *Hae*III-, *Hpa*I- and *Eco*RI-digested *D.b. minor* genomic DNA, as described by Burns & Houlden (1999). Microsatellite loci were amplified using M13 primers. Cycle sequencing was performed using fluorochrome-labelled nucleotides and the dideoxy termination method (Sanger *et al*. 1977). Sequencing was conducted using Pop6™ or Pop4™ acryilamide gels and a 310 capillary system (ABI)®. Primer pairs were designed for 11 loci using PRIMER 0.5 (Lincoln *et al*. 1991) (Table 1). Microsatellite amplification was obtained using 0.5 Units of Red Hot™ thermostable DNA polymerase (Advanced Biotechnologies, Australia), 0.25 mM dNTPs, 1.5–2.5 mM $MgCl_2$, 2 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01%(w/v) Tween-20 (Buffer IV, Advanced Biotechnologies), and 5–15 pmol of each primer in 25 μL. The PCR profile was as follows; (95 °C for 3 min, 58–64 °C for 1 min, 72 °C for 30 s) for one cycle (95 °C for 30 s, 58–64 °C for 20–30 s (Table 1), 72 °C for 30 s) for 30 cycles and (95 °C for 30 s, 58–64 °C for 30 s, and 72 °C for 3 min) for the final cycle. Products were electrophoresed on Pop4™ acryilamide gels on a 310 capillary system (ABI)®. Genescan-500™ TAMRA was used for fragment size calibration. Statistical analysis of microsatellite variation was performed using BIOSYS (Swofford & Selander 1981).

A high degree of allelic diversity remained in the captive populations, despite the recent severe bottleneck experienced by wild founder populations. Between two and seven alleles were detected at each locus (Table 1). The mean number of alleles per locus (± standard error) for the *D.b. minor* ($n = 5$) and *D.b. michaeli* ($n = 2$) subspecies were 3.1 ± 0.3 and 2.6 ± 0.2, respectively, with a mean of 4.2 ± 0.5 for all *D. bicornis* individuals ($n = 7$). Each locus showed moderate to high levels of heterozygosity overall (Table 1). Levels of expected heterozygosity (± standard error) were 0.594 ± 0.068 for *D.b. minor* and 0.682 ± 0.085 for *D.b. michaeli*, and 0.686 ± 0.043 for all *D. bicornis* individuals.

Although our sample size is small, the high level of observed heterozygosity is consistent with those observed in studies by Swart & Ferguson (1997). These results contrast with earlier studies using allozyme analysis (Osterhoff & Keep 1970; Merenlender *et al*. 1989).

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References

Baillie J, Groombridge B (1996) *1996 ICUN Red List of Threatened Animals*. IUCN, Gland, Switzerland and Conservation International, Washington DC, USA.

Burns EL, Houlden BA (1999) Isolation and characterisation of microsatellite markers in the broad-headed snake (*Hoplocephalus bungaroides*). *Molecular Ecology*, **8**, 520–521.

International Rhino Foundation (1998) Rhino Information. *www.rhinos-irf.org/rhinos/black.html*.

Lincoln SE, Daly MJ, Lander ES (1991) *Primer: a computer program for automatically selecting PCR primers*, Version 0.5. MIT Center for Genome Research and Whitehead Institute for Biomedical Research, Cambridge, Massachusetts.

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

Osterhoff DR, Keep ME (1970) Natural variation in the blood proteins of white and black rhinos. *Lammergeyer*, **11**, 50–53.

Sanger F, Niklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Acadamy of Science of the USA*, **74**, 5436–5467.

Swart MKJ, Ferguson JWH (1997) Conservation implications of genetic differentiation in southern African populations of black rhinoceros (*Diceros bicornis*). *Conservation Biology*, **11**(1), 79–83.

Swofford DL, Selander RB (1981) BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *Journal of Heredity*, **72**, 281–283.