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PRIMER NOTE

Characterization of spotted hyena, *Crocuta crocuta* microsatellite loci

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Abstract

We have isolated 10 polymorphic microsatellite loci in the spotted hyena, *Crocuta crocuta*. The loci displayed between eight and 14 alleles in a minimum of 12 individuals tested. These loci will be used to investigate relatedness within social groups, the genetic structure of populations, sexual selection, and mate choice in spotted hyenas.

Keywords: Carnivora, *Crocuta crocuta*, hyena, Hyenidae, microsatellite

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The spotted hyena, *Crocuta crocuta*, is a predator-scavenger that occupies a wide range of habitats in sub-Saharan Africa and lives in social clans that vary in size from a few to up to 80 members in which females are philopatric and males typically disperse (Mills & Hofer 1998). This species displays a suite of unusual traits, including female dominance, facultative siblicide, masculinization of the female clitoris and males that queue for social dominance (East & Hofer 2002). To investigate paternity, reproductive success and mate choice in this unique species, we developed 10 polymorphic microsatellite markers.

We collected hair and/or blood/tissue samples from 46 spotted hyenas, comprising two clans, inhabiting the Serengeti National Park, Tanzania. We extracted DNA from hair samples using Chelex-100, following the Promega DNA extraction protocol (Promega 1996), and from blood, following the protocols of Sambrook *et al*. (1989) or using the QIAamp blood and tissue kit (QIAGEN).

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Three genomic libraries were prepared. For the preparation of the first two libraries (A and B), pooled genomic DNA was digested with the restriction endonucleases *Alu*I, *Hae*III and *Rsa*I. The digest fragments were size-selected (100–600 bp) and ligated into *Sma*I-digested M13mp18/mp19 phage (library A) or *Sma*I-digested pUC18 (library B). Transformed colonies were screened for CA.GT and GA.CT as described in Rassmann *et al*. (1991).

To prepare the third microsatellite library (C), pooled DNA from one male and one female was used. The library was prepared enriched for $(TTTC.AAAG)_n$ following Armour *et al*. (1994) with one modification: to prevent duplicate clones, the DNA fragments were not polymerase chain reaction (PCR)-amplified before the enrichment hybridization. In total, 1200 transformant clones were screened by hybridization to the sequences $(TTTC.AAAG)_n$ (Armour *et al*. 1994) and $(CA.GT)_n$ or $(GA.CT)_n$ (Amersham Pharmacia Biotech) radiolabelled with [α^{32}P]-dCTP. 210 positive clones were obtained. Forty-nine positive clones were sequenced using BIG DYE Terminators on an ABI 377 Sequencer (Applied Biosystems). The majority of clones contained tetranucleotide motifs but several clones contained dinucleotide $(CA.GT)_n$ repeats, despite the library not being enriched for these.

All sequences were checked to determine whether they were unique using GENEJOCKEY software (Biosoft). In total, 38 unique microsatellite sequences were isolated from the three libraries (11, eight and 19, respectively, from libraries

Table 1 Characteristics of 10 spotted hyena (*Crocuta crocuta*) microsatellite loci

Locus	EMBL accession no., library & clone name	Repeat motif (5′–3′)†	Primer sequence (5′–3′) (& 5′ fluorescent primer label, underlined)	Primer T_a (°C)	PCR profile used	$MgCl_2$ (mM)	Exp. allele size (bp)†	*n*	Analysis method used (*n*)	*A*	Obs. allele size range (bp)	H_O	H_E
*Ccroc*01	AJ512845	$(CA)_{18}$	(F) CCTCAATTAGGAACATAAAAAGTG	56	67-55 (B)	1.5	208	24	SS	8	150–200	0.92	0.85
	A, CSH64		(R) GAAGGAAGGAAGCAATATGC	56									
*Ccroc*02	AJ512846	$(TG)_{20}$	(F) GCATGCAGATAATTTGAAGATG	57	67-55 (B & H)	1.5	118	44*	ABI	10	85–111	0.59	0.69
	A, CSH65		(R) (*6-FAM*)-CAAAAAGGAGAAATTTAGCAGA	56									
*Ccroc*03	AJ512850	$(CA)_4(GA)_1(CA)_1(CG)_1$	(F) TTGAAACAAGGATAAATTAATGAAAAGA	60	60-50 (B)	2.0	133	12	SS	8	120–175	0.67	0.75
	B, HY7GH	$(CA)_{18}$	(R) ACAGTAGAAAATAATCCAAGTATAAACC	56									
*Ccroc*04	AJ512851	$(TG)_3(TACG)_1(TG)_{12}$	(F) TGGGCTAAGTCTTTCCCTGA	60	67-55 (B)	1.5	307	42*	ABI	9	305–323	0.86	0.82
	B, HY15GH		(R) (*TET*)-ATGGGGGCTCCTTCACTC	60	62 (H)								
*Ccroc*05	AJ512855	$(CA)_{27}$	(F) ACCAGTGATCTGGACTGGGA	61	67-55 (B & H)	1.5	171	45*	ABI	8	155–175	0.93	0.83
	B, HY27GH		(R) (*TET*)-AAAAGTAATATGACTGCCAAAAGC	58									
*Ccroc*06	AJ512859	$(TTTC)_{22}(TT)_1(TTTC)_{18}$	(F) GATCGTTTTTGACAAGTGCTG	58	58 (B & H)	1.5	308	46*	ABI (38) SS (8)	10	228–328	0.98	0.85
	C, T01A03		(R) (*6-FAM*)-AAACCCACTTCGAGCCTGAT	61									
*Ccroc*07	AJ512860	$(GGAA)_6(GAA)_1(GAAA)_{19}$	(F) TCCCTCAAGTCACTCGGTGT	61	62 (B & H)	1.5	281	45*	ABI	12	273–328	0.96	0.90
	C, T01C09		(R) (*HEX*)-TGCTAATGTTCATTGCAGGG	60									
*Ccroc*08	AJ512866	$(TTTC)_2(GTTC)_1(TTTC)_1$	(F) TTTTTTAAAGTTCTTAGGCAGGAC	58	67-55 (B)	1.5	274	45*	ABI	14	162–276	0.89	0.89
	C, T01H04	$(TT)_1(TTTC)_{22}(TTCTC)_1(TTTC)_{15}$	(R) (*6-FAM*)-GGAGCCTGTTTCAGTCTGTG	58	62 (H)								
*Ccroc*09	AJ512870	$(GT)_{19}$	(F) CAGAATTAATCCATATCACAAACTGC	60	67-55 (B)	1.5	218	43*	ABI 310	8	208–224	0.77	0.77
	C, T02B12		(R) (*TET*)-TAGGAACCTCTTGCCGTCAG	60	62 (H)								
*Ccroc*10	AJ512872	$(TTTC)_5(TC)_2(TTTC)_{18}(TC)_1$	(F) CTCCAGTTTATTAACACCCGC	59	67-55 (B)	1.5	223	46*	ABI (34) SS (12)	11	184–218	0.91	0.85
	C, T02D10	$(TTTC)_1(N)_9(TTTA)_2(T)_4(GA)_7$	(R) (*HEX*)-AAGCTCTTGGAACTAAGCTACTTG	58	62 (H)								

†Of sequenced clone.

T_a = annealing temperature. *n* = number of individuals genotyped; *A* = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; B = blood/tissue, H = hair; *between 22 and 25 of the samples were hair samples; SS = genotypes scored on 6% polyacrylamide gels stained with silver; ABI = genotypes scored on an ABI 377 Sequencer.

PCR profiles used:

Profile 67-55 = 94 °C for 30 s, X °C for 45 s, 72 °C for 45 s 2 cycles per step (X = 67-58 °C decreasing by 3 °C each step) then 94 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s for 35 cycles.

Profile 60-50 = 94 °C for 30 s, X °C for 30 s, 72 °C for 30 s 1 cycles per step (X = 60-55 °C decreasing by 1 °C each step) then 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s for 30 cycles followed by 72 °C for 5 min.

Profile 62 = 95 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min for 43 cycles.

Profile 58 = 94 °C for 30 s, 58 °C for 45 s, 72 °C for 45 s for 40 cycles.

A to C: EMBL accession numbers AJ512839–AJ512876). After submission to the EMBL database, all 38 sequences were confirmed as unique using BLASTN 2.2.4 (Altschul *et al*. 1997). Primers were designed from 17 sequences (five, three and nine, respectively, from libraries A to C) using PRIMER 3 (Rozen & Skaletsky 1996, 1997). The remaining 20 sequences were not used, but all except one (AJ512848) are suitable for primer design.

The loci were tested for polymorphism using a panel of 12–46 individuals. PCR reactions were performed in a 2400 Perkin Elmer thermocycler using 0.2 ml microreaction tubes (Perkin Elmer). DNA was amplified in a 15 μL PCR reaction volume, which contained between 0.5 and 10 ng of template hyena DNA, 200 μM dNTPs, 50 μM of each primer, 0.345 U (0.023 U/μL) of *Taq* DNA polymerase (Amersham Pharmacia Biotech) and 1 × reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0).

Four different cycling profiles were necessary to allow for differences in primer annealing temperatures and the different tissues from which DNA was extracted (Table 1). Each PCR run, for each locus, was ensured contamination-free by including a DNA-free control (sterile ultrapure water). PCR amplification was confirmed on 1.5% agarose gels stained with ethidium bromide.

For eight loci, the genotypes were analysed on an ABI 377 or ABI 310 Sequencer (Applied Biosystems) using fluorescent primers (Table 1) and GENESCAN software (v2.0.2, Applied Biosystems). Loci *Ccroc*01, *Ccroc*03, *Ccroc*06 and *Ccroc*10 were analysed on 6% polyacrylamide gels stained with silver (Promega 1996). Allele sizes were assigned using a ØX174 *Hae*III DNA marker (Applied Biosystems) and two hyena PCR-amplified reference samples. Most genotypes at *Ccroc*06 and *Ccroc*10 (34 and 38, respectively, of the 46) were also checked, and all found to match, on an ABI 310 Sequencer.

Of the 17 primer pairs tested, seven were abandoned. Five loci were abandoned due to nonspecific amplification (CSH18-AJ512839, CSH44-AJ512842, T01D05-AJ512861, T01H06-AJ512867, T02H12-AJ512876); the products at two of these (CSH18 and T02H12) may have resulted from one primer being positioned in a SINE element identified in other carnivore species (Slattery *et al*. 2000). No product was obtained for locus T01D06 (AJ512862), and locus CSH77-AJ512849 (which shows a strong similarity to a human sequence; GenBank AC097478) was monomorphic.

The 10 polymorphic loci displayed between eight and 14 alleles in a minimum of 12 individuals (Table 1). Primer sequences, expected PCR product sizes, allele size ranges and numbers of alleles for the loci are given in Table 1. The observed and expected heterozygosities for each locus were calculated using CERVUS v2.0 (Marshall *et al*. 1998; Table 1). There was no significant deviation from Hardy–Weinberg expected allele frequencies for the samples presented in Table 1 for any locus ($P > 0.05$) and, in a pedigree analysis of 123 known mothers and 463 offspring (111 twin litters), no null alleles were detected at any of the six loci used (*Ccroc*05, *Ccroc*06, *Ccroc*07, *Ccroc*08, *Ccroc*09 and *Ccroc*10; unpublished data).

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