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PRIMER NOTE

Development of 21 microsatellite loci for puma (*Puma concolor*) ecology and forensics

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Abstract

We developed 21 microsatellite (13 dinucleotide and 8 tetranucleotide) primers specifically for pumas (*Puma concolor*). The primers were tested across 243 individuals from California and Nevada, and displayed an average of 5.5 alleles per locus. Previously, domestic cat (*Felis catus*) primers have been adapted for puma genetic studies. Puma-specific loci may reduce concerns associated with ascertainment bias, improve genetic structure resolution and provide additional tetranucleotide loci valuable for forensic applications. These puma-specific microsatellites will aid studies involving parentage, kinship and population structure, along with forensic case applications including poaching and puma-associated injuries to humans and domestic animals.

Keywords: forensics, microsatellites, mountain lion, puma, *Puma concolor*

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The puma (*Puma concolor*) is a large felid distributed throughout North, Central and South America (Cat Specialist Group 2002). Research and applied management applications involving puma genetics are increasingly required to address conservation concerns, puma interactions with endangered species and livestock, and attacks on people. To date, studies have employed microsatellite primers developed from the domestic cat (*Felis catus*) (Menotti-Raymond *et al*. 1999). While adaptation of these cross-species loci has been productive for population and phylogenetic studies (such as Culver *et al*. 2000; Ernest *et al*. 2003), microsatellite primers developed directly from puma may provide additional benefits in genetic applications. Puma-specific primers should reduce ascertainment bias (Ellegren *et al*. 1995) and nonspecific amplification (critical for trace DNA analysis). Marginal samples encountered in forensic cases and noninvasive ecological applications require markers that amplify robustly with minimal artefact peaks. Tetranucleotide microsatellite markers are the legal standard for human identification in the USA because they have the advantage of decreased stutter product formation and greater resolution of alleles by size-based electrophoretic separation methods when compared to dinucleotide markers (Budowle *et al*. 1998).

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Four libraries enriched with microsatellite motifs (CA, GA, CATC, TAGA) were created by Genetic Identification Services with DNA extracted from a male puma following the methods of Jones *et al*. (2002). We used DESIGNERPCR version 1.03 (Research Genetics, Inc.) to design 41 primers with melting temperatures of 60 °C. QIAGEN DNeasy kits (QIAGEN) were used to extract DNA from muscle samples (see Ernest *et al*. 2003 for sample collection details). Primers were first screened with universal M13 tails as in Schuelke's (2000) protocol to reduce the cost associated with fluorescently labelling all potential primers. Those primers that had no more than two alleles per individual and product of expected size were then traditionally fluorescently dye labelled and combined into four multiplex groups (Table 1). The reverse primers for loci with excessive stutter were redesigned with a 5′ PIG-tail (GTTCTT) to facilitate adenylation (Brownstein *et al*. 1996) (Table 1).

Multiplex polymerase chain reaction (PCR) was carried out in 12.5 μL reactions containing 1 × PCR buffer II (Applied Biosystems), 2.5 mM $MgCl_2$, 0.2 mM dNTP (Fisher Scientific), 0.5 U of Thermoprime Plus DNA polymerase (ABgene), 1μL DNA (20–60 ng) and various concentrations of forward fluorescently dye labelled (Applied Biosystems) and reverse primers (BioSource) ranging from 0.05 to 0.15 μM. Parameters for thermal cycling were as follows: 94 °C for 1 min followed by 30 cycles at 94 °C for 45 s, 60 °C for 30 s and 72 °C for 30 s and a final 30 min extension at 72 °C

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Table 1 Characterization of the 21 microsatellite loci developed for pumas, *Puma concolor*. M, multiplex panel; N, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity

Locus	Dye label	PCR product size (bp)	Repeat motif	M	*N*	H_O	H_E	GenBank Accession no.	Primer sequence (5′–3′)†
PcoA106w	PET	237–257	$(AC)_{17}$	1	6	0.3510	0.4029	DQ660984	F: GTTGGAAGGTCACAAAGTCCT R: gttcttCACAAGGTCTGGTTCTACAGTGT
PcoA208w	PET	180–200	$(GT)_{16}$	2	5	0.3633	0.5092	DQ660985	F: TGGCAGCTTTAGTGGAAAGAC R: CCCTATTAAATACCGCCTCACT
PcoA216w	PET	240–260	$(AC)_{21}$	2	4	0.1388	0.1994	DQ660986	F: CTTGGCTCCTTGAATGTGATC R: GCTGTGTGACTGTAAGCAGGTTA
PcoA312w*	PET	261–281	$(CA)_{16}$	1	2	0	0.0081	DQ660987	F: GAACCCTACTGGCTCTACCTTC R: GGATGGGTGTGTTTCATCAC
PcoA339w	VIC	259–277	$(CA)_{19}$	2	7	0.5551	0.6464	DQ660988	F: AGTGGCAAACCTTTCTGGGT R: gttcttGCCTCCCTCTGAAGTGCATAT
PcoB003w	NED	263–289	$(TC)_{14}$	1	7	0.4653	0.6321	DQ660989	F: CCTCAGGTTCCTTGTCTGTAAA R: gttcttCAGTCCTAACAGGGACGTGTAC
PcoB010w	VIC	201–229	$(AG)_{18}$	2	8	0.6816	0.7438	DQ660990	F: TACGGAAAGGTACTGGCAACT R: gttcttCCTGGTAATAGAGGCAGGTGT
PcoB203w	VIC	280–306	$(CT)_{10}$	2	7	0.4571	0.5677	DQ660991	F: GTGGCTCAGTCGGTTAAACTT R: CCATGCTAATCTTCTCTGGGT
PcoB207w	VIC	291–323	$(CT)_{10}$	1	8	0.5878	0.7222	DQ660992	F: GGGTGGCTCAGTTGGTTAAGTA R: gttcttCAGCCTCTTCTTCTGCTGTTTC
PcoB210w	VIC	157–185	$(GA)_{23}$	2	7	0.6245	0.7358	DQ660993	F: CTTGGGATGTCTCATGTGGTT R: CATCTTGGTGTTGATCCTCTAGTC
PcoB303w	6-FAM	246–266	$(GA)_{13}$	2	3	0.1143	0.1318	DQ660994	F: TCCCCAAGAGTTTTACCATAGC R: GGTGGTCAGTCAGTTGAAGGT
PcoB309w*	6-FAM	126–164	$(TC)_{11}$	1	1	—	—	DQ660995	F: AGACCTTAAACATGCTGCTGTCTC R: ATCCCAGAGGGAAAGCCTAAGTA
PcoB316w	6-FAM	249–277	$(TC)_{14}$	1	4	0.3143	0.3598	DQ660996	F: GTCCATTAAGGCGCTGACTCT R: ACCCCCCATCTACTTTTTGTTC
PcoC010w	6-FAM	277–293	$(GGAT)_9$	3A	4	0.3673	0.4160	DQ660997	F: AGGGATTCCTCCTTCTTCTGA R: CTCCTGAGCCAGGTGTAATAGA
PcoC108w	6-FAM	117–141	$(ATCC)_9$	3B	3	0.4694	0.5931	DQ660998	F: CTTGATGATGTTGAGGCAGATC R: ACATGGTAGGTGCTGGGTAAT
PcoC112w	6-FAM	138–170	$(CCAT)_9$	3B	5	0.5265	0.5133	DQ660999	F: TTCACCCTTCTGTCCACCTGT R: GGGCACGTAGTAGGCCAATAA
PcoC209w	VIC	245–269	$(CCAT)_9$	3B	5	0.5102	0.5351	DQ661000	F: TCAGGCAGTATTCATCCCTTTG R: TCCCTCCCTTCCCTGTCTTATA
PcoC217w	VIC	232–252	$(TCCA)_{10}$	3A	5	0.4490	0.5852	DQ661001	F: TCCCCAACTCATCGTTCTTTACTCC R: AAATGGGCTGATAGATGGACAG
PcoD012w	VIC	291–307	$(CTAT)_{11}$	3A	5	0.5224	0.5421	DQ661002	F: CATTGCTCAGACCTGCTTTC R: GGTTTTCCCAAGACCTACGT
PcoD103w	PET	263–287	$(ATAG)_{11}$	3A	6	0.5837	0.7100	DQ661003	F: ACCAAAGACACTTCTGAACCCTAC R: ACAGTAAAGGGATGCTAGGAGACTAC
PcoD217w	PET	236–268	$(TCCA)_{10}$	3B	5	0.4939	0.6181	DQ661004	F: CCCCTGCTTTGAAAAAACTCTC R: CCTGTTCTCCAACCTACAGATAGC

*, primers lacking in polymorphism or heterozygosity and not included in analysis; †, lower case indicates added PIG-tail to facilitate adenylation (Brownstein *et al*. 1996).

followed by 15 °C until further use. PCR products were separated with an ABI PRISM 3730 DNA Analyzer (Applied Biosystems Inc.) with each capillary containing 1 µL of a 1:11 dilution of PCR product and deionized water, 0.05 µL GeneScan-500 LIZ Size Standard and 9.95 µL of HiDi formamide (both products Applied Biosystems Inc.) that was denatured at 95 °C for 3 min. Products were visualized with STRAND version 2.3.69 (Toonen & Hughes 2001).

Of 41 primers tested, 21 successfully amplified and 19 displayed polymorphism across the 243 samples tested. The number of alleles per polymorphic locus ranged from

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three to eight with an average of 5.5. The expected level of heterozygosity ranged from 0.1318 to 0.7438 while the observed level was from 0.1143 to 0.6816 (GENEPOP version 3.4, Raymond & Rousset 1995) (Table 1). We used GENEPOP to test the loci in three known populations across California and Nevada (Ernest *et al*. 2003) for Hardy–Weinberg equilibrium and linkage disequilibrium (LD). After applying the Bonferroni correction for multiple tests (P value = 0.00015), none of the loci deviated from Hardy–Weinberg expectations and one of the three populations contained one locus displaying heterozygote deficiency (PcoB207w) and two instances of significant LD (PcoB203w vs. PcoB210w and PcoD103w vs. PcoD012w). These deviations from expectations may be due to sampling artefact, null allele(s), or other factors. The 19 polymorphic primers have a $P_{(ID)HW}$ of 8.05×10^{-12} and a $P_{(ID)sib}$ of 1.05×10^{-5} (GENECAP version 1–2, Wilberg & Dreher 2004). None of the primers showed significant overlap with previously published domestic cat sequences. These primers have the potential to facilitate the ecological knowledge and forensic identification of pumas.

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