

PRIMER NOTE

Development of 22 new microsatellite loci for fishers (*Martes pennanti*) with variability results from across their range

MARK J. JORDAN,* J. MARK HIGLEY,† SEAN M. MATTHEWS,‡ OLIN E. RHODES,§
MICHAEL K. SCHWARTZ,¶ REGINALD H. BARRETT* and PER J. PALSBØLL*††

*Department of Environmental Science, Policy, and Management, University of California, Berkeley, 137 Mulford Hall 3114, Berkeley, CA 94720–3114, USA, †Hoopa Tribal Forestry, PO Box 368, Hoopa, CA 95546, USA, ‡Wildlife Conservation Society, PO Box 368, Hoopa CA 95546, USA, §Department of Forestry and Natural Resources, Purdue University, 195 Marsteller Street, West Lafayette, IN 47907, USA, ¶USDA Forest Service, Rocky Mountain Research Station, 800 East Beckwith, Missoula, MT 59801, USA

Abstract

We developed 22 new microsatellite loci for the fisher (*Martes pennanti*), a North American mesocarnivore. The loci were developed with samples from the southern Sierra Nevada Mountains in California, and were screened with samples from this population and four other populations. We observed a range of six to 21 polymorphic loci per population, with the Sierra Nevada population exhibiting markedly lower levels of variation compared to the other four.

Keywords: fisher, *Martes pennanti*, microsatellite

Received 23 August 2006; revision accepted 8 January 2007

Fishers (*Martes pennanti*) are carnivorous mammals found throughout forested regions of temperate North America. Recent declines in their distribution have prompted concern for their conservation, particularly in the western USA (Zielinski *et al.* 2005). However, relatively little is known about the patterns of population structure and demography of fishers in this region. To better understand the ecology of this species, we developed microsatellite loci from a population in the Kings River region of the Southern Sierra Nevada Mountains in California. We also screened the loci we developed in four other populations from across the fisher's range in the contiguous USA. These additional populations were in Idaho, Minnesota, Vermont, and a second population in California from the North Coast Range. Here we report on the development of the 22 loci that were variable in at least one of each of these populations.

We isolated microsatellite loci following the protocol of Glenn & Schable (2005). Genomic DNA was obtained from

tissue samples taken by ear punch from live-caught animals and stored in a saturated NaCl solution (6 M) containing 25% dimethyl sulfoxide. Extractions were performed using DNeasy extraction kits following manufacturer's instructions (QIAGEN Inc.). We digested four extracts with *RsaI* (New England Biolabs) and then ligated the digested DNA to universal SNX linkers SuperSNX24F and SuperSNX24+4P (Hamilton *et al.* 1999). We enriched linker-ligated DNA by hybridization to biotinylated oligos using three separate oligo mixes: mix 1: (AT)₄ and (GT)₁₁; mix 2: (TG)₁₂, (AG)₁₂, (AAG)₈, (ATC)₈, (AAC)₈, (AAT)₈, and (ACT)₈; mix 3: (AAAC)₆, (AAAG)₆, (AATC)₆, (AATG)₆, (ACCT)₆, (ACAG)₆, (ACTC)₆, and (ACTG)₆. Enriched DNA was recovered using Dynabeads (DynaL Inc.). Recovered, enriched DNA was ligated into pCR2.1-TOPO plasmids (Invitrogen Inc.) and transformed into TOP 10 cells (Invitrogen Inc.).

We compiled a library of 288 recombinant clones after screening by α -complementation with X-Gal (Invitrogen Inc.; Sambrook & Russell 2001). We lysed 179 colonies by boiling then amplified cloned inserts by polymerase chain reaction (PCR) with M13 primers in a Dyad thermal cycler (MJ Research Inc.). Each 25- μ L reaction contained approximately 10 ng of plasmid DNA, 20 mM Tris-HCl (pH 8.8),

Correspondence: M. J. Jordan, University of California, Berkeley, CA, USA. Fax: +1-510-642-5438; E-mail: mjordan@nature.berkeley.edu
††Present address: Department of Genetics, Microbiology and Toxicology, Stockholm University, S-106 91 Stockholm, Sweden.

2 PRIMER NOTE

10 mM (NH₄)₂SO₄, 10 mM KCl, 2.0 mM MgSO₄, 1.5 mM MgCl₂, 0.1% Triton X-100, 625 µg bovine serum albumin, 0.12 mM each dNTP, 0.5 U *Taq* DNA polymerase (New England Biolabs Inc.), and 250 nM of each primer. Reactions were run with an initial denaturing step of 95 °C for 3 min and then amplified for 28 cycles of 20 s at 95 °C, 20 s at 50 °C, and 1 min 30 s at 72 °C, followed by a 5-min extension step at 72 °C. Amplified products were sequenced with M13 primers following a standard cycle sequencing reaction (BigDye version 3.1, Applied Biosystems).

Out of 179 sequenced clones, we observed 152 sequences (85%) containing microsatellite DNA. Of these, 43 were dinucleotide repeats, 3 were trinucleotide repeats, 64 were tetranucleotide repeats, and 42 were compound or interrupted repeats. We designed primers for 50 of these microsatellites using PRIMER 3 (Rozen & Shaletsky 2000).

To the 5'-end of each forward primer, we added a universal M13 tag (5'-TGTAACACGACGGCCAGT-3').

We screened the 50 loci by amplifying genomic DNA isolated from tissue in an optimized PCR and measuring the fragment lengths. We fluorescently labelled amplification products with a 6-FAM-labelled M13 oligonucleotide primer using one of two methods. In the first method (Schuelke 2000), we set up a 10-µL reaction containing approximately 5 ng DNA template, a PCR cocktail mix [containing 67 mM Tris-HCl (pH 8.8), 2 mM MgCl₂, 16.6 mM (NH₄)₂SO₄, 10 mM β-mercaptoethanol, 0.2 mM of each dNTP, 0.4 U *Taq* DNA Polymerase (New England Biolabs Inc.)], 800 nM reverse primer, 800 nM M13fwd-FAM primer, and 200 nM forward primer. This reaction was run for 2 min at 94 °C, followed by 22–23 cycles of 30 s at 94 °C, 30 s at an optimal annealing temperature (Table 1),

Table 1 Microsatellite loci for *Martes pennanti* including locus name, forward and reverse primer sequences, PCR conditions, and GenBank Accession nos for the clone sequences from which the markers were derived

Locus	Repeat unit	Primer sequences	T _a	Cycles	GenBank Accession no.
MP0018	(GT) ₁₃	F: ATTTGGGGCAACTGTCTTTG R: CTGTGCTTTTGTGGGGATT	58/53†	22/8	EF042875
MP0055	(GC) ₄ (CA) ₁₃	F: GCCCCATGCCTGGTTTAT R: GCTGGTCTAGAACCACCACAC	59/53†	22/8	EF042876
MP0059	(CA) ₁₉	F: CCTCTCCCTCAAACCTTCT R: TTTTCGGTATAAACTCCAACCTACGA	56/53†	22/8	EF042877
MP0084	(GT) ₅ GC(GT) ₁₄	F: GCTGGACTGATGCTTGTTAGA R: GAATCCAAAACCAACGTGCT	59/53†	22/8	EF042878
MP0085	(TG) ₁₂	F: AGGAGTCTCTGCAGGTGAA R: ACAATTGGTGTTCAGGCAATG	59/53†	22/8	EF042879
MP0100	(CATT) ₅	F: CTGGGACAACCTGAACAACCA R: ATCTTATCAGGGGCCCATTC	60/53†	22/8	EF042880
MP0114	(TTTC) ₁₁ TT(TTCC) ₈	F: ATGACACGTTCCCATTTAGC R: TCAGAGAGGCTCAAGAAGAGAAA	60/53†	22/8	EF042881
MP0120	(GTCT) ₅ (CT) ₇ (CA) ₆	F: TCAGTGGATCTCTTGCTTGC R: ATAGAAAAGTCGAGGAAGGAAATG	58/53†	22/8	EF042882
MP0144	(AGAA) ₁₆	F: CCAATCCCTTTGGAAGAAA R: GTGAGTTCAAGCCCCATGTT	57/53‡	20/30	EF042883
MP0175	(CTTT) ₁₁ (CCTT) ₃	F: CAGACCAATGGACCCAATC R: TTCTACATTCATACGTGAGTAAAAGC	56/53†	22/8	EF042884
MP0182	(GAAA) ₄ GAGA(GAAA) ₁₄	F: TTTGCTGTATGGGATGTTGC R: GAACCTGACCCCTATAAACCTAACAGGA	59/53‡	20/30	EF042885
MP0188	(GATT) ₇	F: GCAGAGCCAATCAGAGTTCC R: GGACCTACAGCTCCATCCAA	59/53†	23/8	EF042886
MP0190	(AGAC) ₃ (AG) ₁₂	F: CTTCCAACCCCTGGGTGAGT R: GAACCCGATTAAGTCCAGCA	58/53‡	20/26	EF042887
MP0197	(TTTC) ₁₀ TCTC(TTTC) ₂	F: GCTCAGCCAAAATAAATCCA R: CCACCTTGATCACAATGTATGATCTTT	59/53†	23/8	EF042888
MP0200	(GAAA) ₁₆	F: GCCAATTAAAACCACAGGA R: CCTGAGGGTTCCATTTCTCC	58/53†	22/8	EF042889
MP0227	(AC) ₃ AG(AC) ₃ AG(AC) ₄ AG (AC) ₄ AG(AC) ₉ AG(AC) ₂	F: TTGCCAATGTTGAGACATC R: CCACAGGAAACATAGATAAAAATATGA	58/53†	22/8	EF042890
MP0234	(TGTT) ₇	F: CAACATGCAAAGGTGATGCT R: TTTTCCATGCACTCAGGAA	59/53†	23/8	EF042891

Table 1 Continued

Locus	Repeat unit	Primer sequences	T_a	Cycles	GenBank Accession no.
MP0243	(TGTT) ₇ ... (TGTT) ₄	F: GAGGGTTCTGCAGGGGATA R: CCACGGCATATTTCTAGAGCAG	56/53‡	20/29	EF042892
MP0247	(GAAA) ₃ (GA) ₈ (GAAA) ₁₁	F: GCATTGTGCACCAGCATAAC R: TTCCTTGCCCTTTGCCTCA	59/53‡	20/28	EF042893
MP0263	(TGTC) ₈ TGCC(TC) ₄ (TTTC) ₃	F: GAGTGTCTCTCTCAGGGCTA R: AACAGAACCTTAAGAGAGATAGGAA	58/53†	22/8	EF042894
MP0282	(TGTC) ₈ TGCC(TC) ₄ (TTTC) ₃	F: AGGCAGAGTGTCTCTCTCAG R: TAAACAGAACCTTAAGAGAGATAGGAA	58/53†	22/8	EF042896
MP0288	(ATCA) ₇	F: GGACCTACAGCTCCATCCAA R: GCAGAGCCAATCAGAGTTCC	58/53†	22/8	EF042897

†M13 labelling using first program described in text, adapted from Schuelke (2000).

‡M13 labelling using second program described in text, adapted from Guo & Milewicz (2003).

and 30 s at 72 °C and subsequently for 8–9 cycles of 30 s at 94 °C, 30 s at 53 °C, and 30 s at 72 °C, followed by 10 min at 72 °C. Some loci would not amplify with this method, so we used a second protocol modified from Guo & Milewicz (2003). In this method, we set up a 10- μ L reaction containing approximately 5 ng DNA template, the same PCR cocktail mix as above, and 1.0 mM each of the forward and reverse primers. We ran this reaction for 15 min at 94 °C followed by 20 cycles of 30 s at 94 °C, 30 s at an optimal annealing temperature (Table 1), and 30 s at 72 °C, followed by 10 min at 72 °C. Amplification product from this step was diluted 1000 \times , and 1.0 μ L of this dilution was then used as template in a second PCR that used the same reagents except that we replaced the forward primer with an M13fwd-FAM primer. This reaction was run for 15 min at 94 °C, 28–30 cycles of 30 s at 94 °C, 30 s at 53 °C, and 30 s at 72 °C, followed by 10 min at 72 °C. We tested for variability with 32 samples from the Kings River population, 11 samples from the North Coast population, and 10 samples each from Idaho, Minnesota, and Vermont. Fragment lengths of PCR products were determined with an ABI PRISM 3730 sequencer using LIZ-500 size standard (Applied Biosystems) and analysed using GENOTYPER 3.7 software (Applied Biosystems).

Out of the 50 loci we screened, we were unable to amplify 10. The number of polymorphic loci of the remaining 40 varied by population and in total, 22 were polymorphic in at least one population (six to 21 loci; Table 2). The average number of alleles per locus among variable loci ranged from 2.6 to 3.2 depending on the population. One locus (MP0085) was variable only in the North Coast population, while all other variable loci were polymorphic in more than one population.

To determine if a locus was in Hardy–Weinberg equilib-

rium in a given population, we calculated observed and expected heterozygosities using an unpublished ANSIC program written by PJP. The probability of obtaining H_O by chance (assuming panmixia) was calculated as the proportion of 10 000 simulations of randomized genotypes based on the observed allele frequencies that yield a similar or more extreme estimate of H_O after correcting for multiple tests (Table 2; Rice 1989). The locus MP0120 in the Vermont population and the locus MP0200 in the Idaho population both exhibited lower heterozygosity than expected assuming random mating. The probability of identity for each locus ranged from 0.083 to 0.84 across populations.

We used GENEPOP (version 3.4; Raymond & Rousset 1995) to test for linkage among the loci in each population separately. One pair of loci was significantly linked in the Vermont population after correcting for multiple comparisons (MP0188 and MP0288; $P < 0.0010$), and although the test was not significant in any other population, it did approach significance in the other three populations where these two markers were variable, suggesting that these two loci may in fact be linked. In the Kings River population, we detected significant linkage between MP0059 and MP0144 ($P < 0.0055$), however, this relationship was not significant in any other population (P value range: 0.33–0.72).

These 22 polymorphic loci will be useful in population genetic studies of this species across its range. The low number of variable loci within the Kings River population corroborates previous findings that fishers in the Southern Sierra Nevada have very low genetic diversity relative to their counterparts in other populations (Wisely *et al.* 2004). This discovery underscores the importance these loci will have in studies of the ecology of this imperilled species.

4 PRIMER NOTE

Table 2 Microsatellite loci screening results showing the population(s) in which the locus is variable, the number of individuals sampled (N), the number of samples successfully genotyped, the number of alleles, allele size range, observed heterozygosity (H_O), expected heterozygosity (H_E), P value for the probability of H_O , and probability of identity (I)

Locus	Population	N	N genotyped	No. of alleles	Size range (bp)	H_O	H_E	P value	I
MP0018	MN	10	10	2	197–199	0.10	0.10	< 1.00	0.82
	VT	10	10	3	197–201	0.30	0.52	< 0.059	0.29
MP0055	NC	11	11	2	133–137	0.27	0.24	< 1.00	0.61
	ID	10	7	3	133–137	0.57	0.50	< 0.66	0.32
	MN	10	10	4	133–139	0.70	0.66	< 0.67	0.19
MP0059	VT	10	10	4	133–139	0.40	0.53	< 0.14	0.26
	KR	53	32	2	166–170	0.28	0.46	< 0.033	0.40
	NC	11	11	4	166–176	0.45	0.48	< 0.50	0.32
	ID	10	10	5	166–174	0.40	0.72	< 0.012	0.13
	MN	10	10	4	166–174	0.50	0.59	< 0.26	0.21
MP0084	VT	10	10	4	168–176	0.70	0.54	< 1.00	0.25
	ID	10	9	3	152–156	0.56	0.51	< 0.71	0.32
	MN	10	10	5	144–158	0.90	0.74	< 0.93	0.12
MP0085	VT	10	10	4	144–154	0.90	0.72	< 0.95	0.13
	NC	11	11	2	132–134	0.64	0.48	< 0.94	0.38
MP0100	NC	11	11	2	216–222	0.64	0.43	< 1.00	0.41
	MN	10	10	3	212–220	0.70	0.54	< 0.89	0.29
MP0114	VT	10	10	3	212–220	0.30	0.49	< 0.14	0.34
	NC	11	11	2	206–218	0.55	0.50	< 0.82	0.38
	ID	10	9	3	206–214	0.44	0.48	< 0.53	0.35
	MN	10	10	4	206–218	0.80	0.67	< 0.84	0.17
MP0120	VT	10	10	2	206–214	0.20	0.18	< 1.00	0.69
	NC	11	10	2	112–116	0.70	0.46	< 1.00	0.40
	ID	10	10	3	112–120	0.20	0.54	< 0.013	0.29
MP0144	MN	10	10	4	108–120	0.20	0.55	< 0.0050	0.28
	VT	10	9	3	112–120	0.11	0.55	< 0.0010	0.30
	KR	53	28	4	206–218	0.29	0.31	< 0.39	0.50
	NC	11	11	3	198–214	0.55	0.64	< 0.29	0.20
	ID	10	9	5	198–226	0.44	0.38	< 1.00	0.40
MP0175	MN	10	10	5	186–210	0.70	0.74	< 0.42	0.12
	VT	10	10	4	194–206	0.70	0.73	< 0.41	0.12
	KR	53	31	3	174–182	0.61	0.51	< 0.90	0.35
	NC	11	11	5	186–206	0.73	0.65	< 0.76	0.17
	ID	10	5	4	174–186	0.40	0.70	< 0.057	0.14
MP0182	MN	10	10	5	170–190	0.70	0.78	< 0.25	0.09
	VT	10	10	4	174–186	0.70	0.74	< 0.39	0.12
	NC	11	11	3	204–216	0.45	0.62	< 0.14	0.22
	ID	10	10	2	216–220	0.30	0.46	< 0.30	0.40
MP0188	MN	10	10	5	204–220	0.50	0.55	< 0.34	0.24
	VT	10	10	4	204–216	0.70	0.57	< 0.88	0.24
	NC	11	11	2	129–133	0.36	0.30	< 1.00	0.54
	ID	10	10	2	129–133	0.50	0.46	< 0.80	0.40
MP0190	MN	10	10	2	129–133	0.40	0.48	< 0.49	0.39
	VT	10	10	2	129–133	0.40	0.42	< 0.64	0.42
	NC	11	11	2	197–199	0.45	0.48	< 0.61	0.38
	ID	10	5	2	197–199	0.20	0.50	< 0.24	0.38
MP0197	MN	10	10	2	197–199	0.30	0.38	< 0.50	0.46
	KR	52	31	3	232–240	0.65	0.55	< 0.90	0.29
	NC	11	11	2	232–236	0.64	0.48	< 0.94	0.38
	ID	10	10	3	232–240	0.30	0.27	< 1.00	0.56
	MN	10	10	2	232–236	0.20	0.18	< 1.00	0.69
MP0200	VT	10	10	3	232–240	0.50	0.59	< 0.34	0.26
	KR	52	26	2	173–177	0.23	0.20	< 1.00	0.65
	ID	10	9	6	161–181	0.22	0.77	< 0.0010	0.09
	MN	10	10	4	153–173	0.30	0.60	< 0.023	0.23
VT	10	10	3	161–169	0.40	0.34	< 1.00	0.47	

Table 2 Continued

Locus	Population	<i>N</i>	<i>N</i> genotyped	No. of alleles	Size range (bp)	<i>H_O</i>	<i>H_E</i>	<i>P</i> value	<i>I</i>
MP0227	NC	11	11	2	148–152	0.18	0.17	< 1.00	0.71
	ID	10	9	4	138–154	0.67	0.69	< 0.45	0.15
	MN	10	10	3	148–158	0.30	0.56	< 0.020	0.26
	VT	10	10	6	138–158	0.50	0.78	< 0.016	0.08
MP0234	ID	10	10	3	133–141	0.80	0.64	< 0.89	0.21
	MN	10	10	2	133–137	0.60	0.48	< 0.89	0.39
	VT	10	10	3	133–141	0.50	0.51	< 0.57	0.31
MP0243	ID	10	10	2	214–218	0.50	0.50	< 0.68	0.38
	MN	10	10	2	214–218	0.60	0.48	< 0.90	0.39
	VT	10	10	4	206–218	0.70	0.59	< 0.82	0.26
MP0247	KR	53	28	4	139–159	0.68	0.63	< 0.75	0.20
	NC	11	11	4	139–159	0.73	0.67	< 0.71	0.17
	ID	10	4	4	139–155	0.75	0.66	< 0.78	0.17
	MN	10	10	4	147–163	0.60	0.66	< 0.40	0.18
MP0263	VT	10	10	3	139–155	0.50	0.59	< 0.31	0.24
	NC	11	11	2	125–129	0.09	0.09	< 1.00	0.84
	ID	10	10	2	125–129	0.00	0.32	< 0.0060	0.51
	MN	10	10	2	125–129	0.70	0.50	< 0.96	0.38
MP0282	VT	10	10	2	125–129	0.20	0.42	< 0.14	0.42
	NC	11	11	2	131–135	0.09	0.09	< 1.00	0.84
	ID	10	10	2	131–135	0.10	0.10	< 1.00	0.82
	MN	10	10	2	131–135	0.40	0.48	< 0.48	0.39
MP0288	VT	10	10	2	131–135	0.10	0.46	< 0.021	0.40
	NC	11	11	3	126–134	0.27	0.38	< 0.20	0.42
	ID	10	7	2	126–130	0.57	0.49	< 0.86	0.38
	MN	10	10	2	126–130	0.40	0.48	< 0.45	0.39
	VT	10	10	2	126–130	0.40	0.42	< 0.65	0.42

Populations are abbreviated as follows: Kings River (KR), North Coast California (NC), Idaho (ID), Minnesota (MN), and Vermont (VT). Only populations that were polymorphic are displayed for each locus. Locus-population combinations that were out of Hardy–Weinberg equilibrium are indicated with *P* values in boldface.

Acknowledgements

This work was supported by the University of California, Agricultural Experiment Station (Project 6896MS), the California Department of Fish and Game (contract #S0285008), and the USDA Forest Service, Region 5 and the Sierra Nevada Research Center. Fisher research in Idaho that generated the samples was supported by the Lolo Pass Redevelopment Project funded by the Idaho Transportation Department and the USDA Forest Service, Region 1. Rod Williams helped collect samples from the Minnesota and Vermont populations. We would also like to thank Emily Rubidge, Mary Beth Rew, and Martine Bérubè for invaluable technical assistance on this project.

References

- Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. In: *Molecular Evolution: Producing the Biochemical Data, Part B* (eds Zimmer E, Roalson E), pp. 202–222. Elsevier, San Diego, CA.
- Guo D, Milewicz DM (2003) Methodology for using a universal primer to label amplified DNA segments for microsatellite analysis. *Biotechnology Letters*, **25**, 2079–2083.
- Hamilton MB, Pincus EL, Di Fiore A, Fleischer RC (1999) Universal linker and ligation procedure for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques*, **27**, 500–507.
- Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.
- Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.
- Rozen S, Shaletsky H (2000) PRIMER 3. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, New Jersey.
- Sambrook J, Russell DW (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology*, **18**, 233–234.
- Wisely SM, Buskirk SW, Russell GA, Aubry KB, Zielinski WJ (2004) Genetic diversity and structure of the fisher (*Martes pennanti*) in a peninsular and peripheral metapopulation. *Journal of Mammalogy*, **85**, 640–648.
- Zielinski WJ, Truex RL, Schlexer FV, Campbell LA, Carroll CR (2005) Historical and contemporary distributions of carnivores in forests of the Sierra Nevada, California, USA. *Journal of Biogeography*, **32**, 1385–1407.