Molecular Ecology Resources (2009) **9**, 288–291 doi: 10.1111/j.1755-0998.2008.02234.x

PERMANENT GENETIC RESOURCES NOTE

Tetranucleotide microsatellite loci from the black bear (*Ursus americanus*)

JAMIE SKVARLA SANDERLIN,* BRANT C. FAIRCLOTH,* BRIAN SHAMBLIN* and MICHAEL J. CONROY†

D. B. Warnell School of Forestry and Natural Resources, The University of Georgia, Athens, GA 30602, USA, †USGS, Georgia Cooperative Fish and Wildlife Research Unit, D.B. Warnell School of Forestry and Natural Resources, Athens, GA 30602, USA

Abstract

We describe primers and polymerase chain reaction conditions to amplify 21 tetranucleotide microsatellite DNA loci in black bears (*Ursus americanus*). We tested primers using individuals from two populations, one each in Georgia and Florida. Among individuals from Georgia (n = 29), primer pairs yielded an average of 2.9 alleles (range, one to four) and an average observed heterozygosity (H_O) of 0.50 (range, 0.00 to 0.79). Among individuals from Florida (n = 19), primer pairs yielded an average of 5.7 alleles (range, one to 14) and an H_O of 0.55 (range, 0.00 to 1.00). A comparison of previously developed markers with individuals from Georgia suggests that bear populations in Georgia and Florida have reduced allelic diversity relative to other populations.

Keywords: black bear, microsatellites, primers, SSRs, tetranucleotide repeats, *Ursus americanus*

Received 24 January 2008; revision received 6 April 2008; revision accepted 21 April 2008

The conservation and management of bear populations is facilitated by several molecular techniques, including the use of microsatellite markers in mark–recapture studies (Waits 1999). Mark–recapture studies typically involve noninvasive genetic (NG) samples (e.g. hair, faeces) to estimate demographic parameters (Taberlet *et al*. 1999; Mills *et al*. 2000). Studies have utilized NG samples to investigate demographics (Taberlet *et al*. 1997), habitat relationships (Apps *et al*. 2004), dispersal and/or the effectiveness of corridors (Dixon *et al*. 2006). Reduced quantity or quality of template DNA increases the chances of genotyping errors resulting from dropout or null alleles (Taberlet *et al*. 1999); thus, attention to marker selection is warranted. Twenty-eight dinucleotide bear microsatellite markers are available for use (Paetkau & Strobeck 1994; Paetkau *et al*. 1995; Taberlet *et al*. 1997; Kitahara *et al*. 2000). Dinucleotide markers may be more error-prone (Taberlet *et al*. 1999) than tri- or tetranucleotide repeat arrays; therefore, these markers may be problematic in NG studies. Additional markers may be needed to reach an acceptable probability of identity. We describe the development of species-specific, tetranucleotide microsatellite markers that will facilitate future mark–recapture programmes.

Correspondence: Jamie L. Skvarla Sanderlin, Fax: 706.542.8356; E-mail: jskvarla@uga.edu

We extracted DNA from blood obtained from a Georgia male black bear (*Ursus americanus*) using the DNAzol reagent (Invitrogen) followed by a phenol-chloroform extraction to remove remaining impurities. We digested DNA with *Rsa*I and *Bst*UI (New England Biolabs) and double-enriched for di-, tri-, and tetranucleotide repeats (Glenn & Schable 2005). Product for tetranucleotide repeats was ligated into PCR 2.1-TOPO vector and used to transform OneShot Top 10 chemically competent *Escherichia coli* cells (TOPO TA cloning kit, Invitrogen). We screened colonies for inserts using the β-galactosidase gene, and sequenced 190 positive (white) colony PCR products of 500–1100 base pairs using BigDye (version 3.1, Applied Biosystems) chemistry and a 3730*xl* (Applied Biosystems) sequencer. We assembled and edited sequences using SEQUENCHER 4.2 (Gene Codes Corp.), and we exported 126 contigs to Ephemeris 1.0 (available at http://www.uga.edu/srel/DNA_Laboratory/dnacomputer_programs.htm) to search for microsatellites. Seventy-two unique contigs contained microsatellite repeat arrays, and we developed 40 primers for these loci. We added an M13-reverse or CAG tag to the 5′ end of one of each primer pair using OLIGO 4.0 (Molecular Biology Insights) to facilitate fluorescent size detection on ABI

Table 1 Characteristics of 21 primer pairs amplifying tetranucleotide microsatellite loci from black bears (*Ursus americanus*) individuals of Georgia (n = 29) and Florida (n = 19) populations. Annealing start temperatures (T_a) for each primer pair are reported. For the number of individual genotypes at each locus (N), number of alleles (A), observed heterozygosity (H_O), expected heterozygosity (H_E), and probability that genotype proportions conform to HWE (P_{HW}), the first row contains values for the Georgia population and the second row contains values for the Florida population. P values in bold denote initial values < 0.01 that were not significant, while '*' indicates significance, following Bonferroni correction.

Locus (GenBank Accession no.)	Primer sequence (5′–3′) *†	Dye‡	T_a (°C)	Repeats in cloned allele	Clone size (bp)§	N	A	Size	H_O	H_E	P_{HW}
UA-BM3-P1A08 U	***GGAAACAGCTATGACCAT***CCTCCCATTCCTTTGTAG	FAM	60	$(AATG)_8$	174	25	2	173–177	0.56	0.50	0.694
(EU031659) L	GTTTACAAGCCCTCCTGGTGA					19	1	173	0.00	0.00	—
UA-BM3-P1B04 U	***CAGTCGGGCGTCATCA***GCAGTGTATAAGGGTTTCAAT	FAM	60	$(CTTT)_7$ ... $(CTTT)_5$	419	28	1	427	0.00	0.00	—
(EU031664) L	GTTTCACAGCAAAGAAAACAATCA					19	4	416–427	0.74	0.72	0.126
UA-BM3-P1B05 U	GTTTAGCTGTGCCCTGAGTGTG	FAM	60	$(AAAG)_{13}$	243	29	3	239–249	0.41	0.36	1.000
(EU031665) L	***CAGTCGGGCGTCATCA***TTACTGCTTTTGTGTTTCCTG					18	6	235–262	0.67	0.73	0.782
UA-BM3-P1F04 U	***GGAAACAGCTATGACCAT***ACTCCGCCTCCTGATTTT	FAM	60	$(AAAG)_{19}$	313	29	4	255–315	0.52	0.48	1.000
(EU031682) L	GTTTGTTGCCCTGTGGTTTTC					9	6	232–286	0.67	0.81	0.183
UA-BM4-P1H06 U	GTTTGTGTCTCCTGGTTCCTTTA	FAM	60	$(ATCC)_6$	259	29	3	262–275	0.69	0.61	0.701
(EU031691) L	***CAGTCGGGCGTCATCA***CCTGGCTTTTTCAGTCAG					18	5	262–279	0.50	0.64	0.450
UA-BM4-P1H10 U	GTTTGTAGATGGCAGATTGT	HEX	60	$(ATCT)_{10}$	217	25	2	217–221	0.08	0.15	0.122
(EU031692) L	***CAGTCGGGCGTCATCA***GTGGTAGGCTTCAGAGTTGT					19	5	217–233	0.32	0.63	**0.003**
UA-BM4-P2A03 U	***CAGTCGGGCGTCATCA***TTAAAAGGTTGCTGGTGTG	HEX	60	$(AGAT)_{15}$	273	29	4	276–288	0.59	0.59	0.264
(EU031695) L	GTTTATGGTGTGTTTCTACTAATCTC					19	8	264–296	0.68	0.79	0.108
UA-BM4-P2B05 U	***CAGTCGGGCGTCATCA***CCTCATTTTTTTTAGCAC	HEX	60	$(AGAT)_9$	215	28	1	219	0.00	0.00	—
(EU031705) L	GTTTAATAGGGTTGGGACAGT					17	3	215–223	0.29	0.51	0.079
UA-BM4-P2B06 U	***CAGTCGGGCGTCATCA***GAATACTGCTTGACCT	HEX	60	$(AGAT)_{11}$	210	24	3	201–213	0.79	0.68	0.698
(EU031706) L	GTTTATCAGTTCCTTAAAATACTT					18	4	205–217	0.44	0.57	0.339
UA-BM4-P2C10 U	***CAGTCGGGCGTCATCA***ATAGGGACTCAAATAGATA	HEX	60	$(AGAT)_9$ ... $(AGAT)_{10}$	380	25	3	370–389	0.68	0.64	0.164
(EU031715) L	GTTTAATGGACTCATACAATGTT					17	6	323–382	0.65	0.79	0.262
UA-BM4-P2E11 U	GTTTGGTTTTCTTTCACTTC	HEX	60	$(AGAT)_9$	251	16	3	256–276	0.38	0.54	0.195
(EU031726) L	***CAGTCGGGCGTCATCA***GATTCTCTATTCTTTCTCTA					16	6	256–280	0.50	0.75	0.074
UA-RM3-P2G10 U	GTTTCTGCTGTTTACCAGTGT	FAM	60	$(CTTT)_{14}$ ... $(CTCTCTTT)_5$	384	27	3	382–397	0.15	0.26	0.072
(EU031735) L	***CAGTCGGGCGTCATCA***CCAGAAAGAATACAATCAC					18	14	346–418	1.00	0.89	0.981
UA-RM3-P2H01 U	***CAGTCGGGCGTCATCA***GAAGAAGGAATGGAAAG	HEX	60	$(AAAC)_4$	358	27	2	356–361	0.22	0.20	1.000
(EU031738) L	GTTTAAATATGAGGTTGAATGTG					18	2	356–361	0.11	0.11	1.000
UA-RM3-P2H03 U	***CAGTCGGGCGTCATCA***TTGTTAGACTACCACCAT	HEX	60	$(AAAG)_{24}$	209	29	3	127–208	0.69	0.64	0.710
(EU031740) L	GTTTCTAGGATAGTATGTGCTGT					17	8	127–204	0.71	0.81	0.106
UA-BM3-P1D05 U	***CAGTCGGGCGTCATCA***TTTCACCTAATATACAGC	HEX	60	$(AAAG)_9$	459	28	3	453–460	0.75	0.65	0.309
(EU031675) L	GTTTACTATATCTTTATCCAGACTT					18	7	453–486	0.67	0.66	0.554
UA-BM4-P2A02 U	GTTTCCATTATCAACATTACTTAC	HEX	60	$(AGTT)_4$	193	29	2	196–204	0.48	0.48	1.000
(EU031694) L	***CAGTCGGGCGTCATCA***GTCCAAACACACAGAA					18	3	192–204	0.44	0.68	**0.0003**
UA-BM4-P2A06 U	GTTTGGAATATGGAACAGAGTTA	HEX	60	$(AGAT)_7$	288	27	4	287–317	0.52	0.60	0.676
(EU031698) L	***CAGTCGGGCGTCATCA***GAATCCTTTTAATCTATTA					18	7	276–329	0.72	0.78	0.103
UA-BM4-P2A07 U	***CAGTCGGGCGTCATCA***TCTGTAAGAGTGGATAAAC	FAM	60	$(AGATT)_9$ ... $(GAT)_5$ ... $(AGAT)_{11}$	221	29	1	223	0.00	0.00	—
(EU031699) L	GTTTCTATCACCTATCATCTATC					15	7	183–236	0.87	0.80	0.927
UA-BM4-P2B08 U	***CAGTCGGGCGTCATCA***GGTCCAGTGCTAGGT	HEX	60	$(AGAT)_{11}$ ... $(AGAT)_9$	239	28	3	235–243	0.57	0.52	0.619
(EU031708) L	GTTTGAGGAGGGTGTATAT					18	5	239–256	0.11	0.51	0.000*
UA-BM4-P2C02 U	GTTTGAAATGAAAGATAAATGATA	HEX	60	$(ACAG)_4$	194	26	2	198–207	0.42	0.50	0.450
(EU031711) L	***CAGTCGGGCGTCATCA***CTATTCCTCCTTCTGTTC					18	2	202–207	0.39	0.32	1.000
UA-BM4-P2C05 U	***CAGTCGGGCGTCATCA***TTCTTATTCTCCTCAAAC	FAM	60	$(AGAT)_4$ ... $(AT)_5$	435	23	4	431–446	0.48	0.62	0.325
(EU031713) L	GTTTCCAAACCAAAAAAGTAA					—	—	—	—	—	—

*Sequences used to introduce sites for the universal primer are in bold italics.

†Underlined bases indicate sharing of nucleotides between CAG (5′-CAGTCGGGCGTCATCA-3′) tag, M13R (5′-GGAAACAGCTATGACCAT-3′) tag, or GTTT 'pigtail' and the specific primer binding site.

‡Dyes presented in this table do not refer to directly labelled primers.

§Clone size refers to the predicted size of the PCR product amplified from the clone used to develop each locus.

sequencers (Schable *et al*. 2002; see Table 1 for details). We chose either a CAG or an M13-reverse tag for the upper or lower primer on the basis of minimizing self- or pair-complementarity and secondary structure of each primer or primer pair. We added GTTT 'pigtails' to the 5′ end of each primer lacking either CAG or M13-reverse tag to facilitate the addition of adenosine by *Taq* polymerase (Brownstein *et al*. 1996).

We optimized primer pairs using six DNA samples obtained from wild bears captured in or near Oaky Woods and Ocmulgee Wildlife Management Areas in central Georgia. Prior to extraction, blood and tissue samples were stored in a –20 °C freezer. We extracted DNA from the Georgia tissue samples using the DNeasy Kit (QIAGEN) and from the Florida blood samples with the GenomicPrep DNA isolation kit (GE Healthcare). We performed PCR amplifications in 10 μL volumes using Bio-Rad MyCycler thermal cyclers. Final concentrations for optimizing reactions were 10 mM Tris pH 8.4, 50 mM KCl, 0.5 μM 'pigtailed' primer, 0.05 μM CAG or M13-reverse tagged primer (CAG or M13-reverse + primer), 0.45 μM dye-labelled tag (HEX or FAM + CAG or M13-reverse), 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 U Ampli*Taq* Gold DNA Polymerase (Applied Biosystems), and 50 ng DNA. We ran reactions using one touchdown thermal cycling programme (Don *et al*. 1991), encompassing a 10.5 °C span of annealing temperatures (range, 60–49.5 °C). Cycling parameters were: 21 cycles of 96 °C for 20 s; highest annealing temperature for 30 s minus 0.5 °C per annealing cycle; and 72 °C for 1 min 30 s followed by 14 cycles of 96 °C for 20 s; 50 °C for 30 s; 72 °C for 1 min 30 s; and a final extension period of 10 min at 72 °C. We checked PCR products for amplification and sized fragments using a 3730*xl* DNA sequencer (Applied Biosystems) with GENESCAN Rox500 fluorescent size standard (PE Applied Biosystems). We analysed results using GENEMAPPER software (Applied Biosystems) using the local Southern size-calling method.

Table 1 summarizes the characteristics of 21 primer pairs developed from the black bear and tested using individuals collected from central Georgia ($n = 23$) and northwest Florida ($n = 19$). We calculated observed and expected heterozygosity levels for each locus and total exclusionary power using CERVUS 2.0 (Marshall *et al*. 1998). We used GENEPOP 3.4 (Raymond & Rousset 1995) to test for Hardy–Weinberg equilibrium (HWE) and genotypic linkage disequilibrium (LD). The average number of alleles per locus were 2.9 (range, one to four) and 5.7 (range, one to 14) for Georgia and Florida populations, respectively. Total exclusionary power with both parents unknown was 0.94 and 0.99 in the Georgia and Florida populations, respectively. The probability of identity among siblings (Evett & Weir 1998) for the Georgia population was 5.83×10^{-5}, while P_{IDsib} for the Florida population was 5.27×10^{-7}. In the Georgia population, no loci deviated from HWE. One locus (UA-BM4-P2B08) deviated from HWE (P value < 0.01) in the Florida population following *a posteriori* Bonferroni correction (Rice 1989). No significant LD was detected after Bonferroni correction in either population.

Our data indicate allelic diversity within the Georgia and Florida populations is low. To examine whether this effect can be attributed to the markers described in this study or the population under analysis, we genotyped the same individuals from Georgia using previously developed markers (G1A, G1D, G10B, G10L: Paetkau & Strobeck 1994; G10M, G10P, G10X: Paetkau *et al*. 1995). Typically, black bear studies using these markers with similar sample sizes ($n \cong 23$) report a mean number of alleles per locus between 4.9 and 8.8 (Paetkau & Strobeck 1994; Warrillow *et al*. 2001). In the central Georgia population, we found the mean number of alleles per locus was 2.6, which is different from one of the other studies ($P < 0.001$, *t*-test between Georgia and Cook County, Minnesota, Warrillow *et al*. 2001, for six loci). This comparison, along with alleles potentially unique to other Eastern US populations (data available upon request), suggests that bear populations in central Georgia and northwest Florida have reduced allelic diversity relative to other populations. The markers developed in this study merely reflect this reduction.

Acknowledgements

This research was supported by: Georgia Cooperative Fish and Wildlife Research Unit, jointly sponsored by USGS, USFWS, University of Georgia, Georgia DNR, and the Wildlife Management Institute; Daniel B. Warnell School of Forestry and Natural Resources; and McIntyre-Stennis project GEO-0100-MS and GEO-0136-MS. We thank Travis Glenn and Lisette Waits for comments on earlier drafts. M. J. Yabsley, M. R. Vaughan, C. J. Nairn, and J. P. Carroll contributed to development and testing efforts. Capture, handling, and sedation of bears in Georgia were conducted by the Georgia DNR and approved by the UGA Institutional Animal Care and Use Committee (approval nos: A2003-10148, A2003-10148-m1). Florida bear samples were salvaged from hunter-killed animals. Use of trade names does not imply US Government endorsement of commercial products.

References

Apps CD, McLellan BN, Woods JG, Proctor MF (2004) Estimating grizzly bear distribution and abundance relative to habitat and human influence. *Journal of Wildlife Management*, **68**, 138–152.

Brownstein MJ, Carpenter JD, Smith JR (1996) Modulation of non-templated nucleotide addition by *Taq* polymerase: primer modifications that facilitate genotyping. *BioTechniques*, **20**, 1004–1010.

Dixon JD, Oli MO, Wooten MC *et al*. (2006) Effectiveness of a regional corridor in connecting two Florida black bear populations. *Conservation Biology*, **20**, 155–162.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research*, **19**, 4008.

Evett IW, Weir BS (1998) *Interpreting DNA Evidence: Statistical Genetics for Forensic Scientists*. Sinauer Associates, Sunderland, Massachusetts.

Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. In: *Methods in Enzymology 395, Molecular Evolution: Producing the Biochemical Data, Part B* (eds Zimmer EA, Roalson EH). Academic Press, San Diego, California.

Kitahara E, Isagi Y, Ishibashi Y, Saitoh T (2000) Polymorphic microsatellite DNA markers in the Asiatic black bear, *Ursus thibetanus*. *Molecular Ecology*, **9**, 1661–1662.

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**, 639–655.

Mills LS, Citta JJ, Lair KP, Schwartz MK, Tallmon DA (2000) Estimating animal abundance using noninvasive DNA sampling: promise and pitfalls. *Ecological Applications*, **10**, 283–294.

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

Paetkau D, Strobeck C (1994) Microsatellite analysis of genetic variation in black bear populations. *Molecular Ecology*, **3**, 489–495.

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.

Schable NA, Fischer RU, Glenn TC (2002) Tetranucleotide microsatellite DNA loci from the dollar sunfish (*Lepomis marginatus*). *Molecular Ecology Notes*, **2**, 509–511.

Taberlet P, Camarra J-J, Griffin S *et al*. (1997) Noninvasive genetic tracking of the endangered Pyrenean brown bear population. *Molecular Ecology*, **6**, 869–876.

Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap. *Trends in Ecology & Evolution*, **8**, 323–327.

Waits LP (1999) Molecular genetic applications for bear research. *Ursus*, **11**, 253–260.

Warrillow J, Culver M, Hallerman E, Vaughan M (2001) Subspecific affinity of black bears in the White River National Wildlife Refuge. *Journal of Heredity*, **92**, 226–233.