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TECHNICAL NOTE

Development and characterization of tetranucleotide microsatellite loci for the American alligator (*Alligator mississippiensis*)

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Abstract We isolated and characterized 17 tetranucleotide microsatellite loci in the American alligator, *Alligator mississippiensis*. Loci were screened across 27 individuals from one population and shown to be polymorphic with the number of alleles per locus ranging from 2 to 12. Polymorphic information content ranged from 0.2 to 0.85, and observed heterozygosity ranged from 0.185 to 0.889. One locus showed significant deviation from Hardy–Weinberg equilibrium, and one pair of loci showed evidence of linkage.

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American alligators (*Alligator mississippiensis*) are of ecological and commercial importance throughout their range in the southeastern United States. After having declined severely in the 1960s due to unsustainable harvest practices, their populations have largely rebounded due to improved management. However, on-going research is critical to developing conservation strategies focused at the appropriate spatial scale. Central to this endeavor is determination of population genetic structuring both within habitats and across the species' range (Davis et al. 2001a, b; Ryberg et al. 2002). Highly variable microsatellites can provide detailed insights into many facets of population biology, individual dispersal, and genetic neighborhood sizes.

Most microsatellite markers in American alligators reported to date contain dinucleotide repeat motifs (Glenn et al. 1998; Davis et al. 2002). Generally speaking, such loci are stutter-prone (i.e., *Taq* error causes slippage during amplification), such that discrimination between some heterozygous versus homozygous genotypes, and determination of absolute allele sizes, can be difficult (DeWoody et al. 2006). These scoring errors can also lead to problems with dataset continuity among years for long-term projects. Here we report the development of tetranucleotide microsatellite loci. This marker set contributes additional resolving power for studies concerned with parentage analysis and population structure. Here we describe 14 previously unreported markers, and present complete information for the three new markers reported in Lance et al. (2009).

Table 1 Characterization of 17 microsatellite loci for *Alligator mississippiensis*

Locus	Primer sequence 5′ → 3′	GenBank accession number	Dye	Repeat(s) in cloned allele	T_a	Clone size (bp)	N	k	Size range (bp)	H_O	H_E	PIC
Ami 227 U	*GGAAACAGCTATGACCATG* AAT ACA AAC GGG TAA CCT C	JQ082104	FAM	(AGAT)17	TD60	133	27	10	134–174	0.889	0.873	0.84
Ami 227 L	AGC GGA AAT GTT ATA TCT ATC T											
Ami 229 U	*CAGTCGGGCGTCATC* AAA GCA TTC ACC TCC TAG T	JQ082105	FAM	(ATCC)12	TD60	243	27	8	240–272	0.667	0.813	0.771
Ami 229 L	CAC CCT GTC TAC CTC TCT AC											
Ami 231 U	*GGAAACAGCTATGACCATG* ATT AAC ATT GAT TTG ATT TAC AC	JQ082106	FAM	(ACTC)15	TD60	136	27	10	120–160	0.778	0.777	0.74
Ami 231 L	ACA GAA GAG AGA CTC ACT CAC T											
Ami 232 U	AAA GTC AAC CTC TAT CTA TTT	JQ082107	NED	(ATCC)4	TD60	108	27	12	119–191	0.815	0.881	0.85
Ami 232 L	*GGAAACAGCTATGACCATG* CCA AAG ACC CAG ATG TT											
Ami 233 U	TGA GAC CAG CAA TAC TGT A	JQ082108	NED	(AACC)6	TD60-LN	150	27	6	166–200	0.741	0.675	0.607
Ami 233 L	*CAGTCGGGCGTCATC* ATG GAG GGT AGA GAT TGT C											
Ami 235 U†	*CAGTCGGGCGTCATC* ACT AGG CAC CTT AAC ACT C	JQ082109	HEX	(ACTC)10	TD65	141	27	8	155–191	0.889	0.783	0.743
Ami 235 L	CAC AGG GCC TCA GAT A											
Ami 236 U	AGA AAG AGG CAC AGA TGA C	JQ082110	FAM	(AAGC)5	TD60	134	27	2	156–160	0.185	0.230	0.2
Ami 236 L	*GGAAACAGCTATGACCATG* CCA CTT GTC TCC TTG TAT C											
Ami 237 U	ATG TGT TGC CTG TTA T	JQ082111	NED	(ATCC)9	TD60	88	27	3	102–110	0.519	0.526	0.401
Ami 237 L	*CAGTCGGGCGTCAT* CAG TAA TGG TGG AAT ATA											
Ami 238 U	GTT AGA TGG CAA AGC ATA TT	JQ082112	HEX	(AACC)5	TD65	92	25	2	111–115	0.320	0.490	0.365
Ami 238 L	*GGAAACAGCTATGACCATG* ACC ACT GCC CAA CAA											
Ami 239 U	*GGAAACAGCTATGACCATG* CCC AGA GAT TTC AAA TAG A	JQ082113	NED	(AAGG)12	TD60	146	27	7	144–176	0.815	0.730	0.675
Ami 239 L	TCT TTA AGC TCC CAC ACT											
Ami 241 U	*GGAAACAGCTATGACCATG* ATA CTT CCC TGA CCC TAA TA	JQ082114	FAM	(ATCC)5	TD60	294	27	3	318–326	0.481	0.469	0.394
Ami 241 L	GCA GGT CTT AGC TTA TTC AA											
Ami 242 U	CAG GGT TGG AAT GTC A	JQ082115	NED	(ATCC)13	TD60	155	27	5	160–176	0.593	0.575	0.532
Ami 242 L	*CAGTCGGGCGTCATC* ACA CAG TCC ATA ACA ATT TT											
Ami 243 U†	CAA GTG AGC CTG GTC T	JQ082116	NED	(AAGG)11	TD60	127	27	6	142–162	0.593	0.683	0.618
Ami 243 L	*CAGTCGGGCGTCATCA* TAA GTA GCT TGT AGG ATT TAT TC											
Ami 244 U*	GCT GGT TTG GAT GTG TA	JQ082117	HEX	(ACTC)13	TD65	239	27	7	240–272	0.778	0.806	0.763
Ami 244 L	*GGAAACAGCTATGACCATG* GTG CCA TCT ATG CTC AT											
Ami 245 U	*CAGTCGGGCGTCATCA* CTT TTG GGC TGC TAT TC	JQ082118	FAM	(ATCC)12	TD60	126	27	5	117–133	0.741	0.712	0.653
Ami 245 L	GGG TAA TAT GCC AAG ACT TT											

Table 1 continued

Locus	Primer sequence 5′ → 3′	GenBank accession number	Dye	Repeat(s) in cloned allele	T_a	Clone size (bp)	N	k	Size range (bp)	H_O	H_E	PIC
Ami 246 U	CTA GCC AAA AAT GTC TTA AT	JQ082119	HEX	(AAGG)5	TD55	213	27	4	213–229	0.519	0.599	0.543
Ami 246 L	*CAGTCGGGCGGTCATC* AAA GCA GAA TAA ACC CTA GA											
Ami 247 U	*CAGTCGGGCGGTCATCA* TGG CTC GTT GTC TAC ATA CT	JQ082120	HEX	(ATCC)11	TD60	200	27	4	202–214	0.741	0.654	0.572
Ami 247 L	ATA GTG TGG GCT GTT TTT TA											

Sequences that introduce sites for the universal fluorescent primer are italicized. Underlined bases are shared between the universal and locus-specific primer. 'Dye' refers to the fluorescent dye used for genotyping. Repeats in cloned Allele describe microsatellite characteristics. T_a corresponds to highest annealing temperature in the touchdown PCR profile (LN indicates longer extension time). Clone size is the size of the cloned allele. N is number of individuals genotyped. k is observed number of alleles. Size range indicates the observed distribution of alleles per locus. H_o and H_e are observed and expected heterozygosity, respectively, and PIC is polymorphic information content

* Significant deviation from Hardy–Weinberg equilibrium after sequential Bonferroni correction

† Loci in significant linkage disequilibrium after sequential Bonferroni correction

Genomic DNA was extracted from blood drawn from an alligator from Rockefeller Wildlife Refuge, Louisiana, using a proteinase K digestion. Following Glenn and Schable (2005), DNA was serially enriched twice for microsatellites using three probe mixes (mix 2 = $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$; mix 3 = $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$; mix 4 = $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$). Briefly, DNA was digested with *Rsa*I (New England Biolabs) and simultaneously ligated to double-stranded SuperSNX linkers (SuperSNX24 Forward 5′-GTTTAAGGCCTAGCTAGCAGCAGAATC and SuperSNX24 Reverse 5′-GATTCTGCTAGCTAGGCCTTAAACAAAA). Linker-ligated DNA was denatured and hybridized to biotinylated microsatellite oligonucleotide mixes, and then captured on magnetic streptavidin beads (Dynal). After discarding unhybridized DNA, remaining DNA was eluted from the beads, amplified in polymerase chain reactions (PCR) using the forward SuperSNX24 primer, and cloned with TOPO-TA Cloning Kits (Invitrogen). Clones with inserts were sequenced with M13 forward and reverse primers using the BigDye Terminators v3.1 (Applied Biosystems) on an ABI-377-96 sequencer. Sequences were assembled and edited in Sequencer v4.1 (Genecodes) and exported to Ephemeris v1.0 for microsatellite searching. Primers were designed using Oligo v6.67 (Molecular Biology Insights). A 5′ modification was added to one primer in each pair (CAG tag 5′-CAGTCGGGCGTCATCA-3′) to allow use of a 3rd fluorescently labeled primer (CAG tag) in PCR.

Forty-eight primer pairs were tested using DNA from seven alligators from Rockefeller. Amplifications were performed in 12.5 µl volumes (10 mM Tris pH 8.4, 50 mM KCl, 25.0 µg/ml bovine serum albumin, 0.4 µM unlabeled primer, 0.08 µM tag-labeled primer, 0.36 µM universal dye-labeled primer, 2 mM $MgCl_2$, 0.15 mM dNTPs, 0.5 units JumpStart *Taq* DNA Polymerase (Sigma), and 20–40 ng DNA) using an ABI thermal cycler. Touchdown thermal cycling programs (Don et al. 1991) encompassing a 10°C span of annealing temperatures ranging between 65–55, 60–50 or 55–45°C were used (see Table 1). Cycling parameters were 21 cycles of 96°C for 20 s, highest annealing temperature (decreased 0.5°C per cycle) for 20 s, and 72°C for 30 s; and 15 cycles of 96°C for 20 s, lowest annealing temperature for 20 s, and 72°C for 30 s. Amplicons were run on an ABI-3130xl sequencer and sized with Naurox size standard prepared as in DeWoody et al. (2004), except that unlabeled primers started with GTTT. Results were analyzed using GeneMapper v4.0 (Applied Biosystems). Seventeen primer pairs amplified high quality products that showed polymorphism.

We further assessed variability of these loci in a population sample of 27 alligators from the Joseph W. Jones

Ecological Research Center, Newton, Georgia. Conditions and characteristics of the 17 loci are given in Table 1. We estimated number of alleles per locus (k), observed and expected heterozygosity (H_o and H_e) and Polymorphic Information Content (PIC) using CERVUS v3.0 (Marshall et al. 1998). We tested for null alleles in MicroChecker v2.2.3 (van Oosterhout et al. 2004) and found no evidence for them. Deviations from Hardy–Weinberg equilibrium (HWE) and linkage equilibrium were assessed using GENEPOP v4.1 (Rousset 2008). One locus, Ami244, showed significant deviation from HWE after sequential Bonferroni correction ($P = 0.002$). After sequential Bonferroni correction, one of the 136 possible locus pairs showed non-random association of alleles (Ami 235 and Ami 243; $P < 0.001$). Taken together, this new set of microsatellite loci expands the 'molecular toolbox' available to conservation biologists for generating management-relevant information for *A. mississippiensis*. To this end, we are using these loci to examine fine-scale population structure and landscape-level barriers to dispersal in one portion of the species' range.

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