

## PRIMER NOTE

# Fluorescent dUTP helps characterize 10 novel tetranucleotide microsatellites from an enriched salamander (*Ambystoma texanum*) genomic library

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*Department of Forestry and Natural Resources, Purdue University, West Lafayette, Indiana, 47907–1159, USA***Abstract**

Ten tetranucleotide microsatellite loci were isolated and characterized from Indiana (USA) populations of the smallmouth salamander, *Ambystoma texanum*. As opposed to individually labelling primers that were not yet known to be polymorphic, we used fluorescent dUTP to assess genetic variability and found it to be very effective. Allelic diversity ranged from two to 18 alleles per locus and observed heterozygosity ranged from 0.17 to 0.91 among roughly 25 individuals. One of the 10 markers also amplified and was polymorphic in the tiger salamander (*A. tigrinum*).

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Ambystomatid salamanders typically form large breeding aggregations, and these social arenas can provide a remarkable venue for the study of mating systems (Petranka 1998). To this end, a number of Ambystomatid microsatellites have been cloned recently (Wieczorek *et al.* 2002; Julian *et al.* 2003a,b; Mech *et al.* 2003). Here, we characterize 10 tetranucleotide microsatellite markers that will be useful for studies of the smallmouth salamander. Though we made some notable modifications (see <http://www.agriculture.purdue.edu/fnr/html/faculty/DeWoody/DeWoodyweb/index.html>), we generally followed the microsatellite enrichment protocols described in Hamilton *et al.* (1999) and in Hauswaldt & Glenn (2003).

Tail tissue was collected from a single individual and genomic DNA was extracted by a standard proteinase K/phenol-chloroform procedure (Sambrook & Russell 2001). DNA was digested with *HaeIII*, *NheI*, and *RsaI* to obtain appropriate-sized fragments (200–1000 bp). The digested DNA was dephosphorylated and subsequently ligated to double-stranded SNX linkers according to Hamilton *et al.* (1999). Ligation products were then hybridized to 5' biotinylated oligonucleotides [(GATA)<sub>7</sub>, (GATC)<sub>7</sub>, and (GACA)<sub>7</sub>]. After a 10 min denaturation of the restriction fragments at 95 °C, the biotinylated oligonucleotides were annealed to the restriction fragments by quickly cooling the mixture

to 70 °C and then slowly stepping down 0.2 °C every 10 s until 50 °C. That is, we performed 99 'steps', cooling for 50 s/degree from 70 °C to 50 °C. This was followed by 50 °C for 10 min and a final ramp down 0.5 °C every 10 s for 20 cycles. [This annealing program provides a stringent hybridization environment; the percentage of positive clones isolated from this library (see below) and from a concurrent kangaroo rat library (> 85%; Waser and DeWoody, unpublished data) suggests it worked well.] The hybridized microsatellite-enriched DNA was washed and subsequently amplified according to Hamilton *et al.* (1999). PCR products were then directly ligated into Stratagene's pBS II SK(+) vector and transformed into supercompetent XL2-Blue MRF' cells (Stratagene #200151).

Without further screening, we sequenced 83 recombinant clones and found that 49 (59%) produced sequences that contained microsatellites. Upon sequencing, 35 of the 49 microsatellites (71.4%) were GACA repeats whereas GATA, GATC, and mixed motifs made up a much smaller percentage of microsatellites (22.4%, 0%, and 6.1%, respectively). PCR primers were designed from those sequences containing microsatellites with adequate flanking regions ( $n = 39$ ). Of those 39 putative loci, 14 consistently produced bands of the expected size during 12 µL PCRs that contained ~100 ng template DNA, 1× *Taq* polymerase buffer (5 mM KCL, 1 mM Tris-HCL, pH 9.0, and 0.01% Triton x-100), 1.0 unit *Taq* polymerase (Promega), 1.5 mM MgCl<sub>2</sub>, 0.33 µM of each primer, and 0.2 mM of each dNTP (see below for cycling conditions).

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**Table 1** Characterization of 10 polymorphic tetranucleotide microsatellite loci developed for the smallmouth salamander (*Ambystoma texanum*). The population data are pooled from two distinct wetlands in Tippecanoe County, Indiana

Locus	Accession Number	Primer sequence (5'-3')	Motif	$T_a$ (°C)	Size (bp)	$N$	No. of alleles	$H_O$
Atex 49	AY362352	F: GAGGGGTGCTATATAAAAATCC R: GTCATAGTCTTTGCCTCAATC	(GATA) <sub>20</sub>	54	103–175	22	12	0.64
Atex 63	AY362347	F: TTCCGGCTTTGAAAGAGAGGTA R: TGATGTGTGCCGGTTGTGGT	(GACA) <sub>5</sub>	50	428–440	22	4	0.32
Atex 65	AY362353	F: TTCTGAGCTGTCCATGTTTCATATGC R: CGCTAGGAAGTCACATTTACTTTGTC	(GATA) <sub>19</sub>	55	272–384	23	18	0.91
Atex 74	AY362348	F: TCAACGAAAGAGGTGTGGGT R: TCCAACGACAGCGGTATAAAA	(GACA) <sub>7</sub>	57	211–227	18	5	0.56
Atex 87	AY362354	F: GCGGATTTTGCCTATATAAAA R: ATGATGCTTCAAACCAGAAC	(GATA) <sub>10</sub> GGTA(GATA) <sub>8</sub>	55	135–219	25	14	0.32
Atex 89	AY362349	F: TAAAGCCCTGTCCACAATC R: TCAGTGCCTGGATACCCCTTC	(GATA) <sub>24</sub>	54	213–325	19	11	0.68
Atex 102	AY362355	F: TTCAGGTGGATTACAGTGC R: CTGTGTTAGGGGTTTCCTG	(GATA) <sub>21</sub>	57	149–209	23	13	0.87
Atex 133	AY362350	F: CTTGAGGTTTGTGGTCAAT R: TATCGCCTTCTGGCTCTTA	(GATA) <sub>27</sub>	55	172–280	23	12	0.87
Atex 141	AY362356	F: GCTTCTTTTGCTTGCCTGTT R: TTTTCGCAATTGCTGATAAGG	(GATA) <sub>18</sub>	56	188–304	22	12	0.68
Atex 143	AY362351	F: CTTCTACCCGAGCTGTTTG R: TCTTGTTCCTGGTGACGAAA	(GACA) <sub>6</sub>	53	151–159	24	2	0.17

$T_a$ , annealing temperature;  $N$ , number of individuals screened;  $H_O$ , observed heterozygosity.

As opposed to labelling our primers with radiation or fluorochromes prior to screening for allelic variation, we conducted a final screen of the 14 loci using fluorescent chromatide rhodamine green 5dUTP as a label. The direct incorporation of a labelled nucleotide prior to electrophoresis in an automated sequencing gel allowed us to forego the considerable expense of individually labelling primers that were not yet known to be polymorphic. The only difference in the PCR detailed above was that we added 0.2  $\mu$ M of chromatide rhodamine green 5dUTP (Molecular Probes, catalogue #7629). The main disadvantage to using fluorescent dUTP is simply that loci are indistinguishable on the basis of colour (much as with conventional autoradiography) as they all appear blue when visualized on an automated sequencer such as our ABI377. Thus, we ran different gels for each locus during the population characterization.

For preliminary genotyping, each locus was amplified using genomic DNA extracted from toe clips of 25 individuals representing two populations from Tippecanoe County, Indiana. All PCRs were performed with the following thermal conditions: 2 min at 94 °C (denaturation), followed by 32 cycles of 30 s at 94 °C, 30 s at primer-specific annealing temperature (Table 1), and 30 s at 72 °C; this was followed by a final step of 2 min at 72 °C. All reactions were performed in a PTC-100 thermal controller (MJ Research) and all products were genotyped using an ABI 377 auto-

mated sequencer along with the associated GENESCAN AND GENOTYPER software (Applied Biosystems).

Of the 14 loci that amplified, 10 (71%) proved to be polymorphic in the smallmouth salamander (Table 1). We surveyed two discrete local populations ( $n = 7$  and  $n = 18$ ) for genetic variation. Allelic variability ranged from two to 18 alleles per locus and averaged 10.3. We found no evidence for gametic phase disequilibrium using GENEPOP version 3.1b (Raymond & Rousset 1995).

After optimization in the smallmouth salamander, we assessed the utility of each marker in the tiger salamander (*Ambystoma tigrinum*). One of our loci (Atex 65) proved to be highly variable in tigers, revealing 16 alleles among 15 individuals. While these loci will clearly be informative for studies of the smallmouth salamander, their widespread utility in related species has yet to be determined.

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