Miller ES15-00302R Appendices

APPENDIX A. Detailed description of microsatellite amplification protocol.

A.1 Methods

A.1.1 Primer Selection

Since no previous microsatellite primers were available for *Desmognathus fuscus*, we screened a total of 21 di- and tetranucleotide microsatellite primers developed for related taxa (*Desmognathus auriculatus*, Croshaw and Glenn 2003; *Desmognathus ocoee*, Adams et al. 2005; *Plethodon cinereus*, Connors and Cabe 2003; *Ensatina eschscholtzii*, Devitt et al. 2009). All loci that failed to amplify, or that were monomorphic were excluded from this study. A subset of six tetranucleotide microsatellite markers from *Desmognathus auriculatus* (Croshaw and Glenn 2003) and *Desmognathus ocoee* (Adams et al. 2005) proved consistent amplification and exhibited heterozygosity.

A.1.2 Microsatellite Amplification

Microsatellite loci were optimized for use with RubyTaqTM PCR Master Mix (2X; USB Corp., Cleveland, OH, USA). Total reaction volume amounted to 12.5 µL (6.25 µL RubyTaqTM PCR Master Mix; 3.25 µL deionized H_2O; 1 µL of forward and reverse primer; 1 µL DNA template). Reaction conditions were: (1) *Dau* primers: denaturation at 94°C for two min; 35x (94°C for 30s, primer-specific annealing temperature for 30s, and 72°C for 45s); and a final extension of 72°C for five min; (2) *Doc* primers: denaturation at 95°C for five min; 35x (94°C for one min, primer-specific annealing temperature for one min, and 72°C for two min); and a final extension of 72°C for three min. Primer sequence and annealing temperatures are listed in Table A1.

A.1.3 Fragment Size Analysis

Fragment size analysis was conducted using optimized PCR cycling conditions with fluorescently labeled forward primers and unlabeled reverse primers. Amplicon volumes of 0.6 to 1.0 µL were added to 35 µL of Sample Loading Solution containing Hi-Di Formamide (Applied Biosystems Inc., Foster City, CA, USA) and a Genome Lab DNA Size Standard (400 kit; Beckman Coulter Inc., Brea, CA, USA). Analysis was carried out using the Fragment Analysis Module, which assigned fragment size (base pair length) based on the DNA size standard.

TABLE A1. Sequences of six tetranucleotide microsatellites, dye color, and optimal annealing temperature of PCR reaction used in analysis of *Desmognathus fuscus* genetic population structuring.

Locus	Primer Sequence	Dye Color	Annealing Temperature
Dau6[1]	F: GATCGCACGTTAAATAA R: GGCAGGAAAAGGTTAG	D4-PA (Blue)	53°
Dau8[1]	F: GGAAACACCAGAAAAAGT R: AAGCAGGATTAGGTGAATA	D3-PA (Green)	52°
Dau11[1]	F: GTCCCTCAGGCTTGATAAG R: TGTGCCTATCCAGTCATCTA	D4-PA (Blue)	56°
Dau12[1]	F: CGACTTCTGAAACAACAAC R: CGGTTCTGAATTCCTTAC	D4-PA (Blue)	56°
Doc1[2]	F: TGTGAAGGGTGTTCTCTTACTG R: GCTGTTTGTGCTTTGACTTTAC	D2-PA (Black)	54°
Doc3[2]	F: CTCTCCCACTCTTCCTCAAGTA R: CTTCACCTTCGCTATGACTGT	D4-PA (Blue)	54°

Notes: [1] Croshaw and Glenn (2003), [2] Adams et al. (2005).

LITERATURE CITED

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