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TECHNICAL NOTE

Isolation and characterization of microsatellite markers from the threatened eastern indigo snake (*Drymarchon couperi)*

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Abstract The eastern indigo snake (*Drymarchon couperi*) is the longest snake in North America. Population declines due to extensive loss of habitat led to federal listing as a threatened species in 1978. Knowledge deficiencies regarding the biology of the eastern indigo snake, including population connectivity and gene flow, are impediments to the development of effective conservation and restoration strategies. We describe primers and polymerase chain reaction (PCR) conditions to amplify 22 tetranucleotide and pentanucleotide nuclear microsatellite loci isolated from the eastern indigo snake. We tested primers using 28 shed skins representing 24 individuals from Fort Stewart, Georgia. Primers yielded an average of 4.6 alleles per locus (range 3–7) and an average observed heterozygosity of 0.63 (range 0.46–0.79). These loci should prove useful for individual identification as well as population level analyses of this federally threatened species.

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The eastern indigo snake (*Drymarchon couperi*) is a federally threatened species native to the Coastal Plain of the southeastern United States. This species has experienced a reduction in its overall range as well as population declines due to habitat loss, diminishing gopher tortoise populations, and gassing of gopher tortoise burrows (US Fish and Wildlife Service 1982). A rangewide phylogeographic survey using mitochondrial DNA markers detected the presence of Gulf and Atlantic clades (Krysko et al. 2010), concordant with a biogeographic pattern described for several taxa of plants and animals (Soltis et al. 2006); however the patterns and scale of population connectivity via natal dispersal remain unresolved and may best be studied using microsatellite markers. Nuclear genetic markers capable of individual resolution may supplement traditional mark-recapture monitoring efforts for this species (Stevenson et al. 2009) as well as prove useful in assessments of population structure. We addressed this need through development of a panel of eastern indigo snake specific microsatellite markers.

Genomic DNA was enriched for microsatellite loci using the methods described by Glenn and Schable (2005). DNA was extracted from two freshly shed skins collected at a site in Coffee County, Georgia using a Qiagen® DNEasy Blood and Tissue Kit. DNA was digested using *RsaI* endonuclease (New England Biolabs) at 37°C for 1.5 h. Double stranded SuperSNX linkers (Glenn and Schable 2005) were ligated to the digested genomic DNA overnight at 16°C. Linker-ligated DNA was recovered using PCR on an Applied Biosystems 9,700 thermal cycler and verified using a 1% agarose gel at 100 V for 1 h.

Table 1 Characterization of tetranucleotide and pentanucleotide microsatellite loci from the eastern indigo snake based on 24 shed skins collected at Ft. Stewart, Georgia

Locus	Primary sequence	Genbank Asc #	Motif	*N*	*A*	H_O	H_E	P_{HW}	*Range* (Bp)
Dry05	F: (CAG)[a]CTGCAAGAAAGCCACACATC	GU592703	AATAG^13	23	7	0.74	0.76	0.71	232–262
	R: GTTTGAGCGTATGCACCAAGACAA								
Dry06	F: (CAG)TTCCTACCACCCATCTCCAA	GU592704	AAGG^7	24	6	0.75	0.75	0.41	208–232
	R: GTTTGGCAGCAAATGAATTGAACC								
Dry14	F: (CAG)TGCACCAAAGACAAATTCCTT	GU592705	CTGTT^9	24	3	0.46	0.49	0.96	224–236
	R: GTTTTCAAGAGATTGCCAAATGGA								
Dry24	F: (CAG)GTGAGGAGTGGGTGGAAG	GU592706	ATCT^16	23	5	0.78	0.79	0.02	287–303
	R: GTTTACGAACAGGAAGAAGATAGTG								
Dry28	F: GTTTAATGAACATTTGGGCTTTG	GU592707	AGAT^10	20	3	0.70	0.61	0.05	425–433
	R: (CAG)ACATCATATTGTCACCGCTAC								
Dry30	F: GTTTGATTAGATTGGCAGGTTGAC	GU592709	AGAT^13	24	4	0.62	0.64	0.67	178–190
	R: (CAG)CCATCATCTATCTATCACCATC								
Dry33	F: (CAG)TATAAGCACTCTATGGCCAA	GU989033	AGAT^9… AGAT^6	24	7	0.79	0.83	0.02	232–278
	R: GTTTCAATTTTCCCTCTTTCTCTT								
Dry35	F: (CAG)AAATAGGCACATAGCAAAAAT	GU592708	ATCT^16	24	6	0.71	0.73	0.51	292–312
	R: GTTTAAGCCCCAATTCACAGAG								
Dry40	F: (CAG)TATTTCTCCATTATTTCACCA	GU592710	ATCT^12	16	4	0.50	0.67	0.18	444–456
	R: GTTTTCCCATCTGCTTCTGTTCT								
Dry44	F: (M13)[b]CCCCTGAAAAACCTAAAAG	GU592711	ACTT^10	24	3	0.50	0.50	0.08	193–201
	R: GTTTGGGAAAAAGACGATGAGAG								
Dry45	F: (CAG)GAGGGCATGGCAATAAG	GU592712	ATCT^13	24	4	0.46	0.70	0.02	191–203
	R: GTTTGGTTTATTATCAGGGGATGT								
Dry48	F: (CAG)GGTTCTGAGACTATTGGGAG	GU592713	ACCT^7…ATCT^12	24	4	0.67	0.64	0.15	286–314
	R: GTTTAAATAGCAACCACTTTTCTTC								
Dry55	F: GTTTCCCATTTGGATTTCACTTAA	GU592714	ATCT^16	23	6	0.70	0.69	0.46	343–371
	R: (M13)CTCCCTTTGCCTTTGTCA								
Dry59	F: GTTTGTGCAGATGCTTCCAGATG	GU592716	AGAT^7	24	5	0.83	0.74	0.77	252–282
	R: (CAG)GGGGTGGTGGACTGAAA								
Dry58	F: GTTTCCCTGTTGATTCTGGTGAA	GU592715	AAGG^12…AAAG^15	24	5	0.79	0.71	0.91	340–360
	R: (M13)GGAAAGGATGAGTAAAGTC								
Dry61	F: (M13)ATAAGCCGCCCTGAGTC	GU592717	AATC^12…AAAT^4	24	4	0.54	0.64	0.67	162–214
	R: GTTTGCCTCTGGTTATTCACA								
Dry63	F: (CAG)GATGAAAATGGGACTCTTAGT	GU592718	AGTT^9	24	4	0.46	0.52	0.36	196–244
	R: GTTTAATATTTCGGCTGTTGTGTA								
Dry64	F: GTTTCTTTGGATTTGATTTTGTTC	GU592719	ATCT^13	24	4	0.46	0.65	0.03	295–307
	R: AATTTCCTTTACTCTTCATTGT								
Dry65	F: (CAG)AGGGGGTAGTCCTCATCTC	GU592720	ATCT^14	23	7	0.61	0.72	0.62	164–202
	R: GTTTCAGGGCTTTGGACAATATG								
Dry68	F: (CAG)TTTGAGTCTTATTGGATTGAGT	GU592721	ACAT^10	24	4	0.58	0.57	0.87	205–233
	R: GTTTGAGATGGTGCTGATGAAAT								
Dry69	F: (CAG)ATGTGTGAACCAAATCCTAAT	GU592722	ATGT^9	24	4	0.54	0.60	0.08	428–452
	R: GTTTCACGTATTTGTTTTTCTCCT								
Dry70	F: (CAG)GAGGATTAGGGGATTTTTACA	GU989034	GGAT^4… AGAT^9	24	3	0.58	0.51	0.76	258–266
	R: GTTTAAAGCAAGGGCCAGTAAG								

F Forward primer sequence, *R* reverse primer sequence, *N* number of individuals successfully genotyped; A, number of alleles, H_O observed heterozygosity, H_E expected heterozygosity, P_{HW} probability of Hardy–Weinberg disequilibrium, *Range* amplicon size in base pairs

[a] CAG tag sequence is CAGTCGGGCGTCATCA

[b] M13 tag sequence is GGAAACAGCTATGACCAT

Recovered DNA was hybridized to two different mixtures of biotinylated oligonucleotide probes (Integrated DNA Technologies, Mix 1: $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$ $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTG)_6$; Mix 2: $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$). Probes and hybridized DNA were captured using streptavidin coated magnetic beads (DynaBeads®, Invitrogen®). Enriched DNA was washed and eluted at 95°C, and the enrichment procedure was repeated to increase the efficiency of microsatellite array capture. Microsatellite enriched DNA was ligated into a pCR®2.1-TOPO® vector and transformed into TOP 10® competent cells (Invitrogen).

A total of 384 colonies were screened, treated with ExoSAP-IT® (New England Biolabs) and sequenced using BigDye v3.1 (Applied Biosystems). Sequencing reactions were purified using Sephadex G-50 Fine (Sigma–Aldrich) and analyzed on an Applied Biosystems 3730*xl* DNA Analyzer. Sequences were edited using SeqMan® (Lasergene). Contigs were searched for microsatellite loci using MSATCOMMANDER (Faircloth 2008). We designed primers for 72 loci using Primer3.0 (Rozen and Skaletsky 2000). Amplification was performed in 10 µl reactions consisting of 10 mM Tris pH 8.4, 50 mM KCl, 0.5 µM GTTT "pig-tailed" locus specific primer, 0.05 µM CAG (CAGTCGGGCGTCATCA) or M13 (GGAAACAGCTA TGACCAT) tagged locus specific primer, 0.45 µM fluorescently labeled CAG or M13 tag (Boutin-Ganache et al. 2001), 1.5 mM $MgCl_2$, 0.125 mM dNTPs, 0.5 U Taq polymerase, and approximately 10 ng of template DNA. Fluorophores used were VIC, PET, NED (Applied Biosystems) and FAM (Integrated DNA Technologies). Touchdown cycling parameters (Don et al. 1991) were 95°C for 5 min, 20 cycles of 95°C for 30 s, 60°C minus 0.5°C per cycle for 30 s and 72°C for 1 min, followed by 24 cycles of 95 °C for 30 s, 50°C for 30 s, 72°C for 1 min and a final extension of 72°C for 10 min. Amplicon sizes were analyzed on an Applied Biosystems 3730*xl* DNA Analyzer using LIZ500 size standard (Applied Biosystems). Allele sizes were scored in GENEMAPPER (Applied Biosystems).

Seventy-two primer pairs were screened for amplification consistency and polymorphism on 28 shed skins from Fort Stewart, Georgia, USA. Twenty-two loci amplified consistently and were polymorphic (Table 1). Data were exported from GENEMAPPER using GMCONVERT (Faircloth 2006), and CERVUS 3.0 (Kalinowski et al. 2007) was used to calculate allelic richness (A), observed heterozygosity (H_O), and expected heterozygosity (H_E). We calculated linkage disequilibrium (LD) and deviations from Hardy–Weinberg equilibrium (HWE) using GENEPOP (Raymond and Rousset 1995). No loci deviated significantly from HWE following sequential Bonferroni correction (table-wide alpha = 0.05; Rice 1989). No pairwise loci comparisons exhibited linkage disequilibrium following sequential Bonferroni correction. The number of alleles per locus ranged from 3 to 7, with an average of 4.6 alleles per locus.

Three multi-locus genotypes were recovered more than once in the data set. Given the non-exclusion probability of identity of 2.74×10^{-17} with the combined panel, there is sufficient power to infer that these shed skins represent multiple sampling of the same individual through time. These data clearly demonstrate the utility of this panel in discerning individuals for genetic mark-recapture studies. These loci should also prove useful in addressing population level questions in this threatened species.

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