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Development and characterization of 16 microsatellite markers for the Louisiana pine snake, Pituophis ruthveni, and two congeners of conservation concern

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TECHNICAL NOTE

Development and characterization of 16 microsatellite markers for the Louisiana pine snake, *Pituophis ruthveni*, and two congeners of conservation concern

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Abstract We isolated and characterized 16 microsatellite loci from the Louisiana pine snake, *Pituophis ruthveni*. Loci were screened in 24 individuals from locations throughout its distribution in Louisiana and Texas. The number of alleles per locus ranged from 4 to 12, observed heterozygosity ranged from 0.200 to 0.875, and the probability of identity ranged from 0.043 to 0.298. We examined cross-species amplification at these loci in *P. catenifer* (bullsnakes and gopher snakes) and *P. melanoleucus* (pine snakes). These new markers provide tools for examining the conservation genetics of this species complex. Louisiana pine snakes face numerous threats: population densities are extremely low and their natural habitat has been severely altered and fragmented. In southern Canada, *P. catenifer* is at the northern extreme of its range and limited by the availability of suitable over-wintering sites. Hence, for these two species reduction of heterozygosity, potential for inbreeding, and increased effects of genetic drift are all of considerable conservation concern.

Keywords *Pituophis* · Bullsnake · Pine snake · Louisiana pine snake · Microsatellite · PCR primers · SSR · STR

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The *Pituophis* species complex comprises a group of snakes that includes bullsnakes, gopher snakes, and pine snakes (Rodríguez-Robles and Jesús-Escobar 2000). Some species face conservation threats and are therefore protected by state regulatory agencies (see www.natureserve.org/explorer, keyword *Pituophis*). Perhaps of most concern is the Louisiana pine snake, *P. ruthveni*, a critically rare species that has a limited distribution in south-central United States. Louisiana pine snakes have low reproductive rates (Reichling 1990) and populations are characterized by extremely low density, especially those found in east Texas and Louisiana south of the Red River (Rudolph et al. 2006). Populations face continued habitat loss and fragmentation as road density increases and fire suppression persists within their preferred habitat, long leaf pine of the Gulf Coastal Plain (Rudolph et al. 2006; Rudolph and Burgdorf 1997). Bull snakes, *P. catenifer*, are found in a very small part of southern Canada, and little is known about their ecology or population structure. Their distribution is thought to be limited by the availability of over-wintering sites, and the extent of genetic exchange among breeding groups is unknown. Federally, the bull snake is listed as 'data deficient' in Canada, highlighting the need for genetic data (Committee on the Status of Endangered Wildlife in Canada 2002). Northern pine snakes, *P. melanoleucus*, are found throughout the southeastern U.S. but

Table 1 Details for 16 polymorphic microsatellite loci developed for *Pituophis ruthveni*

Locus	Primer Sequence 5′ → 3′	Repeat motif	T_A	Size (bp)	N	k	H_o	H_e	PI
Piru5	F: GATCTGGCCAACCTTGCAG	$(CATT)_{12}$	TD65	163–205	20	9	0.200†	0.828	0.051
	R: *GTTCCTGCTTCGAATAATCTCC			159–213	23	6	0.304†	0.729	0.115
				167–217	7	8	0.286†	0.837	0.045
Piru8	F: CTCTGCCCAATTGCTGGATG	$(AAGG)_{15}$	62	163–191	24	7	0.667	0.752	0.098
	R: *CTGCAGCCAGCTAGTATTTCC			151–191	24	8	0.583	0.747	0.102
				163–183	8	6	0.625	0.805	0.067
Piru9	F: *GGTTCTGGAGTTTCTTTCAGAC	$(AATG)_{14}$	TD65	194–206	21	4	0.286†	0.694	0.151
	R: TGAGTGCTCAGGAAGAGAGG			194–202	12	3	0.167†	0.486	0.327
				190–206	8	5	0.750	0.680	0.141
Piru12	F: *GTGGGCTACCTGCAAATGG	$(AGAT)_{13}$	TD65	201–213	24	4	0.417	0.545	0.286
	R: ATACCTAAGAGTTGCCCATCC			201–221	23	6	0.522†	0.789	0.077
				193–209	8	5	1.0	0.680	0.141
Piru13	F: TAAGCTGCTCACAGTTGCC	$(AGAT)_{13}$	TD65	176–216	24	9	0.875	0.779	0.076
	R: *GGGTAGCCAAGAATGTCTG			160–280	23	13	0.870	0.881	0.026
				184–220	7	7	0.857	0.704	0.112
Piru15	F: *GAGAGAACAGAGCATTTGCC	$(ATCT)_{12}$	TD65	217–249	22	7	0.682	0.754	0.091
	R: GTATTGGGAGCTGTCCAGAG			221–269	21	9	0.667	0.824	0.051
				225–249	8	6	0.375	0.797	0.070
Piru16	F: *ACCAGACAACATCCCTGC	$(ATCT)_{15}$	TD65	208–254	23	12	0.739	0.834	0.043
	R: GACTGTGGGAAGCTGTAACC			208–252	24	9	0.792	0.863	0.034
				208–238	8	9	0.875	0.836	0.043
Piru23	F: GCCTCATTCTGCCTGGAAAC	$(GTTT)_{11}$	TD65	285–301	24	5	0.625	0.535	0.290
	R: *CGAGCTTCGCCCATCTTTATG			261–385	24	10	0.833	0.780	0.077
				285–309	7	5	0.857	0.673	0.148
Piru25	F: *GAGCTTTGCAGTCGTGG	$(ATCT)_{11}$	TD65	295–323	24	9	0.583	0.795	0.066
	R: AGCATTAGATATGCTTGCAGCC			299–321	24	6	0.625	0.701	0.134
				301–317	4	5	1.0	0.781	0.083
Piru27	F: CACAAGTAGGGTTCTCGGTG	$(CATT)_{14}$	TD65	301–317	24	7	0.750†	0.736	0.113
	R: *TTGTAAGCTGCCCAGAGTCC			293–325	23	7	0.826	0.827	0.053
				301–317	8	4	0.625	0.648	0.191
Piru31	F: *TTCAGGAGCCTGCTTTACC	$(CATT)_{11}$	68	298–336	23	9	0.261†	0.554	0.217
	R: AGGGATAAGTCCTGGTAGTTGC			298–330	23	7	0.696	0.805	0.062
				298–334	8	7	0.875	0.750	0.088
Piru33	F: *TTCCTTGTGTGTCCAATCAC	$(CTGT)_{11}$	TD65	280–358	24	4	0.542	0.604	0.220
	R: CACACTGGAGGAGCAAATAC			280–366	23	9	0.957	0.846	0.042
				284–288	6	2	0.500	0.375	0.461
Piru34	F: *TTACCCTTCTCCAAACTGTC	$(AGAT)_{16}$	62	330–354	24	6	0.542	0.683	0.137
	R: TAAGATTTGGCCTACTGCTG			332–352	22	9	0.682	0.825	0.053
				332–356	8	7	0.750	0.813	0.059
Piru35	F: CAGGGTCACCTGAATAGTGC	$(ATCT)_{16}$	TD65	351–363	20	4	0.450	0.654	0.187
	R: *GCCAAGAGGAACATACATGCC			355–375	24	5	0.583	0.656	0.169
				355–371	8	5	0.375	0.625	0.180
Piru42	F: *TGGGTCCTAAAGGCGATCAG	$(AGTT)_{11}$	TD65	366–378	22	4	0.591	0.505	0.298
	R: GCTCTAATGTTTGCACCTGGG			358–402	24	8	0.750	0.808	0.063
				358–378	4	4	0.500	0.563	0.229

Table 1 continued

Locus	Primer Sequence 5′ → 3′	Repeat motif	T_A	Size (bp)	*N*	*k*	H_o	H_e	PI
Piru48	*AACCTTGGCAATCTGGC	$(ATCT)_{11}$	TD65	356–428	15	11	0.467†	0.829	0.047
	GTTGGAACCCTGAACATCC			362–428	24	14	0.875	0.880	0.025
				364–430	8	10	1.0	0.891	0.022

For each locus the information in the top row refers to *P. ruthveni,* the second row refers to *P. cantenifer,* and the third row refers to *P. melanoleucus*. The number of individuals genotyped is *N*; size indicates the range of observed alleles in base pairs and includes the length of the CAG tag; T_A refers to the annealing temperature (°C) with TD65 indicating a touchdown protocol with a highest annealing temperature of 65; *k* is number of alleles observed; H_o and H_e are observed and expected heterozygosity, respectively; PI is the probability of identity for each locus

* Indicates CAG tag (5′-CAGTCGGGCGTCATCA-3′) label

† Indicate loci that deviated from HWE after Bonferroni corrections

they are patchily distributed and associated with habitat types, such as the longleaf pine ecosystem, that have been drastically reduced (Simberloff 1993).

We extracted total DNA from one individual *P. ruthveni*, using the Gentra Puregene Mousetail Kit, with minor modifications of the suggested protocol (Qiagen, Valencia, CA). We followed the enrichment procedure of Glenn and Schable (2005) with some exceptions. Briefly, DNA was digested with restriction enzyme *Rsa*I (New England Biolabs), ligated to double-stranded linkers, denatured and hybridized to biotinylated microsatellite oligonucleotide mixes then captured on magnetic streptavidin beads (Dynal). Unhybridized DNA was washed away and remaining DNA was eluted from the beads, and amplified in polymerase chain reactions (PCR) using the forward SimpleX-5 as a primer. There were two primary changes to the Glenn and Schable (2005) protocol. First, a new linker was used (SimpleX-5 Forward 5′-AAAACGAGCAGCGG AACT and SimpleX-5 Reverse 5′-pAGTTCCGCTGCT CG). Second, the enriched libraries were sequenced on a 454 using titanium chemistry following standard Roche 454 library protocols (454 Life Sciences, a Roche company, Branford CT). Sequences were subjected to a 3′ quality trim where only one base in the last 25 bases of the sequence contains a quality score less than 20 or alternatively contains one ambiguous base. CAP3 [33] was then used to assemble sequences at 98% sequence identity using a minimal overlap of 75 bp. Along with singlets, contigs of two or three sequences were searched for the presence of microsatellite DNA loci using the program MSATCOMMANDER version 0.8.1 (Faircloth 2008) and primers designed with Primer3. One primer from each pair was modified on the 5′ end with an engineered sequence (CAG tag 5′-CAGTCGGGCGTCATCA-3′) to enable use of a third primer in the PCR (identical to the CAG tag) that was fluorescently labeled for detection.

Forty-eight primer pairs were tested for amplification and polymorphism using DNA obtained from eight individuals of *P. ruthveni*. PCR amplifications were performed in a 12.5 μL volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0 μg/ml BSA, 0.4 μM unlabeled primer, 0.04 μM tag labeled primer, 0.36 μM universal dye-labeled primer, 3.0 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 units JumpStart Taq DNA Polymerase (Sigma), and ~20 ng DNA template) using an Applied Biosystems GeneAmp 9700. Standard thermal cycling parameters consisted of 40 cycles of 96°C for 30 s, annealing temperature for 30 s, and 72°C for 30 s. Touchdown thermal cycling programs (Don et al. 1991) encompassing a 10°C span of annealing temperatures ranging between 65–55°C were used for most loci (Table 1). Touchdown cycling parameters consisted of 20 cycles of 96°C for 30 s, highest annealing temperature of 65°C (decreased 0.5°C per cycle) for 30 s, and 72°C for 30 s; and 20 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR products were run on an ABI-3130xl sequencer and sized with Naurox size standard prepared as described in DeWoody et al. (2004), except that unlabeled primers started with GTTT. Results were analyzed using GeneMapper version 3.7 (Applied Biosystems). Sixteen of the tested primer pairs amplified high quality PCR product that exhibited polymorphism.

We assessed the variability of 16 polymorphic loci in 24 specimens that came from sites throughout the distribution of *P. ruthveni* that can be generalized into three regions: East Texas ($n = 8$), Louisiana north of the Red River ($n = 10$), and Louisiana south of the Red River ($n = 6$). We performed the same assessment for 23 *P. catenifer* from the Grasslands National Park area of southern Saskatchewan, Canada, and 8 *P. melanoleucus* from the Savannah River Site in Aiken and Barnwell Counties, South Carolina, USA. Conditions and characteristics of the 16 loci are given in Table 1. We estimated number of alleles per locus (*k*), observed and expected heterozygosity (H_o and H_e), probability of identity (PI) using GenAlEx v6.0 (Peakall and Smouse 2006). Tests for deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium were conducted using GENEPOP v4.0 (Rousset 2008). After Bonferroni correction for multiple comparisons 5 loci showed significant deviations from expectations under HWE and no linkage disequilibrium

was detected for any of the 105 paired loci comparisons. Deviation from HWE at some loci for Louisiana pine snakes may result from sampling throughout the species' range; if genetic structure exists within this range, random mating assumptions may not be met (i.e., the Wahlund effect). However, deviations from HWE are not unexpected given that Louisiana pine snake population densities are extremely low (Rudolph et al. 2006), and likely experiencing inbreeding and/or genetic drift. If low heterozygosity is observed within populations due to inbreeding, perhaps reintroduction of new individuals should be considered in future recovery plans. In Canada, the new markers for *P. catenifer* will be used to assess population subdivision among hibernacula, long term patterns in snake movement, and appropriate units for conservation. Given the broad distribution of *Pituophis* and recent conservation concerns about some species within the complex, the genetic markers described here are invaluable for further study of *Pituophis* ecology and conservation.

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