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TECHNICAL NOTE

Isolation and characterization of microsatellite loci in two species-at-risk in British Columbia: Great Basin spadefoot (*Spea intermontana*) and Western painted turtle (*Chrysemys picta bellii*)

Anders Gonçalves da Silva · Kathleen E. Williams · Stephanie L. Kirk · Christine A. Bishop · Karen E. Hodges · Michael A. Russello

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Abstract Seventeen polymorphic microsatellite loci were characterized in two Canadian species-at-risk, Great Basin spadefoot (*Spea intermontana*, $N = 9$) and the Western painted turtle (*Chrysemys picta bellii*, $N = 8$), from GT_n enriched genomic libraries and cross-taxa amplification. Number of alleles per locus ranged from 2 to 9, with an average of 3.9 and 4.3 alleles/locus in Great Basin spadefoot and Western painted turtle, respectively. Mean expected heterozygosities were comparatively high (0.43–0.52), as was the power to distinguish between individuals relative to the number of loci characterized per species (multilocus $P_{ID} = 9.2 \times 10^{-5}$–$1.6 \times 10^{-5}$). These loci will be fundamental in generating historical population information in support of conservation efforts for these two imperiled species.

Keywords Amphibians · Dinucleotides · Microsatellites · Polymorphic · Primers · Reptiles · Tetranucleotides

Amphibians and reptiles are important biodiversity indicator species due to their sensitivity to environmental change (Dobson et al. 1997; Gibbon et al. 2000). The major causes of population declines of reptiles and amphibians worldwide are habitat loss, fragmentation, competition and predation by invasive species, and climate change (Gardner et al. 2007). Remarkably, this group has received substantially less attention from the conservation community than birds and mammals, even though approximately 30% of the assessed species of amphibians ($N = 6{,}260$ assessed out of 6,347 described species) and reptiles ($N = 1{,}385$ assessed out of 8,734 described species) were classified as threatened in the latest IUCN wildlife assessment (Vié et al. 2009).

Status assessments performed by the Canadian government and the province of British Columbia suggest that the Great Basin spadefoot (*Spea intermontana*) and Western painted turtle (*Chrysemys picta bellii*) have recently experienced population declines mainly due to habitat loss and fragmentation (COSEWIC 2006; British Columbia Southern Interior Reptile and Amphibian Recovery Team 2008). Both species have the northern peripheries of their geographic ranges in British Columbia, representing valuable components of the province's biodiversity. In addition, these peripheral populations are uniquely positioned to help inform conservation decisions across the whole distribution of each species, as they can provide data on the limits of adaptation and potential constraints on responses to climate change (Bridle and Vines 2007). Here, we characterize polymorphic microsatellite markers in the Great Basin spadefoot and the Western painted turtle to enable population-level genetic studies of these species-at-risk.

Genomic libraries enriched for GT_n repeat sequences were constructed following Hamilton et al. (1999). Total genomic DNA was isolated using the Qiagen DNeasy kit (Qiagen) from two Great Basin spadefoot tadpoles from different ponds (hereafter spadefoot), and one blood sample from an adult Western painted turtle (hereafter painted turtle). Samples were collected in the Okanagan Valley in

A. G. da Silva · K. E. Williams · S. L. Kirk · K. E. Hodges · M. A. Russello (✉)
Department of Biology and Centre for Species at Risk and Habitat Studies, University of British Columbia Okanagan, 3333 University Way, Kelowna, BC V1V 1V7, Canada
e-mail: michael.russello@ubc.ca

C. A. Bishop
Science and Technology Branch, Environment Canada, Delta, BC, Canada

south-central British Columbia. Spadefoot total DNA was digested with *Nhe* I, *Xmn* I and *Rsa* I (New England Biolabs), and painted turtle total DNA was digested with *Nhe* I, *Xmn* I and *Alu* I (New England Biolabs) resulting in a majority of fragments within the 200–1,000 bp size range. Following enrichment, fragments were cloned into pCR2.1-TOPO vector using the TOPO® TA cloning kit (Invitrogen) and transformed into *E. coli* TOP10 cells (Invitrogen). A total of 144 and 64 clones were PCR amplified using T3 and T7 primers for spadefoot and painted turtle, respectively, and sequenced on an Applied Biosystems 3130XL DNA Analyzer using the BigDye Terminator Cycle Sequencing Kit. Consensus contigs were constructed using SEQUENCHER 4.7 (Gene Codes Corporation). Many of the clones had high GT content, but lacked suitable microsatellite repeat units, suggesting that GT/CA repeat motifs might not be common in these species (Beckman and Weber 1992; Lagercrantz et al. 1993). Overall, twenty and six primer pairs were designed flanking microsatellite regions of ≥7 GT/CA repeats using PRIMER3 software (Rozen and Skaletsky 1999) for the spadefoot and painted turtle, respectively. An additional four tetranucleotide loci isolated in New Mexico spadefoot (*S. multiplicata*, Van Den Bussche et al. 2009) were tested in our samples of Great Basin spadefoot. Three dinucleotide loci isolated in midland painted turtles (*C. p. marginata*) sampled from Illinois, USA (Pearse et al. 2001) and four tetranucleotide loci isolated from Diamondback terrapin (Malaclemys terrapin, Hauswaldt and Glenn 2003) were also tested in our samples of Western painted turtles.

PCRs were performed using an ABI Veriti™ 96-well Thermal Cycler and carried out in a 12.5 µl volume containing: ~20–50 ng of DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.8 µM of each primer and 0.5 U of AmpliTaq® Gold DNA polymerase (Applied Biosystems). Reaction conditions for all primers consisted of a 'touchdown' cycling program: 95°C for 10 min; 30 cycles of 95°C for 30 s, annealing for 30 s, and 72°C for 1 min; and a final extension step of 72°C for 10 min. The annealing step in the touchdown program decreased 1°C per cycle from 59°C to 51°C at which point the remaining cycles continued with a 51°C annealing temperature.

Variation at each locus was assessed in fourteen and eight wild-caught and putatively unrelated individuals of spadefoot and painted turtle, respectively. All forward primers were 5′-tailed with an M13 sequence [5′-TCCC AGTCACGA-CGT -3′] to facilitate automated genotyping. Specifically, the M13-tailed forward primer was used in combination with an M13 primer of the same sequence 5′-labeled with one of four fluorescent dyes: (6-FAM, VIC, NED, PET), effectively incorporating the fluorescent label into the resulting PCR amplicon (Schuelke 2000). In addition, reverse primers were modified following Brownstein et al. (1996) to reduce stutter and improve scoring accuracy. Fragments were separated on an ABI 3130XL Genetic Analyzer and scored using GENEMAPPER® 4.0 (Applied Biosystems). Expected heterozygosity at each locus and tests for deviations from Hardy–Weinberg and linkage equilibriums were calculated using GENEPOP (Raymond and Rousset 1995). Probability of identity for each locus was calculated using GENALEX 6 (Peakall and Smouse 2006), and the test for null alleles was performed with MICRO-CHECKER (Van Oosterhout et al. 2004). Type I error rates for tests of departure from Hardy–Weinberg expectations and linkage disequilibrium were corrected for multiple comparisons using the sequential Bonferroni procedure (Rice 1989).

Eleven and nine polymorphic loci were successfully and consistently amplified across our samples of spadefoot and painted turtles, respectively (Table 1). Neither set of loci showed evidence for departure from linkage equilibrium. Evidence for departure from Hardy–Weinberg equilibrium was found for the spadefoot locus SpeaD106 ($P \leq 0.0041$). Evidence for null alleles was found in spadefoot loci SiGT074 (Genbank accession # GQ902951) and SpeaD125 (Van Den Bussche et al. 2009), and the painted turtle locus TerpSH7 (Hauswaldt and Glenn 2003). Estimates of variability did not include loci with null alleles, and are not reported in Table 1.

The spadefoot loci exhibited a mean of 3.9 alleles/locus (range of 2–8 alleles) with a mean expected heterozygosity of 0.43 ± 0.27 (Table 1). The most powerful locus for differentiating among individuals was SpeaD106 ($P_{ID} = 0.05$) and the least powerful was SiGT038 ($P_{ID} = 0.87$). Across all loci, the power to discriminate spadefoot individuals at the observed allele frequencies was 1.6×10^{-5}. In the painted turtle, we observed a mean 4.3 alleles/locus (range 2–9 alleles) and a mean heterozygosity of 0.52 ± 0.25. Painted turtle P_{ID} values ranged from 0.11 (TerpSH2) to 0.79 (Cp10), with a total across loci of 9.2×10^{-5}.

We thus characterized 17 polymorphic loci in two species-at-risk that include eight novel loci developed specifically for *S. intermontana* and *C. p. bellii* and an additional nine loci previously isolated in closely related taxa (Pearse et al. 2001; Hauswaldt and Glenn 2003; Van Den Bussche et al. 2009). Currently, information on connectivity among populations within British Columbia and across the species' distribution is lacking but urgently needed (COSEWIC 2006; British Columbia Southern Interior Reptile and Amphibian Recovery Team 2008). These marker sets will enable population-level studies directed towards filling such knowledge gaps to inform provincial management strategies.

Table 1 Polymorphic microsatellite loci characterized for Great Basin spadefoot (*Spea intermontana*) and Western painted turtle (*Chrysemys picta bellii*)

Species	Locus	Primer sequences (5′–3′)	Repeat structure	T_a (°C)	No. of alleles	Product size ranges (bp)	H_O	H_E	P_{ID}	GenBank accession No.	Reference
Spea intermontana	SiGT023	ATACACAGAATATAAAAGG CAGGAGGGAGCATAAACACC	$(AT)_4(AC)_{11}$	59 → 51	2	117–119	0.29	0.25	0.60	GQ902948	This study
	SiGT038	GGAGTCCGTAATGAAAACACC GCAGATACAATACACAGGGTTC	$(GT)_7GA(GT)_5$	59 → 51	2	155–159	0.07	0.06	0.87	GQ902949	This study
	SiGT061	AGATCCAACGCCAAGTCATC CCCTATTAAAGTATCAGCTTCAACAT	$(GT)_{27}$	59 → 51	8	181–201	0.77	0.77	0.07	GQ902950	This study
	SiGT085	TCCAATAAAAGGCAGCATGA CACATTCCCTTCCAATATACTCTT	$(CA)_4CG(CA)_6$	59 → 51	3	162–168	0.29	0.36	0.44	GQ902952	This study
	SiGT158	GACCACAAGGTGGTGCTT CAAAATGGTGCCAGATTTGC	$(CA)_3TA(CA)_{12}$	59 → 51	2	175–177	0.43	0.34	0.50	GQ902953	This study
	SiGT202	CCAGCCTGCTTAAAGAACGA GGGCCCTAATGTAGAAAGG	$(GT)_9$	59 → 51	2	205–207	0.14	0.13	0.76	GQ902954	This study
	SpeaC7*	TGACCATTGAGGGAGGTG GTCCAGGCAGAGCAGAGA	CATC	59 → 51	4	247–267	0.46	0.51	0.29	–	Van den Bussche et al. (2009)
	SpeaD103*	TGGTGATACCGTTTTAACTACG TAGAAAATGTCGCCAGTCTG	TAGA	59 → 51	4	194–202	0.40	0.65	0.17	–	Van den Bussche et al. (2009)
	SpeaD106*	CCAACTTTGAAACCAACAA TCAGGTAGCTCCATCTCC	TAGA	59 → 51	8	293–337	0.67	0.82	0.05	–	Van den Bussche et al. (2009)
	Total				3.9 (± 2.5)		0.39 (± 0.24)	0.43 (± 0.27)	1.6E-05		
Chrysemys picta bellii	CpGT108	CCTAGAAAGTAAGAACCAATTTCAG CCACCAACAGAAGGAAGTTAGTG	$(CA)_4CT(CA)_{11}$	59 → 51	3	265–285	0.63	0.65	0.20	GQ902943	This study
	CpGT124	TCGGGGAGCACACTATACC CTCAGCCCCAAAATGAAC	$(GT)_{31}(GC)_5$	59 → 51	6	214–240	0.38	0.51	0.26	GQ902944	This study
	Cp2*	CTCTAAGGGTTGCACTTCTCAAA GAGGTGGCATCAAAACATCAT	Dinucleotide	59 → 51	3	222–238	0.25	0.23	0.61	–	Pearse et al. (2001)
	Cp3*	ATCTTTAAGTCTGTGAACTTCAGGG CTGTCTCATGCAAAGCTGGTAG	Dinucleotide	59 → 51	3	159–165	0.50	0.57	0.25	–	Pearse et al. (2001)
	Cp10*	GGTGCAGCAAGTTCAGGAGAC GGTGTTAATGCACTGGAGAATCA	Dinucleotide	59 → 51	2	198–210	0.13	0.12	0.79	–	Pearse et al. (2001)
	TerpSH2*	TGGCCAGCAGGAGTAATG CTATTAGGGCAGAGACGAG	AGAT	59 → 51	6	181–205	0.75	0.73	0.11	AY156710	Hauswaldt and Glenn (2003)
	TerpSH3*	TCCCCCAATGCACAC CTGCCCAATCCATTTAGA	CAAA	59 → 51	2	289–297	0.57	0.49	0.38	AY156711	Hauswaldt and Glenn 2003
	TerpSH5*	TTGCTGCTATATGCTTAAT CCTCCCTGCCTATTGA	CTAT	59 → 51	9	117–173	1.00	0.86	0.35	AY156713	Hauswaldt and Glenn 2003
	Total				4.3 (±2.49)		0.53 (±0.30)	0.52 (±0.25)	9.2E-05		

Primer sequence, repeat structure, annealing temperature (T_a), numbers of alleles, product size range (bp), observed heterozygosity (H_O), expected heterozygosity (H_E) and probability of identity (P_{ID}) are listed for each locus. Genbank accession numbers were provided when available

Locus in bold exhibits significant heterozygote deficit

* Loci isolated in previous studies

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