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Characterization of seven cross-amplifying microsatellite markers in *Crotalus adamanteus* (Serpentes: Viperidae)

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The Eastern Diamondback Rattlesnake, *Crotalus adamanteus* Palisot de Beauvois, 1799, is the largest rattlesnake species native to North America, reaching an average adult length of 130 cm (Conant & Collins 1991). The species is distributed throughout the southeastern United States, although populations are mostly located in Florida, Alabama, and southern Georgia. Despite a significant population decline since the 1950s (Timmerman & Martin 2003), mostly as a result of habitat loss, habitat fragmentation, and indiscriminate killings by humans (e.g., "rattlesnake roundups", Means 2009), the species has not been afforded specific protection. Additionally, limited data are available on the most fundamental aspects of *C. adamanteus* biology, including population biology, life history, and population genetics (Hoss et al. 2010). As urbanization and other anthropogenic effects continue to degrade and destroy viable habitat, small and isolated biological preserves may be the last resort for this and similar species. However, snakes with large home ranges (such as *C. adamanteus*) may not benefit from small preserves because they are unlikely to meet the requirements for stable populations (Dodd et al. 1988, Timmerman 1995) and natural population genetic mechanisms may be compromised. For example, the population on our university campus, Florida Gulf Coast University (FGCU, Fort Myers, Florida, USA), may be rather small and isolated because of surrounding development. If so, inbreeding could lead to lowered heterozygosity and fitness.

Despite the need for conservation of Eastern Diamondbacked Rattlesnakes, few genetic studies have been performed. For example, only two polymorphic microsatellite markers have been identified for *C. adamanteus* (Bushar et al. 2001) and none have been developed specifically for this species. Because of successful cross-species amplification of PCR primers in *Crotalus* and other snake taxa (Bushar et al. 2001, Stapley et al. 2005, Anderson 2006, Lukoschek & Avise 2011, Pozarowski et al. 2012), we aimed to test published microsatellite loci from four congeneric rattlesnakes with *C. adamanteus* DNA and characterize the successful loci in the FGCU population. Establishing polymorphic molecular markers in this species will allow advanced studies in behavioral ecology and conservation genetics.

We extracted adult genomic DNA from scale clips, blood, or liver samples with standard phenol-chloroform procedures. We initially tested one DNA sample for cross-amplification success with 25 published microsatellite primer pairs from four other rattlesnake species (*C. atrox* and *C. scutulatus*, Pozarowski et al. 2012; *C. tigris*, Goldberg et al. 2003, Munguia-Vega et al. 2009; and *C. viridis*, Oyler-McCance et al. 2005). Oligonucleotide primers were labelled for fluorescent detection using a three-primer reaction system as in Boutin-Ganache et al. (2001) and Croshaw & Glenn (2003). The forward primer of each pair had a 5' M13 tag added (5'-GGAAACAGCTATGACCATG-3'), and a third dye-labeled primer (6FAM) was included in each reaction. We used standard touchdown polymerase chain reaction (PCR) (Don et al. 1991) with the following reaction conditions: 25.0 µL total reaction volume with 12.5 uL GoTaq Master Mix (2x, Promega), 0.16 µM forward primer, 0.16 µM reverse primer, 0.2 µM 6FAM-labeled M13 primer, and 20–50 ng DNA template. Based on visualization of PCR products with 2% agarose gels, we selected 10 loci for further study. Reactions were

Table 1. Characterization of seven microsatellite markers from N = 15 individual *Crotalus adamanteus* in southwest Florida, USA. Each forward primer had the M13 tag (5'-GGAAACAGCTATGACCATG-3') appended to the 5' end. The sample size (n), number of alleles observed (K), expected and observed product sizes (bp), TouchDown temperature (TD) in thermal regime, observed heterozygosity (H_o), expected heterozygosity (H_e), and Nei's unbiased gene diversity (H_s) are also listed for each locus. P is the p-value based on the results of a statistical test for Hardy-Weinberg equilibrium in the FGCU population (Genepop). Expected product size comes from the original source publication. Locus Ca274 was monomorphic and therefore offered no information for statistical analyses.

Locus	Source	Species	Repeat motif	Primer sequence (5'–3')	n	K	Expected product size (bp)	Observed product size (bp)	P	TD	H_o	H_e	H_s
CS234	Pozarowski et al. 2012	*C. scutulatus*	$(GAAA)_5(GGAA)_4$ $(GAAA)_{20}GGAA$ $(GAAA)_4$	F: CAGATGGTTAAAAGGGCATCA R: GGAATTCAACCATAAAAGTGACA	14	10	438–546	455–508	1.000	55	0.929	0.886	0.919
Ca122	Pozarowski et al. 2012	*C. atrox*	$(TG)_{15}$	F: CCCCCAGGAAAGAATATGTATG R: CACATCTTCAGGTTGCCAAA	15	2	187–225	189–197	0.983	60	0.667	0.515	0.533
Ca281	Pozarowski et al. 2012	*C. atrox*	$(GAAA)_{19}TAAA$ $(GAAA)_4$	F: CCCAGCACACTCACAATGTC R: GCTCCTAGCCAGGAATGTCA	12	7	253–455	322–355	0.372	60	0.583	0.786	0.820
Ca238	Pozarowski et al. 2012	C. atrox	$(AGGC)_{11}(AGGA)_7$	F: CTCTGCTCTGCCATTTCACA R: GAGCCAGTGCTTTGTTTTCC	15	6	315–411	356–384	0.599	60	0.867	0.818	0.846
Crti23	Munguia-Vega et al. 2009	*C. tigris*	$(GT)_{12}(GA)_{12}$	F: GATTGTGTGGTGTTTATTGTTGC R: GGATGCTCCATCACTAGACG	15	2	224–234	199–203	0.060	60	0.133	0.515	0.533
Ca131	Pozarowski et al. 2012	*C. atrox*	$(TC)_{19}$	F: GGGATTCAGGCCAATTTTTA R: TGGTAATTTTGCATTTCAGCA	14	9	187–340	227–275	0.426	55	0.714	0.767	0.795
Ca274	Pozarowski et al. 2012	*C. atrox*	$(GT)_{21}$	F: AGCCGCTTGTCTCAAATTGT R: TGTCCTCTGATTTGCCCATT	15	1	292–334	295	N/A	65	0	0	0

then run on polyacrylamide gels (Mini-Protean Precast, Biorad) to visually screen for polymorphism. Because three primer pairs did not show consistent amplification in the expected size range, we ran the samples from just seven loci on an ABI 3130 automated DNA sequencer (University of Florida's Interdisciplinary Center for Biotechnology Research) with LIZ 600 as an internal size standard. We called alleles with Peak Scanner v1.0 (Applied Biosystems).

After confirming the presence of scorable peak patterns, we then optimized thermal and reaction conditions before genotyping 15 total snakes collected in southwest Florida, USA [11 adults and one offspring from the Florida Gulf Coast University campus (FGCU) two adults from Florida Panther National Wildlife Refuge, and one adult from Koreshan State Park]. Reactions were run with three different touchdown PCR thermal cycling programs on T100 Thermal Cyclers (Biorad). The amplification conditions consisted of programs using a range of annealing temperatures from either 65–55°C, 60–50°C or 55–45°C. Each program consists of an initial denaturation of 96°C for 3 min, followed by 5 cycles of 96°C for 20 s, the highest annealing temperature for 30 s, and extension of 72°C for 1 min, followed by 21 cycles of 96°C for 30 s, the highest annealing temperature minus 0.5°C each cycle for 30 s, and 72°C for 1 min, and finally 10 cycles of 96°C for 30 s, the lowest annealing temperature for 30 s, and 72°C for 1 min.

Finally, the complete genotypic data set (N = 15 snakes) was used to characterize the loci in Cervus 2.0 (Marshall et al. 1998). We tested each locus for Hardy-Weinberg equilibrium (HWE, exact test) and linkage disequilibrium in the FGCU population (N = 11 adult snakes) with Genepop v4.1.4 (Raymond & Rousset 1995) using all default settings.

Of the seven consistently amplifying microsatellite loci, six were polymorphic (Table 1). For polymorphic loci, 2 to 10 alleles (mean = 6.0) were amplified. Observed heterozygosity ranged from 0.13 to 0.93 (mean = 0.65) and Nei's unbiased gene diversity ranged from 0.53 to 0.92 (mean = 0.74). HWE was not rejected in any case (p-value ranged from 0.06 to 1.00) but one locus in particular (Crti23) showed high heterozygote deficiency and had a high estimated frequency of null alleles (0.58). The null hypoth-

esis of independence of genotypes could not be rejected for any locus pair (p-value ranged from 0.22 to 1.00). The entire marker panel showed low non-exclusion probabilities (0.015 for the second parent and 0.000003 for identity).

Our results are consistent with the relatively high cross-species amplification success reported within the *Crotalus* genus and in other snakes (King 2009, Lukoschek & Avise 2011, Rice et al. 2017). Most of the 25 primer pairs we tested generated clear products that appeared to be in the expected size range on agarose gels. Further assessment of the applicability of existing primers should be performed for additional species within the Crotalinae and other groups to provide markers useful in population genetics. Moreover, with six of the seven markers we assayed being polymorphic in *C. adamanteus*, it is likely that establishing panels of robust, informative microsatellite loci will not be especially difficult in this species. Additional advanced research applications in population genetics, conservation genetics, and behavioral ecology of this species, which are sorely lacking, are possible with these markers. However, one locus (Crti23) had substantial heterozygote deficiency, likely because of null alleles. This primer pair should be tested in other populations and with larger samples to determine its utility. Nevertheless, our study offers the possibility for neutral evolutionary processes to be added to the extensive work on the adaptive evolution of venom proteins in this species (e.g., Margres et al. 2014, 2016).

Despite our concern that the small campus population of *C. adamanteus* at FGCU could have low genetic variation, our results revealed levels of gene diversity (mean = 0.74 for FGCU snakes) and polymorphism (mean = 4.5 alleles per locus) that were consistent with other population genetic studies of *Crotalus* (King 2009). Perhaps this population is either larger than we know or is connected to healthy populations on the east side of the FGCU campus, where development is less extensive. Alternatively, if population declines were recent they might not yet have resulted in detectable losses of genetic variation. Much more data are needed before robust population genetic conclusions can be made, and further ongoing analysis may reveal answers to these questions. The microsatellite DNA loci reported here allow for future studies concerning conservation and population genetics, paternity analysis, and individual relatedness within wild *C. adamanteus* populations.

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