MICROSATELLITE MARKER DEVELOPMENT IN THE WESTERN DIAMONDBACK (*CROTALUS ATROX*) AND MOHAVE (*CROTALUS SCUTULATUS*) RATTLESNAKE SPECIES

By

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Abstract

We developed novel microsatellite markers for the *Crotalus atrox* and *Crotalus scutulatus* species of rattlesnake for future use in population studies. We identified 20 loci from *Crotalus atrox* and 12 from *Crotalus scutulatus* to test for amplification and variability. We finally selected loci that showed variation with a number of alleles in diversity panel sample DNA for higher resolution testing. Future directions of this project include assessing allelic richness and variability with fragment analysis, and assessing observed and expected heterozygosity as well as deviations from Hardy-Weignberg equilibrium using the program Genepop 3.4 (Raymond and Rousset, 1995). These assessments will tell us how useful the developed markers will be for future studies in population and conservation genetics.

Introduction

In this study, we developed novel genetic markers for two species of rattlesnake: the Western Diamondback rattlesnake (*Crotalus atrox*) and the Mohave rattlesnake (*Crotalus scutulatus*). The Western Diamondback usually inhabits arid environments, and is often found in North American deserts (Castoe et al, 2007). It is responsible for more human deaths than any other snake in its range (Castoe et al, 2007). The Mohave rattlesnake can be found in areas of high desert and lower mountain slopes, the most abundant populations inhabiting the Mohave and Sonoran Deserts. It can also be found in grasslands, and is commonly found close to plants such as the creosote bush (Brennan, 2008). Like the Diamondback rattlesnake, the Mohave rattlesnake delivers a sizeable and effective dose of venom (Brennan, 2008).

The desert habitats of these two rattlesnakes are comparable, and their appearances are similar, distinguishable by minor morphological differences like the angle of the eye stripe and the width of the black bands around the tail (Brennan, 2008).

Our development of novel microsatellite marker sets for the Western Diamondback and Mohave rattlesnake species will have subsequent applications in studies of population fragmentation along roadways and in other conservation and population genetic studies. To check the informativeness of the new markers, we used a fifteen-specimen diversity panel from different populations for each marker to assess amplification and variability.

Results regarding variability and divergence are dependent on mutations or polymorphisms in genetic markers (Bushar et al, 2001). Gene flow between populations will influence allele frequencies in the different populations under study (Shine, 2008). Cross species amplification tests (not performed here) may also show amplification with these new microsatellite markers (Bushar et al, 2001). The utilization of high-resolution genetic markers is a vital tool in the study of population structure and the effects of human-made barriers on rattlesnakes.

Methods

Blood and Tissue Samples

We collected blood samples from the caudal vein of live specimens and stored them in lysis buffer at -20ºC. We took liver samples from salvaged specimens found dead on roads, placed them into lysis buffer and stored them at -20ºC.

DNA Extraction and Quantification

We added approximately 50-100µL of sample blood in ethanol to 850µL of lysis buffer and 50µL of proteinase K solution to lyse the cells and digest membranes. These amounts were

sufficient because reptile red blood cells are nucleated, so the darker the sample, the higher the DNA concentration upon extraction. Extraction of genomic DNA took place using a silica magnetic bead-based robotic technique with Thermo Biosprint96 and Kingfisher96 automated extraction robots with multiple elutions of the blood and tissue resulting in high quality genomic DNA. This process was carried out using the Qiagen BioSprint96 Blood DNA Extraction Kit. We quantified newly extracted samples using PicoGreen, a fluorochrome that selectively binds to double-stranded DNA, and measured fluorescence with an Flx 800 Fluorimeter. PicoGreen shows a very high fluorescence enhancement when bound to double-stranded DNA fragments, and is very stable to photobleaching, which allows it to be exposed to UV light longer for analysis (Ahn et al, 1996). All quantified DNA was then normalized to a concentration of 5ng/µL with Low T.E. (1x Tris EDTA buffer: 10mM Tris and 0.1mM EDTA) and stored at -20ºC.

Construction of STR Enriched DNA Library

We probed Genomic DNA for the Western Diamondback rattlesnake and the Mohave rattlesnake using a modified version of the Hamilton Protocol described in Glenn and Schable, 2005. A restriction enzyme digest fragmented DNA into approximately 500bp segments, followed by ligation of double-stranded SuperSNX-24 linker to the fragments in order to provide primer binding sites for polymerase chain reaction (PCR). Ligation of the linker also incorporated a GTTT pigtail so that a 3' overhang A would be present in the PCR product for insertion into a plasmid vector. We captured fragments of digested DNA that were complementary to our 5'-biotinylated simple sequence repeat oligonucleotide probes using the protocol described in Glenn and Schable, 2005. We recovered successful hybridizations of DNA on streptavidin-coated paramagnetic beads (Dyna1) using PCR with an Invitrogen Topo TA

Cloning kit in order to insert the PCR product into the plasmid vector. This process is successful because the plasmid contains a single T overhang on the 3' end, which successfully attaches to the 3'A left by the pigtail sequence (Glenn and Schable, 2005). We harvested white colonies which had incorporated into the *E. coli* vector with sterile toothpicks and performed PCR with universal T7 and M13 Reverse primers to amplify the insert. Agarose gel electrophoresis on the PCR products determined the specific size and variability of the colony DNA. Subsequently, we examined insert sequences for microsatellite. If we found a microsatellite marker, we recorded its motif and repeat number, as well as 10-15 bases of its immediate 5' flanking region. We compared flanking regions for markers having the same motif and repeat number. If the 10-15 base flanking regions were identical, we used only one of the clones for marker development and discarded putative copies.

Primer Development and Optimization

We used the following criteria for further development of microsatellites present in cloned DNA fragments: the type of repeat (di, tri, or tetranucleotide) and the length of the repeat motif (a minimum of six repeats when considering variability). For example, we preferred a tetranucleotide that repeated about ten times. For the most part, tetranucleotide and trinucleotide motifs were considered far superior to dinucleotide repeats, even when comparing microsatellites such as a tetra repeat of 8 and a di repeat of 16. We ordered candidates for both species by the above criteria, and used the program Primer3 in order to examine flanking regions of the microsatellites and design primers to amplify the desired microsatellite sequence (Rozen and Skaletsky, 2000). When an input sequence is entered into the Primer3 program and a centered target sequence (here, the microsatellite marker) is identified, the following conditions are automatically examined in regions on either side of the target: a similar melting temperature for

the forward and reverse primer and a low propensity to form a dimer or hairpin structure, as excessive dimerization would interfere with annealing and amplification of the target sequence. This can be avoided by ensuring that the primers are not closely complementary to one another. For the purpose of later analysis using fragment analysis (which cannot handle fragments larger than 500 bp) we restricted the product fragment sizes in the range of 100-450 bases total. Meeting these conditions is crucial in order to create optimal forward and reverse primers of about 20 nucleotides in length. Universal M13 primer sequences (TGTAAAACGACGGCCAGT) were added to the 5' end of the forward primer sequence to allow the attachment of an M13 fluorescent dye by PCR (Schuelke, 2000), and pigtail sequences (GTTTCTT) were added to the 5' end of the reverse primer sequence in order to promote Taq polymerase polyadenylation in order to reduce variability in products. If adenylation was not completed, double peaks or stutter appeared in the allelic signal from 3'A overhangs in the DNA fragments (Brownstein et al, 1996).

We rehydrated oligonucleotide primers with 1x Low T.E. to stock concentrations of 200µM. I made working concentrations of all primers by dilution of stocks to a concentration of 20µM with HPLC grade water. For each primer pair at each locus selected, I performed gradient PCR optimization reactions using a variable magnesium concentration in eight buffers: Buffer A containing 10mM magnesium through Buffer H containing 45mM magnesium (a 5mM stepwise change, see Appendix 1), and a temperature gradient of 50- 62ºC (See Figure 2). In each individual optimization reaction, 2µL of sample DNA (CA005, CA016, or CS005: Table 1) was combined with 5.7µL of HPLC grade water, 1µL buffer, 0.8µL of 10mM dNTPs, 0.2µL of 20µM forward and reverse primer, and 0.1u Taq polymerase. Reactions were incubated in a Biorad DNA Engine 2 Tetrad Thermo Cycler with the following cycler protocol: initial

denaturation at 94ºC for 3 minutes, 35 cycles of 94ºC for 30 seconds, a gradient annealing temperature of 50-62ºC for 30 seconds, and an extension step with a temperature of 72ºC for 30 seconds. This was followed by a final extension step of 72ºC for 10 minutes. We determined optimal buffer and temperature conditions for each locus' primer pair individually using 2% agarose gel electrophoresis with the following specifications: a clean and strong band at the correct size for each marker, with no additional bands that represented nonspecific amplification (usually observed at lower temperatures and higher magnesium concentrations).

	50.0 1	50.3 2	51.0 3	52.0 4	53.4 5	55.1 6	57.2 7	58.9 8	60.2 9	61.1 10	61.8 11	62.0 12
Buffer A	CA005	blank	blank	CA005	CA005	CA005	CA005	CA005	CA005	CA005	blank	CA005
Buffer B	CA005	blank	blank	CA005	CA005	CA005	CA005	CA005	CA005	CA005	blank	CA005
Buffer C	CA005	blank	blank	CA005	CA005	CA005	CA005	CA005	CA005	CA005	blank	CA005
Buffer D	CA005	blank	blank	CA005	CA005	CA005	CA005	CA005	CA005	CA005	blank	CA005
Buffer E	CA005	blank	blank	CA005	CA005	CA005	CA005	CA005	CA005	CA005	blank	CA005
Buffer F	CA005	blank	blank	CA005	CA005	CA005	CA005	CA005	CA005	CA005	blank	CA005
Buffer G	CA005	blank	blank	CA005	CA005	CA005	CA005	CA005	CA005	CA005	blank	CA005
Buffer H	CA005	blank	blank	CA005	CA005	CA005	CA005	CA005	CA005	CA005	blank	CA005

Figure 1: Optimization PCR layout for gradient temperature and buffer magnesium concentrations, populated with *Crotalus atrox* sample CA005. Columns labeled 'blank' did not have a significant temperature difference when the gradient program was entered into the Thermo Cycler, and therefore were not tested.

Diversity Panel Testing

With optimized conditions that met the above specifications, we sequenced PCR products in order to ensure that the amplified products actually contain the desired microsatellite. Subsequently, I tested the primers against a diversity panel of samples made up of various populations from and for each species. For this technique, I performed PCR reactions using the M13 fluorescent dye 6FAM, which in later stages of the PCR reaction becomes the driving forward primer (Schuelke, 2000). In these reactions, 2µL of each different DNA samples was

added to 5.4µL of HPLC grade water, 1µL of specified buffer (see Optimized Conditions, Table), 0.8µL of 10mM dNTPs, 0.10µL of 20µM forward and reverse primer, 0.40µL of 20µM fluorescently labeled M13 primer 6FAM, 0.10µL of 0.2% Bovine Serum Albumin (BSA), and 0.1u Taq polymerase. I ran confirmation gels using 2% agarose gel electrophoresis on all PCR products. We obtained higher resolution data by utilization of the Applied Biosystems 3730 Genetic Analyzer to perform capillary electrophoresis (fragment analysis) on the amplified DNA samples. We processed raw fragment analysis data using the program Gene Marker (Softgenetics) and analyzed individual samples by assessing allelic variability. The increase or decrease in the motif repeats, and thus fragment length, serves as the basis for the observed variability individual samples and populations.

Results

Crotalus atrox

All *Crotalus atrox* loci from the generated primers (Tables 2 and 3) were optimized using CA005 and CA014 sample DNA, both specimens from the Cattletank study site. Those that produced a single, clean band at the correct fragment size were selected for testing with a diversity panel. Visualizations of good and bad optimization gels are shown in Appendix 2.

Out of all markers designed for the Western Diamondback rattlesnake in this experiment, 20 were chosen for diversity panel testing, having successfully produced a single, correctly sized band in optimizations: Ca1_5, Ca1_14, Ca1_17, Ca1_20, Ca1_22, Ca1_31, Ca1_39, Ca1_43, Ca1_48, Ca2_23, Ca2_27, Ca2_38, Ca2_39, Ca2_51, Ca2_64, Ca2_71, Ca2_74, Ca2_81, Ca2_90, and Ca2_92 (Tables 2, 3). A list of the specific temperature and buffer magnesium concentrations chosen for these markers is shown below (Figure 3):

Primer Name	Temperature (°C)	Buffer
Ca1_5	61	C
Ca1_14	61	B
Ca1_17	59	A
Ca1_20	60	E
Ca1_22	61	C
Ca1_31	61	E
Ca1_39	59	C
Ca1_43	59	C
Ca1_48	60	E
Ca2_23	53	B
Ca2_27	53	C
Ca2_38	55	B
Ca2_39	50	D
Ca2_51	53	D
Ca2_64	55	C
Ca2_71	53	F
Ca2_74	59	B
Ca2_81	59	D
Ca2_90	59	C
Ca2_92	55	A

Figure 2: Optimized temperature and buffer conditions for *Crotalus atrox* loci. Specific magnesium concentrations for Buffers A-H can be found in Appendix 1.

From the set of markers with the above optimized conditions, loci Ca1_5, Ca1_17, Ca1_48, Ca2_51, and Ca2_92 showed little to no amplification of panel samples and were therefore discarded. For the remaining 15 markers, amplification was successful on all or most samples in the chosen diversity panel (sample descriptions found in Table 1), as shown below:

	1	2
A	1. CA001	9. CAtv001
B	2. CA005	10. CAsz041
C	3. CA021	11. CAsz075
D	4. CA029	12. CAPR001
E	5. CA032	13. CAPR005
F	6. CA036	14. CAPR010
G	7. CAx004	15. CAPR011
H	8. CAx008	H2O

Figure 3: Diversity Panel sample selection and plate mapping for testing against chosen *Crotalus atrox* loci. Samples from different populations/locations have been colored as follows: Cattletank (green), Benson, Cascabel Road (grey), Sasabe (blue), Tanque Verde Wash (teal), Suizo Mountains (purple), Ina Road (red), Organ Pipe Cactus National Monument (yellow), Route 85 and Ajo Way (pink).

A Map of sample collection is shown below:

Figure 4: Map of *atrox* sample collection sites in Tucson, AZ and surrounding areas.

Diversity panel amplification using gel electrophoresis is shown for some markers below:

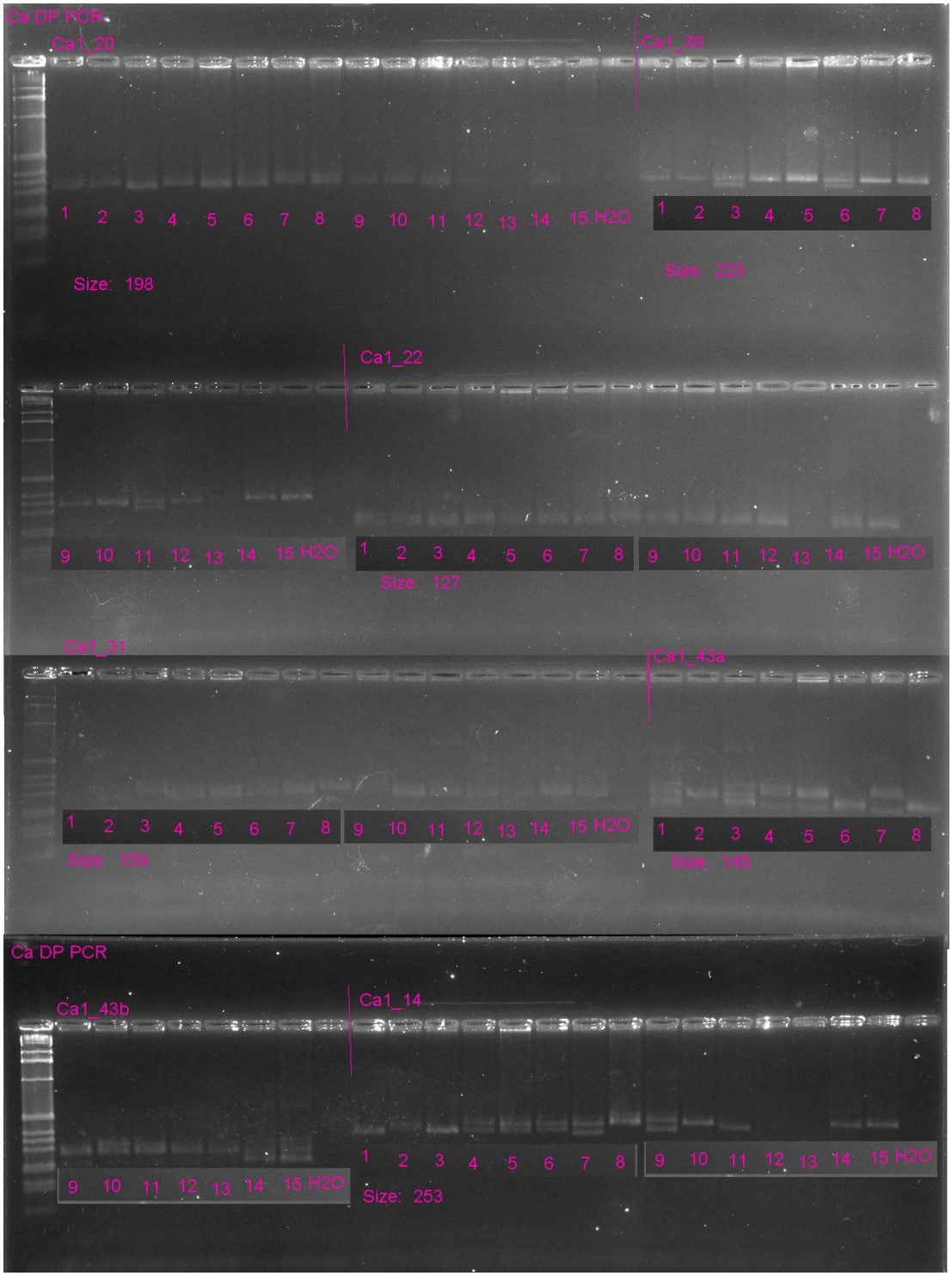

Figure 5: The 2% Agarose Gel for the diversity panel of *Crotalus atrox* loci Ca1_14 through Ca1_43. Numbers correspond to diversity panel sample names found in Figure 4.

Crotalus scutulatus

All *Crotalus scutulatus* loci from the generated primers (Table 4) were optimized using CS005 sample DNA, a specimen from the Cattletank study site. Those that produced a single, clean band at the correct fragment size were selected for testing with a diversity panel. (Optimization gels shown in Appendix 2).

Out of all markers designed for the Mohave rattlesnake in this experiment, 12 were chosen for diversity panel testing, having successfully produced a single, correctly sized band in optimizations: Cs115, Cs1213, Cs1214, Cs2112, Cs2116, Cs2127, Cs2153, Cs234, Cs2319, Cs2322, Cs2340, and Cs2342. A list of the specific temperature and buffer magnesium concentrations chosen for these markers is shown below:

Primer Name	Temperature (°C)	Buffer
Cs115	53	C
Cs1213	53	D
Cs1214	55	B
Cs2112	55	A
Cs2116	53	B
Cs2127	50	C
Cs2153	50	E
Cs234	55	C
Cs2319	50	D
Cs2322	59	B
Cs2340	55	C
Cs2342	50	D

Figure 6: Optimized temperature and buffer conditions for *Crotalus scutulatus* loci. Specific magnesium concentrations for Buffers A-H can be found in Appendix 1.

From the set of markers with the above optimized conditions, loci Cs2127, Cs2153, Cs2342, Cs115, Cs1213, and Cs2112 showed little to no amplification of panel samples and were therefore discarded. For the remaining 6 markers, amplification was successful on all or

most samples in the chosen diversity panel (sample descriptions found in Table 1), as shown below:

	1	2
A	1. CRSC005	9. CS011
B	2. CRSC006	10. CS013
C	3. CRSC007	11. CS014
D	4. CS003	12. CS015
E	5. CS004	13. CSx001
F	6. CS005	14. CSx002
G	7. CS006	15. CSx003
H	8. CS009	H2O

Figure 7: Diversity Panel sample selection and plate mapping for testing against chosen *Crotalus scutulatus* loci. Samples from different populations/locations have been colored as follows: Cochise County (teal), Campbell Road (light green), Cattletank (green), Park Link Road (purple), Interstate 10 (pink), Near Bonita (blue), Benson, Cascabel (grey), Route 181 (aqua), Cap (yellow), Frontage Road (red).
A Map of sample collection is shown below:

Figure 8: Map of *scutulatus* sample collection sites in Tucson, AZ and surrounding areas.

Diversity panel amplification using gel electrophoresis is shown for some markers below:

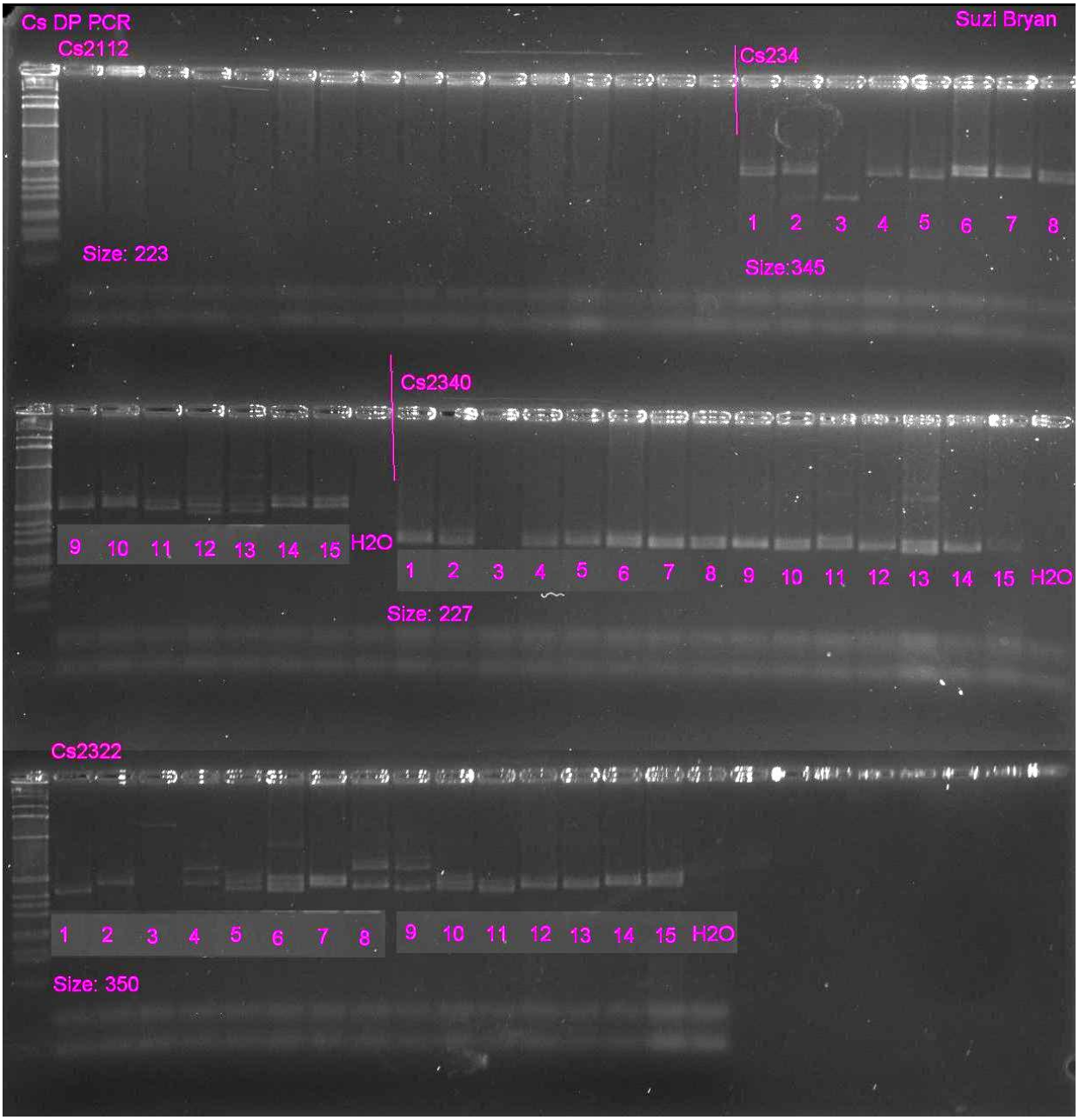

Figure 9: The 2% Agarose Gel for the diversity panel of *Crotalus scutulatus* loci Cs2112 through Cs2322. Numbers correspond to diversity panel sample names found in Figure 8.

Future Directions

We will examine results obtained from fragment analysis for both *Crotalus atrox* and *Crotalus scutulatus* diversity panels with regard to different populations in the following ways: allelic variability based on fragment length (variable between individuals as microsatellite repeat number changes), allelic richness (the number of alleles per locus), observed and expected heterozygosity, and deviations from Hardy-Weinberg equilibrium. Heterozygosity and Hardy-Weinberg deviations will be examined using the program Genepop 3.4 (Raymond and Rousset, 1995). Adjusted P values from Genepop will help to assess deviations from Hardy-Weinburg equilibrium: a significant difference between observed and expected heterozygosity will result in a substantial deviation from Hardy-Weinberg equilibrium ($P<0.05$) (Raymond and Rousset, 1995). A decrease in heterozygosity may be explained by inbreeding within a specific population. Genetic drift will cause random changes frequencies of genes, and will have a more significant effect if the population is small (Munguia-Vega et al., 2009). After evaluating the microsatellite markers using these appraisals, we can confidently assess the usefulness of our newly developed STR libraries will have for applications in population genetic studies.

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References

Ahn, Susan J., Costa, Jose, and Emanuel, Janet, 1996. "PicoGreen Quantitation of DNA: Effective Evaluation of Samples Pre- or Post-PCR." *Nucleic Acids Research*. 24:2623-2625.

Anderson, Corey Devin, 2006. "Utility of a Set of Microsatellite Primers Developed for the Massasauga Rattlesnake (*Sistrurus catenatus*) for Population Genetic Studies of the Timber Rattlesnake (*Crotalus horridus*)." *Molecular Ecology Notes*. 6:514-517.

Brennan, Thomas C., 2008. "Mohave Rattlesnake: *Crotalus scutulatus.*" *Online Field Guide to The Reptiles and Amphibians of Arizona*. Arizona Game and Fish Department Phoenix, AZ.

Brownstein, M.J., Carpten J.D., Smith J.R., 1996. "Modulation of Non-templated Nucleotide Addition by Taq DNA Polymerase: Primer Modifications that Facilitate Genotyping." *BioTechniques.* 20:1004-1010.

Bushar, Lauretta M., Maliga, Marianne, Reinert, Howard K., 2001. "Cross-Species Amplification of Crotalus horridus Microsatellites and Their Application in Phylogenetic Analysis." *Journal of Herpetology*. 35.3:532-537.

Castoe, Todd A., Spencer, Carol L., and Parkinson, Christopher L., 2007. "Phylogeographic Structure and Historical Demography of the Western Diamondback Rattlesnake (*Crotalus atrox*): A perspective on North American Desert Biogeography." *Molecular Phylogenetics and Evolution*. 42:193-212.

Glenn, T.C. and Schable, N.A, 2005. "Isolating Microsatellite DNA Loci." *Methods in Enzymology*. 395:202-222.

Goldberg, Karen S., Edwards, Taylor, Kaplan, Matthew E., and Goode, Matt, 2003. "PCR Primers for Microsatellite Loci in the Tiger Rattlesnake (*Crotalus tigris,* Viperidae)." *Molecular Ecology Notes.* 3:539-541.

Kjoss, Victoria A. and Litvaitis, John A., 2001. "Community Structure of Snakes in a Human-Dominated Landscape." *Biological Conservation*. 98:285-292.

Mendelson, Joseph R. and Jennings, W. Bryan, 1992. "Shifts in the Relative Abundance of Snakes in a Desert Grassland." *Journal of Herpetology.* 26.1:38-45.

Munguia-Vega, A., Pelz-Serrano, K., Goode, M., and Culver, M., 2009. "Eleven New Microsatellite Loci for the Tiger Rattlesnake (*Crotalus tigris).*" *Molecular Ecology – Permanent Genetic Resources Note*. 1267-1270.

Raymond, M. and Rousset, F., 1995. "Genepop Verson 1.2: Population Genetics Software for Exact Tests and Ecumenicism." *Journal of Heredity,* 86:248-249.

Rozen, S. and Skaletsky, H, 2000. "Primer 3 on the WWW for General Users and for Biologist Programmers." In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. (eds Krawetz, S. and Misener, S.), 365-386. Humana Press, Totowa, NJ.

Schuelke, M, 2000. "An Economic Method for the Fluorescent Labeling of PCR Fragments." *Nature Biotechnology*. 18:233-234.

Secor, Stephen M., 1994. "Ecological Significance of Movements and Activity Range for the Sidewinder, *Crotalus cerastes*." *Coperia*. 3:631-645.

Shine, Richard, 2008. "Tracking Elusive Timber Rattlers with Molecular Genetics." *Molecular Ecology*. 17:715-718.

Tables:

Table 1: *Crotalus atrox* and *Crotalus scutulatus* Samples

Species	Sample	Location	Type
C. atrox	CA001	Cattletank	Blood
C. atrox	CA005	Cattletank	Blood
C. atrox	CA021	CAP	Blood
C. atrox	CA029	I10	Blood
C. atrox	CA032	I10	Blood
C. atrox	CA036	Cap	Blood
C. atrox	CaPR 001	Ina Rd. E. of Harold	Blood
C. atrox	CaPR 005	Organ Pipe Ntl. Park	Blood
C. atrox	CaPR 010	Organ Pipe	Blood
C. atrox	CaPR 011	Rt. 85 & Ajo Way	Blood
C. atrox	CaSZ 075	Suizo Mountains	Blood
C. atrox	CaSZ 041	Suizo Mountains	Blood
C. atrox	CaX003	Benson, Cascabel Road	Blood
C. atrox	CaX004	Benson, Cascabel Road	Blood
C. atrox	CaX008	Sasabe	Blood
C. atrox	Catv 001	Tanque Verde Wash near Cloud Rd/Sabino Canyon Rd.	Blood
C. scutulatus	CRSC005	AZ: Cochise Co: Hwy 80: w/in 5 mi of jct w/ Rucker Rd	Blood
C. scutulatus	CRSC006	AZ: Cochise Co: Hwy 80: w/in 5 mi of jct w/ Rucker Rd	Blood
C. scutulatus	CSx001	Near Bonita	-
C. scutulatus	CSx002	Benson, Cascabel Road	-
C. scutulatus	CSx003	Route 181	-
C. scutulatus	CRSC007	Cambell RD Ben SD4	Blood
C. scutulatus	CS003	CattleTank	Blood
C. scutulatus	CS004	Park Link	DOR, tissue
C. scutulatus	CS005	Cattletank	DOR, tissue
C. scutulatus	CS006	I10	Blood
C. scutulatus	CS009	-	Blood
C. scutulatus	CS011	I10	Blood
C. scutulatus	CS013	Cap	Blood
C. scutulatus	CS014	Cap	Blood
C. scutulatus	CS015	Frontage Rd	liver

Table 2: *Crotalus atrox* Primers I

Primer Name	Size	Motif	Forward	Reverse
Ca1_01	156	(AAAG)15	CCCACAGGTTGAG AACCACT	ACACTGGCAGCAC ATCTCC
Ca1_03	243	(GT)20	CCTTGTGAAAGGC CAACAAG	TTCCTCCTGAGTTG TGCTGA
Ca1_04	166	(AG)18 TG(14)	TGGGCCAAATTAC CTTGAAC	CCACCTGCATACCA TCTTCC
Ca1_05	221	(TG)10	GAATCCTCTCTACC AACTGCATC	CACAGCTGGCAAA AGTGAGA
Ca1_06	117	(CA)17+	CCTAGCTAGCAGA ATCACACAAA	TCCCTTTTCCTCACT TGCAC
Ca1_12	297	(CT)18	TGGCAACACACTTT TTCAGC	CAAGGCATAGGGC ACAATTT
Ca1_14	253	(AGA)35+	GATGCGACACCCA GAAAAAT	CCAATAGATGGTT GGGGAAA
Ca1_17	142	(GA)25	GATGGATGGATGG ATGGATG	GGGGCCTCAGAGG TCTTCTA
Ca1_18	272	(GA)6 (GT)6 AT (GT)10 (CT)14	GAAGGCCTATTAA GCAGAATCA	TCCACCATTCGAGT TCTTCC
Ca1_20	198	(TC)20 (TG)7	GAATCCTTCACCAA AGCTAGAAA	TTGGGTGGCAATC AGTGTTA
Ca1_22	127	(AC)15	CCCCCAGGAAAGA ATATGTATG	CACATCTTCAGGTT GCCAAA
Ca1_25	269	(AC)3 TC (AC)11	CTAGAAACCCAGC CATCCAG	TGGAGTGTAGCAC GTTCCAG
Ca1_28	260	(CA)2 TA (CA)2 TG (CA)14	GGGTTGGGAGATG GGATATT	GCCAAGATGTCAG GCTTCTC
Ca1_31	159	(AG)16 AT (AG)19 TC (AC)18	GGGATTCAGGCCA ATTTTTA	TGGTAATTTTGCAT TTCAGCA
Ca1_32	247	(CT)12	ATGCTGGCTGAGG AATTCTG	TTAAGCTGGCAAG TTTGGAA
Ca1_33	349	(CA)2 CC (CA)14	CCTGGAAAGGATT TTGCTCA	GCGTTTGGAAGCC GTATTTA
Ca1_34	105	CA…CT	TCCTATTAAGCAGA ATCCTTTGC	AAGAGGGAGGGG AAGAGTGA
Ca1_36	291	(TG)4 TT (TG)3 TC (TG)10 TC (TG)3 TC (TG)5	ATGGCCCACAATC CTCAAG	GATTCAATGGAGG GAGCTGA
Ca1_37	193	(AG)27	CCTATTAAGCAGA ATCACTGACACA	AGGCAATGGTGCA TTTCTTC

Ca1_39	223	(AAT)3 GAT (AAT)4 (AAC)3 AGC (AAC)3 AAT (AAC)2 (AAT)2	AATCACAACTTCAT CAGTAACATCTAA	AGGACAAGAAGCG ATGGATG
Ca1_40	311	(CT)10 (GT)4 (GA)18	GCCACGGTGGTTA AGTGAAT	CTCTAGGGCACAG GTCGGTA
Ca1_42	345	(ATC)11 TTC (ATC)13	TGGACAGTGGTGA GATTCAAA	GCACCACTTGAAC ACCATTG
Ca1_43	145	(TG)8 TA (TG)6 TA (TG)5 TA (TG)19	TCAAAACAACCTGT GGCACT	CCTGATCCTCTCAG GAAAGC
Ca1_44	107	(CA)7 (CT)20	TGTGGCTCTTTGA GCCTTCT	AGCAGAAGCACCA GGATCAG
Ca1_45	185	(TCTC)3 TTTC	GGGGGAAACCAG ATTCCTTA	TCCTCTCCCATTGT CCACTC
Ca1_48	178	(CT)2 CC (CT)6 TT (CT)15	GCCATCATTGGCTA CTGGAG	AATCTTCCCCTGCT TTGGAT

Table 3: *Crotalus atrox* Primers II

Primer Name	Size	Motif	Forward	Reverse
Ca2_4	282	(AC)23 AA (AC)4	TGCTTTTTCAAGAG GCAGGT	CGGCTTCCTAACA GAATCACA
Ca2_16	230	(ATCT)10 (GTCT)15	CCTTTGGTTTTTCC TGCCTA	CTGGCAGAATCCT GACATGG
Ca2_23	259	(CTAT)2 CTGC CT CTAT CTGT (CTAT)3 (CTGT)3 CTAC (CTAT)15	TCTGTCCATGCAAC ATCCAT	ATGCAAGCCGTTCT CAGTTT
Ca2_26	220	(CT)21	GCAGAATCACTAC TATTCTCTAGCATC	GGCAAACAGAAAG TGGAAGC
Ca2_27	319	(AAG)32 AGAA AAG(15)	AATCCTTCCATCCT GCAATG	ATCACTTTTGGGTG CAGAGC
Ca2_31	277	(TAAG)8	TATTGCTGCTTTTT GCATGG	GATCCTTGCATTTC CTGAGC
Ca2_37	311	(ACAG)11 (ATAG)9	GATCTGCCTGTCAC AGTTGG	CTCCAAAGCTTCCT CCTTCC
Ca2_38	307	(TCCG)11 (TCCT)7	CTCTGCTCTGCCAT TTCACA	GAGCCAGTGCTTT GTTTTCC
Ca2_39	216	(GA)24	AGCAGAATCCTAG GGAGCAA	CACAACGTTGGTG GAACAAT
Ca2_48	185	(CTCA)10	TGAGAGAAACCCA GGACAGG	TTGTTGCAATTTCT GTCACCA
Ca2_51	220	(CTT)11 CAT (CTT)14	ATCACCCCACGGTC CTTT	AGAGGGGGTGGA TTTACTGG

Ca2_63	264	(GACG) (GACA)9 (GATA)15	CCTTGTGCCTTGG GTGTATT	GCTGACAGAATCA CCTTTTGC
Ca2_64	295	(TTTC) TTC (TTTC)19	CCGGTTAATGAAC CAAAAATG	AAGCAGAATTGGC TTGACCA
Ca2_65	182	(AC)23	TGAGATCATAGAC AGATGAAGCAA	GAATCCTTGTTTAA TATTGGCATT
Ca2_71	176	(CA)20	AGCCTGAAGATGA CGAATGG	CCAGCCTTTCCTTT TTCCTT
Ca2_74	270	(CA)21	AGCCGCTTGTCTCA AATTGT	TGTCCTCTGATTTG CCCATT
Ca2_79	213	(ACGG)8 (ATAG)16 (ATGG)14	TGGTTGCCAATTAT GTCACC	CGGGTTTTAGGTG TTGTTCG
Ca2_81	297	(CTTT)5 CTTA (CTTT)20	CCCAGCACACTCAC AATGTC	GCTCCTAGCCAGG AATGTCA
Ca2_88	262	(CCTA)2 (CTAT)16 (CCAT)10 (CTAT)4	TCATCTGCCCTCAT ACAGCA	CCAAAATCTTGCTG GCTCTT
Ca2_90	204	(AAAG)21	TTGCTGACTGAAG GAGCATTT	AACCTGCAAGACC ACAAAGG
Ca2_92	175	(TC)13 (TTTC)16	TGTTTTTGTGGGA GGGTTTC	CCTGTCAGGAGAA CTATGAGAACTT

Table 4: *Crotalus scutulatus* Primers

Primer Name	Size	Motif	Forward	Reverse
Cs115	369	CTTT(11)	TGACTGTGCCAAA GAAGAGG	TTTAGCCATGGGA ACTTGCT
Cs118	244	AC(10)	TCCGGGCAGAGAA TTTAATG	CGTATGATGGATG CTTTTGC
Cs1213	148	TG(19)	GAAGGAGGAAAC AGCACTGG	AATATTCTTAAAGG ACCATGGAAA
Cs1214	322	GTA(2) ACA GTA(7)	GATCAAGACAGGG CAGGGTA	TCCGAAAATCCAA GAGATGC
Cs1215	157	ATT(9)	GCTGGTGAGGCTT ACTTCCA	CAAAAATGCCCAA TCCAATC
Cs2112	223	AAAT(2) AAGT(7) AAAT(8)	AGGATGGCAACAG AAACCAC	CAGCCCCTCAAATA CTGGAA
Cs2116	226	AG(23)	AGGCCTGCTTTAAC CCTTGT	CACAGACCCAGAA TTAAATCCA
Cs2127	316	CA(17)	CATCTTTGGGGAA AGCTTCA	TCAGGCAGATTAG CGTGAGA
Cs2129	296	GT(14)	CCTCTTCCTCACAC CACACA	TTGGCTGGATCCAT ATTTCA
Cs2135	200	CTA(9)	CAGGGTGCCTTTTC CACATA	TGAATCCAGCTTCA ATGCAA

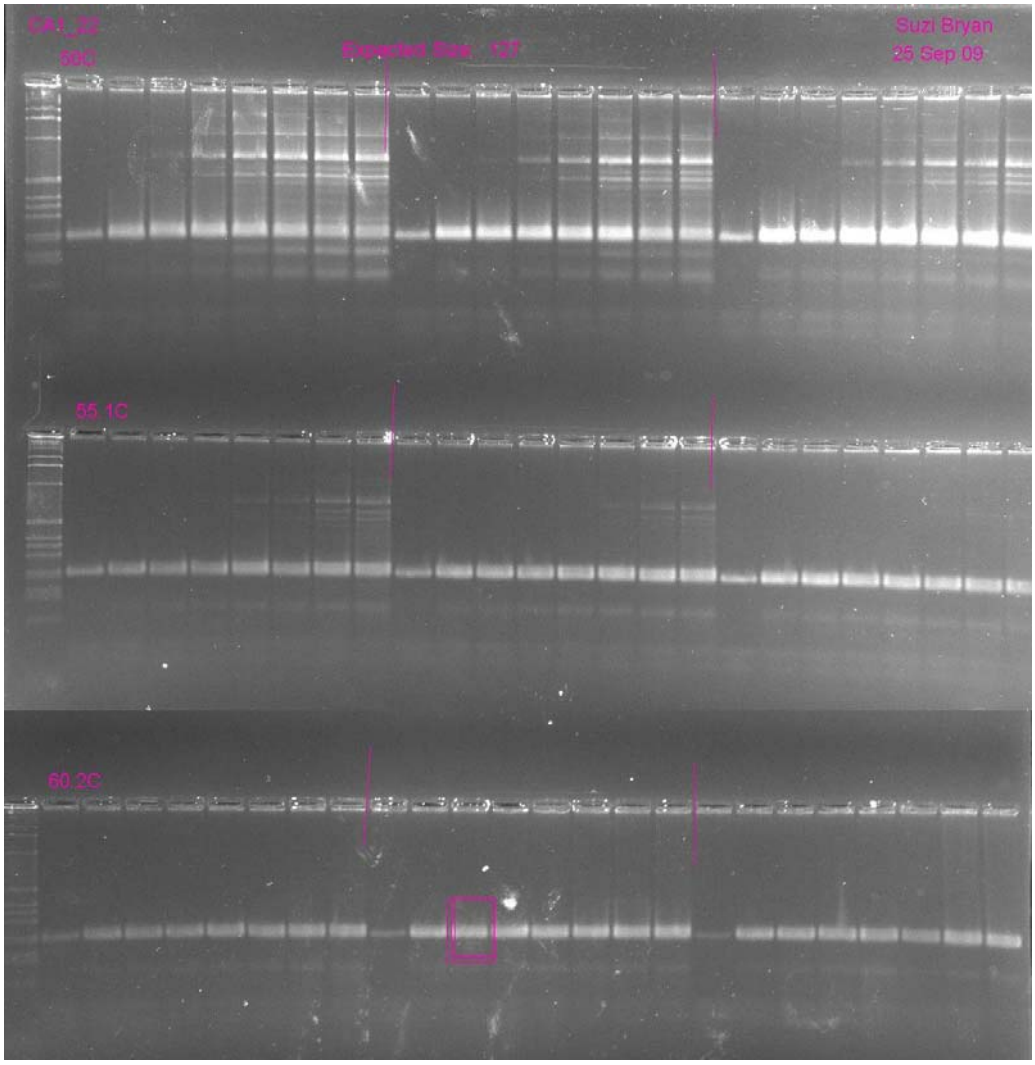
Cs2136	309	TC(21)	CTCAAGAAGACCC TGCAGAA	CTCCCTAAGGGGG TTCATTC
Cs2153	335	CT(18) TT CT(3)	AAATCCTGCAGAA TCCGATG	CAGGCATTTCAAAT TGCTTTT
Cs2232	278	AG(25)	TGACTTTGAAGGA AGGCACA	GCAAGACAGAAGG CCAAAGT
Cs234	345	CA CG CA(11)	CAGATGGTTAAAA GGGCATCA	TGGAATTCAACCAT AAAAGTGACA
Cs236	266	GAAA(7) GGAA GAAA(6) AAAA GAAA(2) GAAC	CAGGGTTTTTGTG GGAAAGA	CCGTTCTCTGCTGC TGTGTA
Cs2319	199	GT AT GT(2) GG GT(16)	CTGGCTCACTGGG AAAACAT	TGGGATTAGTTTAC TTTCAGGTCTC
Cs2320	258	GT(10)	CAGAGGAAGTCGA AGCAACC	TCCGTGTGCCTTTT GATACA
Cs2322	350	CTAT(17)	CTGTGTGTGGCAC CTTTCAG	AGTGTAAGCATGC CCAGTCC
Cs2325	327	TTTC(14)	ATGGCAAGGCCAC TCATAAC	CATCCACTACCCGC AATCTT
Cs2336	149	AC GC AC(10) AA AC(3) AA	AGATGACGAATGG GACTTCG	GCAGAATCCTGGG GTGTTAT
Cs2340	227	AC(4) AG TC AC(2) A AC(18)	TTTCCCTGCTCGAG GTAATG	AAAGTTTTGCCCTG TCATGC
Cs2342	323	CTAA(5) CTA CTAA(3) CTAC(2) TAAA CTAA A CTAA	CGGTTTGTGTCTGC CCTAAT	TGAGCAGAAGCCA ACAAGTG

Appendices:

Appendix 1: Buffers Concentrations A-H

10x Buffer	Tris (pH 8.3)	KCl	MgCl2
A	100mM	500mM	10mM
B	100mM	500mM	15mM
C	100mM	500mM	20mM
D	100mM	500mM	25mM
E	100mM	500mM	30mM
F	100mM	500mM	35mM
G	100mM	500mM	40mM
H	100mM	500mM	45mM

Appendix 2: Examples of Gradient Optimization visualized using 2% Agarose Gel Electrophoresis



The 2% Agarose Gel for optimization of *Crotalus atrox* locus Ca1_22. Conditions are grouped in order of increasing temperature and buffer magnesium concentration. The first group begins with 50°C and 10mM Mg, progressing to 50°C and 45mM Mg. The following groups are at 52°C, 10-45mM Mg, 53.4°C, 10-45mM Mg, and so on. Amplification follows the gradient pattern, with an area of nonspecific priming occuring at low temperatures and high magnesium concentrations, while rigorous conditions exist at high temperatures and low magnesium concentrations. The boxed band shows an optimal, clean amplicon. (This condition proved too stringent for Diversity Panel samples, and was relaxed).