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Phylogeographic Patterns in the Eastern Diamondback Rattlesnake (Crotalus adamanteus)

Karalyn Aronow

ABSTRACT

The aim of this project was to analyze relationships among populations of *Crotalus adamanteus* (Eastern Diamondback Rattlesnake) and to compare phylogeographic patterns across different classes of genes. The objective was to determine the effect of selective pressures on the detection of barriers to gene flow. For example, venom genes are under strong positive selection and allow an analysis of the rate of allele exchange in a gene that directly affects the fitness of an individual. This selection might drive alleles through phylogeographic barriers that limit the spread of neutral alleles. The relationship between nontoxin genes and fitness is not as clear and can be difficult to establish. However, most variation in these genes is neutral and determined by stochastic events. The type of selection on a gene will affect the phylogeographic pattern displayed. Prior phylogeographic studies have used either neutral markers (e.g., microsatellites) or genes not directly involved in specific adaptations (e.g., mitochondrial genes). One venom gene, two mitochondrial genes, one nuclear gene, and one pseudogene were compared for patterns of adaptation and the presence of geographic barriers to gene flow. This novel approach to studying gene flow could have an impact on defining taxonomic groups and conservation efforts, as well as serve as a guide for choosing appropriate genetic markers for other species. In the venom gene that was analyzed, it appeared the high level of selection led to alleles being driven across the barriers to gene flow of alleles under neutral selection.

KEYWORDS: Eastern Diamondback Rattlesnake, Venom, Phylogeography

THE FLORIDA STATE UNIVERSITY

COLLEGE OF ARTS & SCIENCES

PHYLOGEOGRAPHIC PATTERNS IN THE EASTERN DIAMONDBACK RATTLESNAKE *(Crotalus adamanteus)*

By

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The aim of this project was to analyze relationships among populations of *Crotalus adamanteus* (Eastern Diamondback Rattlesnake) and to compare phylogeographic patterns across different classes of genes. The objective was to determine the effect of selective pressures on the detection of barriers to gene flow. For example, venom genes are under strong positive selection and allow an analysis of the rate of allele exchange in a gene that directly affects the fitness of an individual. This selection might drive alleles through phylogeographic barriers that limit the spread of neutral alleles. The relationship between nontoxin genes and fitness is not as clear and can be difficult to establish. However, most variation in these genes is neutral and determined by stochastic events. The type of selection on a gene will affect the phylogeographic pattern displayed. Prior phylogeographic studies have used either neutral markers (e.g., microsatellites) or genes not directly involved in specific adaptations (e.g., mitochondrial genes). One venom gene, two mitochondrial genes, one nuclear gene, and one pseudogene were compared for patterns of adaptation and the presence of geographic barriers to gene flow. This novel approach to studying gene flow could have an impact on defining taxonomic groups and conservation efforts, as well as serve as a guide for choosing appropriate genetic markers for other species. In the venom gene that was analyzed, it appeared the high level of selection led to alleles being driven across the barriers to gene flow of alleles under neutral selection.

INTRODUCTION

The Eastern Diamondback Rattlesnake (*C. adamanteus*) is a large snake, averaging between 1 and 2 meters long, with large venom glands (Klauber, 1997). Its diet consists mainly of small mammalian prey such as rats, mice, and squirrels (Klauber, 1997). It is moderately common across its range, which spans from south Florida to southeastern North Carolina and west to Mississippi (Conant and Collins, 1998). The majority of snakebite deaths in the United States can be accounted for between the Eastern and Western Diamondback Rattlesnakes (*C. atrox*) (Gold et al. 2002).

Displays of phyogeographic patterns may vary across different classes of genes. For example, in the absence of geographical barriers, panmixia would be expected when looking at genes under negative selection, such as mitochondrial or nontoxin nuclear genes, because any observed variation should be neutral. Positive selection could lead either to a polytomy with no variation across the whole range due to species-wide selective sweeps or to local adaptations creating smaller polytomies within the larger phylogeny due to local, limited selective sweeps in particular populations.

Venomous snake species have been found to have high intraspecific variation in toxin expression patterns (Gibbs et al. 2009), DNA sequences (Soto et al. 2006), and protein composition (Creer et al. 2003). This variation could be the result of local adaptation in areas where populations of prey have been found to evolve resistance to snake venom (Biardi et al. 2005). Variation could also result from geographic barriers, however few are apparent in the range of *C. adamanteus*, especially given the propensity of this species to cross waterways (Lillywhite et al. 2008).

The venom gene used in the study, Myotoxin, has been shown to be under positive selection with dN/dS=2.8 (Rokyta et al. 2011). The dN/dS ratio measures the relative rate of nonsynonymous change to synonymous change, indicating the type of selective pressure on a gene. Recent strong positive selection can lead to selective sweeps. Nonetheless, thorough sampling across a species' range is required to determine whether there has been a selective sweep or small pockets of local adaptation. In addition, different venom genes may show different evolutionary patterns.

MATERIALS AND METHODS

Blood and tissue sampling

When possible, road-salvaged specimens were used for sample collection to reduce the impact on natural populations. Of the 71 samples used for Cytochrome b (see below), 55 were tissue samples, and 16 were blood samples.

DNA extraction

DNA extraction was attempted from tissues and blood of 86 Eastern Diamondback Rattlesnakes using the Omega Biotek E.Z.N.A. tissue DNA kit. Of those samples, 71 produced high-quality genomic DNA of concentrations varying from 20ng/μL to 267.77ng/μL. Extracted DNA was quantified by both UV/Vis and fluorescent spectrometry methods (nanodrop and Qubit (Invitrogen), respectively). These samples cover the entire range of the species, with the most dense sampling from the Apalachicola National Forest (see Figure 1).

Gene selection and primer design

To identify genes for use in this project, a venom-gland transcriptome was sequenced from a single *C. adamanteus* female, using 454 GS FLX technology (Rokyta et al. 2011). We chose to study Cytochrome b and Sodium dehydrogenase because of their popular use in phylogeographic studies of other species. Protein phosphatase was chosen as a good example of a nuclear gene not under strong positive selection. The two venom genes, Myotoxin and Phospholipase A_2, were chosen based on their different dN/dS values as found in Rokyta et al., 2011 (2.8 and .79, respectively). Primers were designed to amplify the entire coding sequence of each gene using SeqBuilder from the DNAStar sequence analysis software package. A pseudogene was inadvertently discovered when attempting to amplify the Phospholipase A_2 gene. We attempted to use twenty primer pairs, with the successful sets listed below. To be considered a successful primer set, the oligos must have successfully amplified the targeted gene region in eleven individuals spanning the species' range.

Gene Function

Cytochrome b is a subunit of cytochrome bc1, which functions in mitochondrial respiration (Pierre et al. 1995). Sodium dehydrogenase subunit 5 (NADH5) is a member of a family which acts as a catalyst to transfer two electrons from NADH to ubiquinone in a reaction associated with protein translocation across the membrane (Walker, 1992). Protein

Phosphatase is a family of intracellular proteins that regulate an array of cellular processes, including enzyme activity and creation of protein docking sites (Virshup, 2000). Myotoxin functions by producing proteins that cause skeletal muscle spasms and spastic paralysis in mice hind limbs (Oguiuta et al. 2006).

Primer sequences

Gene	**Forward Primer sequence (5'->3')**	**Reverse primer sequence (5'->3')**
Cytb	ATGACCCACCAACATCTACTCAC	TTAGGTTTGTAGACCTGAGACTT
NADH5	GGTGCAAGTCCAAGTGATA	GGTCTTGTTTTCTGTTTTAGTTA
PP	CCTCTCTATGATCAGGGTGGTCC	TTTGTACAGAGTCAGTTGGCACCT
Pseudo	GCCACATCGTTGCCATTTTC	TTGTGTAATTTGAGGGGTTTTTCC
Myotoxin	GTCCAGAACCAGTCTCAGCATGAAGAT	TGGAGATGGCATTATTTACACT

Gene amplification and sequencing

The genes of interest were amplified using polymerase chain reaction (PCR). The 25μL PCR mix contained final concentrations of 1X buffer, 0.8mM dNTPs, 0.4mM forward and reverse primers, 0.625U Takara ExTaq, and DNA (4 to 110 ng). The table below shows the PCR conditions for Cytochrome b (Cytb), NADH 5, Protein Phosphatase (PP), Pseudogene (Pseudo), and Myotoxin. The denaturation, annealing, and extension steps went through 35 cycles.

Gene	Initial Denaturation Temp&Time	Denaturation Temp&Time	Annealing Temp&Time	Extension Temp&Time	Final Extension Temp&Time	Hold
Cytb	95ºC 2:00	95ºC 0:30	47ºC 0:30	72ºC 1:30	72ºC 4:00	4ºC ∞
NADH5	95ºC 2:00	95ºC 0:30	55ºC 0:30	72ºC 1:30	72ºC 4:00	4ºC ∞
PP	95ºC 2:00	95ºC 0:30	57ºC 0:30	72ºC 1:30	72ºC 4:30	4ºC ∞
Pseudo	95ºC 2:00	95ºC 0:30	50ºC 0:30	72ºC 1:30	72ºC 5:00	4ºC ∞
Myotoxin	95ºC 2:00	95ºC 0:30	47ºC 0:30	72ºC 0:45	72ºC 7:00	4ºC ∞

The products from the initial PCRs for each primer pair were assayed using a 0.7% agarose gel electrophoresis containing 7 x 10^{-5} mg/ml ethidium bromide to determine success of the primer. These reactions were subsequently optimized for each primer pair by performing PCRs with a range of annealing temperatures (47-60 ºC). Once the optimum annealing temperature was determined, each DNA sample was then amplified. Amplified gene products for Cytb, PP, and Myotoxin were purified using the Qiagen QIAquick PCR Purification Kit. The amplified products for NADH5 and the pseudogene required gel purification which was performed using the Omega Biotek E.Z.N.A gel extraction kit.

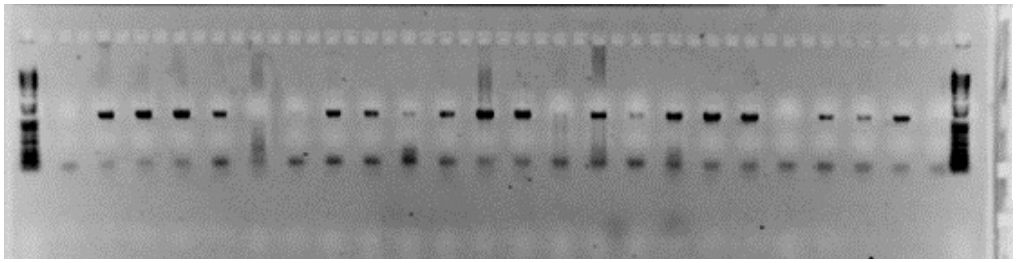

Image 1. Agarose gel image from a Cytochrome b amplification.

Sequencing

Sequencing was performed by Florida State University's DNA Sequencing Facility and the DNA Analysis Facility on Science Hill at Yale University. The sequences were edited and assembled into contigs using the SeqMan package of DNAStar Lasergene software suite. The edited sequences were then aligned using the MegAlign package of the DNAStar Lasergene software suite.

Analysis

The maximum-likelihood phylogenies were estimated using PAUP*, version 4.0b10 following the approach outlined in Rokyta et al., 2011.

RESULTS/DISCUSSION

Pseudogene Identification

The pseudogene used for this project was discovered during an attempted amplification of the Phospholipase A_2 gene. It was identified as a pseudogene by its stop codons in the coding sequence, indels not of lengths that are multiples of three (relative to the original), and by its lack of introns.

Variation

GENE	Number of samples (n)	Number of alleles	Number of Variable Sites	Amplified Fragment length (bp)
MITOCHONDRIAL				
Cytochrome b	71	6	6	915
NA dehydrogenase 5	11	8	18	1741
NUCLEAR				
Protein phosphatase	13	3	2	915
PSEUDOGENE				
Phospholipase A2 pseudo	24	4	2	468
VENOM				
Myotoxin	15	1	0	1085

Table 1. Measure of variation throughout the five genes we looked at.

This pattern of variation is consistent with the hypothesis based on selection of gene type: low in strong positive selection(venom), and present in the genes under negative selection(mitochondrial and nuclear) and neutral selection(pseudogene). Interestingly, there was a vast difference in amount of variation between Cytochrome b and NADH 5, despite their close proximity within the mitochondrial genome. We attribute this, in part, to the difference in length of the two genes.

Figures

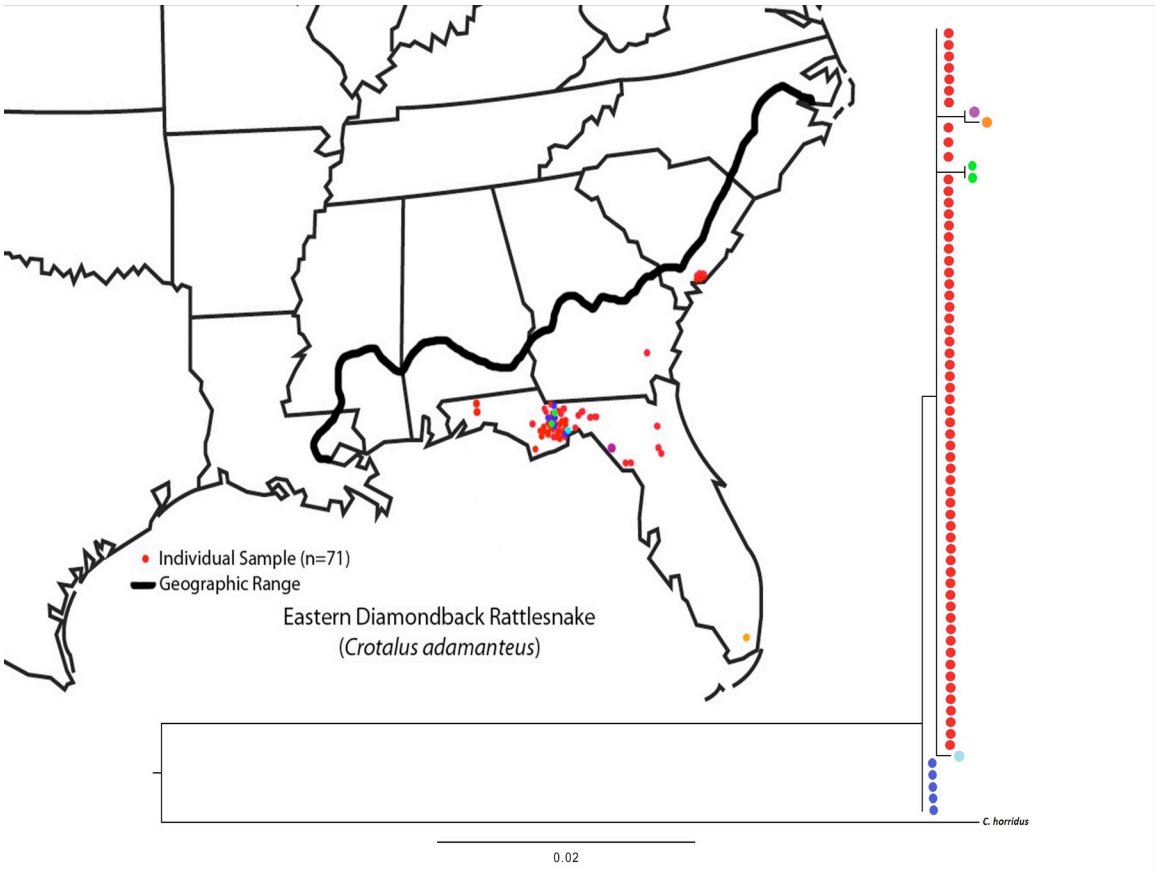

Fig 1. Map showing the locations of the 71 samples and 6 alleles in our cytochrome b analysis. Each color represents a unique allele.

The sample from South Florida had a unique allele, as did the sample from Dixie County (purple dot). The South Carolina population had no unique alleles when compared to the Apalachicola National Forest(ANF) population, and there are no known barriers to gene flow between the two. The most dense sampling was in the ANF, where three alleles were identified.

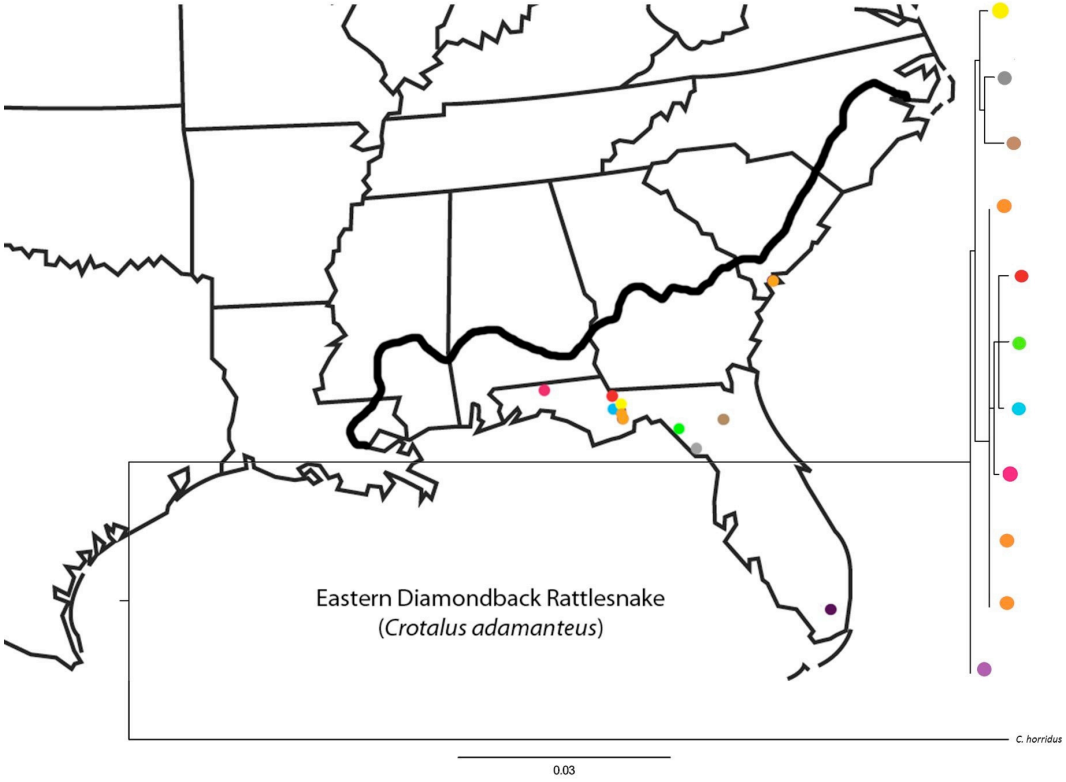

Fig 2. Map showing the locations of the 11 samples and 8 alleles in our NADH 5 analysis. Each color represents a unique allele.

The variation in NADH 5 was very high. These 11 samples were included in each of the other genes' subsamples, as they are representative of localities from across the species' range. Only three of the eleven samples shared a common allele, with two being located in the ANF and one in the South Carolina population. In the nontoxin genes, we found variation suggestive of a phylogeographic pattern, but our current sampling is not sufficient to test this hypothesis.

Venom genes directly influence the fitness of individuals due to their critical role in feeding. In particular, Myotoxin functions by producing proteins which cause skeletal muscle spasms and spastic paralysis in mice hind limbs (Oguiuta et al. 2006). Myotoxin is under strong positive selection, with a reported dN/dS ratio of 2.8 when compared with *C. durissus* (Rokyta et al. 2011). Selective sweeps have therefore occurred since the divergence of these two species and may be the cause of the low variation. Another explanation for the low variation includes our limited sampling missing small segments of the population containing local adaptation. Our current sampling is sparse, with only populations from the Apalachicola National Forest and South Carolina well represented.

This project established a starting point for future work with this species. We now have a known set of functional primers, genes with variation, and extracted DNA from 86 individuals. The original goal was to look at the effect of selection on observed phylogeographic patterns. Due to the time restrictions associated with an undergraduate thesis, the volume of data collected was less than expected. There were a number of steps that slowed the project including: needing to do multiple extractions on many of the blood samples, problems with the sequencing reads, and issues optimizing primers. As with all phylogeographic studies, the development of successful genetic markers was our main rate-limiting step. In the interest of collecting as much data as possible, we decided to run all samples possible for Cytrochrome b and a subset of individuals covering the range for the remaining genes (see Fig 2). While this allowed for the maximum number of genes to be analyzed, it also put a limitation on our conclusions.
This project represents a novel approach to phylogeographic studies. A tissue-specific transcriptome was used to identify functionally diverse genes for analysis. This demonstrates a technique for using genomic approaches on nonmodel species.

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