PHYLOGEOGRAPHY OF THE COTTONMOUTH, *AGKISTRODON PISCIVORUS*, USING AFLP AND VENOM PROTEIN PROFILES

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PHYLOGEOGRAPHY OF THE COTTONMOUTH, *AGKISTRODON PISCIVORUS,*

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ABSTRACT

The objective of this study was to examine population structure in cottonmouths (*Agkistrodon piscivorus*) using Amplified Fragment Length Polymorphism (AFLP) and compare genetic and venom protein profiles in Texas. AFLP profiles using 622 fragments were generated for 105 individuals to understand the level of variation within *Agkistrodon*. In Texas, there was a significant lack of gene flow detected and support for the isolation of Concho Valley individuals. Cottonmouths showed the greatest genetic variation when compared to other *Agkistrodon* species but there was not complete support for two species of cottonmouths as currently proposed. RP-HPLC was used to examine venom protein profiles in 86 Texas cottonmouths. Relative peak heights were analyzed using PCA and the MANOVA demonstrated separation of populations based on profiles ($p<0.001$). Genetic and venom variation did not follow the same pattern indicating that there may be other selection pressures acting on the venom proteins.

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INTRODUCTION

The cottonmouth or water moccasin, *Agkistrodon piscivorus*, is a semi-aquatic pit-viper that occurs in the southeastern United States (Fig. 1). Owing to the large geographic range and observed morphological variation, the cottonmouth has been under systematic scrutiny the past 20 years to determine the number of species and the relationships among species in the *Agkistrodon* clade (Knight et al., 1992; Parkinson et al., 1997; Parkinson et al., 2000). Currently, there are two proposed classifications. The historic view is that there is one species with three subspecies; *Agkistrodon piscivorus piscivorus* (eastern cottonmouth) found in Virginia, North Carolina, South Carolina, northern Georgia, and Alabama, *A. piscivorus leucostoma* (western cottonmouth) found in Texas, Arkansas, Missouri, Louisiana, Mississippi, and Alabama, and *A. piscivorus conanti* (Florida cottonmouth) found in Florida and southern Georgia (Gloyd and Conant, 1990; Knight et al., 1992; Castoe and Parkinson, 2006). Mitochondrial DNA (mtDNA) data indicate that there are two monophyletic lineages, a Florida clade and a continental clade. (Guiher and Burbrink, 2008; Douglas et al., 2009). These two papers suggested that nuclear data be used to determine if there are in fact two species of cottonmouths in the United States.

The cottonmouth's distribution in the United States extends into Texas where it occupies the eastern third of the state. It also inhabits the area along the Colorado River drainage which is what the Concho River connects with in west Texas (Fig. 2). The Concho Valley cottonmouths are a disjunct population with the nearest population being 65 km (40 miles) away over land with no permanent water in between (Werler and Dixon, 2000). The nearest population following the river is on

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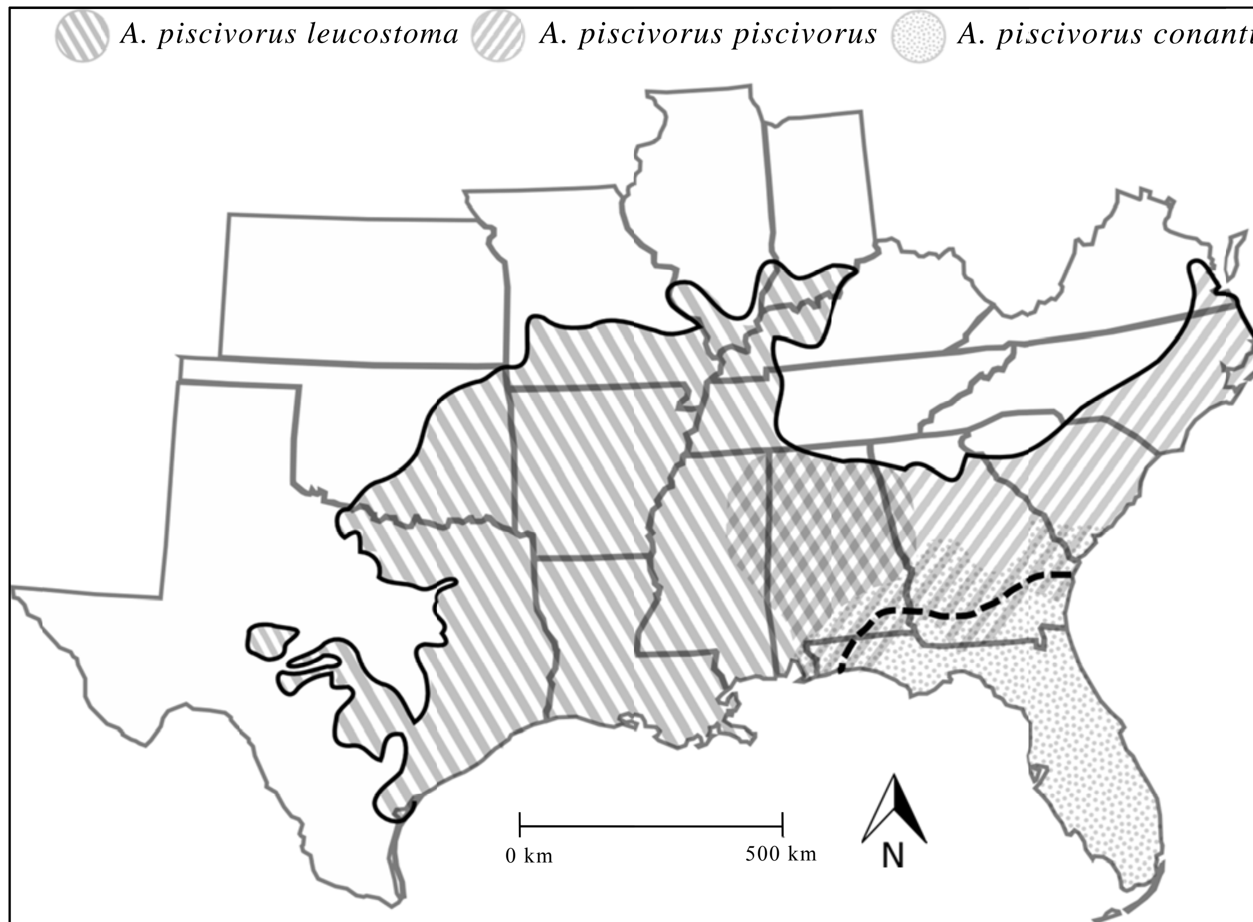

FIG. 1. Distribution map of the cottonmouth, *Agkistrodon piscivorus*, showing the two current views on its taxonomy. The three subspecies are shown as patterns and the proposed species split would be on either side of the dashed line (Follows Werler and Dixon, 2000; Campbell and Lamar, 2004).

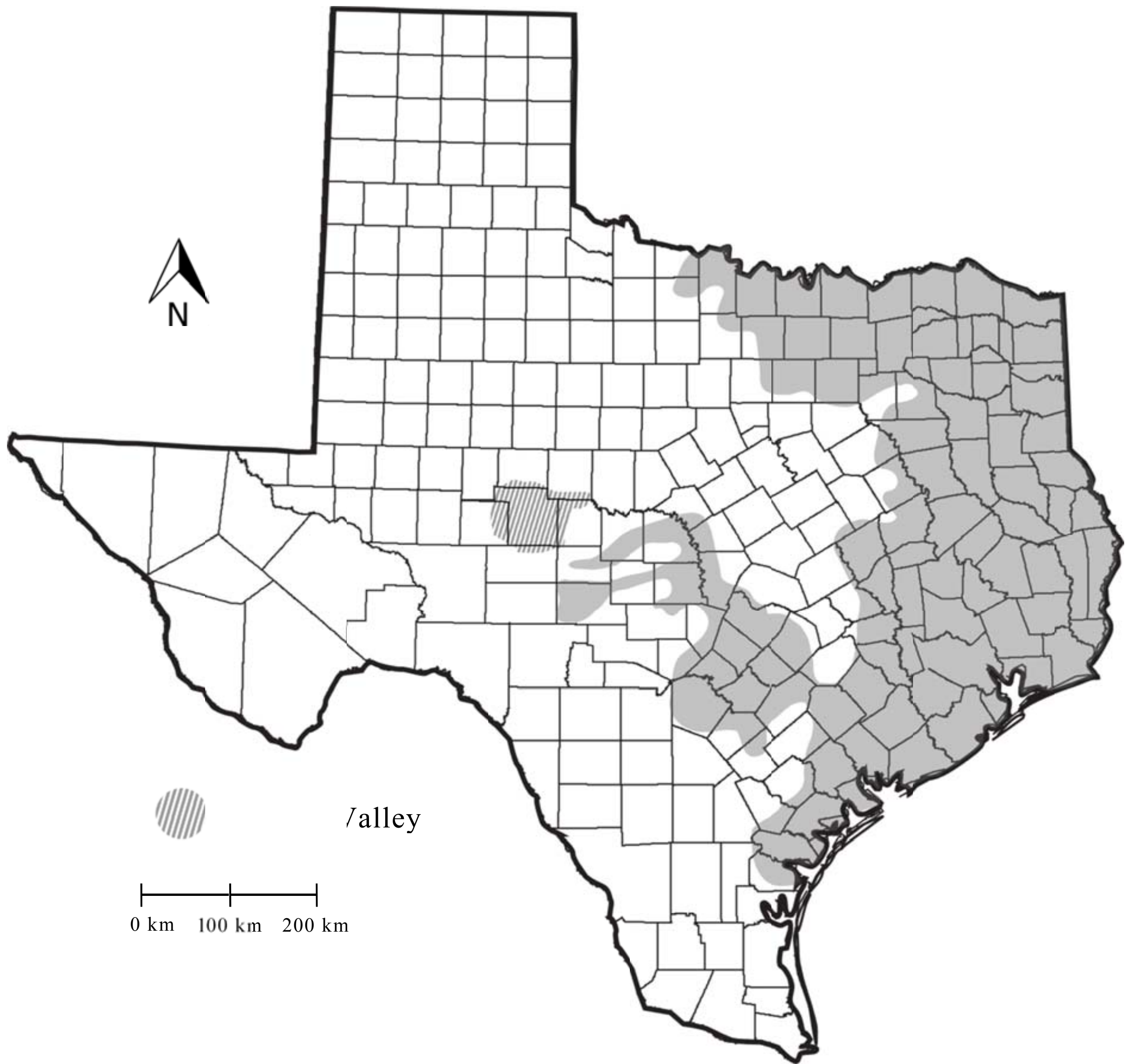

FIG. 2. Texas distribution map of the cottonmouth, *Agkistrodon piscivorus*, showing the disjunct population in the Concho Valley in west-central Texas (Follows Werler and Dixon, 2000).

the Colorado River approximately 230 km (140 miles) away. The average home range of individuals in this species is small enough that gene flow would not be expected between populations unless they were in continuous contact (Roth, 2005). Most of the previous genetic studies on *A. piscivorus* have had few samples from Texas, none have included the western limit in the Concho Valley, and nearly all have used mitochondrial DNA to determine phylogeographic relationships (Knight et al., 1992; Guiher and Burbrink, 2008; Douglas et al., 2009). In my study, two different approaches were used to examine variability in cottonmouths. The first, was Amplified Fragment Length Polymorphism (AFLP) data that made it possible to examine gene flow directly between this presumably isolated population in west Texas and the remainder of individuals in Texas as well as look at population structure in the entire species. The second approach was venom protein variation among populations that was only used to compare populations in Texas.

Genetic variation among the cottonmouths was determined using AFLP which is a predominately nuclear DNA marker. AFLP selectively amplifies certain parts of the genome that can then be compared among snakes to examine gene flow, variation, and the population structure (Vos et al., 1995; Bensch and Akesson, 2005; Meudt and Clarke, 2006). AFLP has been used more often in recent years and, although it is not reliable when examining relationships at larger taxonomic levels (Giannasi et al., 2001; Ogden and Thorpe, 2002; Campbell et al., 2003; Simmons et al., 2007), it is very reliable when comparing organisms at the population and species level (Creer et al., 2004; Mendelson and Simons, 2006; Althoff et al., 2007; Makowsky et al., 2009). This technique has been attempted on three populations of

cottonmouths in Florida with marginal success due to small sample area and a limited number of primer combinations (Roarke, 2003). My study sampled the entire range of the species and used six more primer combinations to avoid the problems from Roarke's study (2003). The advantage of AFLP is that it works by creating an anonymous multilocus DNA profile for each individual based on the fragments that are generated. This means that no prior knowledge is needed to use this technique. The disadvantage is that it is not possible to know where in the genome the fragment comes from or what the sequence is (Vos et al., 1995). AFLP is a reliable and reproducible method that allows profiles to be compared between individuals run at different times as well as in different labs (Savelkoul et al., 1999). It is a cheap and effective way to elucidate relationships among populations and is able to detect subtle structure that may not be picked up using other techniques such as gene sequencing (Bensh and Akesson, 2005).

A technique that has not been applied to cottonmouths is to examine variation in venom proteins and put that variation into context with patterns revealed by molecular data. Snake venom is a highly stable liquid that can handle many different types of treatment and still remain viable as a research tool (Munekiyo and Mackessy, 1998). Venom variation has two main sources: genetic or ecological. Due to the function of venom, studies tend to focus on the ecological side in relation to diet. The venom proteins are coded for genetically, but ecological studies assess how well the venom subdues the prey rather than understanding the proteins individually. A smaller number of studies have looked at interspecific and intraspecific venom variation and how it matches with phylogenetic studies (Daltry

et. al., 1996; Wuster et al., 1999; Fox and Serrano, 2008). Daltry et al. (1996) were able to eliminate all variables except for diet as the cause of venom variation. Several studies have demonstrated geographic variation within a species in venom proteins and profiles (Creer et al., 2003; Alape-Giron et al., 2008). Different species of snakes within a genus also show variation with weak correlation to genetic variation and in some instances may resolve species boundaries such as those seen in vipers in the genus *Bitis* that are found in Africa (Calvete et al., 2007). Few studies have examined venom variation in a species even though it has great medical implications with regards to antivenin production (Gutierrez et al., 2010).

Geographic, ontological, and dietary differences could all play a role in leading to variation in snake venoms among individuals and populations (Chippaux et al., 1991; Sasa, 1999; Eskew et al., 2009; Gibbs and Mackessy, 2009). With the cottonmouth, diet may be very important. They are generalists in their feeding behavior across the range, but smaller populations may be specializing on certain food items such as frogs or mice (Gloyd and Conant, 1990). The venom of pygmy rattlesnakes was shown to be most effective on a single prey item. For populations that fed on multiple prey, venom was not highly effective on a single prey type. Rather, it was sufficient for multiple prey types (Gibbs and Mackessy, 2009). Kanavage et al. (2006) demonstrated that there was variation between individual western diamondback rattlesnakes (*Crotalus atrox*) both geographically and ontologically using reverse-phase high performance liquid chromatography (RP-HPLC). With species as widespread as the cottonmouth or western diamondback, venom variation could play a key role in the effectiveness of antivenin use.

Currently, antivenin is produced by milking snakes, pooling the venom, and then creating the antivenin (Kanavage et al., 2006). If a person was bitten by a snake in Texas, and the antivenin was produced using venom from snakes from Florida there could be problems associated with its use. This would occur if the proteins used to make the antivenin did not match the proteins in the snake bite. The antivenin would not neutralize all of the proteins in the snake bite. This could also be the case if a juvenile snake bit someone and antivenin produced using adult snake venom was used.

Venom is under direct natural selection pressure due to diet and other environmental factors. Because they are proteins, they also have a genetic component (in their expression). The natural selection pressure may be too great to allow there to be a correlation between genetic variation and venom variation. If there is no correlation, then that implies the natural selection forces in the environment, such as diet, are causing the variation in venom and it is not simply lack of gene flow. Venom protein variation was used as another tool to examine the variation in cottonmouths, but only performed within Texas. Variation in venom samples has not previously been quantified in this way. These data were directly compared to the variation exhibited in the AFLP profiles to test the hypothesis that protein variation is congruent with genetic variation.

The first objective of this study included evaluating the proposed elevation of the Florida cottonmouth subspecies to test the hypothesis that they are two distinct species. The second objective was to look at population structure and gene flow in Texas to determine if the Concho Valley population is genetically isolated based on

its current geographic isolation. I tested the hypothesis that there would be little to no gene flow but the level of genetic variation should be similar to other populations in Texas. The final objective was to compare genetic and venom variation in Texas to test the hypothesis that patterns of venom variation match patterns of genetic variation.

MATERIALS AND METHODS

Sample Collection:

Snakes collected include cottonmouths as well as copperheads and rattlesnakes that were used as outgroups for the analysis (Appendix 1). They were collected throughout Texas under permit number SPR-0390-029. In locations in which voucher specimens were allowed, 1-3 specimens were sacrificed and placed in the Angelo State Natural History Collection (ASNHC) at Angelo State University (ASU) in San Angelo, TX once demographic data and tissues were collected. All other snakes were released at the collection location after data, blood, and venom samples were collected. All snakes were handled humanely and carefully to maximize safety for both the collector and the snake. Snakes were initially captured using tongs and then placed in 5-7 gallon buckets for secure transport to a location to handle the snakes and safely collect data. All available and accessible locations in a given area were sampled in the morning and at night to maximize the number of snakes captured. Captured snakes were maintained in a cool dark area to minimize stress.

Demographic data including head length, snout-to-vent length (SVL), tail length, sex, weight, and relative age was collected. Length measurements were determined using a squeeze box (Quinn and Jones, 1974). Weight was determined using a scale and relative age was determined based on yellow coloration on the tail. A snake with yellow under the tail is considered less than three years old (Eskew et al., 2009) and snakes with less than a 45cm SVL were considered juveniles (Ford et al., 2004). The snake was then placed in an acrylic tube and the sex was determined

using a cloacal probe. After this information was collected, approximately 1 cc of blood was taken from the caudal vein using an insulin syringe. This was stored in a modified Tris-EDTA Longmire lysis buffer and placed at -80°C (Longmire et al., 1997). The modifications were that the NaCl was removed and the SDS (Sodium dodecyl sulfate) was increased from 0.5% to 1.0%. To collect venom, the snake was manipulated until its head was outside the tube. The snake's head was placed near a sterile collection cup and the snake was allowed to voluntarily bite and inject venom into the cup. The venom was collected and stored at -80°C. The snake was then either sacrificed using Nembutal and placed in the ASNHC or released back at the site of capture.

To supplement samples from outside of Texas, tissue loans were used. For the analysis, 75 cottonmouths (*Agkistrodon piscivorus*) (Fig. 3), 24 copperheads (*A. contortrix*), 2 Mexican cantils (*A. bilineatus*), 2 Taylor's cantils (*A. taylori*), 1 western diamond-backed rattlesnake (*Crotalus atrox*), and 1 black-tailed rattlesnake (*C. molossus*) were used (Appendix 2). This sample represented all four species within *Agkistrodon* and at least two individuals of all currently recognized subspecies of cottonmouths and copperheads.

Amplified Fragment Length Polymorphism Analysis:

Whole genomic DNA was extracted using a Qiagen DNA extraction kit (Valencia, CA) following the protocol in the kit for blood or tissue samples stored in lysis buffer or 95% ethanol. A 50 µL elution was obtained for each sample and then 5 µL of that was used in a 0.8% agarose gel to determine quality of DNA extracted. For AFLP, the DNA must not be degraded because the process requires the entire

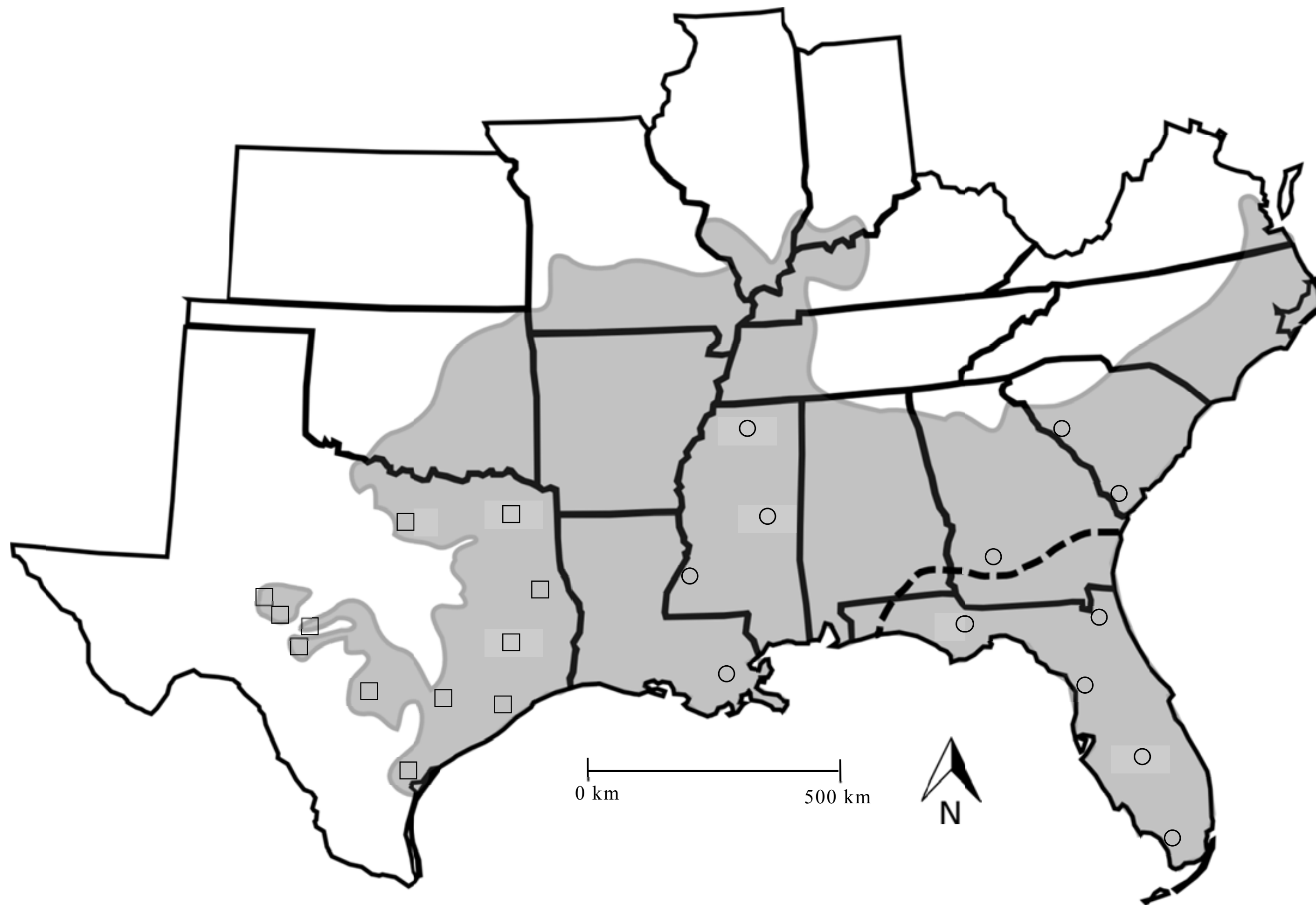

FIG. 3. Map of cottonmouth samples collected in Texas that were used in both the genetic and venom protein analysis shown as squares (□) and those acquired via tissue loan for the genetic analysis only shown as circles(○).

genome to be intact. If there is degradation, the homoplasy risk increases (Robinson and Harris, 1999).

To quantify the amount of DNA extracted, the Quant-iT ds DNA BR assay kit was used (Invitrogen, Carlsbad, California). This made it possible to determine how much DNA would be needed for the AFLP protocol that followed Phillips et al. (2007) based on modifications from Vos et al. (1995) who initially described the method. Restriction enzymes (*EcoRI*, *AseI*, and *TaqI*) were used to digest approximately 200 ng of total genomic DNA into fragments of different lengths. Generally, only two restriction enzymes are used and all primer combinations are created based on those. For this study, there were three restriction enzymes so each sample underwent two separate protocols (*EcoRI* and *AseI*; *EcoRI* and *TaqI*).

For this analysis, there were a total of nine primer combinations used in the cottonmouth analyses and eight used in the analysis with all taxa. Each sample in the analysis had two treatments because there were three total restriction enzymes used. All of them used 20 units of *EcoRI* (New England Biolabs, Ipswich, Massachusetts) which was used as the end to attach the fluorescent label in the final step. Each sample then was subjected to two different treatments. One treatment used 20 units of *AseI* (New England Biolabs, Ipswich, Massachusetts) as the second enzyme and the other treatment used 20 units of *TaqI* (New England Biolabs, Ipswich, Massachusetts) as the second enzyme. For all restriction digestions, 1X enzyme buffer was added to the reaction and the restriction digest was placed at 37 ^{o}C for three hours. Next, 75 pmoles of the appropriate enzyme adapter was ligated to the ends of the fragments that were created by the restriction digest using T4 DNA

Ligase and 4 µL of 10X Ligase Buffer (New England Biolabs, Ipswich, Massachusetts). The reaction was incubated at 16 ºC for 16 hours and then diluted with 160 µL of 10mM Tris HCl which results in the creation of "sticky ends" on the fragments.

The sticky ends provided a region for the PCR (polymerase chain reaction) primers to bind and begin synthesizing the double stranded fragments. The first PCR, called the pre-selective step, decreased the number of fragments because of an additional base pair on the primer. With the additional base, the number of fragments was reduced to approximately 1/16 of those that were initially created in the restriction digest (Meudt and Clarke, 2007). The pre-selective step was conducted by taking 10 µL of the ligation reaction product and combining that with 15 pmoles of each of the pre-selective primers, 1.5 mM $MgCl_2$, 2.5 units of *Taq* DNA polymerase, 1X *Taq* buffer, and 0.8 mM deoxynucleoside triphosphates for a total reaction volume of 50 µL. This reaction was amplified in the thermal cycler (MyCycler, BioRad) which was programmed to go through the following protocol: initial step at 72 ºC for 60 seconds, 20 cycles of amplification (denaturation 94 ºC for 50 seconds, anneal at 56 ºC for 60 seconds and extension at 72 ºC for two minutes) followed by a final extension at 72 ºC for five minutes.

The second PCR, the selective step, lowered the number of fragments even more depending on how many bases were added to the primer. This step also attached a fluorescent dye onto each fragment for detection by the Beckman-Coulter CEQ 800 Genetic Analysis System (Beckman-Coulter, Inc., Fullerton, California). Before this step, 10 µL of the pre-selective reaction was diluted into10mM Tris HCl.

For the selective PCR, a 25 μL reaction volume was used that contained 5 pmoles of each of the selective primers, 1.5mM $MgCl_2$, 1.25 units of *Taq* DNA polymerase, 1X *Taq* buffer, 1.2 mM deoxynucleoside triphosphates and 5 μL of the diluted pre-selective reaction. This was amplified in the thermal cycler (MyCycler, BioRad) which was programmed to go through the following protocol: initial step at 72 ºC for 60 seconds, one cycle of amplification (denaturation 94 ºC for 50 seconds, anneal at 65 ºC for 60 seconds and extension at 72 ºC for two minutes) followed by 12 touchdown cycles just as the first except that the annealing temperature was lowered by 0.7 ºC each time. After this, there were 23 cycles of amplification (denaturation 94 ºC for 50 seconds, anneal at 56 ºC for 60 seconds and extension at 72 ºC for two minutes) followed by a final extension at 72 ºC for five minutes.

Restriction enzymes and primer combinations are listed in Table 1. A total of nine combinations were used to yield a large number of fragments to give an accurate measure of polymorphic loci in the genome. Bonin et al. (2007) suggested that at least 200 total fragments be used in an AFLP analysis to be able to show population structure. In essence, the more fragments that are scored, the higher the resolution and the better statistical support there should be for the analyses (Albertson et al., 1999; Ogden and Thorpe, 2002; Bensch and Akesson, 2005). The fragments in the selective PCR reactions were separated by loading 0.8 μL of the reaction with 0.25 μL of 400 base pair (bp) size standard in the CEQ8000 (Beckman-Coulter, Inc., Fullerton, California). The fragments were scored as present (1) or absent (0) using the software available on the CEQ8000. Once the initial scoring was complete,

TABLE 1. List of restriction enzymes, adapters, pre-selective primer, and selective primer sequences for the PCR used in the AFLP analysis of cottonmouths and outgroup taxa. Asterisk (*) indicates the primer with the fluorescent label attached. Primers used in combination with *EcoRI*-CAC are indicated with † and those used with *EcoRI*-CAT are indicated by ‡. *TaqI*-TTG was only used in the cottonmouth analysis.

Name	Sequence
Restriction Enzymes	
EcoRI	5'-…G|AATC…-3'
AseI	5'-…AT|TAAT-3'
TaqI	5'-…T|CGA…-3'
Adapters	
EcoRI	5'-CTCGTAGACTGCGTACC-3'
	3'-CATCTGACGCATGGTTAA-5'
AseI	5'-GACGATGAGTCCTGA-3'
	3'-TACTCAGGACTCAT-5'
TaqI	5'-CGGTCAGGACTCAT-3'
	3'-AGTCCTGAGTAGCAG-5'
Pre-Selective Primers	
EcoRI	5'-ACTGCGTACCAATTCC-3'
AseI	5'-GATGAGTCCTGAGTAATT-3'
TaqI	5'-ATGAGTCCTGACCGAT-3'
Selective Primers	
EcoRI*-CAC	5'-ACTGCGTACCAATTCCAC-3'
EcoRI*-CAT	5'-ACTGCGTACCAATTCCAT-3'
***AseI*-TAG†**	5'-GATGAGTCCTGAGTAATTAG-3'
***AseI*-TCC†**	5'-GATGAGTCCTGAGTAATTCC-3'
***AseI*-TGA†**	5'-GATGAGTCCTGAGTAATTGA-3'
***AseI*-TGC‡**	5'-GATGAGTCCTGAGTAATTGC-3'
***AseI*-TCT‡**	5'-GATGAGTCCTGAGTAATTCT-3'
***AseI*-TAT‡**	5'-GATGAGTCCTGAGTAATTAT-3'
***TaqI*-TCA‡**	5'-ATGAGTCCTGACCGATCA-3'
***TaqI*-TTC‡**	5'-ATGAGTCCTGACCGATTC-3'
***TaqI*-TTG‡**	5'-ATGAGTCCTGACCGATTG-3'

fragments were evaluated by eye to ensure proper scoring of fragments. Any fragments that were scored inconsistently or were too close to other fragments were removed from the analysis leaving only unambiguous fragments. All individuals were scored in a random order to minimize the bias in the results (Bonin et al., 2005; Bonin et al., 2007).

Population Genetic Analysis:

The binary matrix created for the fragments and individuals was imported into Microsoft Excel© (Appendix 3). The program GenAlEx ver. 6.41 was used to statistically analyze the data and visualize population structure (Peakall and Smouse, 2006). GenAlEx initially created a genetic distance matrix based on Nei-Li distances from the binary matrix (Appendix 4) (Nei and Li, 1979). Both inter- and intraspecific Nei-Li genetic distances were calculated for all four species of *Agkistrodon*. That information was used in Principal Coordinate Analysis (PCoA) to visualize the population divergence. This analysis does not require groups be assigned before the test (*a priori*) and makes it possible to examine relationships in either two dimensional or three dimensional space depending on how many eigen vectors are used. PCoA was performed on all samples, then only cottonmouths, and finally on populations of cottonmouths from Texas to visualize the pattern at each level. The genetic distance matrix was also analyzed via Analysis of Molecular Variance (AMOVA). The AMOVA made it possible to compare variation between the populations to variation within the populations to determine if there was population differentiation based on the AFLP profiles for each snake.

For binary data, Φ_{pt} was calculated which is analogous to F_{st}. Both are measures of genetic differentiation between populations (Andrade et al., 2007). The Φ_{pt} values were calculated based on 1,000 replicates (α=0.05). This gave a statistical measure of gene flow among the populations and made it possible to examine the variation in *Agkistrodon*. The data matrix was then formatted for the program STRUCTURE ver. 2.3.3 (Pritchard et al., 2000; Falush et al.,2003; Falush et al., 2007) using the program AFLP-SURV ver. 1.0 (Vekemans, 2002). STRUCTURE estimated the highest degree of genetic structure between the populations (Lee et al., 2010). STRUCTURE calculated the number of populations (K) that were in the entire sample based on the genetic distance. For the STRUCTURE analysis, the admixture model was used with a burn in of 30,000 followed by 100,000 iterations. This was done for K values of 1-10 with 10 replications at each K value. The resulting log likelihood scores were averaged for each K. The admixture model was chosen because there was not any reason to assume gene flow was limited in these populations. If there was gene flow, STRUCTURE results should show the proportions of each individual's genome that was shared with each of K populations. This meant that if STRUCTURE estimated that there were two populations, then you would see the proportion of markers that had their ancestry in each of the two populations. If there was no gene flow, there should not be individuals that have mixed markers. All markers will come from one of the proposed populations. With the log likelihood scores, I determined ΔK and then used that to find the true number of groups, K* (Evanno et al., 2005). The final test used to examine the population structure in all cottonmouths sampled was the Isolation by Distance (IBD) Mantel

test. This is a pairwise test that looks for correlation between the genetic matrix created in GenAlEx and a geographic distance matrix created based on the straight line distance between the points (Jensen et al., 2005).

Phylogenetic Analysis:

The binary matrix was imported into PAUP* (phylogenetic analysis using parsimony) ver. 4.0b10 (Swofford, 2001) to create a neighbor joining phylogram using the Nei-Li genetic distances (Saitou and Nei, 1987). The two rattlesnakes were used as outgroup taxa and the trees were statistically tested with 1,000 bootstrap pseudoreplicates. Parsimony methods were not used because they are not appropriate for binary AFLP data according to Robinson and Harris (1999) and Sullivan et al., (2004). For AFLP analysis, bands may be lost independently in more than one lineage and could result in poorly resolved trees if parsimony is used (Dasmahapatra et al., 2009). Moreover, analysis of discreet characters could result in the situation where a few markers determine the phylogenetic pattern whereas the neighbor-joining analysis takes into account overall similarity (Dasmahapatra et al., 2009).

Venom methods:

Venom protein variation was determined using reverse-phase high performance liquid chromatography (RP-HPLC). This technique uses a short column packed with beads that have a long carbon chain attached. The long carbon chains bind the proteins as they pass through the column. A gradient of methanol and water was used to break the bonds between the proteins and beads. As the percentage of methanol in solution goes up, more and more proteins are washed through the column and detected by a spectrophotometer (Kanavage et al., 2006). Using this

technique, it was possible to obtain a venom profile of absorbance peaks for each individual to compare among and within populations.

The venom samples were diluted into a 90% water/10% methanol solution to match starting conditions of the gradient. Trifluoroacetic acid (TFA) was added to lower the pH of the samples to prevent degradation of the column. Ten µL of venom was diluted into 1 mL of solution. Venom samples were filtered through Spin X centrifuge tubes (VWR, Radnor, PA) to remove any cell fragments or anything else that could clog the column. The samples were then placed in an HPLC auto sampler vial and loaded into the auto-sampler. Each sample ran through the HPLC machine (Waters Corp., Milford, MA) and separation column (SUPELCO SUPELCOSIL™ LC-18 Column 50x4.6mm 3µm, Sigma-Aldrich, St. Louis, MO) following the gradient (Table 2) with each sample giving absorbance readings at 210 nm and 280 nm. The absorbance at 280 nm was used for the analysis. Each sample took approximately 60 minutes to run through the HPLC machine. There were 86 venom samples used and all of them came from cottonmouths from Texas. Duplicates were run for several individuals during the sampling as well as afterward to make sure results were repeatable. Samples that were collected over time from the same individual were also run to make sure there was not large variation in one individual's venom over time.

The resulting venom profiles were analyzed using the Breeze v.3.30 software published by Waters Corp. (Milford, MA) because it produced data that could be exported and used for statistical analysis. The relative peak heights were compared

TABLE 2. Gradient method used in the RP-HPLC of 86 cottonmouth venom samples in the SUPELCO C18 HPLC column. Each step is a linear gradient (Curve 6) in which the percentage of water and the percentage of methanol change the same amount for each unit of time.

Step	Time (min)	Flow Rate (mL/min)	Water %	Methanol %	Curve
	0	1	90	10	
1	1	1	90	10	6
2	3	1	75	35	6
3	33	1	35	75	6
4	37	1	10	90	6
5	47	1	10	90	6
6	50	1	90	10	6
7	60	1	90	10	6

via Principal Component Analysis (PCA) which does not need *a priori* groups defined using SYSTAT 12.0 (SYSTAT software, Inc., San Jose, California). The first two principal components were then graphed to visualize the venom variation pattern in Texas. MANOVA was used to statistically test the population means to determine the possibility of differentiating populations based on venom samples. This type of analysis to compare snake venom samples has not been done before.

RESULTS

AFLP Results:

There were eight primer combinations used to create 622 AFLP fragments for all taxa. Of these, 498 (80%) were polymorphic and between 59 and 102 fragments were scored from each primer combination (Table 3). These fragments were used in GenAlEx which generated a PCoA for all 105 individuals used. The first three axes explained 83.7% of the variation (Fig. 4). In this PCoA, the two rattlesnake species were pulled out of the *Agkistrodon* plane based on the third axis. The cottonmouths had the largest number of polymorphic loci (44.5%) which can be visualized by the amount of spread in the cottonmouth cluster. The copperheads (32.53%) and Texas cottonmouths (31.36%) had a similar level of polymorphism as indicated by the similarity in shape of their clusters on the PCoA. Because there were only two samples from each of the two cantil species, it was not possible to determine the amount of variation seen in each of those species.

For the neighbor-joining analysis, branches recovered in more than 50% of bootstrap pseudoreplicates are shown (Fig. 5). Branches with over 70% support were considered to be significantly supported (Felsenstein 1985; Hillis and Bull, 1993). This analysis was able to identify the species level relationships as currently understood, but there was little resolution within any of the species. When Nei-Li genetic distances were calculated, cottonmouths had the highest amount of intraspecific variation (6.4%) based on the Nei-Li genetic distances and the two cantil species were the closest in genetic distance (Table 4).

TABLE 3. Number of fragments scored and the percentage of polymorphic loci for each of the eight primers used for all samples in the analysis including outgroup taxa.

Primer Combination	# of Fragments Scored	% of Fragments Polymorphic
EcoRI-CAC/*AseI*-TAG	87	86%
EcoRI-CAC/*AseI*-TCC	60	80%
EcoRI-CAC/*AseI*-TGA	87	85%
EcoRI-CAT/*AseI*-TGC	62	79%
EcoRI-CAT/*AseI*-TCT	101	87%
EcoRI-CAT/*AseI*-TAT	102	65%
EcoRI-CAT/*TaqI*-TCA	64	78%
EcoRI-CAT/*TaqI*-TTC	59	83%

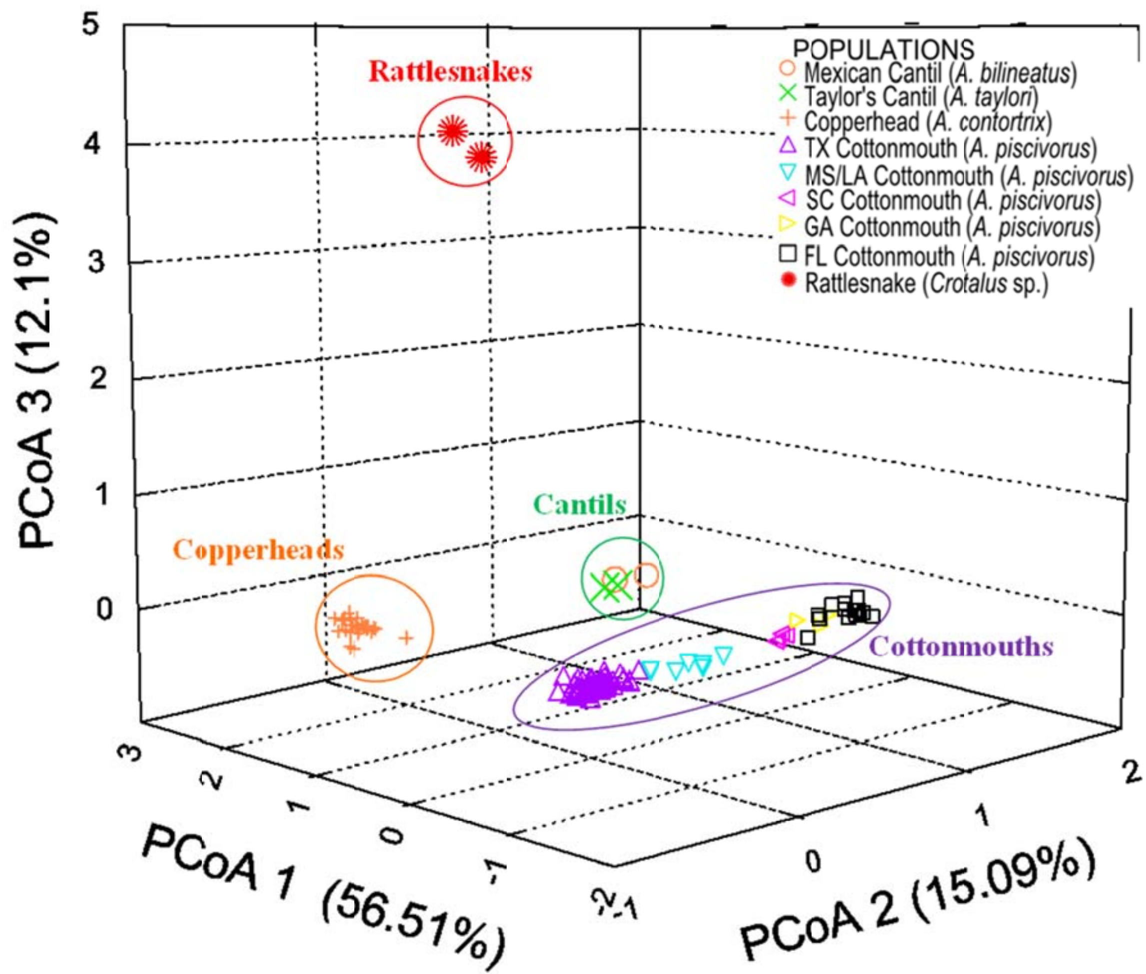

FIG. 4. Three-dimensional PCoA of all samples used in the analysis. Rattlesnakes fall out on the third axis away from the four species within *Agkistrodon*.

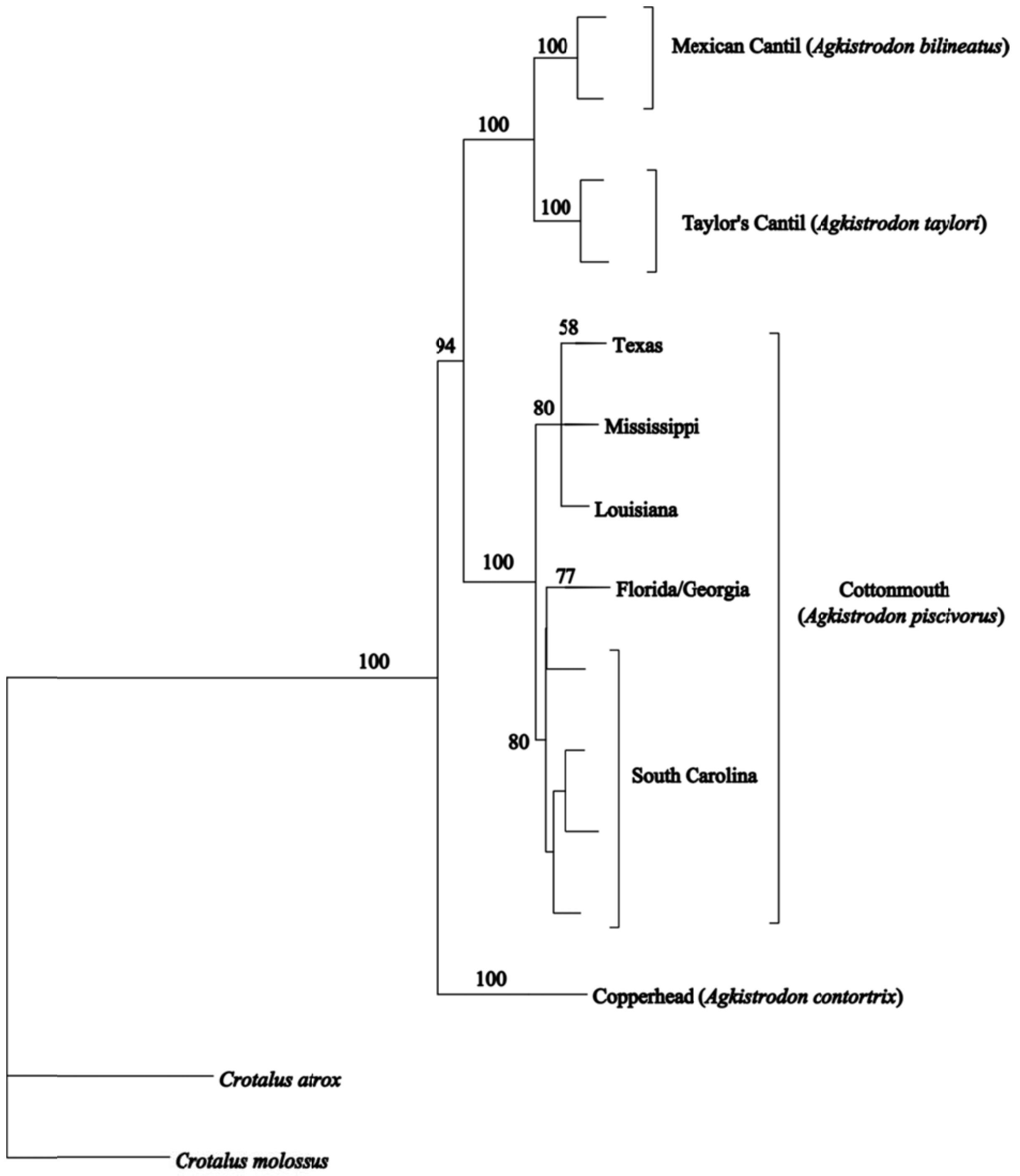

FIG. 5. Neighbor-joining phylogram of all samples (n=105) using the AFLP data with terminal branches condensed. One thousand bootstrap pseudoreplicates were performed and those with support over 50 percent are shown.

TABLE 4. Average Nei-Li genetic distances within and between all four species of *Agkistrodon* based on the AFLP data.

	A. piscivorus	***A. contortrix***	***A. taylori***	***A. bilineatus***
A. piscivorus	6.4%			
A. contortrix	17.8%	5.1%		
A. taylori	17.6%	22.1%	3.9%	
A. bilineatus	18.3%	22%	9.5%	5.1%

After outgroups were removed, nine primer combinations were used and all cottonmouths were analyzed. There were 481 fragments used in the analysis with 44.7% of them (215) polymorphic (Table 5). The average population level of polymorphism was 10.07% ± 1.42%. The AMOVA indicated that there was a significant lack of gene flow (Φ_{pt}=0.466, p<0.001). For this PCoA, only the first two axes were used (73.34% variation explained) but it did not show any clustering and there was an east to west pattern for all cottonmouths (Fig. 6). The Mantel Isolation by Distance test showed that genetic distance was correlated with geographic distance (r^2=0.731, r=0.8551, p<0.0001; Fig. 7). Using STRUCTURE, a value of K=2 was determined for the number of groups within cottonmouths. There appeared to be a geographic cline based on the pattern observed in the STRUCTURE output (Fig. 8). Samples from the middle of the distribution had some proportion of their genes estimated to be from both of the populations. The neighbor-joining tree did not support the two groups of cottonmouths proposed in the previous studies, but there is support for two groups of cottonmouths -- an Eastern and a Western clade (Fig. 5).

For the final analysis, only cottonmouths from Texas were used. Once again, all nine primer combinations were used and it yielded 440 total fragments with 31.36% polymorphic (138) (Table 6). The average population level of polymorphism was 8.23% ± 0.95%. The AMOVA analysis indicated significant lack of gene flow between populations (Φ_{pt}=0.348, p<0.001). The PCoA indicated that there was a cluster of individuals from the Concho Valley that was separated from the other populations. Individuals from the Llano River in Junction, Texas (Kimble Co.) were

TABLE 5. Number of fragments scored and the percentage of polymorphic loci for each of the nine primers used for all cottonmouth samples in the analysis.

Primer Combination	# of Fragments Scored	% of Fragments Polymorphic
***EcoRI*-CAC/*AseI*-TAG**	56	55%
***EcoRI*-CAC/*AseI*-TCC**	32	28%
***EcoRI*-CAC/*AseI*-TGA**	54	54%
***EcoRI*-CAT/*AseI*-TGC**	39	31%
***EcoRI*-CAT/*AseI*-TCT**	76	47%
***EcoRI*-CAT/*AseI*-TAT**	76	16%
***EcoRI*-CAT/*TaqI*-TCA**	44	41%
***EcoRI*-CAT/*TaqI*-TTC**	45	62%
***EcoRI*-CAT/*TaqI*-TTG**	58	66%

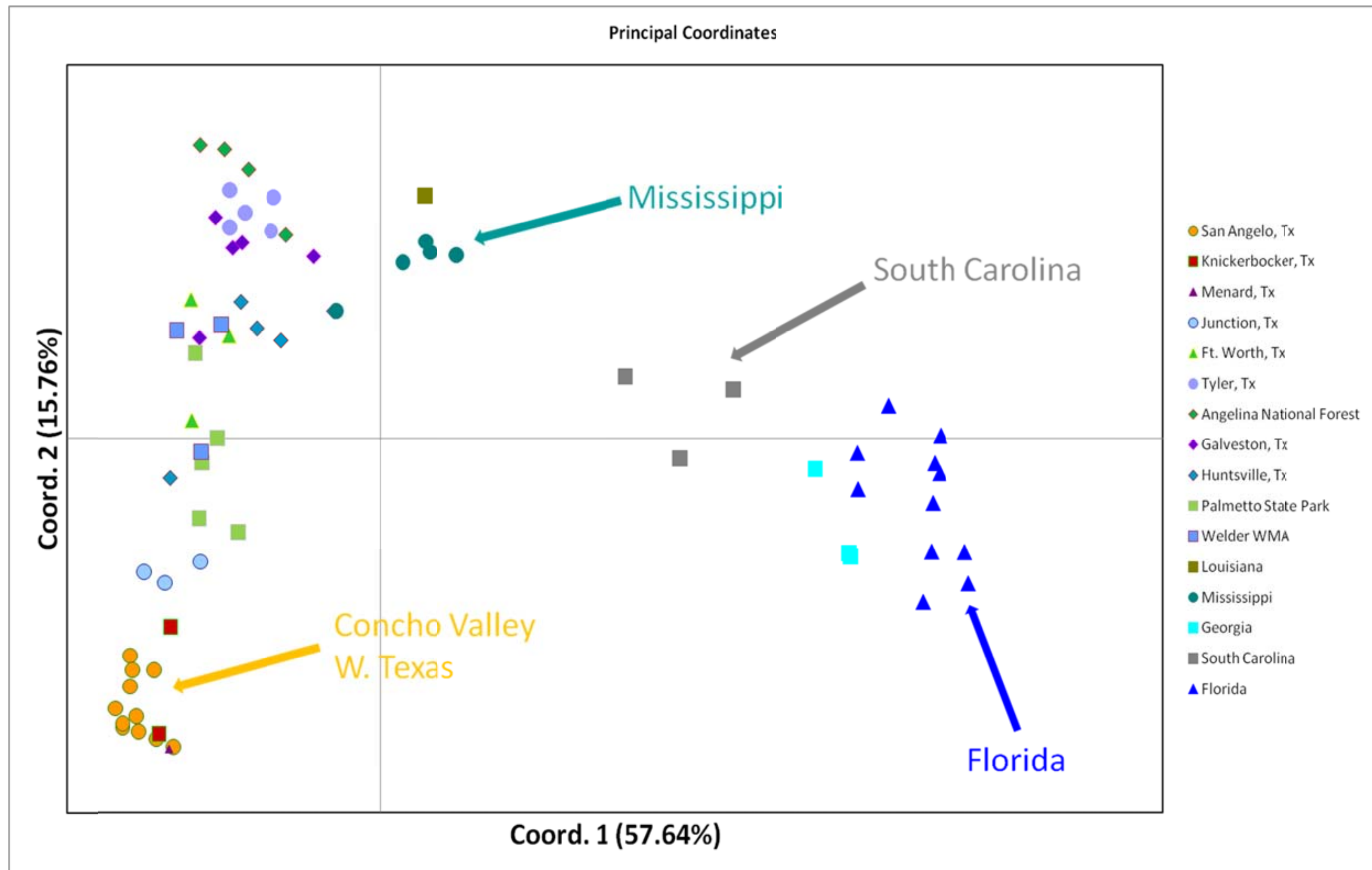

FIG. 6. Two-dimensional PCoA of all cottonmouths from their entire range based on AFLP data. The first two axes explain 73.40% of the variation and the pattern indicates a west to east trend in genetic variation.

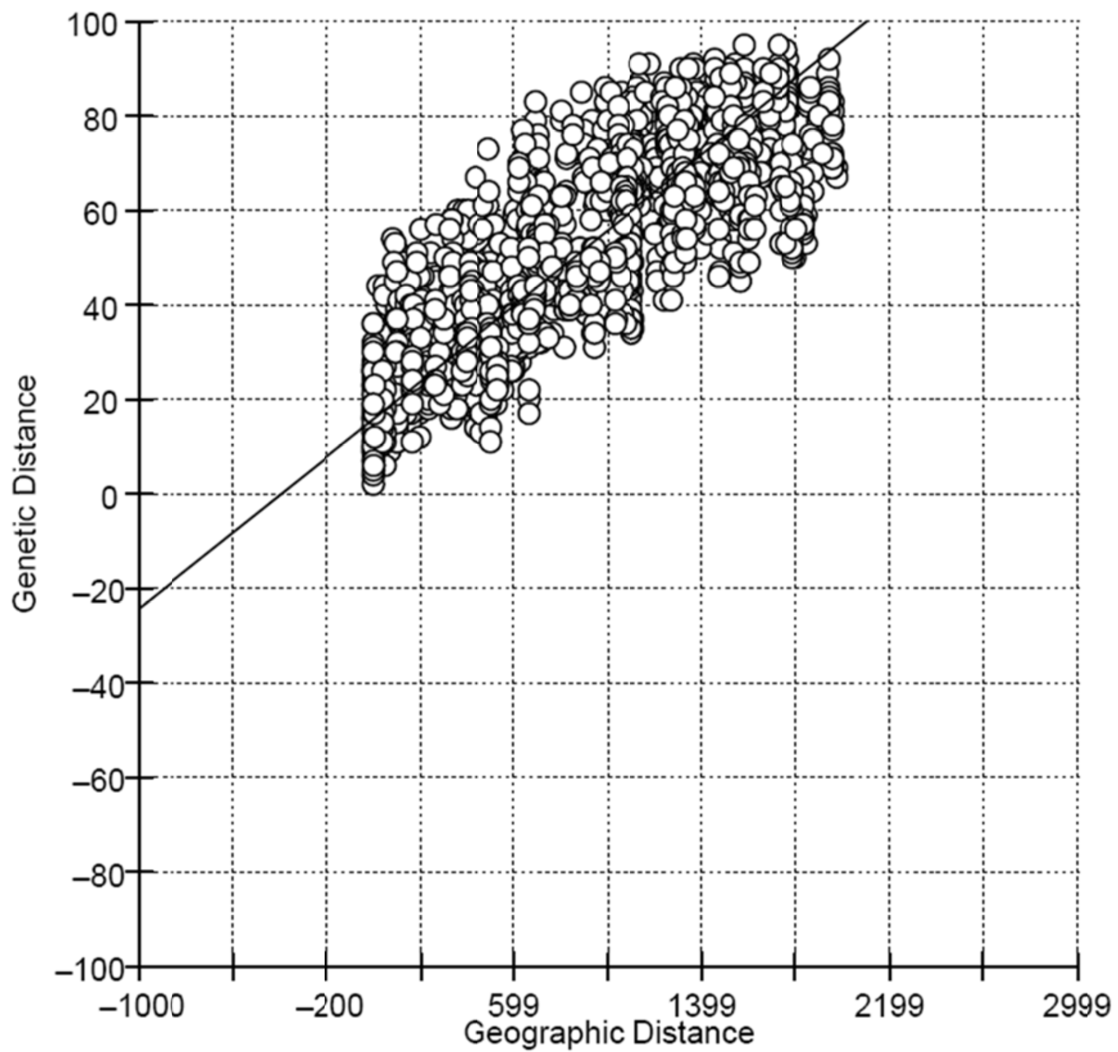

FIG. 7. Isolation by Distance Mantel test using the geographic and genetic pairwise distances for the cottonmouth range indicating significant correlation ($r=0.8551$, $r^2=0.731$, $P<0.0001$). This indicates that as geographic distance increases, so does genetic distance (Jensen et al., 2005).

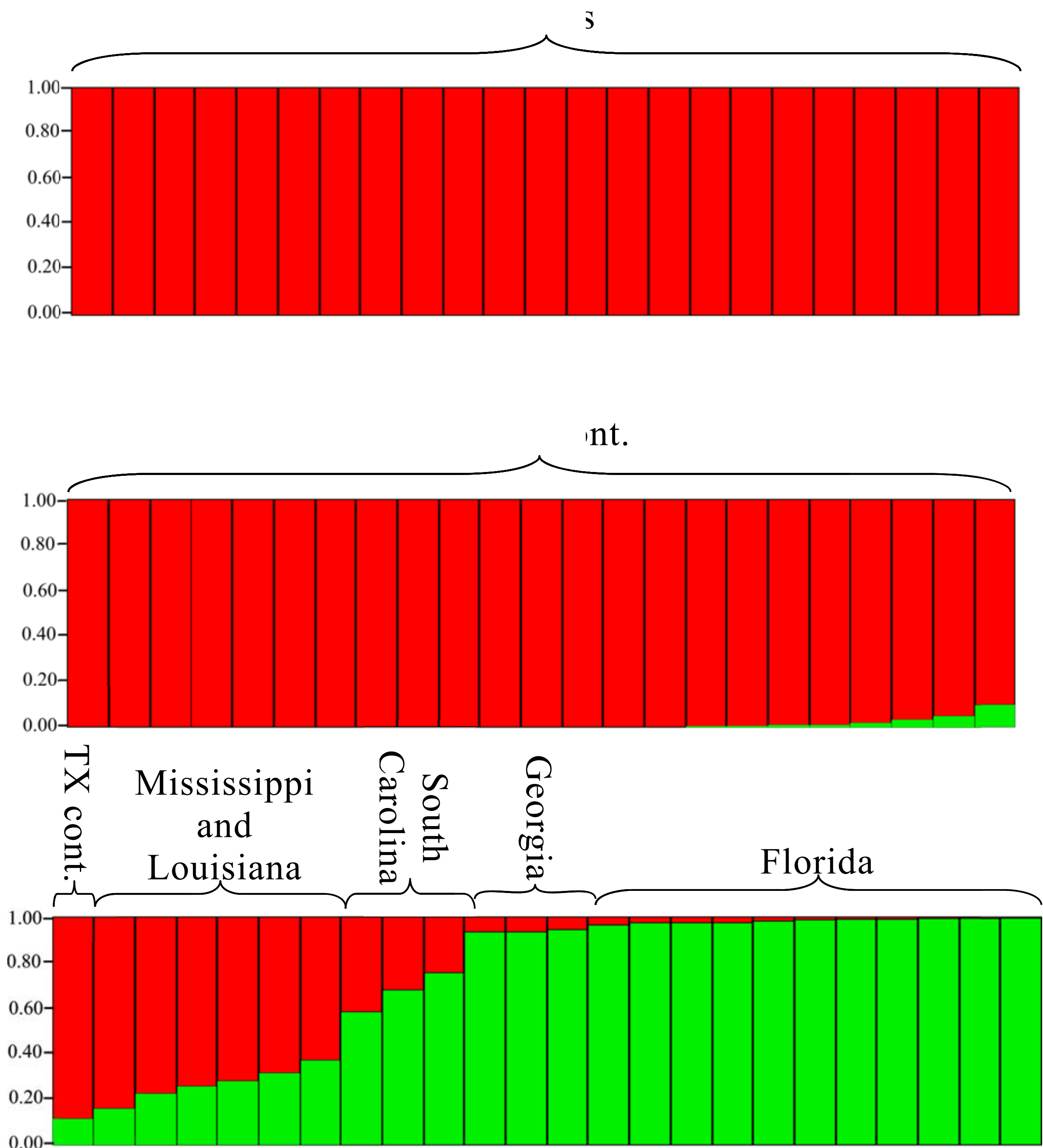

FIG. 8. Posterior mean estimates of the proportion of each individual's genome that belongs to each of the two estimated populations from STRUCTURE. An individual is represented by a single bar and all of these individuals were run in a single analysis using STRUCTURE.

TABLE 6. Number of fragments scored and the percentage of polymorphic loci for each of the nine primers used for only Texas cottonmouth samples.

Primer Combination	**# of Fragments Scored**	**% of Fragments Polymorphic**
***EcoRI*-CAC/*AseI*-TAG**	47	32%
***EcoRI*-CAC/*AseI*-TCC**	31	19%
***EcoRI*-CAC/*AseI*-TGA**	47	45%
***EcoRI*-CAT/*AseI*-TGC**	35	23%
***EcoRI*-CAT/*AseI*-TCT**	72	36%
***EcoRI*-CAT/*AseI*-TAT**	73	11%
***EcoRI*-CAT/*TaqI*-TCA**	42	31%
***EcoRI*-CAT/*TaqI*-TTC**	41	41%
***EcoRI*-CAT/*TaqI*-TTG**	52	46%

also isolated (Fig. 9). STRUCTURE analysis indicated that there were 4 groups. One of the groups was comprised of solely the Concho Valley individuals and the other three groups were split up in east Texas. All groups had mixing of genes from other populations (Fig. 10). The neighbor-joining analysis did not detect any splits in Texas that would indicate separate populations (Fig. 5).

Venom Protein RP-HPLC Results:

For the 86 cottonmouth venom samples used (Appendix 1), there were up to 18 peaks scored for a single individual. All samples had the initial two large peaks and then contained between 9 and 16 additional peaks. Using the PCA scores for each sample, they were plotted on a graph with the 75% confidence interval around the mean (Fig. 11). Using the mean for each population, they were compared using three test statistics (Wilks's Lambda, Pillai's Trace, Hotelling-Lawley Trace) and all indicated that the populations were significantly different from each other (p=0.000). Overall, there was not a pattern visible in the PCA and the first two axes only explained 45.59% of the variation.

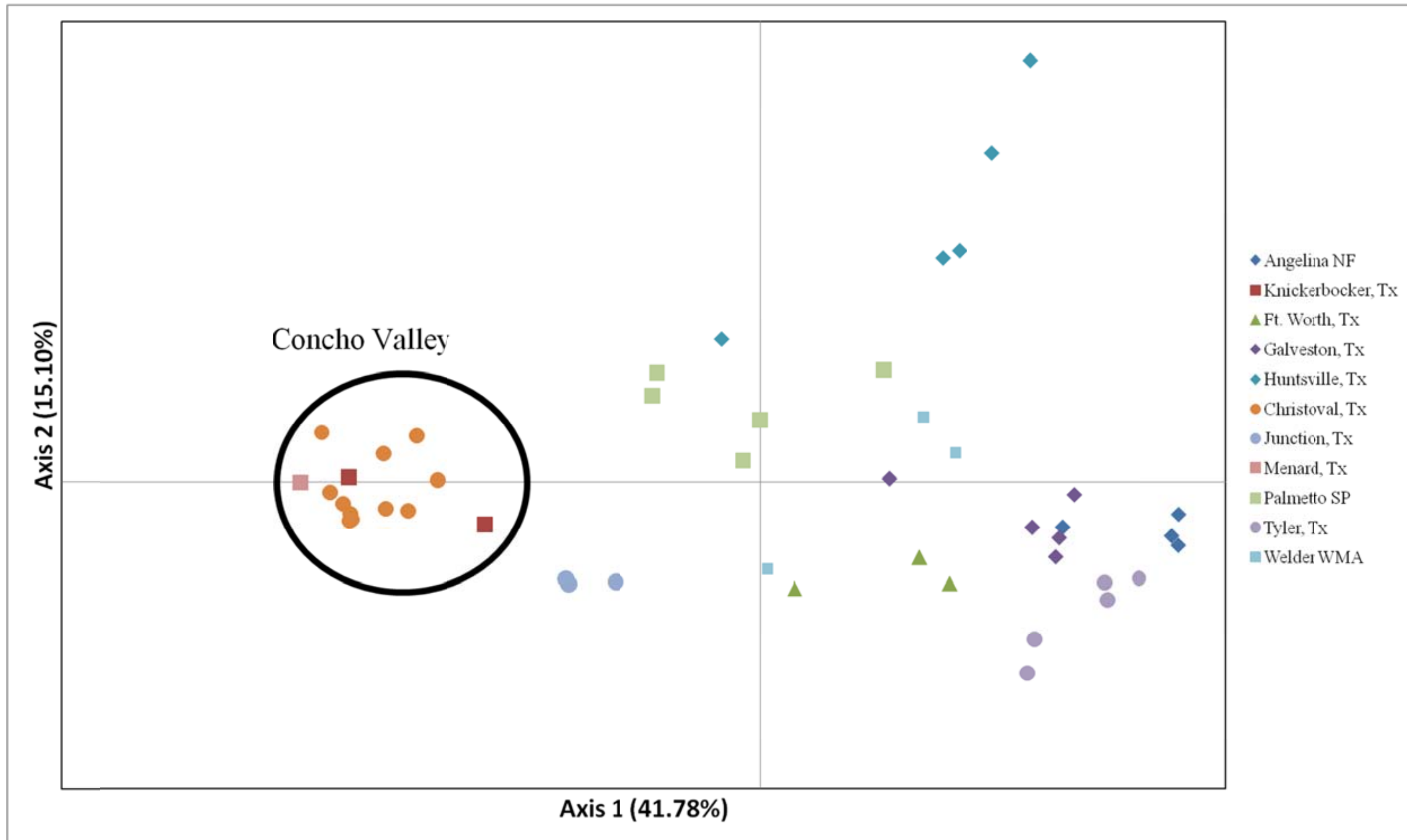

FIG. 9. Two-dimensional PCoA of only Texas cottonmouths based on AFLP data. The first two axes explain 56.88% of the variation and the pattern indicates that the Concho Valley population is less genetically variable than other populations in Texas.

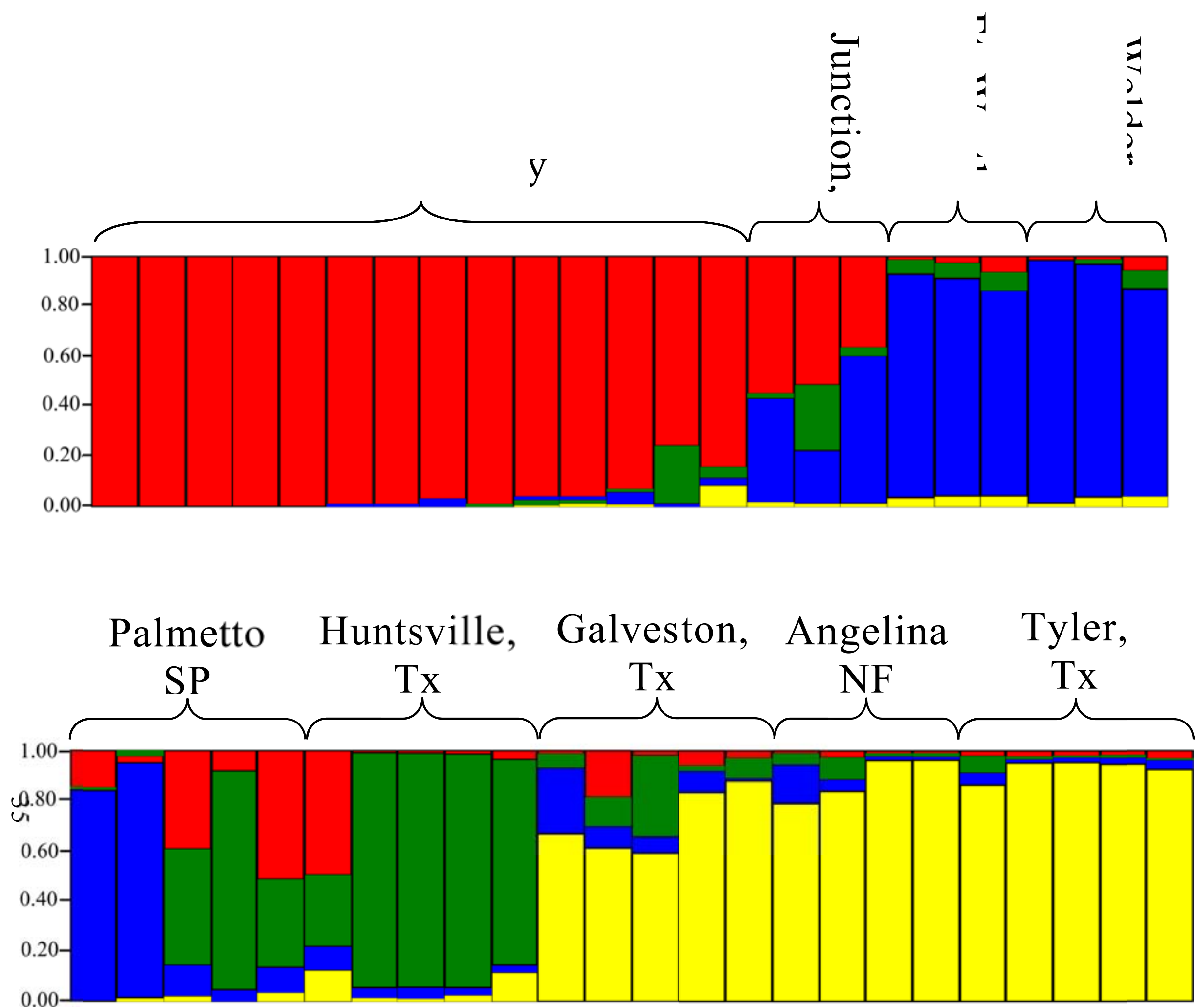

FIG. 10. Posterior mean estimates of the proportion of each individual's genome that belongs to each of the four estimated populations in Texas from STRUCTURE. Each bar represents an individual and all are from one structure analysis. SP indicates State Park, NF indicates National Forest, and WMA indicates Wildlife Management Area.

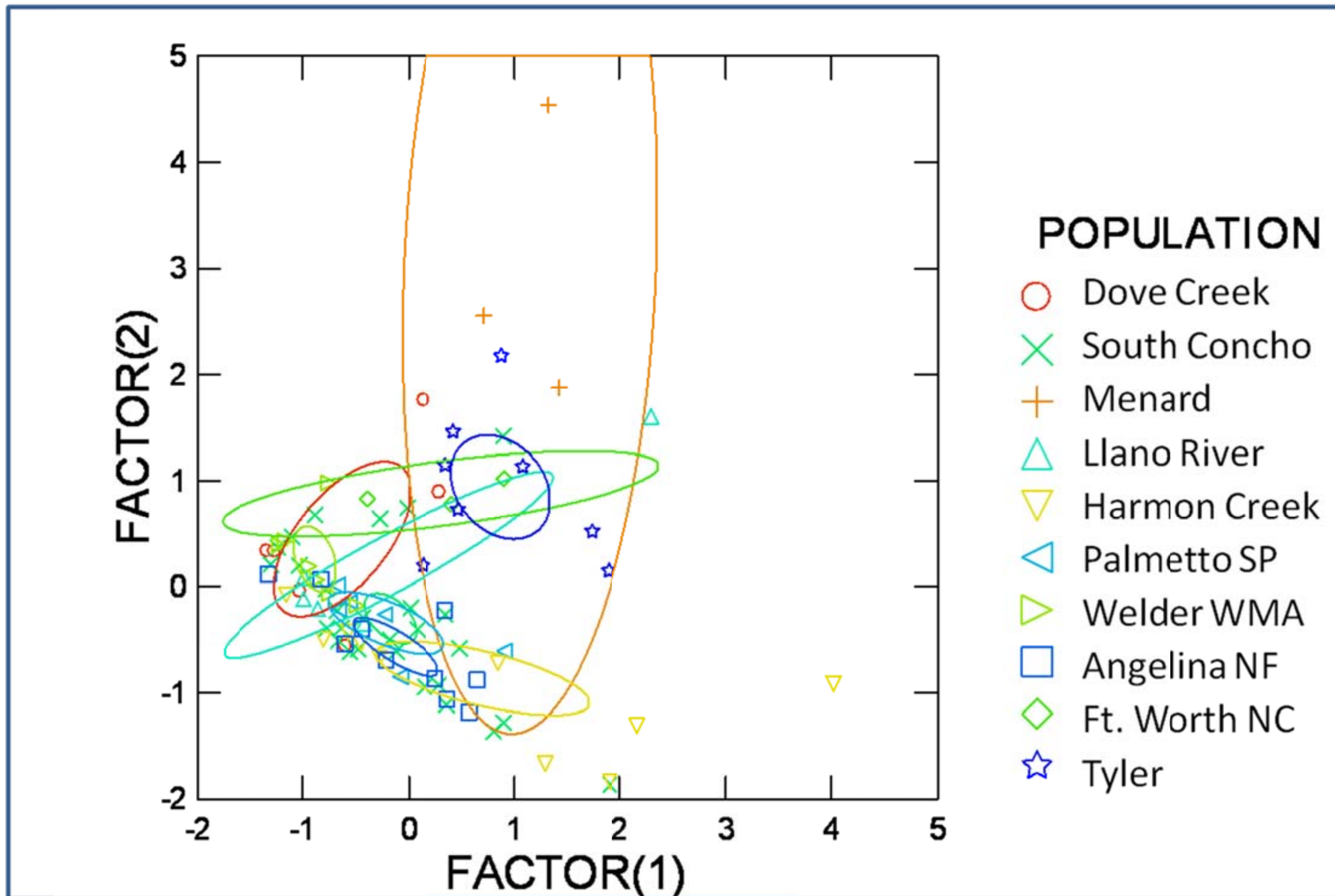

FIG. 11. First two principal components of 86 Texas cottonmouth venom samples based on relative peak heights from RP-HPLC analysis. The first two axes explain 45.59% of the variation. Ellipses are 75% confidence intervals on the mean for each population that was compared via MANOVA.

DISCUSSION

The AFLP data generated an overall pattern that is consistent with the current understanding of *Agkistrodon*. The PCoA (Fig. 4) reveals that the rattlesnakes fall out on a third axis relative to the four currently recognized species of *Agkistrodon*. Because it is not possible to statistically test a PCoA, a neighbor-joining phylogram was created. The species relationships were consistent with those of Parkinson et al. (2000). There was significant support for the monophyly of *Agkistrodon* as well as support for each of the four species currently recognized within *Agkistrodon*. The hypothesized relationships were confirmed with copperheads falling separately from the cottonmouth and cantil sister relationship. The neighbor-joining tree also showed that cantils and copperheads were monophyletic and that there was significant support for the two species of cantils. Within cottonmouths, there was significant support for two groups. There was a clade that included the Texas, Mississippi, and Louisiana individuals and a clade that included the Florida, Georgia, and South Carolina individuals. Within the second clade, there was also significant support for the Georgia and Florida individuals as a monophyletic group.

There does not seem to be enough evidence from this study to conclude that there are two distinct cottonmouth species as currently proposed (Douglas et al., 2009; Guiher and Burbrink, 2008). The two previous studies proposed that Florida cottonmouths were separate from the rest of the cottonmouths in the United States. The results from the AFLP data suggest an east to west split with the Florida individuals falling out with those that are on the East coast. There was also no evidence for three subspecies of cottonmouths based on the groupings returned by

STRUCTURE (Fig. 8) and the support in the neighbor-joining tree (Fig. 5). The Mantel test showed a significant correlation with geographic and genetic distance meaning that cottonmouths show isolation by distance (Fig. 7). Because the cottonmouths had this pattern, STRUCTURE should be interpreted with caution (Pritchard et al., 2000). STRUCTURE has a tendency to return a greater number of populations than what are actually present when the species shows isolation by distance (Frantz et al. 2009). STRUCTURE and the K* calculations both indicated two populations (Evanno et al. 2005). Based on the value of two populations, STRUCTURE was used to look at the genes shared between the population for the two populations it predicted (Falush et al., 2003). This indicated that markers were being shared from Florida to Texas (Fig. 8). The markers from the East coast found in Texas only just made it into the eastern side of Texas and markers from Texas found in the eastern populations made it into Florida but did not make it all the way throughout Florida. This suggests considerable gene flow across the range and does not support separation of the cottonmouth into two species. If STRUCTURE did overestimate the number of populations, this would yield the same interpretation. Therefore, the STRUCTURE analysis is useful as an interpretive tool even though it is possible that it could be predicting more populations than what are actually present.

The PCoA for all cottonmouth samples also showed an East to West pattern in population structure (Fig. 9). There is also somewhat of a gap visible when looking at the distribution of cottonmouths in the PCoA (Figs. 4 and 9). This could mean that there are two groups within cottonmouths, but that seems unlikely given

the overall pattern of gene flow predicted by STRUCTURE and the fact that there are not any samples from the center of the distribution of cottonmouths where the gap appears to be. The pattern seen in the PCoA was the same relationship seen in the neighbor-joining analysis. There was significant support for two groups within cottonmouths (Fig. 5). There are several possibilities for the incongruence in the data and the difficulties in interpreting the results; one of the main ones being a lack of samples from each area of the distribution. Another possibility is that the current biogeographical hypothesis is not correct in explaining what actually happened since the last glaciation event.

Douglas et al. (2009) proposed that the cottonmouth/cantil ancestor rafted to northern Florida 3-4 million years ago. After this, it colonized southern Florida and during interglacial periods, it moved into the southeastern United States. During periods of glaciation, Florida served as a refuge for cottonmouths that was stable over long periods of time and allowed for the eventual speciation event that is proposed based on their mtDNA data. Douglas et al. (2009) did not assert that there are two distinct species, but found some support for the consideration of the Florida subspecies as a distinct species. Guiher and Burbrink (2008) also used mtDNA sequence and found similar results. North Carolina individuals fell out closer to individuals from Texas than from Florida in their parsimony tree. They also concluded that the Florida cottonmouth might be a separate species but did not have enough evidence to elevate it. The phylogenetic trees presented in these two papers were consistent with one another, but are not consistent with my findings.

The inconsistencies may have been a result of the samples collected for cottonmouths. The samples in my study did not cover the entire range and may not have sampled enough of the areas that are needed to clearly understand the population structure of cottonmouths. In Fig. 3, there are areas where samples were not collected that could be very important in understanding the level of gene flow in cottonmouths. The areas that are not adequately sampled can be found in the middle of the distribution (Alabama) and at the northeastern edge of the distribution (North Carolina and Virginia). In the PCoA for all cottonmouths, the gap located in the center may correspond with Alabama. If individuals from that area were added, they might fill the gap in both the PCoA analysis and the neighbor-joining tree. The results based on AFLP do not support the relationships based on the mtDNA data (Guiher and Burbrink, 2008; Douglas et al. 2009). When compared to the other members of the genus, the cottonmouth does show the highest level of genetic variation but it follows a geographic gradient as indicated by the Mantel test. In my study, the individuals from South Carolina were in the same group as the individuals from Florida and Georgia whereas in the previous studies they fell out with individuals from Texas and Mississippi. It is possible that the proposed species boundary could be farther north than presented in either Guiher and Burbrink, (2008) or Douglas et al. (2009) and would actually include the samples from South Carolina used in my study. The separation seen in my study based on AFLP does show evidence for an alternative biogeographic hypothesis than what was proposed by Dougles et al. (2009).

If at any point in the cottonmouth's history it was separated into geographic isolates, it does not appear that that split was between Florida and the rest of the United States. Considering the history proposed by Douglas et al. (2009), it seems possible that Texas could have also served as a refuge for cottonmouths during the glacial periods along with Florida. If this happened, the results from my AFLP study would be consistent with the expansion of the two refuge populations (Texas and Florida) of cottonmouth into the southeastern United States that came into secondary contact with each other resulting in the currently continuous distribution. Given the exchange of markers in the STRUCTURE results (Fig. 8), it appears that the time that they were separated was not sufficient to cause genetic or reproductive isolation. Once they came into contact, genes were shared and have moved across the range. Enough time may not have passed for the markers to make it into the eastern and western ends of the distribution. Based on the large integrade zone proposed by Gloyd and Conant (1990) and seen in Fig. 1, this would be a possible scenario. The neighbor-joining phylogram and the significant Φ_{pt} value from the AMOVA may be reflecting the historic separation whereas the PCoA and the STRUCTURE results are demonstrating the exchange of genes that has occurred since they have come back into contact. Overall, there does not appear to be enough evidence from the AFLP data to consider the Florida cottonmouth (*A. piscivorus conanti*) as a separate species of cottonmouth. Further samples need to be collected and other analyses such as nuclear gene sequencing need to be used to test the proposed history of the cottonmouth in the United States.

In regard to gene flow and genetic variation of cottonmouths in Texas, the objective of the study was met. The population in the Concho Valley is isolated and exhibits less genetic variation than any other population in the state. The majority of the other populations that were sampled in Texas were from a much smaller geographic area than the area sampled in the Concho Valley but still had higher levels of genetic variation based on the visualization of populations in the PCoA (Fig. 9). The Concho Valley population is on the western limit of the range and is geographically isolated. Eckert et al. (2008) analyzed data from 134 studies and showed that 60-70% of plant and animal species had lower genetic variability at the periphery of the species' distribution, especially when isolated.

The pattern seen in the PCoA (Fig. 9) suggests either a founder event or bottleneck occurred in the Concho Valley as opposed to solely a fragmentation event. If it would have been strictly a fragmentation event, then the variation seen in the Concho Valley should be similar to that seen in other populations. A founder event would show the pattern seen in the PCoA because a few individuals with a limited gene pool would have invaded the Concho River system and then colonized the area. This would show low genetic variation over a relatively large geographic area. The bottleneck could have occurred in conjunction with a fragmentation event. Historically, there were at least eight springs south of the headwater springs of the South Concho River (Brune, 1975). These springs would have connected the Concho Valley with the San Saba River near Menard, Texas (Menard Co.). Given the close proximity of the one individual from the headwaters of the San Saba to those in the Concho Valley in the PCoA (Fig. 9), it is possible that the drying period over the

last 200 years has slowly shrunk the western population of cottonmouths. Now all that remain are those isolated in the Concho Valley and the population at the head of the San Saba River.

The STRUCTURE results showed there were 4 groups within Texas, one of which was the Concho Valley population. The other three groups represent geographic areas in Texas. There are more shared characters between the three eastern groups than in the Concho Valley population (Fig. 10). This, along with the significant Φ_{pt} value indicates that the population has recently become genetically isolated. To understand the history of cottonmouths in the Concho Valley, a more thorough sample will be needed from areas directly surrounding the Concho Valley. This would make it possible to determine if cottonmouths entered the area from the south when there were other springs that extended the South Concho River or if they entered from the Colorado River and traveled upstream into the Concho River system.

The results of the analysis of the venom samples from Texas were not consistent with the genetic variation as expected. At best, it was possible to differentiate populations based on venom samples. Beyond that result, no pattern was discernable other than the lack of any pattern at all. Because only Texas samples were used in the study, it is not possible to draw conclusions for the entire species. However, it appears that there is not much venom variability in cottonmouths in Texas. There are several possible explanations for this. First, venom is under direct natural selection pressures based on diet which restricts variability (Daltry et al., 1996; Wuster et al.1999; Fox and Serrano, 2008).

Cottonmouths are generalist feeders and have been known to eat mice, fish, lizards, and frogs. Based on the dietary breadth of cottonmouths, the venom may have to be less specialized (Gibbs and Mackessy, 2009). It would be in the snake's best interest to have venom that is effective on several prey types as opposed to one type. This could explain the lack of variation in Texas.

A second possible reason for low venom variability that is somewhat related to the first is that the type of proteins required in a venom sample may be similar (Chippaux et al., 1991). Venom samples contain many enzymes in them, particularly proteases. If a venom sample does not have "housekeeping" proteins contained within it, the proteins may degrade each other causing the venom to be ineffective in neutralizing a prey item. This would force the venom samples to be similar.

The final possibility for little variation seen is that this method is not useful for examining venom variation. As mentioned previously, this method of comparing relative peak heights has not been used to try to examine variation in venom samples before. Each peak in the profile could potentially be many proteins causing there to be low resolution in trying to compare entire profiles at once. Trying to then compare variation to genetic variation has added complications. For instance, AFLP markers fall predominately in areas that are non-coding areas where there are not any genes (Cooper and Hausman, 2007). This is due to the fact that there are much more non-coding portions of the genome than there are coding portions. Venom proteins are coded for by genes that the AFLP markers are not likely measuring. Accurately comparing venom and genetic variation would require sequencing the genes that are coding for the proteins in the venom as well as the amino acid

sequence of each of the proteins and then measuring variation in that manner. The previous methods of looking at a particular protein or enzyme activities may be better suited for examining variation in a species (Alape-Giron et al., 2008). Further analyses on other species using this technique will be needed to evaluate its usefulness in comparing venom samples before it is possible to put the variation into a phylogenetic context.

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APPENDIX I

Specimens collected in Texas. An * indicates samples used in the AFLP analysis. ASK numbers are associated with the tissue vouchers and ASNHC numbers are the specimen vouchers. An † indicates samples used in the venom analysis.

ASK	Snake ID	ASNHC	Species	County	UTM	Block	Northing	Easting
9044*	JLS 11†	14264	*Agkistrodon piscivorus*	Tom Green	14	R	3459114	342280
	JLS 12†	14265	*Agkistrodon piscivorus*	Tom Green	14	R	3459114	342280
9045	JLS 13†		*Agkistrodon piscivorus*	Walker	15	R	3404871	263071
9046	JLS 14†		*Agkistrodon piscivorus*	Walker	15	R	3404871	263071
9047	JLS 16		*Agkistrodon piscivorus*	Walker	15	R	3404871	263071
9048*	JLS 17†		*Agkistrodon piscivorus*	Walker	15	R	3404871	263071
9049*	JLS 18†		*Agkistrodon piscivorus*	Walker	15	R	3404871	263071
9050*	JLS 19†		*Agkistrodon piscivorus*	Walker	15	R	3404871	263071
9051	JLS 20†		*Agkistrodon piscivorus*	Walker	15	R	3404871	263071
9052	JLS 21†		*Agkistrodon piscivorus*	Walker	15	R	3404871	263071
9053*	JLS 22†		*Agkistrodon piscivorus*	Walker	15	R	3404871	263071
9054	JLS 23†		*Agkistrodon piscivorus*	Walker	15	R	3404871	263071
9055*	JLS 24†		*Agkistrodon piscivorus*	Walker	15	R	3404871	263071
9056*	JLS 25		*Agkistrodon contortrix*	Walker	15	R	3404871	263071
9057	JLS 26		*Agkistrodon contortrix*	Walker	15	R	3404871	263071
9058*	JLS 27		*Agkistrodon contortrix*	Walker	15	R	3404871	263071
9059*	JLS 28		*Agkistrodon contortrix*	Walker	15	R	3404871	263071
9060	JLS 29		*Agkistrodon contortrix*	Walker	15	R	3404871	263071
9061*	JLS 30		*Agkistrodon contortrix*	Walker	15	R	3404871	263071
9191	JLS 31	14266	*Agkistrodon piscivorus*	Irion	14	R	3455779	338146
9191	JLS 32†	14267	*Agkistrodon piscivorus*	Irion	14	R	3456435	339203
9062*	JLS 33†		*Agkistrodon piscivorus*	Gonzales	14	R	3273919	637144
9063*	JLS 34†		*Agkistrodon piscivorus*	Gonzales	14	R	3273543	637158
9064	JLS 35†	14281	*Agkistrodon piscivorus*	Gonzales	14	R	3273528	637455
9065*	JLS 36†		*Agkistrodon piscivorus*	Gonzales	14	R	3773919	637144
9066*	JLS 37†	14284	*Agkistrodon piscivorus*	Gonzales	14	R	3773919	637144
9067*	JLS 38†	14286	*Agkistrodon piscivorus*	Gonzales	14	R	3273993	636759
9068*	JLS 39†	14279	*Agkistrodon piscivorus*	San Patricio	14	R	3111944	658172
9069	JLS 40†	14282	*Agkistrodon piscivorus*	San Patricio	14	R	3111695	663663
9070	JLS 41†		*Agkistrodon piscivorus*	San Patricio	14	R	3111695	663663
9071*	JLS 42†		*Agkistrodon piscivorus*	San Patricio	14	R	3111695	663663
9072*	JLS 43†		*Agkistrodon piscivorus*	San Patricio	14	R	3111695	663663
9074	JLS 44†	14280	*Agkistrodon piscivorus*	San Patricio	14	R	3111695	663663
9075	JLS 45†		*Agkistrodon piscivorus*	San Patricio	14	R	3111695	663663
9193	JLS 46		*Agkistrodon piscivorus*	Aransas	14	R	3129134	708193
	JLS 47	14273	*Agkistrodon contortrix*	Angelina	15	R	3442848	374178
9076	JLS 48†		*Agkistrodon piscivorus*	Angelina	15	R	3433297	374318

9077*	JLS 49	14274	*Agkistrodon contortrix*	Angelina	15	R	3442848	374178
9078	JLS 50		*Agkistrodon contortrix*	Jasper	15	R	3433297	374318
9079	JLS 51†		*Agkistrodon piscivorus*	Jasper	15	R	3433297	374318
9080	JLS 52†		*Agkistrodon piscivorus*	Jasper	15	R	3433297	374318
9081	JLS 53†	14283	*Agkistrodon piscivorus*	Jasper	15	R	3433297	374318
9082*	JLS 54†		*Agkistrodon piscivorus*	Angelina	15	R	3440919	374830
9083*	JLS 55†	14276	*Agkistrodon piscivorus*	Angelina	15	R	3440432	377952
9084*	JLS 56†		*Agkistrodon piscivorus*	Jasper	15	R	3436807	378353
9085*	JLS 57†		*Agkistrodon piscivorus*	San Augustine	15	R	3473297	381483
9086	JLS 58†		*Agkistrodon piscivorus*	San Augustine	15	R	3471690	381420
9087	JLS 59		*Agkistrodon contortrix*	Jasper	15	R	3433297	374318
9088*	JLS 60		*Agkistrodon contortrix*	Angelina	15	R	3440426	377324
9089	JLS 61†	14285	*Agkistrodon piscivorus*	Jasper	15	R	3434758	377857
9090	JLS 62†		*Agkistrodon piscivorus*	Kimble	14	R	3373641	427083
9091*	JLS 63†		*Agkistrodon piscivorus*	Kimble	14	R	3373641	427083
9092*	JLS 64†		*Agkistrodon piscivorus*	Kimble	14	R	3373641	427083
9093*	JLS 65†		*Agkistrodon piscivorus*	Kimble	14	R	3373641	427083
9094	JLS 66†		*Agkistrodon piscivorus*	Kimble	14	R	3373641	427083
9095*	JLS 67†		*Agkistrodon piscivorus*	Smith	15	S	3570656	294259
9096	JLS 68†		*Agkistrodon piscivorus*	Smith	15	S	3570656	294259
9097	JLS 69†		*Agkistrodon piscivorus*	Smith	15	S	3608400	280324
9098*	JLS 70†		*Agkistrodon piscivorus*	Smith	15	S	3608521	280410
9099	JLS 71†		*Agkistrodon piscivorus*	Smith	15	S	3609224	279945
9194	JLS 72		*Agkistrodon contortrix*	Smith	15	S	3603907	283090
9100*	JLS 73	14272	*Agkistrodon contortrix*	Smith	15	S	3609143	281189
9101*	JLS 74†	14275	*Agkistrodon piscivorus*	Smith	15	S	3608525	280398
9102	JLS 75†		*Agkistrodon piscivorus*	Smith	15	S	3570306	293924
9103*	JLS 76†		*Agkistrodon piscivorus*	Smith	15	S	3570831	292801
9104	JLS 77†		*Agkistrodon piscivorus*	Smith	15	S	3607603	276345
9105*	JLS 78		*Agkistrodon piscivorus*	Smith	15	S	3607603	276345
9106	JLS 79		*Agkistrodon piscivorus*	Smith	15	S	3607603	276345
9107	JLS 80†		*Agkistrodon piscivorus*	Tarrant	14	S	3635934	640930
9108*	JLS 81†		*Agkistrodon piscivorus*	Tarrant	14	S	3635934	640930
9109*	JLS 82†		*Agkistrodon piscivorus*	Tarrant	14	S	3635934	640930
9110*	JLS 83†		*Agkistrodon piscivorus*	Tarrant	14	S	3635934	640930
9112*	JLS 85		*Agkistrodon contortrix*	Pecos	13	R	3420696	780558
9113	JLS 86		*Agkistrodon contortrix*	Pecos	13	R	3420696	780558
9114	JLS 87		*Agkistrodon contortrix*	Pecos	13	R	3420696	780558
9115	JLS 88		*Agkistrodon contortrix*	Pecos	13	R	3420696	780558
9116*	JLS 89		*Agkistrodon contortrix*	Pecos	13	R	3420696	780558
9117*	JLS 90		*Crotalus atrox*	Pecos	13	R	3420696	780558
9118*	JLS 91		*Crotalus molossus*	Pecos	13	R	3420696	780558
9119*	JLS 92		*Agkistrodon contortrix*	Brewster	13	R	3257619	644841
9121*	JLS 94†		*Agkistrodon piscivorus*	Tom Green	14	R	3458882	341789
9122	JLS 95		*Agkistrodon contortrix*	Jeff Davis	13	R	3377969	611799

9123*	JLS 96	*Agkistrodon contortrix*	Jeff Davis	13	R	3413020	612494
9124*	JLS 97	*Agkistrodon contortrix*	Brown			No GPS Locality	
9125	JLS 98†	*Agkistrodon piscivorus*	Menard	14	R	3411854	394058
9126	JLS 99†	*Agkistrodon piscivorus*	Menard	14	R	3411854	394058
9127*	JLS 100	*Agkistrodon piscivorus*	Menard	14	R	3411854	394058
9128	JLS 101†	*Agkistrodon piscivorus*	Menard	14	R	3411854	394058
9129	JLS 102†	*Agkistrodon piscivorus*	Tom Green	14	R	3458882	341789
9073	JLS 103†	*Agkistrodon piscivorus*	Tom Green	14	R	3467218	344135
9130	JLS 104	*Crotalus lepidus*	Brewster	13	R	3265451	706748
9131*	PIT 114938716A†	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9132	PIT 114409631A	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9133	PIT 114625792A†	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9134	PIT 114625691A†	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9135	PIT 114626164A†	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9137*	PIT 115222097A	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9141	PIT 114973520A†	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9147	PIT 114625256A	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9150*	PIT 114979652A	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9170	PIT 115235247A	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9146	PIT 115313251A	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9149	PIT 114956610A	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9145*	PIT 114952455A†	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9144*	PIT 115317467A†	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9143*	PIT 114967277A	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9142	PIT 114966190A†	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
9145	PIT 114625445A†	*Agkistrodon piscivorus*	Tom Green	14	R	3446925	357057
N/A	PIT 114969127A†	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9157	PIT 114619730A†	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9158	PIT 114624595A†	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9154*	PIT 114616122A†	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9159	PIT 114629526A	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9166	PIT 115136320A	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9153	PIT 113932567A	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9169*	PIT 115322477A†	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9167	PIT 115221756A†	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9162	PIT 114945673A	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9163	PIT 114948663A†	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9156	PIT 114617670A	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9164*	PIT 114949391A†	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9152*	PIT 114954121A†	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544
9161*	PIT 114633364A†	*Agkistrodon piscivorus*	Tom Green	14	R	3445671	357544

APPENDIX II

Samples received via tissue loan, tissue purchases, or individuals borrowed from their owner. An * indicates samples used in the analysis, an ‡ indicates samples that were too degraded to use for AFLP analysis, and an † indicates a sample that did not work properly during the AFLP procedure. JLS is my field abbreviation, CLP indicates tissue loaned by Christopher Parkinson at the University of Central Florida, MF indicates tissue loaned from Michael Forstner from Texas State University, KW indicates tissue loaned by Ken Wray from Florida State University, BG indicates samples collected by Brian Greene at Missouri State University, NCSM indicates samples loaned from North Carolina Museum of Natural Sciences, TNHC indicates those loaned from the Texas Natural History Collection, LSUMZ indicates tissues loaned from Louisiana State University Museum of Natural Science, and samples with no abbreviation were purchased from the Venom Research Center.

Specimen ID	Species	Country	State	County	UTM	Northing	Easting
JLS 84*	*Agkistrodon taylori*	Mexico	Tamaulipas			No GPS Location	
CLP 140*	*Agkistrodon taylori*	Mexico	Tamaulipas			No GPS Location	
CLP 159*	*Agkistrodon piscivorus*	USA	FL	Collier	17R	2867258	468888
CLP 160†	*Agkistrodon piscivorus*	USA	FL	Collier	17R	2864020	473753
CLP 984*	*Agkistrodon piscivorus*	USA	GA	Grady	16R	3433116	773608
CLP 986*	*Agkistrodon piscivorus*	USA	GA	Thomas	17R	3426148	228246
CLP 989*	*Agkistrodon piscivorus*	USA	GA	Grady	16R	3433116	773608
MF 2931†	*Agkistrodon piscivorus*	USA	FL	Collier	17R	2892830	465527
MF 3943†	*Agkistrodon piscivorus*	USA	TX	Franklin	15S	3685245	301641
MF 17541†	*Agkistrodon piscivorus*	USA	TX	Concho	14R	3488009	401969
011-310-839*	*Agkistrodon contortrix*	USA	TX	Tarrant		No GPS Location	
058-843-771*	*Agkistrodon contortrix*	USA	TX	Colorado		No GPS Location	
058-557-565*	*Agkistrodon contortrix*	USA	KY	Wolf		No GPS Location	
058-594-037*	*Agkistrodon contortrix*	USA	MO	Cole		No GPS Location	

058-375-116*	*Agkistrodon contortrix*	USA	MO	Boone		No GPS Location	
011-367-560*	*Agkistrodon contortrix*	USA	TX	Midland		No GPS Location	
058-625-027†	*Agkistrodon contortrix*	USA	TX	Pecos		No GPS Location	
011-311-367*	*Agkistrodon piscivorus*	USA	FL	N/A		No GPS Location	
010-820-563*	*Agkistrodon piscivorus*	USA	TX	Galveston		No GPS Location	
010-325-361*	*Agkistrodon piscivorus*	USA	TX	Harris		No GPS Location	
KW0548*	*Agkistrodon piscivorus*	USA	FL	Glades	17R	2980990	487878
KW0549*	*Agkistrodon piscivorus*	USA	FL	Glades	17R	2988305	497561
KW0602*	*Agkistrodon piscivorus*	USA	FL	Madison	17R	3374591	243791
KW0655*	*Agkistrodon piscivorus*	USA	FL	Columbia	17R	3357972	358132
KW0661*	*Agkistrodon piscivorus*	USA	FL	Columbia	17R	3350773	353292
KW0670†	*Agkistrodon piscivorus*	USA	FL	Alachua	17R	3270599	378055
KW0728*	*Agkistrodon piscivorus*	USA	FL	Baker	17R	3347081	361934
KW0253†	*Agkistrodon piscivorus*	USA	FL	Levy	17R	3249658	302021
KW0679*	*Agkistrodon piscivorus*	USA	FL	Levy	17R	3249658	302021
KW0693†	*Agkistrodon piscivorus*	USA	FL	Levy	17R	3253174	335392
KW0727*	*Agkistrodon piscivorus*	USA	FL	Jefferson	17R	3341918	788037
KW0579*	*Agkistrodon piscivorus*	USA	FL	Jefferson	17R	3361897	214009
KW0660*	*Agkistrodon piscivorus*	USA	FL	Liberty	16R	3378646	694603
KW0662†	*Agkistrodon piscivorus*	USA	FL	Liberty	16R	3378646	694603
KW0791*	*Agkistrodon piscivorus*	USA	FL	Wakulla	16R	3344522	731050
KW0805*	*Agkistrodon piscivorus*	USA	FL	Wakulla	16R	3354557	743918
KW0752†	*Agkistrodon piscivorus*	USA	MS	Lafayette	16S	3812469	280185
KW0759*	*Agkistrodon piscivorus*	USA	MS	Lafayette	16S	3812469	280185
KW0769*	*Agkistrodon piscivorus*	USA	MS	Lafayette	16S	3812469	280185
KW0629*	*Agkistrodon piscivorus*	USA	SC	Barnwell	17S	3667094	440962
KW0631*	*Agkistrodon piscivorus*	USA	SC	Barnwell	17S	3667094	440962
KW0648*	*Agkistrodon piscivorus*	USA	SC	Jasper	17S	3579393	488555
KW0623*	*Agkistrodon piscivorus*	USA	SC	Aiken	17S	3676256	430431
BG1020	*Agkistrodon piscivorus*	USA	MO	Stone	15S	4087699	447132
BG4600†	*Agkistrodon piscivorus*	USA	MO	Stone	15S	4087699	447132
BG4947†	*Agkistrodon piscivorus*	USA	MO	Stone	15S	4087699	447132
BG0225	*Agkistrodon piscivorus*	USA	MO	Stone	15S	4087699	447132
BG2D41†	*Agkistrodon piscivorus*	USA	MO	Stone	15S	4087699	447132
BG397E	*Agkistrodon piscivorus*	USA	MO	Stone	15S	4087699	447132
BG4C53	*Agkistrodon piscivorus*	USA	MO	Stone	15S	4087699	447132
BG5E25	*Agkistrodon piscivorus*	USA	MO	Stone	15S	4087699	447132

BG711E†	*Agkistrodon piscivorus*	USA	MO	Stone	15S	4087699	447132
BG265B	*Agkistrodon piscivorus*	USA	MO	Stone	15S	4087699	447132
NCSM 75992‡	*Agkistrodon piscivorus*	USA	NC	Moore	17S	3887970	630020
TNHC 65313*	*Agkistrodon piscivorus*	USA	TX	Fort Bend	15R	3252039	243601
TNHC 65358*	*Agkistrodon piscivorus*	USA	TX	Jefferson	15R	3299012	388744
TNHC 66514*	*Agkistrodon piscivorus*	USA	TX	Chambers	15R	3283335	356025
TNHC 68394‡	*Agkistrodon piscivorus*	USA	TX	Kimble	14R	3373641	427083
TNHC 65357‡	*Agkistrodon piscivorus*	USA	TX	Travis	14R	3356854	627207
TNHC 74705‡	*Agkistrodon piscivorus*	USA	TX	Travis	14R	3346294	638002
TNHC 65352‡	*Agkistrodon contortrix*	USA	OK	Pottawatomie	14S	3880774	678734
TNHC 58828*	*Agkistrodon contortrix*	USA	TX	Edwards	14R	3299034	364753
TNHC 61851*	*Agkistrodon contortrix*	USA	TX	Lee	14R	3353109	719479
TNHC 84300*	*Agkistrodon contortrix*	USA	TX	Travis	14R	3369368	610454
LSUMZ H-6416*	*Agkistrodon bilineatus*	Mexico				No GPS Location	
LSUMZ H-20951*	*Agkistrodon bilineatus*	Mexico				No GPS Location	
LSUMZ H-2240*	*Agkistrodon contortrix*	USA	MS	Forrest	16R	3451142	286596
LSUMZ H-9234*	*Agkistrodon contortrix*	USA	IL	Jersey	15S	4320257	712483
LSUMZ H-18955‡	*Agkistrodon contortrix*	USA	KS	Elk	14S	4148918	743703
LSUMZ H-18958‡	*Agkistrodon contortrix*	USA	KY	Hardin		No GPS Location	
LSUMZ H-18959*	*Agkistrodon contortrix*	USA	KY	Hart	16S	4127712	598361
LSUMZ H-1888‡	*Agkistrodon piscivorus*	USA	NC	Dare	18S	3900238	449837
LSUMZ H-2020*	*Agkistrodon piscivorus*	USA	MS	Perry	16R	3437640	305743
LSUMZ H-2367*	*Agkistrodon piscivorus*	USA	MS	Wilkinson	15R	3466137	667029
LSUMZ H-2368*	*Agkistrodon piscivorus*	USA	MS	Wilkinson	15R	3466137	667029
LSUMZ H-18953‡	*Agkistrodon piscivorus*	USA	KY	Daviess		No GPS Location	
LSUMZ H-19042*	*Agkistrodon piscivorus*	USA	LA	East Baton Rouge	15R	3375547	690463
LSUMZ H-19697‡	*Agkistrodon piscivorus*	USA	FL	Liberty	16R	3378646	694603
LSUMZ H-19879‡	*Agkistrodon piscivorus*	USA	LA	East Carroll	15S	3630649	671028
LSUMZ H-19883‡	*Agkistrodon piscivorus*	USA	LA	East Carroll	15S	3630649	671028
LSUMZ H-20010‡	*Agkistrodon piscivorus*	USA	LA	Lafourche	15R	3309355	715174
LSUMZ H-20648‡	*Agkistrodon piscivorus*	USA	LA	St. Tammany	15R	3362370	782106

APPENDIX III

Nei-Li genetic distance matrix calculated in GenAlEx. All individuals are left justified and then the comparisons are read from left to right and then down. The comparisons are in the same order as the individuals in the left column.

ASK 9082	0										
ASK 9083	19	0									
ASK 9084	21	22	0								
ASK 9085	21	18	20	0							
ASK 9044	34	37	35	41	0						
ASK 9121	32	29	27	31	14	0					
011-367-560	81	83	78	91	83	82	0				
KW 0548	82	85	77	91	80	80	43	0			
KW 0549	81	86	76	90	83	81	36	19	0		
KW 0579	74	75	65	77	78	76	46	38	33	0	
KW 0602	72	71	63	75	76	72	37	40	31	24	0
KW 0679	67	66	62	74	73	67	54	47	46	41	33
0											
KW 0727	82	83	77	86	88	86	56	60	59	44	44
49	0										
KW 0728	84	87	79	91	88	86	51	50	47	40	40
	47	22	0								
KW 0791	70	73	69	79	80	78	47	50	49	40	36
39	40	40	0								
KW 0805	64	67	61	71	74	72	47	50	47	34	36
33	42	46	24	0							
CLP 159	65	66	62	74	69	67	38	45	40	39	37
38	53	45	41	35	0						
ASK 9108	31	24	26	22	33	27	83	79	84	75	71
64	79	81	73	65	64	0					
ASK 9109	32	27	33	27	36	34	88	84	87	84	76
73	90	92	82	74	73	13	0				
ASK 9110	28	29	33	31	32	30	85	82	83	82	76
73	86	90	76	74	69	23	26	0			
010-325-361	27	24	30	20	39	31	85	85	84	75	73
74	83	87	79	71	72	28	31	25	0		
010-820-563	27	24	26	26	37	29	81	85	82	75	71
68	85	83	79	71	68	30	33	35	24	0	
TNHC 66514	25	22	26	22	35	29	81	77	78	67	65
70	85	85	73	69	66	28	25	29	20	26	0
TNHC 65358	21	26	28	30	37	33	74	75	74	65	63
64	77	79	71	69	60	34	39	33	24	30	22
0											
TNHC 65313	24	23	25	31	26	22	80	78	81	76	68
69	86	86	78	70	67	25	26	28	29	29	27
29	0										
CLP 984	65	62	60	70	69	63	46	53	46	43	35
46	53	51	39	35	28	62	69	65	66	70	64
62	65	0									

CLP 986	63	70	60	72	69	65	51	51	42	41	35
36	49	45	41	37	30	66	75	67	74	70	70
64	69	34	0								
CLP 989	67	68	62	74	67	67	45	49	44	39	37
40	45	49	31	31	36	62	71	69	74	70	70
66	67	28	30	0							
ASK 9048	32	27	33	33	40	34	84	88	85	80	74
73	82	84	82	74	71	31	32	36	33	39	35
39	30	67	73	71	0						
ASK 9049	33	32	36	34	41	39	77	81	78	67	65
66	71	75	73	61	66	36	41	41	34	40	36
38	31	62	62	64	23	0					
ASK 9050	27	22	26	30	29	27	81	81	82	69	67
66	77	79	73	65	66	28	33	37	32	32	26
32	23	64	66	62	21	26	0				
ASK 9053	24	25	27	31	22	22	84	82	85	76	72
71	80	80	78	72	69	29	26	26	29	31	25
33	20	67	69	67	24	27	19	0			
ASK 9055	32	33	33	35	36	40	79	80	77	76	70
69	78	78	76	66	67	35	40	34	33	31	33
35	30	67	65	65	32	25	31	26	0		
ASK 9154	35	36	34	40	9	17	86	81	82	81	77
74	88	91	81	75	72	34	39	27	40	40	36
38	27	72	68	70	39	42	32	23	35	0	
ASK 9150	44	43	39	45	20	22	89	84	83	78	78
79	90	90	86	78	77	39	44	34	37	39	37
41	36	73	71	69	42	47	35	30	40	19	0
ASK 9143	34	31	27	33	16	12	86	80	81	74	70
69	84	84	80	72	71	27	30	30	31	31	27
33	22	67	67	65	32	35	25	16	36	15	16
0											
ASK 9152	33	30	26	30	15	13	88	85	86	77	75
70	83	83	79	71	72	24	29	25	28	30	30
38	25	70	68	68	35	36	26	17	33	14	17
9	0										
ASK 9161	34	31	31	33	12	10	83	80	83	80	74
73	86	86	80	74	69	23	28	22	31	33	29
33	22	67	67	65	34	37	29	16	32	11	16
8	7	0									
ASK 9164	34	35	35	35	14	14	81	80	83	82	76
73	86	86	80	74	69	27	32	22	31	33	31
31	26	67	69	65	38	39	33	20	32	13	18
12	11	4	0								
ASK 9169	32	33	33	33	10	14	85	82	85	80	78
75	84	86	82	76	71	27	28	24	31	33	29
33	26	69	71	67	32	35	29	16	32	15	16
10	9	6	8	0							
ASK 9131	35	30	30	34	11	9	84	81	84	75	73
72	87	87	81	73	70	26	29	27	32	30	26
32	23	66	70	66	35	38	26	17	35	14	17
9	8	5	9	7	0						
ASK 9145	44	39	41	45	22	20	92	89	92	87	82
80	95	95	89	80	78	35	37	34	43	41	36
43	32	73	78	75	44	47	37	26	44	23	27
18	19	14	16	18	13	0					

ASK 9144 32 31 31 35 12 12 83 80 83 82 76
73 86 86 80 74 69 25 30 20 33 33 31
33 22 67 67 65 34 39 29 16 30 9 16
10 9 2 4 6 7 16 0
ASK 9137 34 29 27 33 14 8 83 82 83 76 72
73 86 88 82 72 71 27 32 24 29 31 27
35 22 65 69 69 32 39 27 18 36 11 16
10 9 6 10 10 5 16 6 0
ASK 9091 38 37 33 33 26 24 83 82 85 84 80
75 90 90 84 74 75 27 30 32 33 31 37
45 30 71 73 71 42 45 39 28 42 29 32
22 17 18 20 20 19 30 20 20 0

ASK 9092 27 28 30 28 17 19 84 83 86 83 77
70 85 85 77 71 68 20 23 23 32 30 32
38 23 66 66 64 37 40 34 21 31 20 25
17 14 11 13 13 16 25 11 17 17 0
ASK 9093 29 32 30 32 21 25 78 73 76 75 73
66 83 83 73 67 64 24 23 23 34 34 30
34 27 62 68 62 37 40 34 23 35 24 29
19 18 15 17 15 18 27 15 21 23 14 0
LSUMZ H-19042 34 31 29 35 46 40 72 69 67 62
54 53 70 72 58 52 59 31 36 40 31 35
31 31 34 56 59 57 40 45 37 42 42 45
48 38 39 40 42 44 39 48 42 38 44 39
39 0

ASK 9127 37 38 36 42 9 17 84 79 80 79 77
72 87 89 79 73 72 34 37 29 38 40 34
36 29 70 68 68 39 42 34 25 39 6 17
11 16 11 13 13 14 23 11 13 27 18 22
41 0
KW 0762 33 34 34 40 37 35 75 74 74 67 65
64 79 79 71 63 66 42 43 39 34 42 32
38 31 64 72 70 41 44 32 35 43 38 41
35 34 37 39 37 34 45 37 35 43 38 38
37 36 0
KW 0769 49 50 46 50 55 49 85 78 80 68 71
71 83 81 69 66 67 48 51 51 48 52 42
48 43 66 74 69 55 54 48 49 53 56 57
49 50 51 55 53 46 59 53 49 57 52 52
41 52 36 0
LSUMZ H-2020 45 42 36 46 49 43 74 67 66 61
57 60 73 77 67 57 62 42 41 45 44 44
38 42 39 61 64 56 51 44 44 45 43 50
53 43 46 45 47 49 44 53 47 45 50 44
48 33 46 36 41 0

LSUMZ H-2367 42 41 39 47 46 42 77 72 72 68
66 63 76 78 70 60 63 43 46 46 43 43
39 43 36 62 71 61 46 49 41 42 44 45
48 42 45 44 44 46 43 52 42 40 49 39
43 30 43 33 40 27 0

LSUMZ H-2368 38 39 33 41 48 44 74 68 68 66
60 57 74 76 64 56 61 37 42 40 45 43
39 43 34 60 63 55 46 47 41 40 40 45
48 40 43 42 44 46 43 52 40 42 47 39
41 30 43 33 37 27 26 0
ASK 9062 31 32 30 36 25 27 79 79 80 73 71
68 71 73 73 67 68 28 35 29 34 34 34
32 25 66 62 56 35 34 28 23 29 26 27
19 22 19 21 19 24 33 19 25 29 18 28
41 22 36 52 40 41 39 0
ASK 9063 28 29 27 31 20 22 81 82 83 74 72
67 80 80 74 68 67 29 34 28 29 27 29
31 26 65 61 59 32 33 23 22 30 23 22
16 17 18 20 16 19 30 18 20 26 19 23
38 19 31 47 43 40 38 15 0
ASK 9065 42 39 35 41 36 36 91 89 94 84 80
75 90 88 86 76 73 35 40 42 42 41 41
49 36 68 79 77 42 45 41 34 42 43 42
34 29 32 34 30 31 40 34 34 34 31 35
42 41 45 57 49 52 50 35 34 0
ASK 9066 24 23 25 25 22 22 77 78 81 76 70
63 78 78 70 64 61 17 26 20 27 29 29
29 22 59 63 59 30 29 25 22 28 23 28
22 17 14 16 18 19 28 14 20 24 13 17
34 23 33 47 41 40 36 19 14 30 0
ASK 9067 25 22 26 24 27 25 82 85 86 73 71
68 77 81 75 65 68 24 27 27 24 28 24
34 25 62 72 66 27 26 24 21 33 32 33
25 20 23 27 21 22 33 25 21 27 20 24
35 30 34 46 40 39 43 22 19 29 11 0
KW 0629 62 63 55 67 68 62 58 58 55 48 42
55 60 58 42 44 59 63 68 66 65 69 63
61 60 54 49 49 68 59 57 62 66 67 72
64 63 64 68 70 65 74 66 64 70 67 65
52 65 53 57 51 58 50 59 58 72 56 59
0
KW 0631 50 47 45 55 54 50 60 62 57 60 46
49 54 58 48 46 53 47 54 52 53 53 49
41 52 51 47 43 58 57 49 54 54 55 58
50 53 52 54 54 51 60 52 52 60 53 53
34 53 49 55 43 48 40 45 48 64 46 49
36 0
KW 0648 57 56 52 62 59 53 60 59 56 51 41
38 57 57 49 47 54 58 63 61 60 60 58
58 55 56 50 46 65 62 56 57 63 58 63
55 56 57 57 61 58 67 57 57 61 56 62
45 56 52 63 48 49 47 54 49 69 51 58
47 37 0
ASK 9095 14 15 17 19 32 26 80 80 81 70 66
67 80 84 70 60 63 25 26 28 23 19 21
23 18 59 65 65 30 33 25 24 32 33 38
24 27 28 30 28 27 36 28 26 28 23 27
26 31 31 43 37 34 34 27 26 34 26 23
60 48 55 0

ASK 9098	21	18	24	24	37	33	76	81	82	73	67
66	83	85	71	61	62	28	33	33	28	28	30
30	25	60	64	64	39	36	32	29	31	36	47
37	32	33	35	37	32	41	33	31	35	30	34
31	40	40	52	44	41	43	34	33	41	27	28
59	53	58	17	0							
ASK 9101	20	19	21	23	30	24	80	78	81	74	68
65	84	86	72	64	65	25	24	26	27	25	23
27	18	61	65	63	34	41	27	26	36	31	38
30	27	28	30	28	25	36	26	24	30	23	25
28	33	27	41	35	30	32	31	26	38	24	25
60	46	51	14	21	0						
ASK 9103	19	22	20	26	31	27	75	73	76	71	65
62	83	83	65	61	60	26	29	25	26	28	22
20	21	58	64	62	35	40	26	29	37	32	37
31	30	29	31	29	28	39	27	27	35	28	26
27	32	26	38	38	33	29	30	25	37	21	26
55	41	54	17	26	11	0					
ASK 9105	17	12	22	22	31	31	81	77	78	73	69
64	83	81	69	65	62	24	27	27	26	22	24
26	21	60	64	62	35	38	26	25	25	32	39
31	28	29	31	29	30	39	27	31	35	24	26
31	36	34	48	42	39	35	30	27	39	23	28
63	49	54	15	18	17	20	0				
ASK 9068	28	27	29	25	30	28	85	88	89	76	76
75	80	84	80	70	73	27	26	28	23	25	27
37	24	67	75	69	30	31	29	24	34	37	34
28	23	26	30	22	25	36	28	26	28	23	21
40	35	37	49	45	42	44	27	22	32	20	11
64	58	63	24	31	24	29	27	0			
ASK 9071	24	25	29	29	32	30	79	86	85	74	70
69	80	80	74	64	65	27	30	24	27	25	31
37	24	61	67	65	32	29	33	24	30	35	38
30	27	26	28	28	27	36	26	26	28	23	23
36	35	41	51	47	40	40	29	28	36	22	19
66	56	61	20	23	26	31	25	12	0		
ASK 9072	25	28	30	34	27	27	79	77	78	81	75
66	83	81	75	69	68	24	27	19	30	26	30
36	25	64	66	64	37	40	32	23	27	26	33
27	22	19	21	21	22	31	17	23	27	18	18
39	28	36	50	46	41	39	24	23	37	17	24
63	49	56	25	28	23	26	22	23	19	0	

VITAE

Jason Layne Strickland was born in Andrews, Texas, and grew up in Seminole, Texas. He went to Seminole High School and graduated as salutatorian in 2005. Jason attended Angelo State University (ASU) as an undergraduate and graduated *summa cum laude* in 2009 with a Bachelor's Degree in Biology. In 2009, Jason was awarded by the SGA as the "Most Outstanding Leader" on campus and won the Presidential Award given to the most outstanding undergraduate. During his undergraduate career, Jason began working with cottonmouths as his research project under Dr. J. Kelly McCoy. Jason won many awards for his presentations of his research including the best undergraduate oral presentation at the Texas Academy of Science (TAS), Brooks Award for the best presentation at the βββ Regional Convention and second place at the National Convention.

Jason continued his education and entered the graduate program at ASU in pursuit of a Masters degree in Biology. Jason expanded his project on cottonmouths to look at genetic and venom variation. Dr. McCoy was joined by Drs. Loren K. Ammerman and John Osterhout as advisors for Jason. As a student in the Biology Department, Jason was active in helping with projects in the ASNHC as well as working with other students to help them with or to develop their projects. In the department, Jason was a teaching assistant for Man and the Environment and Human Biology. Jason gave several presentations of his research and was awarded 3rd place at TAS and the Wilks Award for the best presentation given at the Southwestern Association of Naturalist's meeting. In his final semester at ASU, Jason was nominated by the Biology Dept. and won the College of Sciences Distinguished Graduate Award. After Jason graduates from ASU, he will pursue his Ph.D. in Conservation Biology at the University of Central Florida with Dr. Christopher Parkinson as his advisor.

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