Phylogeography of *Agkistrodon piscivorus* with Emphasis on the Western Limit of Its Range

Jason L. Strickland[1,2], Christopher L. Parkinson[2], J. Kelly McCoy[1,3], and Loren K. Ammerman[1]

The Cottonmouth, *Agkistrodon piscivorus*, is a semi-aquatic pitviper that occupies the southeastern U.S. west into Texas. Several previous studies have investigated the biogeographic history of *A. piscivorus*. It has been hypothesized that *A. piscivorus* was split into two separate populations during the last glacial maximum and climate change has impacted its distribution. Additionally, a geographically isolated population of *A. piscivorus* occurs at the western limit of the species' range in the Concho Valley of Texas. To investigate biogeography and population structure within *A. piscivorus* in Texas and throughout its range, we utilized amplified fragment length polymorphism (AFLP) and sequence data from cytochrome *b* (cyt-*b*). The AFLP data indicate a lack of gene flow between the population of *A. piscivorus* in the Concho Valley and other nearby populations. However, based on cyt-*b*, there is no genetic differentiation. The AFLP data for the entire species show a signature of two historic populations that have recently come into secondary contact. Finding two historic populations is consistent with previously published data based on mitochondrial DNA analyses; however, due to the rapid evolution rate of AFLP data, we were able to detect a high level of gene flow between these populations. We conclude that it is possible Texas and Florida served as refugia for *A. piscivorus* during the last glacial maximum, and, as the glaciers receded, the two populations expanded, coming into secondary contact. The subsequent gene flow has resulted in shared loci across the two populations. The difference between the conclusions drawn between our two markers and previous research is due to the different time scales that AFLP and cyt-*b* markers measure. The AFLP data provided a contemporary marker and cyt-*b* indicated historic separation.

THE Cottonmouth, *Agkistrodon piscivorus*, is a semi-aquatic pitviper that occurs in the southeastern United States into Texas (Fig. 1). Within the last 500 years, the western limit of its distribution has been contracting due to the drying and desertification of west Texas (Brune, 1975; Werler and Dixon, 2000). This has led to a geographically isolated peripheral population in the Concho River Valley (Werler and Dixon, 2000). Historically, the species distribution has expanded and contracted due to the advancement and retreat of the Laurentide ice sheet during Pleistocene glaciation (Gloyd and Conant, 1990; Guiher and Burbrink, 2008; Douglas et al., 2009). Previous research, using mitochondrial DNA (mtDNA), has suggested that fluctuations in the distribution resulted in two distinct lineages of Cottonmouths. This hypothesis does not reflect the currently accepted taxonomy that there is one species with three subspecies: *Agkistrodon piscivorus piscivorus* (Eastern Cottonmouth), *A. piscivorus leucostoma* (Western Cottonmouth), and *A. piscivorus conanti* (Florida Cottonmouth; Gloyd and Conant, 1990; Knight et al., 1992; Castoe and Parkinson, 2006; Fig. 1). Guiher and Burbrink (2008) and Douglas et al. (2009) concluded that additional genetic markers, particularly nuclear loci, were needed before taxonomy can be addressed. Using range-wide sampling with emphasis on populations in Texas, we sought to determine if the remote peripheral population in Texas is genetically isolated and if there are two lineages within *A. piscivorus* using amplified fragment length polymorphisms (AFLPs) supplemented with additional mtDNA cyt-*b* sequences.

AFLP markers are advantageous because they are representative of the nuclear genome and it is easy generate a large number of polymorphic loci with enough power to differentiate populations (Bensch and Akesson, 2005). AFLPs can be applied to populations sampled throughout the range of a species to determine overall population structure. Additionally, AFLPs have been used to differentiate sister taxa and determine relationships among species (Creer et al., 2004; Mendelson and Simons, 2006; Althoff et al., 2007; Makowsky et al., 2009). Another marker that has been used traditionally to distinguish species-level relationships in a variety of taxa is mitochondrial DNA (mtDNA; Rosenberg et al., 2002; Jacobsen et al., 2010). Results from AFLP markers and mtDNA sequence data such as cyt-*b* have been compared for a variety of taxa. When analyzing small portions of a species' distribution or examining population structure within an entire species, AFLP markers yield more fine-scale information compared to cyt-*b* sequence data (Mendelson and Simons, 2006; Egger et al., 2007; Phillips et al., 2007; García-Pereira et al., 2011).

Given the geographic isolation of the peripheral Concho Valley population on the western edge of the species' range, we would expect genetic isolation. Thus, the initial goal of our study was to evaluate the fine-scale population structure in Texas, and we hypothesized that the population in the Concho Valley is genetically distinct and would have a lower level of genetic variation than other populations throughout Texas. The second goal of our study was to test the hypothesis proposed by Guiher and Burbrink (2008) and Douglas et al. (2009) that the Florida population of *A. piscivorus* is genetically isolated. We expected to see genetic separation between the Florida population and other populations in our sample consistent with the presence of

[1] Department of Biology, Angelo State University, 2601 W. Avenue N., San Angelo, Texas 76909; E-mail: (LKA) Loren.Ammerman@angelo.edu.
[2] Department of Biology, University of Central Florida, 4000 Central Florida Blvd., Orlando, Florida 32816; E-mail: (JLS) jason.strickland@knights.ucf.edu; and (CLP) parkinson@ucf.edu. Send reprint requests to JLS.
[3] College of Arts and Sciences, Georgia Southwestern State University, 800 Georgia Southwestern State University Drive, Americus, Georgia 31709; E-mail: kelly.mccoy@gsw.edu.
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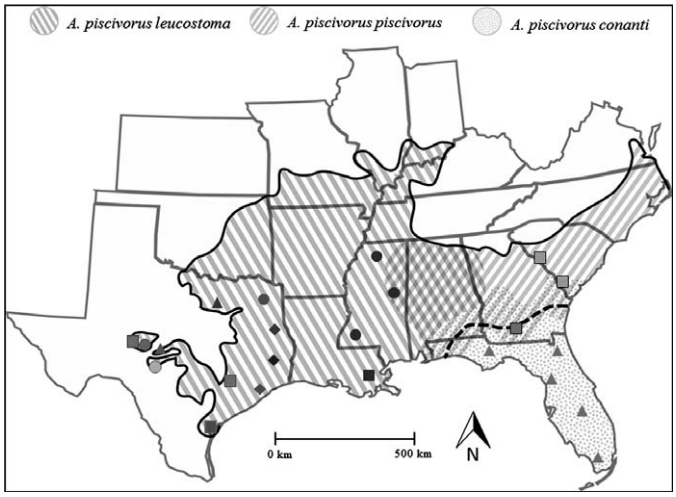

Fig. 1. Distribution map of the Cottonmouth, *Agkistrodon piscivorus*, showing the two current views on its taxonomy. The patterns designate the subspecies view and the dashed line splits the Florida and continental groups discussed in text. Shapes indicate sampling localities and correspond to the shapes in Figure 4.

two lineages. To test our predictions, we generated AFLP data for the entire distribution of *A. piscivorus* and added mtDNA sequence data to a previously published mtDNA matrix (Guiher and Burbrink, 2008). We discuss our results in the context of the biogeographic history of Texas and the southeastern United States as well as to previous studies on *Agkistrodon*.

MATERIALS AND METHODS

Taxon sampling and DNA extraction.—Through collection and tissue loans, we sampled 75 *A. piscivorus* from 24 separate localities as our ingroup (Fig. 1; Appendix 1). We also sampled 24 *A. contortrix*, two *A. bilineatus*, two *A. taylori,* one *Crotalus atrox,* and one *C. molossus* for our analysis as outgroups (Appendix 1; Castoe and Parkinson, 2006). For samples we collected, blood was taken from the caudal vein using an insulin syringe, and stored in modified Tris-EDTA Longmire lysis buffer which increased DNA yield (Longmire et al., 1997; removed NaCl and increased sodium dodecyl sulfate from 0.5% to 1.0%.). We deposited voucher specimens in the Angelo State Natural History Collection at Angelo State University in San Angelo, Texas (Appendix 1).

We extracted whole genomic DNA using a Qiagen DNA extraction kit (Valencia, CA) following the kit protocol for blood or tissue samples stored in lysis buffer or 95% ethanol.

Amplified fragment length polymorphism (AFLP) protocol.—We followed the AFLP protocols of Phillips et al. (2007) and Lee et al. (2010) based on modifications of Vos et al. (1995) who initially described the method. Restriction enzymes (*EcoRI*, *AseI*, and *TaqI*) were used to digest approximately 200 ng of genomic DNA into fragments of different lengths. Generally, only two restriction enzymes are used and all primer combinations are created based on those. In this study, each sample underwent two separate protocols to increase the number of polymorphic loci scored. We used a total of nine primer combinations in the analyses of *A. piscivorus* and eight in the analysis with all taxa (Table 1). All reactions used 20 units of *EcoRI* (New England Biolabs [NEB], Ipswich, MA). One treatment used 20 units of *AseI* (NEB) as the second enzyme and the other treatment used 20 units of *TaqI* (NEB) as the second enzyme. For all restriction digestions, 1X enzyme buffer was added to the reaction and the restriction digest was placed at 37°C for three hours. Next, 75 pmoles of the appropriate enzyme adapter (Table 1) were 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 (NEB).

The pre-selective PCR decreased the number of fragments because of an additional base pair on the primer (Table 1). With the additional base, the number of fragments were reduced to approximately 1/16 of those that were initially created in the restriction digest (Meudt and Clarke, 2007). The second PCR, the selective step, lowered the number of fragments even more depending on how many bases were added to the primer (Table 1). This step also attached a fluorescent dye onto each fragment for detection by Beckman-Coulter CEQ 8000 Genetic Analysis System (Beckman-Coulter, Inc., Fullerton, CA).

Nine primer combinations were used to yield a large number of fragments, giving a representative 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 show population structure and to get enough polymorphic loci to differentiate populations. The more total fragments that are scored, the higher the resolution and the better statistical support for analysis (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. Fragments greater than 80 bp were scored as present (1) or absent (0) using software available on the CEQ8000, which created a binary matrix. Once the initial scoring was complete, fragments were evaluated by eye to ensure proper scoring. Any fragments scored inconsistently or that were too close to other fragments were removed from the analysis, leaving only unambiguous fragments. All individuals were scored in random order to minimize bias in results (Bonin et al., 2005, 2007).

Population genetic analysis.—GenAlEx ver. 6.41 was used to 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 (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 population divergence (Orlóci, 1975). This analysis does not require groups assigned *a priori* and makes it possible to examine relationships in space (usually two or three dimensional) depending on how many eigenvectors are used. PCoA was performed first on all samples, then on only *A. piscivorus*, and finally on populations of *A. piscivorus* from Texas to visualize the pattern at different geographic scales. Average heterozygosity was calculated for each population using Hickory ver. 1.1 which relaxes Hardy-Weinberg assumptions and uses Bayesian statistics to calculate heterozygosity from dominant markers (Holsinger et al., 2002). The genetic distance matrix was analyzed via Analysis of Molecular Variance (AMOVA) to compare variation between and within populations to determine if there was population differentiation. We calculated Φpt which is an analogous measure of F_{st}

Table 1. List of restriction enzymes, adapters, pre-selective primer, and selective primer sequences for the PCR used in the AFLP analysis of *Agkistrodon piscivorus* 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 analysis of *A. piscivorus*.

Name	Sequence
Restriction enzymes	
EcoRI	5′-- . . . G|AATTC . . . --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′
Cyt-*b* sequencing primers (Burbrink et al., 2000)	
L14910 (Forward)	5′--GACCTGTGATMTGAAAAACCCAYCGTT--3′
H16064 (Reverse)	5′--CTTTGGTTTACAAGAACAATGCTTTA--3′

specifically for binary data. Both are measures of genetic differentiation between populations (Andrade et al., 2007). The Φpt values were calculated based on 1000 replicates (α = 0.05). This estimate gave a statistical measure of gene flow among the populations and made it possible to examine variation in *Agkistrodon*.

To test for isolation by distance (IBD), a Mantel test, which is a pairwise comparison to determine correlation between geographic and genetic distances, was used to examine population structure in all *A. piscivorus* sampled (Mantel, 1967; Jensen et al., 2005). The data matrix was formatted for the program STRUCTURE ver. 2.3.3 (Pritchard et al., 2000; Falush et al., 2003, 2007) using the program AFLP-SURV ver. 1.0 (Vekemans, 2002). STRUCTURE estimated the highest degree of genetic structure between the populations and calculated the number of populations (K) in the entire sample based on genetic distance. For the STRUCTURE analysis, the admixture model was used with a burn in of 30,000 followed by 100,000 iterations. This process was applied for K values of 1–10 with ten replications at each K value. The resulting log likelihood scores were averaged for each K. The admixture model was chosen because we did not want to bias the results toward finding a lack of gene flow. With the log likelihood scores, we determined ΔK and then used that to find the true number of groups, K* (Evanno et al., 2005).

To determine the phylogenetic position of *A. piscivorus*, we created a neighbor joining phylogram from the Nei-Li genetic distances (Saitou and Nei, 1987) in PAUP* (phylogenetic analysis using parsimony) ver. 4.0b10 (Swofford, 2003). The two rattlesnakes were used as outgroup taxa and nodal support was calculated with 1000 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 discrete 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).

DNA sequencing and analysis.—To test for isolation in west Texas and to compare AFLP to mtDNA data, eight cyt-*b* sequences from *A. piscivorus* in Texas were generated (GenBank accession KC431019–KC431026) and added to the previously published tree of Guiher and Burbrink (2008).

Table 2. Mean heterozygosity and descriptive statistics of *Agkistrodon piscivorus* based on 479 AFLP loci. Global F_{st} = 0.294±0.016.

Population	*n*	H_e±SE	No. private alleles	Polymorphic within (%)	Ave. no. bands±SE
Angelina Nat. Forest (TX)	4	0.151808±0.0098	0	5.22	260.8±1.70
Knickerbocker, TX	2	0.156785±0.0113	0	2.71	254.5±0.50
Florida	14	0.208125±0.0071	5	15.45	256.8±2.24
Ft. Worth, TX	3	0.165072±0.0106	2	5.01	262.7±0.33
Galveston, TX	5	0.15604±0.0093	0	7.10	259.4±1.81
Georgia	3	0.187233±0.0102	0	6.47	255.3±0.33
Huntsville, TX	5	0.156731±0.0092	1	7.72	258.8±1.24
Junction, TX	3	0.115658±0.0079	0	4.80	254.8±2.47
San Angelo, TX	12	0.150483±0.0107	1	3.13	259.0±1.00
Menard, TX	1	—	0	—	264.0±0.00
Louisiana	1	—	0	—	258.0±0.00
Mississippi	5	0.186057±0.0009	3	10.86	258.8±2.78
Palmetto State Park (TX)	5	0.1603±0.0095	2	7.52	260.0±1.30
South Carolina	4	0.197022±0.0098	0	8.98	259.3±5.63
Tyler, TX	5	0.141247±0.01	0	4.80	260.4±0.93
Welder WMA (TX)	3	0.152452±0.0105	0	3.34	259.3±2.19

These samples included seven individuals from the isolated population in the Concho Valley and one from south Texas. One additional sequence of *A. piscivorus* from southern Georgia and one from South Carolina were also added to ensure accuracy of sequence comparison (GenBank accession KC431027 and KC431028). PCR amplification was accomplished using primers L14910 and H16064 designed by Burbrink et al. (2000; Table 1), following the protocol described by Castoe and Parkinson (2006). PCR product was sequenced in both directions at Arizona Research Laboratories, Division of Biotechnology, University of Arizona Genetics Core Facility (http://uagc.arl.arizona.edu/). Sequences were edited using Sequencher ver. 4.2 (Gene Codes) and novel sequences were aligned with those from Guiher and Burbrink (2008; GenBank accession EU483411–EU483493) in GeneDoc ver. 2.7.0 (Nicholas et al., 1997).

To determine phylogenetic position of the samples from Texas, we used the same parameters for Bayesian inference (BI) as Guiher and Burbrink (2008). For BI, the GTR + I + Γ model was used in MrBayes ver. 3.1.2 with default parameters (Huelsenbeck and Ronquist, 2001). Chains were run for 5×10^6 generations with a burn-in period of 5×10^5 generations and sampling every 1000th generation. Tracer v 1.4 (Ronquist and Huelsenbeck, 2003) was used to ensure stationarity was reached during the burn-in period.

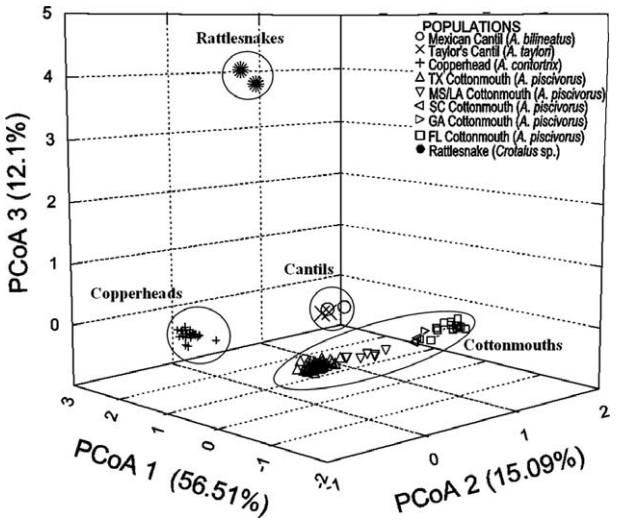

Fig. 2. Three-dimensional principal coordinate analysis (PCoA) of all samples used in the analysis. Rattlesnakes fall out on the third axis away from the four species within *Agkistrodon*. Ellipses were used to help delineate groups.

RESULTS

Eight primer combinations were used to create 622 AFLP fragments for all taxa (Table 2). Of these, 498 (80%) were polymorphic and between 59 and 102 fragments were scored from each primer combination. In the PCoA for all 105 individuals, the first three axes explained 83.7% of the variation (Fig. 2). In this PCoA, the two rattlesnake species (*Crotalus*) were separated from *Agkistrodon* along the third axis. *Agkistrodon piscivorus* had the largest number of polymorphic loci (44.5%) which can be visualized by the amount of spread in the *A. piscivorus* cluster. *Agkistrodon contortrix* (32.53%) collected from throughout their range and Texas *A. piscivorus* (31.36%) had a similar level of polymorphism as indicated by the similarity in shape of their clusters in Figure 2. Because there were only two samples from each of the two cantils (*A. bilineatus* and *A. taylori*), it was not possible to determine the amount of variation seen in each of those species. For the neighbor-joining analysis, a 50% majority rule consensus tree was created (Fig. 3). Branches with over 70% support were considered to be significantly supported (Felsenstein, 1985; Hillis and Bull, 1993). When Nei-Li genetic distances were calculated, *A. piscivorus* had the highest amount of intra-specific variation (6.4%) and the two cantil species had the closest genetic distance between any two species (9.5%).

After outgroups were removed, nine primer combinations were used and all *A. piscivorus* were analyzed (Table 2). There were 479 fragments used in the analysis with 44.9% (215) polymorphic. The average amount of polymorphism within a population was 10.07±1.42%. The AMOVA indicated a significant lack of gene flow among *A. piscivorus* across their entire range (Φ_{pt} = 0.466, $P < 0.001$). For this PCoA, only the first two axes were used (73.4% variation explained); this ordination did not show any clustering and there was an east to west pattern for all *A. piscivorus* (Fig. 4). The Mantel

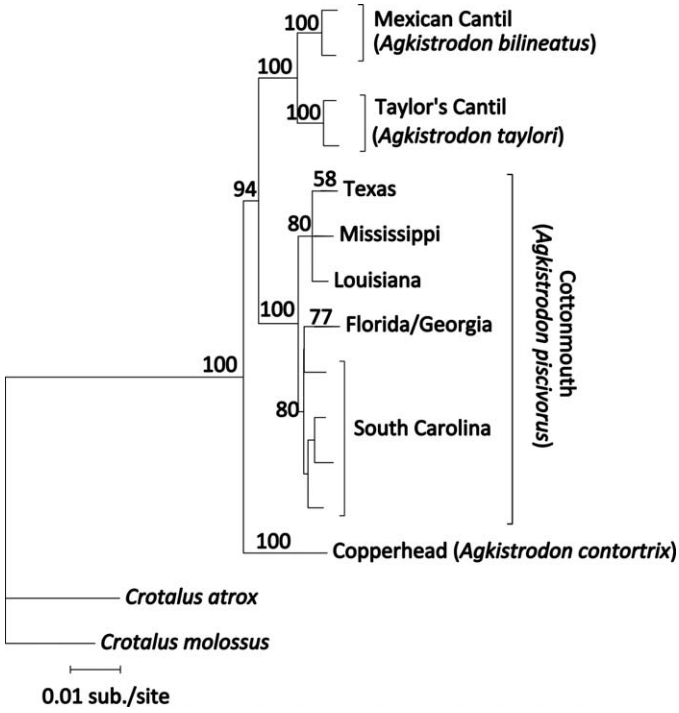

Fig. 3. Neighbor-joining phylogram with terminal branches condensed from all samples using AFLP data. One thousand bootstrap pseudo-replicates were performed, and those with support over 50 percent are shown.

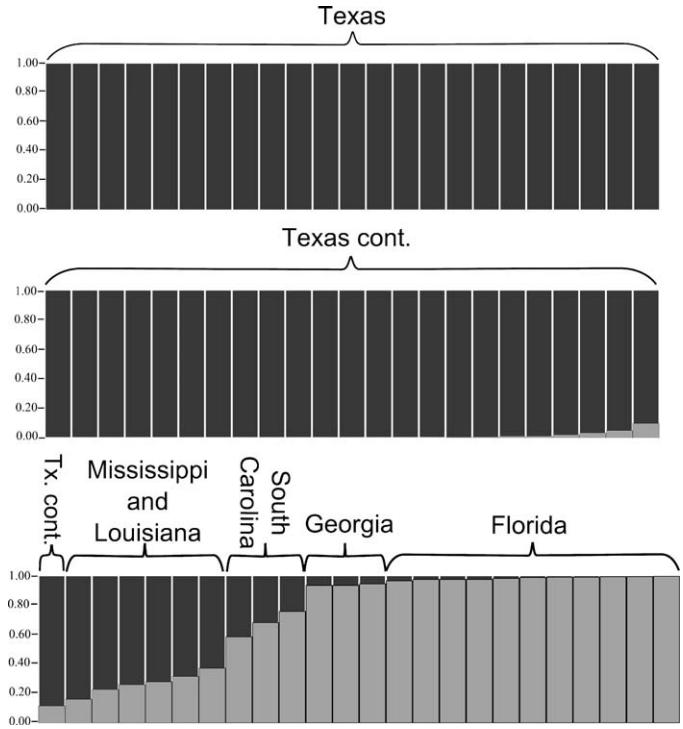

Fig. 5. *Agkistrodon piscivorus* posterior mean estimates of the proportion of each individual's genome that belongs to each of the two estimated populations from STRUCTURE.

IBD test indicated that genetic distance was significantly correlated with geographic distance ($R^2 = 0.731$, $P < 0.0001$). A value of K = 2 was determined using STRUCTURE for the number of groups within *A. piscivorus*. There appeared to be a geographic cline based on the pattern observed in the STRUCTURE output (Fig. 5). Samples from the middle of the distribution had some proportion of their genes estimated to be from both of the populations. Within *A. piscivorus*, there was significant support for two clades from both markers but high gene flow made it difficult to determine the geographic location of the divergence. The neighbor joining tree based on the AFLP markers had a clade that included Texas, Mississippi, and Louisiana individuals and a clade that included the Florida, Georgia, and South Carolina individuals (Fig. 3). We recovered a Florida clade and a continental clade from our Bayesian tree based on cyt-*b*, which was consistent with the tree from Guiher and Burbrink (2008). Both our STRUCTURE and phylogenetic analyses determined there were two populations of *A. piscivorus* throughout their range (Fig. 5).

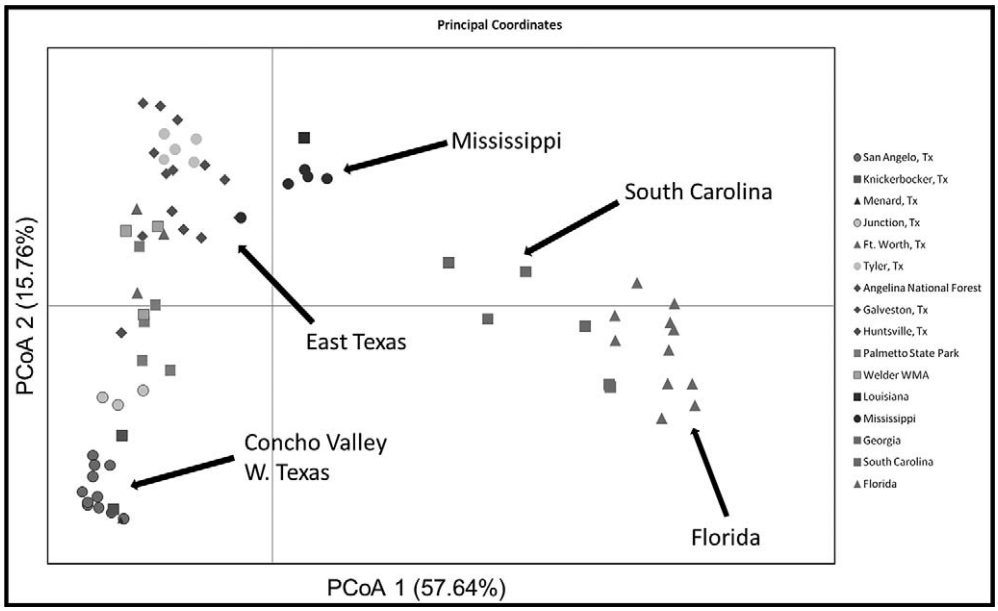

Fig. 4. Two-dimensional PCoA of *Agkistrodon piscivorus* from their entire range based on AFLP data. The first two axes explain 73.40% of the variation, and the pattern indicates a west to east trend in genetic variation.

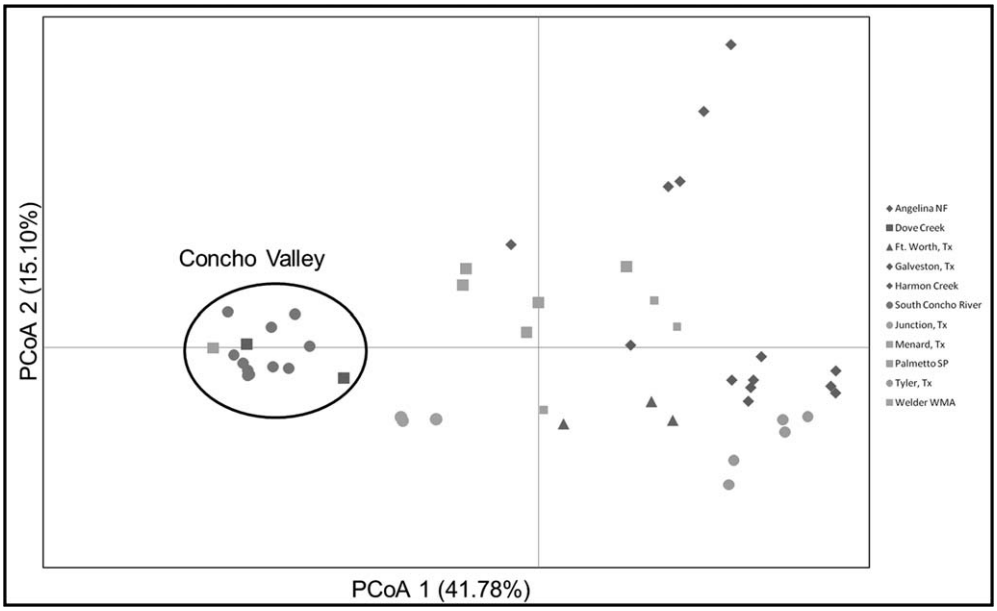

Fig. 6. Two-dimensional PCoA of only Texas *Agkistrodon piscivorus* based on AFLP data. The first two axes explain 56.88% of the variation, and the pattern indicates that the Concho Valley population is less genetically variable than other populations in Texas.

For the final analysis, only *A. piscivorus* from Texas were used. Once again, all nine primer combinations were used and yielded a total of 440 fragments with 31.36% of them being polymorphic (138). The average population level of polymorphism was 8.23±0.95% with the Concho Valley population at 3.13%. The number of fragments decreased from the previous analysis because fragments that were all 0 were removed. The AMOVA analysis indicated significant lack of gene flow between populations ($\Phi_{pt} = 0.348$, $P < 0.001$). 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 also isolated (Fig. 6). STRUCTURE analysis indicated four groups. One of the groups was comprised of solely Concho Valley individuals and the other three groups were split up throughout the remaining distribution in Texas. All groups had mixing of genes from other populations.

Using the mtDNA sequence data we were not able to distinguish population-level separation between the isolated Concho Valley population and the rest of Texas. The individuals from the Concho Valley used in the analysis were recovered throughout the topology of the continental clade as presented in Guiher and Burbrink (2008). The cyt-*b* phylogeny (not shown) recovered using BI did not differ from the tree published in Guiher and Burbrink (2008) or the NJ tree generated based on AFLP data (Fig. 3). There was support for the currently recognized relationships within *Agkistrodon* as well as two lineages within *A. piscivorus* that generally correspond to the continental and Florida clades (Guiher and Burbrink, 2008).

DISCUSSION

The population structure of *A. piscivorus* based on AFLP and mtDNA sequence data indicates a complex history in the southeastern U.S. We were able to determine the population structure in Texas and the entire distribution of *A. piscivorus* to address the goals of our study. In Texas, the AFLP markers indicated that the geographically isolated Concho Valley population is also genetically isolated. The PCoA (Fig. 6) indicates distinct separation from the remaining individuals in Texas, whereas BI based on cyt-*b* did not resolve distinct lineages in Texas. Amplified fragment length polymorphism markers work at finer taxonomic and temporal scales than cyt-*b* and are able to detect genetic changes in populations sooner (Bensch and Akesson, 2005; Meudt and Clarke, 2007). These AFLPs are predominately neutral and can accrue changes much more quickly than cyt-*b*, which makes it possible to use them to determine if gene flow is occurring between populations in close proximity (Andrade et al., 2007). Even with just 15 individuals from the Concho Valley, the analyses demonstrated that this population had lower genetic variation. Most of the other populations 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. 6).

Approximately 50–100 years ago, there were at least eight springs south of the headwater springs of the South Concho River that have since run dry (Brune, 1975). These springs provided habitat corridors for *A. piscivorus* between the Concho Valley and the San Saba River near Menard, Texas (Brune, 1975). It is possible that the drying period occurring over the last 200 years has slowly shrunk the western population of *A. piscivorus* leaving populations isolated in the Concho Valley and at the head of the San Saba River. Our STRUCTURE results are consistent with an isolated Concho Valley population. The three eastern populations inferred by STRUCTURE have more characters in common with each other than any has with the Concho Valley populations. This, along with the significant genetic structure demonstrated by the Φpt value, indicates that the population has recently become genetically isolated. To fully understand the history of *A. piscivorus* in the Concho Valley, a more thorough sampling effort will be needed from areas directly surrounding the valley.

The overall genetic variation in Texas was not different from the rest of the range of *A. piscivorus*. The neighbor joining (Fig. 3) and Bayesian phylograms show species

relationships consistent with those of Parkinson et al. (2000). In agreement with recent work, there was significant support for the monophyly of *Agkistrodon* as well as support for each of the four species currently recognized within *Agkistrodon* (Knight et al., 1992; Castoe and Parkinson, 2006). Also in agreement, we found strong support for the sister relationship of *A. contortrix* to the *A. piscivorus* and cantil clade. These groupings were supported by PCoA for all individuals based on AFLP markers (Fig. 2).

Our results indicate that there are two lineages within *A. piscivorus,* and there was no evidence for three subspecies as presented in Gloyd and Conant (1990). The AMOVA indicated significant genetic structure throughout the range which showed *A. piscivorus* does not form a panmictic population. The PCoA for all samples of *A. piscivorus* showed an east to west pattern in population structure (Fig. 4). Our STRUCTURE analysis demonstrates that there are many markers shared throughout the range from east Texas to north Florida of the two proposed populations, likely indicating high levels of gene flow, but it is not possible to rule out incomplete lineage sorting (Fontenot et al., 2011). Incomplete lineage sorting is unlikely because the large number of randomly distributed markers throughout the genome effectively neutralize differential lineage sorting (Avise, 2004; Koblmuller et al., 2010). The Mantel test showed a significant, positive correlation between geographic and genetic distance. Because *A. piscivorus* 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 are actually present when the species shows isolation by distance (Frantz et al., 2009). We conclude that there are two lineages within *A. piscivorus*, but there is a high amount of gene flow between the two. Our inferred groupings are similar to those presented in previous studies with a few exceptions.

In this study, individuals from South Carolina were in the same lineage as the individuals from Florida and Georgia based on AFLP data, whereas they clustered with individuals from Texas and Mississippi based on cyt-*b*. 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 the Florida lineage presented in those two papers should include the samples from South Carolina used in this study. The difference in inferred relationships when comparing AFLP with sequence data is likely caused by the difference in temporal scale reflected by the two markers (Egger et al., 2007; Fontenot et al., 2011). Cyt-*b* is expected to retain the signature from when the continental and southern populations were isolated during Pleistocene glacial cycles (Douglas et al., 2009). Once in Florida, *A. piscivorus* colonized southern Florida and during interglacial periods, it moved into the southeastern United States. Florida could have served as a glacial refuge for *A. piscivorus* that was stable over long periods of time and may have allowed for the mtDNA lineage differentiation. It is likely that Texas served as the continental refuge for *A. piscivorus* during glacial periods (Swenson and Howard, 2005). In his unpublished dissertation, Guiher (2011) used additional sequence data from the nuclear genome, ecological niche modeling, and morphological data and found evidence for a large hybrid zone. The markers used by Guiher were biparentally inherited which allowed him to detect the area of gene flow. AFLP markers detect the finest scale which explains why these data exhibit the largest area of gene flow of all studies of *A. piscivorus*. The results from this study are consistent with the explanation that Texas and Florida refuge populations expanded into the southeastern United States eventually coming into secondary contact resulting in the current continuous distribution (Barrowclough et al., 2011). After secondary contact, gene flow occurred and allowed for AFLP markers to move between Texas and Florida.

Since colonizing the southeastern U.S., *A. piscivorus* has been influenced by glacial and interglacial periods. This species carries the genetic signature of historic separation, but contemporary markers indicate that gene flow is occurring between formerly isolated lineages. Guiher (2011) proposed that speciation has occurred and there are two species within *A. piscivorus* (above and below the dashed line in Fig. 1). He detected an area of gene flow that spread from Mississippi along the Florida/Georgia border and into South Carolina. Our data confirm the hybrid zone but indicate it is larger than was proposed by Guiher. The incongruence between these studies is likely the result of difficulty in defining when speciation has occurred (de Queiroz, 2007; Nosil, 2008). It is difficult to determine when a speciation event has occurred and exactly how much gene flow is allowable to recognize distinct species (Nosil, 2008). The incongruence is also influenced by the historical biogeography of the southeastern United States since the last glacial cycle in the Pleistocene (Hewitt, 1996, 2000; Swenson and Howard, 2005; Soltis et al., 2006; Fontanella et al., 2008) and by the natural history of the *A. piscivorus* lineage (Gloyd and Conant, 1990). Future work with *A. piscivorus* will be able to use genetic structure to better understand its biogeography. *Agkistrodon piscivorus* is a good model species to test finer scale markers such as single nucleotide polymorphisms to determine the extent of gene flow throughout their range and to determine if secondary contact will remove the evidence of two lineages.

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APPENDIX 1. Locality information for all individuals used in the AFLP portion of this study including outgroups. Museum number and tissue collection abbreviations are as follows: ASK (Angelo State Karyotype), ASNHC (Angelo State Natural History Collection), CLP (Christopher L. Parkinson), JLS (Jason L. Strickland), KW (Kenneth Wray), LSUMZ (Louisiana State University Museum of Zoology), NNTRC (National Natural Toxins Research Center), PIT (Passive Integrated Transponder), TJL (Travis J. Laduc), and TNHC (Texas Natural History Collection). All individuals with GenBank accession numbers were added to the individuals used by Guiher and Burbrink (2008) to build the cyt-*b* phylogeny. The four individuals at the end with an * were only used to generate cyt-*b* sequence data and were not in the AFLP portion of this study.

Tissue ID	Snake ID	Museum ID	Species	Country	State	County	Accession no.
		LSUMZ H-20951	*A. bilineatus*	Mexico			
		LSUMZ H-6416	*A. bilineatus*	Mexico			
ASK 9056	JLS 25		*A. contortrix*	USA	TX	Walker	
ASK 9058	JLS 27		*A. contortrix*	USA	TX	Walker	
ASK 9059	JLS 28		*A. contortrix*	USA	TX	Walker	
ASK 9061	JLS 30		*A. contortrix*	USA	TX	Walker	
ASK 9077	JLS 49		*A. contortrix*	USA	TX	Angelina	
ASK 9088	JLS 60		*A. contortrix*	USA	TX	Angelina	
ASK 9100	JLS 73		*A. contortrix*	USA	TX	Smith	
ASK 9112	JLS 85		*A. contortrix*	USA	TX	Pecos	
ASK 9115	JLS 88		*A. contortrix*	USA	TX	Pecos	
ASK 9116	JLS 89		*A. contortrix*	USA	TX	Pecos	
ASK 9119	JLS 92		*A. contortrix*	USA	TX	Brewster	
ASK 9123	JLS 96		*A. contortrix*	USA	TX	Jeff Davis	
ASK 9124	JLS 97		*A. contortrix*	USA	TX	Brown	
		LSUMZ H-18959	*A. contortrix*	USA	KY	Hart	
		LSUMZ H-2240	*A. contortrix*	USA	MS	Forrest	
		LSUMZ H-9234	*A. contortrix*	USA	IL	Jersey	
		TNHC 58828	*A. contortrix*	USA	TX	Edwards	
	TJL 922	TNHC 61851	*A. contortrix*	USA	TX	Lee	
	TJL 1953	TNHC 84300	*A. contortrix*	USA	TX	Travis	
	011-310-839	NNTRC	*A. contortrix*	USA	TX	Tarrant	
	011-367-560	NNTRC	*A. contortrix*	USA	TX	Midland	
	058-375-116	NNTRC	*A. contortrix*	USA	MO	Boone	
	058-557-565	NNTRC	*A. contortrix*	USA	KY	Wolf	
	058-594-037	NNTRC	*A. contortrix*	USA	MO	Cole	
	058-843-771	NNTRC	*A. contortrix*	USA	TX	Colorado	
ASK 9044	JLS 11	ASNHC 14264	*A. piscivorus*	USA	TX	Tom Green	
ASK 9048	JLS 17		*A. piscivorus*	USA	TX	Walker	
ASK 9049	JLS 18		*A. piscivorus*	USA	TX	Walker	
ASK 9050	JLS 19		*A. piscivorus*	USA	TX	Walker	
ASK 9053	JLS 22		*A. piscivorus*	USA	TX	Walker	
ASK 9055	JLS 24		*A. piscivorus*	USA	TX	Walker	
ASK 9062	JLS 33		*A. piscivorus*	USA	TX	Gonzales	
ASK 9063	JLS 34		*A. piscivorus*	USA	TX	Gonzales	
ASK 9065	JLS 36		*A. piscivorus*	USA	TX	Gonzales	
ASK 9066	JLS 37	ASNHC 14284	*A. piscivorus*	USA	TX	Gonzales	
ASK 9067	JLS 38	ASNHC 14286	*A. piscivorus*	USA	TX	Gonzales	
ASK 9068	JLS 39	ASNHC 14279	*A. piscivorus*	USA	TX	San Patricio	
ASK 9071	JLS 42		*A. piscivorus*	USA	TX	San Patricio	
ASK 9072	JLS 43		*A. piscivorus*	USA	TX	San Patricio	
ASK 9082	JLS 54		*A. piscivorus*	USA	TX	Angelina	
ASK 9083	JLS 55	ASNHC 14276	*A. piscivorus*	USA	TX	Angelina	
ASK 9084	JLS 56		*A. piscivorus*	USA	TX	Jasper	
ASK 9085	JLS 57		*A. piscivorus*	USA	TX	San Augustine	
ASK 9091	JLS 63		*A. piscivorus*	USA	TX	Kimble	
ASK 9092	JLS 64		*A. piscivorus*	USA	TX	Kimble	
ASK 9093	JLS 65		*A. piscivorus*	USA	TX	Kimble	
ASK 9095	JLS 67		*A. piscivorus*	USA	TX	Smith	
ASK 9098	JLS 70		*A. piscivorus*	USA	TX	Smith	
ASK 9101	JLS 74	ASNHC 14275	*A. piscivorus*	USA	TX	Smith	
ASK 9103	JLS 76		*A. piscivorus*	USA	TX	Smith	
ASK 9105	JLS 78		*A. piscivorus*	USA	TX	Smith	
ASK 9108	JLS 81		*A. piscivorus*	USA	TX	Tarrant	
ASK 9109	JLS 82		*A. piscivorus*	USA	TX	Tarrant	

APPENDIX 1. Continued.

Tissue ID	Snake ID	Museum ID	Species	Country	State	County	Accession no.
ASK 9110	JLS 83		*A. piscivorus*	USA	TX	Tarrant	
ASK 9121	JLS 94		*A. piscivorus*	USA	TX	Tom Green	KC431020
ASK 9127	JLS 100		*A. piscivorus*	USA	TX	Menard	
ASK 9131	PIT 114938716A		*A. piscivorus*	USA	TX	Tom Green	
ASK 9137	PIT 115222097A		*A. piscivorus*	USA	TX	Tom Green	
ASK 9143	PIT 114967277A		*A. piscivorus*	USA	TX	Tom Green	
ASK 9144	PIT 115317467A		*A. piscivorus*	USA	TX	Tom Green	
ASK 9145	PIT 114952455A		*A. piscivorus*	USA	TX	Tom Green	
ASK 9150	PIT 114979652A		*A. piscivorus*	USA	TX	Tom Green	
ASK 9152	PIT 114954121A		*A. piscivorus*	USA	TX	Tom Green	KC431023
ASK 9154	PIT 114616122A		*A. piscivorus*	USA	TX	Tom Green	
ASK 9160	PIT 114631146A	ASNHC 14289	*A. piscivorus*	USA	TX	Tom Green	
ASK 9161	PIT 114633364A		*A. piscivorus*	USA	TX	Tom Green	
ASK 9164	PIT 114949391A		*A. piscivorus*	USA	TX	Tom Green	KC431025
ASK 9169	PIT 115322477A		*A. piscivorus*	USA	TX	Tom Green	
		LSUMZ H-2020	*A. piscivorus*	USA	MS	Perry	EU483465
		LSUMZ H-2367	*A. piscivorus*	USA	MS	Wilkinson	EU483466
		LSUMZ H-2368	*A. piscivorus*	USA	MS	Wilkinson	EU483467
		LSUMZ H-19042	*A. piscivorus*	USA	LA	East Baton Rouge	
	TJL 1487	TNHC 65313	*A. piscivorus*	USA	TX	Fort Bend	EU483474
	TJL 1476	TNHC 65358	*A. piscivorus*	USA	TX	Jefferson	EU483473
	TJL 990	TNHC 66514	*A. piscivorus*	USA	TX	Chambers	
	010-325-361	NNTRC	*A. piscivorus*	USA	TX	Harris	
	010-820-563	NNTRC	*A. piscivorus*	USA	TX	Galveston	
	011-311-367	NNTRC	*A. piscivorus*	USA	FL		
	CLP 159		*A. piscivorus*	USA	FL	Collier	
	CLP 984		*A. piscivorus*	USA	GA	Grady	
	CLP 986		*A. piscivorus*	USA	GA	Thomas	KC431026
	CLP 989		*A. piscivorus*	USA	GA	Grady	KC431027
	KW 0548		*A. piscivorus*	USA	FL	Glades	
	KW 0549		*A. piscivorus*	USA	FL	Glades	
	KW 0579		*A. piscivorus*	USA	FL	Jefferson	
	KW 0602		*A. piscivorus*	USA	FL	Madison	
	KW 0623		*A. piscivorus*	USA	SC	Aiken	
	KW 0629		*A. piscivorus*	USA	SC	Barnwell	
	KW 0631		*A. piscivorus*	USA	SC	Barnwell	
	KW 0648		*A. piscivorus*	USA	SC	Jasper	
	KW 0655		*A. piscivorus*	USA	FL	Columbia	
	KW 0660		*A. piscivorus*	USA	FL	Liberty	
	KW 0661		*A. piscivorus*	USA	FL	Columbia	
	KW 0679		*A. piscivorus*	USA	FL	Levy	
	KW 0727		*A. piscivorus*	USA	FL	Jefferson	
	KW 0728		*A. piscivorus*	USA	FL	Baker	
	KW 0759		*A. piscivorus*	USA	MS	Lafayette	
	KW 0769		*A. piscivorus*	USA	MS	Lafayette	
	KW 0791		*A. piscivorus*	USA	FL	Wakulla	
	KW 0805		*A. piscivorus*	USA	FL	Wakulla	
	CLP 140		*A. taylori*	Mexico	Tamaulipas		AY223613
ASK 9111	JLS 84		*A. taylori*	Mexico	Tamaulipas		
ASK 9117	JLS 90		*C. atrox*	USA	TX	Pecos	
ASK 9118	JLS 91		*C. molossus*	USA	TX	Pecos	
ASK 9070*	JLS 41		*A. piscivorus*	USA	TX	San Patricio	KC431019
ASK 9132*	PIT 114409631A		*A. piscivorus*	USA	TX	Tom Green	KC431021
ASK 9133*	PIT 114625792A		*A. piscivorus*	USA	TX	Tom Green	KC431022
ASK 9153*	PIT 113932567A		*A. piscivorus*	USA	TX	Tom Green	KC431024