AN EVALUATION OF THE GENETIC VARIATION WITHIN SELECTED POPULATIONS OF *VAEJOVIS COAHUILAE* WILLIAMS 1968 USING MITOCHONDRIAL DNA ANALYSES

A Thesis

by

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ABSTRACT

AN EVALUATION OF THE GENETIC VARIATION WITHIN SELECTED POPULATIONS OF *VAEJOVIS COAHUILAE* WILLIAMS 1968 USING MITOCHONDRIAL DNA ANALYSES.

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The species *Vaejovis coahuilae* Williams 1968 is the second most common scorpion species in New Mexico and Texas. This species is a generalist that can inhabit a wide array of habitats. Based on the reported range of this species, we predicted that it originated in northern Mexico and spread north through Texas and New Mexico. The aim of this study is to gain a better understanding of the historical dispersal pattern of the species *V. coahuilae* from Texas through New Mexico. A molecular approach was utilized to discover deeper relationships among populations of this species. Two partial sequences from the mitochondrial genome including the 16S rDNA and the cytochrome c oxidase subunit I (COI) gene are used to make a molecular comparison between populations. Isolated PCR products were sequenced using the LI-COR Global Edition IR2 DNA analyzer or at the DNA Core Facility at Cornell University using the Applied Biosystems Automated 3730 DNA analyzer. Three closely relates species were used as outgroups for sequence comparison. All sequences (39 total) were aligned using the BioEdit program to produce the finished products including a partial (360 bp) sequence

of the mitochondrial 16S rDNA and a partial sequence (696 bp) of the COI gene. These two sequences were appended to produce a 1046 bp sequence that was also used to evaluate the dispersal pattern of *V. coahuilae.* These three data sets were analyzed separately using PAUP 4.0. One of the equally parsimonious trees was chosen for each data set to compute a 50% majority rule consensus tree and the topology was evaluated with 1,000 bootstrap replicates. Analysis of the 16S rDNA was mostly inconclusive producing a large polytomy but three populations from BBNP each fell out together and the populations from Albuquerque, San Antonio and one specimen from Deming grouped as a monophyletic subgroup. However, the arrangement of the tree suggests that all populations are equally related. Analysis of the COI gene was slightly more informative than the 16S rDNA analysis suggesting some geographically local populations to be more closely related to each other than to other populations. A large branch contains a polytomy with four branches; one of which contains one individual from GMNP and two individuals from the Roswell population; the other three branches contain individuals from BBNP. Outside of this polytomy are two branches containing a specimen from GMNP. The topology of both the COI/16s rDNA combined sequence data tree suggests possibly a high elevation specimen from GMNP (G3) has a more recently experienced gene flow with other populations. The branching pattern of the tree produced from the COI sequence data suggests the other specimen from GMNP collected at a much lower elevation on the eastern side of the mountain range has more recently experienced gene flow with populations in Roswell. In the topology of the COI tree and the combined sequence data tree the Albuquerque population appears more genetically related to

populations from BBNP which represents the most northern and southern reaches of the sampled range. This suggests an accessible route for gene flow between the populations.

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INTRODUCTION

Scorpions are among the most ancient arachnids who debatably evolved during the mid-Silurian (~420-450 mya) from the giant water scorpions (Eurypterida). The fossil record reveals that the first land dwelling scorpions arose sometime within the late Devonian or early Carboniferous periods (~325-350 mya) (Polis 1990). Throughout the course of their evolution the basic scorpion body plan has remained relatively static. However, scorpions have developed distinctive physiological, biochemical, and ecological adaptations reflected by their ability to inhabit a large array of terrestrial habitats. In addition to other characteristics, the retention of an ancient body plan has enabled scorpions to endure ever-changing habitats throughout the millennium (Polis 1990). Subsequently, extant scorpion species vary little in terms of their basic morphology. This trend makes analyses of scorpion systematics difficult when based solely on physiological, morphological, and biogeographical attributes (Polis 1990).

Since the late twentieth century scientists have sought to reveal the medicinal and the ecological significance of scorpions. Many scorpion species can deliver very painful stings, although most have not been shown to be dangerous to humans in good health. However, there are several species of the family *Buthidae* Koch 1837 whose venom is of medicinal concern. Peptides isolated from the venom of *Centruroides noxious* Koch 1837 have been shown to adversely affect potassium currents vital to proper heart function (Corona et al. 2002). The sting of *Centruroides excilicauda*

Wood 1863 has been attributed to numerous deaths in Mexico and the United States (Corona et al. 2002). For this reason, research into the physiological and biochemical actions of scorpion venom will continue to be of medicinal significance. It is of utmost importance that researchers can determine the origin of venom injected into an individual so that they can be properly treated. Therefore, there is a great need for an understanding of systematic relationships of both potentially hazardous scorpion species as well as all other species.

Scorpions are also ecologically significant, usually representing the dominant predators within their habitats in terms of population density and diversity (Polis 1990). Therefore, studying the community structure of scorpions reveals relationships with other species as well as relationships between individual scorpions. Recent studies indicate several scorpions possess species specific venom, having more adverse affects on species of mammals rather than other vertebrate and invertebrate species (Corona et al. 2002). Additionally, when considering conservation issues pertaining to scorpions, it is important to delineate within particular region each habitat that all species inhabit so that we will know when or if they disappear due to poor habitat husbandry. Finally, if we consider that terrestrial scorpions likely evolved from the giant water scorpions some 350 mya, they are potentially excellent models in the study of evolution. Scorpions are considered key to our understanding of chelicerate evolution because of their stratigraphic age (Dunlap & Braddy 2001).

Research is currently underway which aims to better understand community structure, systematics and evolutionary history of scorpions. The evolutionary history and systematics of scorpions are poorly understood but not due to a lack of interest.

Although new classification schemes utilizing genetic analysis are more often being employed many of the extant scorpion groups are still solely classified by internal and external morphology. The heavy reliance on morphological character analysis has become problematic over the past several decades because many scorpion characters typically used are now considered somewhat questionable character states for reliably determining taxonomic distinctions. Many cases exist showing that such morphological character analysis has provided us with a general understanding of scorpion relationships in the higher taxa but it is becoming evident that at the genus and species levels such analyses can only provide clues about the relationships that exist at these levels. Therefore, systematists are now turning to molecular biology to help decipher the true nature of scorpion phylogeny while scorpiologists are searching for more determinative morphological characters.

The true phylogeny of the family Vaejovidae Thorell 1876 has been debated for decades. Its taxanomic rank and breadth has evolved as genera where shifted to separate families or superfamilies (Soleglad & Fet 2008). According to most authorities the family Vaejovidae, consists of ten genera, 154 species and 35 subspecies. There are no subfamilies currently recognized and all taxa are of North or Central American origin (Sissom 1990 & Fet et. al 2000). Prendini and Sissom are presently revising the family using both morphological and genetic analyses (R. Henson, Personal Communication). The largest Vaejovidae genus of North American scorpions is *Vaejovis* Koch 1836 whose type species is regarded to be *Vaejovis mexicanus* Koch 1836 (Sissom 2000). The genus *Vaejovis* contains a diverse group of scorpions, whose origin according to the common consensus is not monophyletic, and

its 66 species are currently broken into five species groups (Stockwell 1992). The focus of this study is on the species currently recognized by most authorities as *Vaejovis coahuilae* Williams 1968. The placement of this scorpion into the eusthenera species group like many others species in this genus is in need of re-evaluation; as are the criteria for the species groups designations themselves (Sissom 2000).

This species is one of the most common scorpions in the family Vaejovidae, is sympatric with the most common North American scorpion, *Centruroides vittatus* Say 1901 throughout most of its range, but it is not as widespread as *C. vittatus* (Sissom 2000). *Vaejovis coahuilae* also inhabits regions in conjunction with other members of its family throughout New Mexico and Texas. In the United States, *Vaejovis coahuilaes*' reported range according to Fet et al. (2000) reaches as far northeast and southeast as Randall and Val Verde Counties in Texas, respectively; and as far southwest as Cochise County Arizona and northwest as San Juan County, Utah (R. Henson Personal Communication) (Figure 1). In Texas this species has been reported in at least18 counties and in at least seven counties in New Mexico (Stockwell 1986). In Mexico, *V. coahuilae* has been found as far south as Durango then northeast and northwest through Coahuilae and Chihuahua respectively (Fet et al. 2000). *Vaejovis coahuilae* is found in nearly every habitat, up to the Alpine tree-line (over 3,000 meters), although most are found in arid and semi-arid habitats (to 70 m below sea level) (Polis 1990).

While *V. coahuilae* has been described in detail morphologically very little research has been done in regards to the molecular makeup. Williams (1968) first described *V. coahuilae* from a specimen collected in the Cuatro Cienegas basin in

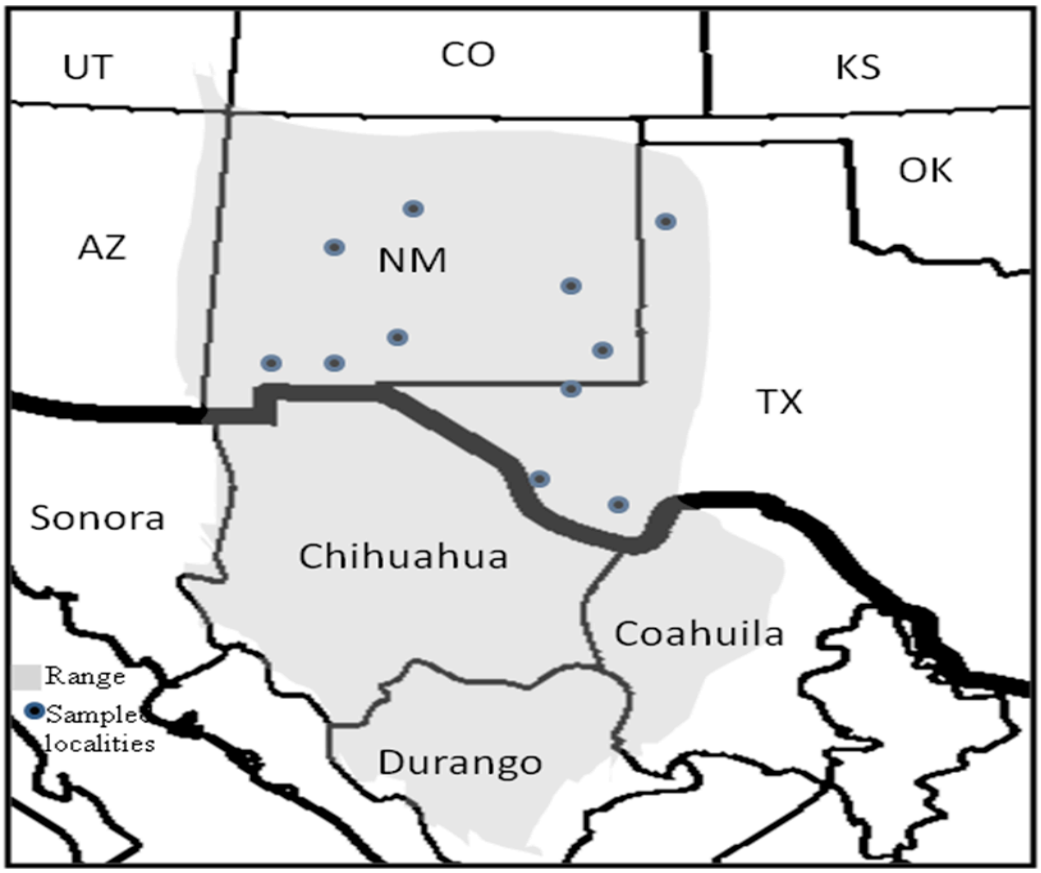

Figure 1: Range and collection localities for *Vaejovis coahuilae.*

Coahuilae, Mexico. The holotype for the species was described as "base color of body brownish-yellow, this slightly darker on dorsum, pedipalps, and telson; pectines almost white; carapace with area of distinct dusky pigmentation" (Williams 1968). This distinct coloration feature is found to be exhibited more or less depending on the substrate to which the scorpion is adapted (Williams 1968). Some of the populations of *V. coahuilae* sampled for this study have been observed to differ from each other in subtle ways (such as variations in color) depending on a particular groups' locality, especially in Big Bend National Park in Texas. Molecular analyses that use different species and take into account a populations' biogeography suggest differences most likely arose in response to geological events that have occurred since the origin of their

founding population and, more recently, in response to anthropological actions (Gantenbein et al. 2000).

Vaejovis coahuilae is ecomorphologically adapted to a generalized habitat. Stanke (1974) classified a scorpion as "errant" if they remained active while foraging as opposed to those scorpions that remain relatively inactive and if they were dispersed among a variety of habitats. He described this ecomorphotype as one with slender pedipalps and a long, slender body. This body plan has likely enabled the species to readily disperse from their founding population across diverse habitats in its North American range. Molecular studies may provide clues into the relationships that exist between and within populations of *V. coahuilae* where morphological character states that have been used in the recreation of this species life history have failed to explain such discrepancies. Recent molecular research shows that there are nuclear and mitochondrial markers that can be used to shed light on the phylogeny and evolutionary history of a species (Gantenbein et al. 1999). Gantenbein et al. (1999) produced a new phylogeny of four species of the genus *Euscorpius* based on an analysis of mitochondrial DNA and nuclear gene variation. The phylogeny revealed patterns of genetic divergence that coincide with geographical events (Gantenbein et al. 1999). The same group of researchers has used these identical genetic markers to reveal a divergence of two parapatric scorpion species in the Alps (Gantenbein et al. 2000). An understanding of the genetic variations that exist within populations of *V. coahuilae* collected in specific habitats and geographies could be complemented by comparing them to those that exist within populations of this species throughout its range. This molecular analysis could provide an estimate of the degree of genetic variation between

V. coahuilae populations and could allude to the occurrence and amount of gene flow. At the very least my research will reveal any variation of sequence data among populations of *V. coahuilae* and it may be able to show the spatial distributions of particular sequence aberrations that may be present in some populations.

The rate of evolution experienced by different populations of *V. coahuilae* is not known. If populations experience different rates of evolution it may be possible to discover underlying causes for the variation. In order to characterize genetic variation, the amount of variation must be described in terms of how it is organized among populations. If *V. coahuilae* exhibits a variable evolutionary rate then it may be possible to explain this variation in terms of life history strategies, ecology and demographics. Owing to the fact that *V. coahuilae* is more common and widespread than many other species it is expected that they have experienced a high rate of evolution. *Vaejoivs coahuilae* is more or less a generalist species but its distribution appears discontinuous depending on habitat suitability. Periods of geographic isolation among populations likely promoted morphologically and genetically different populations (Yamashita & Polis 1995a). Indeed, there are slight morphological variations depending on the substrate that the specimens where collected on.

This study set out to compare the amount of genetic variation and possibly gene flow between and within populations of a representative of the family Vaejovidae to gain a more complete understanding of their evolutionary histories. Analysis of a highly conserved regions of DNA including a 500 bp section of a mitochondrial 16S rDNA gene and a 710 bp section of the cytochrome c oxidase subunit I (COI) gene will reveal any substitutions that have taken place over time. These mitochondrial DNA

markers are widely used to decipher phylogenetic relationships among taxa including many scorpion species because of their conserved nature. Theoretically, among DNA sequence the number of mismatches between populations should correlate to the time that each population has been isolated from the others and the location of these mismatches should reveal relationships that exist between them (Gantenbein et al. 1999). A recent study by Gantenbein et al. (2005) demonstrated compelling evidence for recombination in the mitochondrial genome of scorpions in the family Buthidae Koch 1837. Cytological observations have revealed that spermatogenesis of several buthid scorpions is unique in that it involves two different segregation mechanisms. Formation of a structure formed by mitochondrial fusion known as Nebenkern structures have been observed in later phases of spermatogenesis in some buthid scorpions as well as many other invertebrates. A second mode of recombination that occurs at the very beginning of meiosis, which results in a ring shaped Nebenkern which undergoes two subsequent divisions, has only been observed in the scorpion family, Buthidae (Gantenbein et al. 2005). Cytological studies of spermatogenesis in the family Vaejovidae suggest no such recombination occurs. Incidentally, the Buthidae, believed to be an ancient scorpion lineage, is the only family whose venom exhibit mammal specific neurotoxins. Gantenbein et al. (2005) used the same mitochondrial DNA markers utilized in this study in relation to the estimated physical distance between them using the complete mitochondrial genome sequenced from the species *Mesobuthus gibbosus*. They observed a marked decline of linkage-disequlibrium correlated to physical distance between the loci as is commonly observed

for nuclear recombining genes suggesting recombination does occur in the scorpion mitochondrial genome (Gantenbein et al. 2005).

Molecular data combined with toxin, allozyme, cytological, as well as detailed morphological data of extant and fossil scorpions, could reveal a truer taxonomic and genetic structure of populations of *V. coahuilae* in Texas and New Mexico. We predicted phylogenetic analysis of the mitochondrial markers used in this study would indeed reveal sequence variation between populations of *V. coahuilae* and likely even among populations of this common species. Based on the current reported range of this species we predicted that there would be a moderate amount of gene flow between populations due to its generalist nature. Any variations in the sequence data may also provide some insight into general migratory pathways taken by this species from Texas through New Mexico. A previous study on sand scorpion populations revealed that populations become less dense, more isolated, and less variable genetically the farther they are from the center of a species' range (Yamashita & Polis 1995b). The latter may be the case with populations of *V. coahuilae* allowing one to determine which of the selected populations is likely to be the most recently evolved. Comparisons of the genetic variation observed for populations of *V. coahuilae* to those observed for other species could reveal how *V. coahuilae* differs in its ability to adapt to ever changing environmental conditions. Genetic differences between sampled populations may be related to the differences in habitat location or substrate composition and reveal that some of these populations may exist on more of a continuum than others. This study may also serve as a conservation model to study the effects of habitat fragmentation, which is becoming increasingly problematic in the twenty-first century.

MATERIALS AND METHODS

Specimens of *Vaejovis coahuilae* were collected in May and June 2003 and 2004 as well as in July of 2005. The collection sites covered a 1,200 mile range from the trans-pecos area in Texas, which includes Big Bend National Park (BBNP) and Guadalupe Mountain National Park (GMNP) as well as the most western parts of Texas. Collections were also made throughout New Mexico (Figure 1). The latitude and longitude at the site of each collection was obtained with a Garmin GPS 12XL personal navigator. General habitat descriptions were recorded at each locality. Collection sites ranged from rocky to consolidated sand substrates with various types and density of vegetation. A black light was used to locate the scorpions at night when the moon was waning or new. Each scorpion was stored alive overnight. The next morning the specimens were identified then placed in 95% ethanol. One pedipalp was cut off of each scorpion so that the internal tissues could be preserved and the DNA fixed for analysis.

Collection Sites

Specimens were collected throughout much of the know range of *Vaejovis coahuilae* in the United States (Figure 1). Collections were made in Big Bend National Park, Texas; Palo Duro Canyon, Texas; Los Palomas Wildlife Management Area, Texas; Guadalupe Mountains National Park, Texas; Roswell, New Mexico; Albuquerque, New Mexico; San Antonio, New Mexico; and Oliver Lee Memorial State Park, New Mexico and Deming, New Mexico. The collections made in Deming, NM

were the most western collections, while the most eastern and northern collections were made in Palo Duro Canyon in Randall County, TX and Albuquerque, NM, respectively.

Big Bend National Park (B)**, Texas**

Big Bend National Park is located in Brewster County, Texas (29° 15' 0" N latitude, 103° 15' 0" W longitude). Specimens of *V. coahuilae* used in this analysis were collected from 10 different localities within the park. The habitats in the park vary tremendously, ranging from riparian along the Rio Grande to coniferous forests in the Chisos mountains with desert, grassland and deciduous forests in between (R. Henson Personal Communication & Moss 1983). The elevation in the park ranges from about 610 meters (m) along the river to 2358 m at Emory Peak, covering five life zones. Two specimens were collected from Grapevine Hills; one from Grapevine Hills circle (B) (29° 24'27.3" N latitude, 103° 11' 29.7" W longitude) and one from Grapevine Hills trail (B2) (29° 24'37.8" N latitude, 103° 12' 27.6" W longitude) all three locations consisting of consolidated sand. Also in a consolidated sand habitat, two additional specimens were collected from La Harmonia west of Castolon (B3 and B4) (29° 08'20.9" N latitude, 103° 31' 27.2" W longitude). Two specimens were collected from Dugout Wells (B5 and B6) (29° 16' 16.7" N latitude, 103° 08' 10.1" W longitude) which is a consolidated sand habitat with some hardpan. Three specimens used for analysis were collected from Dagger Flats circle in consolidated sand and a moderately rocky habitat (B7, B8, and B9) (29° 31'31" N latitude, 103° 02' 57.2" W longitude). Three more specimens were collected from a consolidated sand habitat at

Sam Nail Ranch off of Ross Maxwell highway (B10, B11, and B12) (29° 16' 45.2" N latitude, 103° 22' 07.3" W longitude).

One specimen was collected at each of the following sites: Hot Springs (B13) on consolidated sand (29° 10' 39.7" N latitude, 102° 59' 57.8" W longitude); Boquillas Canyon also on consolidated sand (B14) (29° 12'0.82" N latitude, 102° 54' 49.7" W longitude); Santa Elena Road on consolidated sand with some rocky areas (B15) (29° 08' 37.7" N latitude, 103° 31' 46.0" W longitude); Rio Grande Village west of RV park (B16) (29° 11' 10.3" N latitude, 102° 58' 11.3" W longitude); and at Green Gulch off of Basin Road in a habitat consisting of hardpan and rocky substrates (B17) (29° 18' 34.2" N latitude, 103° 15' 42.8" W longitude).

Palo Duro Canyon (P)**, Texas**

One specimen (P) used for analysis was collected in a rocky area at Palo Duro Canyon, located in Randall County, Texas. This specimen was collected by Kari J. McWest, approximately seven miles north east of the canyon off of FM 1541.

Los Palomas Wildlife Management Area (L)**, Texas**

Los Palomas Wildlife Management Area is located approximately one half mile west of Ruidosa Texas at Gate #1of highway 170 in Texas (Presidio County). Two specimens (L and L1) were collected about one half mile from the Rio Grande River in a consolidated sand habitat, with a lot of vegetation (30° 00' 49" N latitude, 104° 41' 17" W longitude).

Guadalupe Mountain National Park (G)**, Texas**

Guadalupe Mountain National Park is located in Culberson and Hudspeth counties in Texas along the border of New Mexico. Two specimens used in the analysis were collected at the Ship on the Desert in a rocky habitat with moderate vegetation (G and G2) (31° 57' 29.7" N latitude, 104° 45' 31.8" W longitude). One specimen was collected on the northeast side of the park outside the boundary off of highway 62/180 (Culberson County) in a hill-cut with a lot of vegetation (G3) (31° 47' 55" N latitude, 104° 51' 40" W longitude).

Roswell (R)**, New Mexico**

Four specimens used for analysis were collected in Roswell, New Mexico (Chavez County) approximately six miles west of town, off of US highway 380. Three specimens (R, R2, and R3) were collected in a rocky hillcut on the east side of the Pecos River (33° 23' 47.7" N latitude, 104° 23' 11.8" W longitude). Another specimen (R4) was collected on the west side of the Pecos River alongside the sandy river bank which had a lot of vegetation (33° 23' 36.4" N latitude, 104° 23' 56.4" W longitude).

Albuquerque (A)**, New Mexico**

Two specimens used for analysis were collected in Albuquerque, New Mexico (Bernalillo County). These specimens (A and A1) were collected off of Unser Road in a consolidated sand habitat with a considerable amount of vegetation (35° 10' 47.6" N latitude, 106° 43' 40.4" W longitude).

San Antonio (S)**, New Mexico**

One specimen (S) was collected in San Antonio, New Mexico (Socorro County) approximately one and a half miles from interstate 25S at the historical marker for the birth place of Conrad Hilton. The substrate type was consolidated sand with heavy vegetation cover (33° 55' 01.9" N latitude, 106° 52' 24.3" W longitude).

Oliver Lee Memorial State Park (O)**, New Mexico**

Oliver Lee Memorial State Park is located approximately 10 miles south of Alamagordo, NM (Otero County). Two specimens were collected outside the entrance to the park; one (O) was collected just outside the gate to the park (32° 44' 50.0" N latitude, 105° 55' 11.7" W longitude) and the other (O2) was collected about three-quarters of a mile from the gate (32° 44' 50.0" N latitude, 105° 55' 11.7" W longitude).

Deming (D)**, New Mexico**

One specimen used in this analysis was collected in Deming, New Mexico (Luna County). The sample was collected approximately one mile from interstate 10, in the town of Deming, off Country Club Drive, in consolidated sand and with a lot of vegetation cover comprised of mainly of acacia thickets and many low-lying cacti (D) (32° 15' 13.8" N latitude, 107° 44' 18.7" W longitude).

Out-Group

Specimens of *Vaejovis crassimanus* Pocock 1898 (VCr)*, Vaejovis globosus*

Borelli 1915 (VG), *Vaejovis chisos* Sissom 1990 (VCh), and *Paruroctonus gracilior* Hoffman 1931 (PG) were also collected between 2003 and 2005 for outgroup comparison. Two *V. crassimanus* specimens were used for the analysis that were collected from the same locality listed for specimens of *V. coahuilae* from the northeast side of the Guadalupe Mountains National Park and from Rio Grande Village in Big Bend National Park. The *V. globosus* specimen was also collected at the aforementioned Rio Grande Village location in Big Bend National Park. *Vaejovis chisos* was collected at Cattail Falls in Big Bend National Park (29° 16' 42.4" N latitude, 103° 20' 32" W longitude). While *P. gracilior* was collected outside the entrance to living Desert State Park in New Mexico (Eddy County).

Total DNA Isolation

Total DNA extraction from *Vaejovis coahuilae* was performed using a DNeasy animal tissue kit from Qiagen (Valencia, CA). One or two preserved pedipalps were frozen in liquid nitrogen then ground to a fine powder in a chilled pestle and mortar. The powder was transferred to an Eppendorf tube and according to the protocol ATL buffer and proteinase K (20 mg/ml) were added before being placed in a 55°C water bath for one hour. RNase (100 mg/ml) was added to each sample and allowed to incubate for two minutes at room temperature and then AL buffer was added and each sample was allowed to incubate for 10 minutes in a 70°C water bath. Following this, 100% ethanol was added to the sample which was vortexed. The resulting homogenate was transferred to a DNeasy mini-column and centrifuged for one minute at 6000 x g in an Eppendorf centrifudge model 5417C (Eppendorf, North Anerica Westburg, NY).

AW1 buffer was added and the samples were centrifuged as above. Buffer AW2 was added and the samples were spun for three minutes at maximum speed. If the membrane of the mini-column was not noticeably dry the sample was spun for an additional minute at full speed. Finally, AE buffer was added directly to the membrane, allowed to incubate for one minute at room temperature, and then centrifuged at 6000 x g for one minute. The column was removed and the collected DNA was stored in a -20°C freezer until needed.

Three to five microliters (ul) of the isolated DNA was analyzed for relative concentration on a 0.8% agarose gel with *Lambda Hin*d III size markers. Each gel was allowed to run for one hour at 100 volts using a BRL Life Technologies Model 250 power supply, stained with ethidium bromide (2 ug/ml) then visualized with an Alpha Innotech Digital Imaging and Analysis System (Alpha Innotech Corporation, San Leandro, CA).

PCR Amplification of Mitochondrial DNA

16S rDNA

A 500 base pair (bp) section of the mitochondrial 16S rDNA gene was amplified by PCR (Polymerase Chain Reaction) with the use of scorpion specific primers, *38* (5'GTGCAAAGGTAGCATAA TCA3') and *40* (5'CGATTTGAACTCAGATCA3') which have been shown to work with scorpion specific sequences (Ganteinbein et al. 1999). These primers were synthesized at the Core Facility of Marshall University (Huntington, WV) as a 300 uM stock solution and were diluted to a 10 uM working solution and stored at -20°C. Amplification of the

selected sequences was achieved with the use of the Takara Ex Taq DNA Polymerase kit (Takara Bio, Madison, WI) following the recommended protocol and component concentrations. In each 25 µl PCR reaction, there was 5 ul of isolated template DNA, 0.125 ul Takara Ex Taq DNA polymerase, 2 ul dNTP mixture (2.5 mM each dNTP), 2.5 ul 10X Ex Taq buffer (containing 20 mM $MgCl_2$), 1 ul each of the working primer, and 13.375 ul of sterile distilled water (dH_2O). Using a GeneAmp 9600 Thermocycler (Perkin Elmer, Norwalk, CT), DNA was first denatured at 95°C for 5 minutes, then 45 cycles were run as follows: 95°C for 1 minute for denaturation; 50°C for 1 minute to allow primer annealing; and 72°C for 1 minute of sequence elongation. This was followed by a final 5 minute sequence elongation at 72°C then a 4°C hold. The PCR products were stored at -20°C until analyzed to assess size, concentration and purity. Two microliters of each PCR product and *PhiX* 174 *Hae* III size markers were loaded onto a 1% agarose gel allowed to run for one hour at 100 volts, then stained with ethidium bromide (2 ug/ml), and visualized with the Alpha Innotech Digital Imaging and Analysis System.

Cytochrome c Oxidase Subunit I (COI)

For the partial amplification of the cytochrome *c* oxidase subunit I gene the primers *LCO* (5'GGGTCAACAAAATCATAAAGATATTGG3') and *HCO* (5'TAAACTTCAGGGTGACCAAAAAATCA3') were utilized, which were reported to consistently amplify a 650 bp fragment (Folmer, et al. 1994). These primers were also synthesized at the Core Facility of Marshall University as a 300 uM stock solution and then diluted to a 10 uM working solution. The cycle sequencing parameters were

modified from a protocol used successfully on scorpion specimens designed by Gantenbein et al. (2003). Using the GeneAmp 9600 Thermocycler, DNA was first denatured at 94°C for 4 minutes, followed by 40 cycles each consisting of: 94°C for 30 seconds; 50°C for 20 seconds; and 72°C for 90 seconds. This was followed by a final 5 minute sequence elongation at 72°C then a 4°C hold. The PCR products were stored at -20°C until analyzed by gel electrophoresis as described for the PCR products of the 16S rDNA gene.

DNA Sequencing

The PCR products were prepared for sequencing with the YM-100 Microcon centrifugal filter device (Millipore Corporation, Bedford, MA). Following the protocol from the manufacturer, each PCR product was transferred to the spin filter reservoir with distilled water and then centrifuged at room temperature for 15 minutes at 500 x g. Twenty microliters of Tris EDTA buffer was added to the filter device which was subsequently inverted into a new Eppendorf tube and centrifuged at room temperature for three minutes at a speed of 1000 x g. The cleaned PCR products were stored at -20°C until sequenced.

PCR products were sequenced either using a LI-COR Gene Reader 4200 (LI-COR Inc., Lincoln, NE) with IR700 and IR800 dye labeled primers or at the DNA Core Facility at Cornell University using same primers used for PCR amplification. Sequences produced with the LI-COR Gene Reader 4200 were prepared with a USB thermosequenase kit (USB Corp., Cleveland, OH) and cycle sequencing was performed in a Perkin-Elmer Gene Amp 9600 Thermocycler. Each sequencing reaction was

prepared according to LI-COR recommendations and contained template DNA (50-100 ng/ul), 2 ul of each respective IR dye labeled primer, and 2.5 mM of each dNTP nucleotide mixed with 7 deaza-dGTP (Roche Molecular Biochemicals, Indianapolis, IA). The cycle sequencing protocol began with an initial denaturation at 92°C for 30 seconds, followed by 30 cycles of 94°C for 30 seconds for denaturation; 50°C cycle for 30 seconds of primer annealing and 72°C elongation cycle for 1 minute ending with a 4°C hold. Upon completion LI-COR stop solution was added to each reaction tube and samples were stored at -20°C until sequenced.

The samples were sequenced on a 5.5% polyacrylimide gel using 41 cm glass plates and a 48-well sharks-tooth comb. Samples were melted at 92°C for 3 minutes before being loaded onto the gel. Upon completion, sequences were examined using e-Seq DNA sequencing and analysis software (LI-COR Inc., Lincoln, NE). Sequences were then assessed for accuracy of the base calls and edited where necessary.

Samples sent to the Core Facility at Cornell University were sequenced using the Applied Biosystems Automated 3730 DNA Analyzer (Applied Biosystems, Applera Corp., Foster City, CA) and Big Dye Terminator chemistry (Applied Biosystems, Applera Corp., Foster City, CA) with AmpliTaq-FS DNA Polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ).

Sequence Alignment

Multiple copies of individual sequences were aligned with the AlignIR alignment software (LI-COR Inc., Lincoln, NE). All sequences for each respective

gene were aligned using the available Windows based shareware BioEdit (BioEdit 2001) and only sequenced sections found in all samples were used for further analysis.

Phylogenetic Analysis

Statistical analysis was done individually on each gene and a separate analysis was done by combining the 16S rDNA and COI fragments together. The software program Phylogenetic Analysis Using Parsimony or PAUP (version 4.06b, Swafford 1991) was utilized to perform a bootstrap analysis using a fast-heuristic search and parsimony criteria with 1,000 bootstrap replicates present at each node. The assumptions of the PAUP analysis however, are not explicitly designed to account for data at the population level.

RESULTS

Total DNA was isolated from 39 scorpions including 34 specimens of *Vaejovis coahuilae*, four outgroup species including two from *Vaejovis crassimanus* (VCr), and one each of, *Vaejovis globosus* (VG), *Vaejovis chisos* (VCh), and *Paruroctonus gracilior* (PG). Isolations were analyzed by gel electrophoresis using *Lambda Hin*d III size markers and revealed a single high molecular weight DNA band (Figure 2).

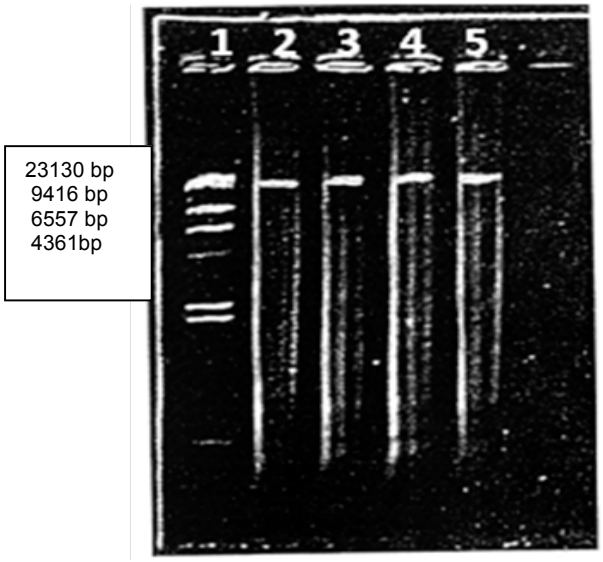

Figure 2. Total DNA isolation from *Vaejovis coahuilae* (lanes 2-5) with *Lamda Hin*d III size marker (lane 1).

Mitochondrial 16S rDNA

Approximately 500 base pairs of the mitochondrial 16S rDNA was amplified by PCR and confirmed by gel electrophoresis using *PhiX* 174 *Hae* III size markers (Figure 3). A total of 35 PCR products, including 33 specimens of *Vaejovis coahuilae* and one from each of the designated outgroup species, *Vaejovis crassimanus* and

Vaejovis globosus, were successfully sequenced. Sequences were aligned and trimmed to the same length of characters, analyzed and found to be parsimony-informative (Appendix A).

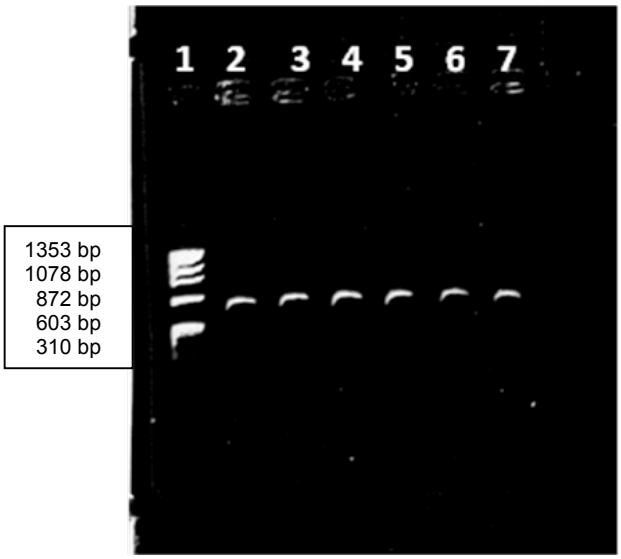

Figure 3. Mitochondrial 16S rDNA. 500 bp PCR product of 16S rDNA region of mitochondrial genome from *Vaejovis coahuilae* (lane 2-7) with *PhiX 174 Hae* III size marker (lane 1).

The partial sequence of the 16S rDNA was generated with a bootstrap analysis using a fast-heuristic search and parsimony criteria with 1,000 bootstrap replicates present at each node (Figure 4). The bootstrap consensus tree places all populations of *V. coahuilae* as a monophyletic clade supported by 99% of the bootstrap replicates distinctly separating the species from the outgroups *V. crassimanus* (VCr) and *V. globosus* (VG). The *V. coahuilae* clade contains a large group of unresolved populations. Within this large polytomy 22 specimens lie at the base of the branch and represent samples from populations distributed throughout the collected range. There are four monophyletic

subclades within this group supported by varying bootstrap values. The Dagger Flats population consisting of three individuals (B7, B8 and B9) forms a monophyletic subclade with bootstrap support of 61%. The topology indicates that the specimens from Dagger Flats are more closely related to each other than to any of the other populations. The second monophyletic subclade consists of one individual (B16) from Rio Grande

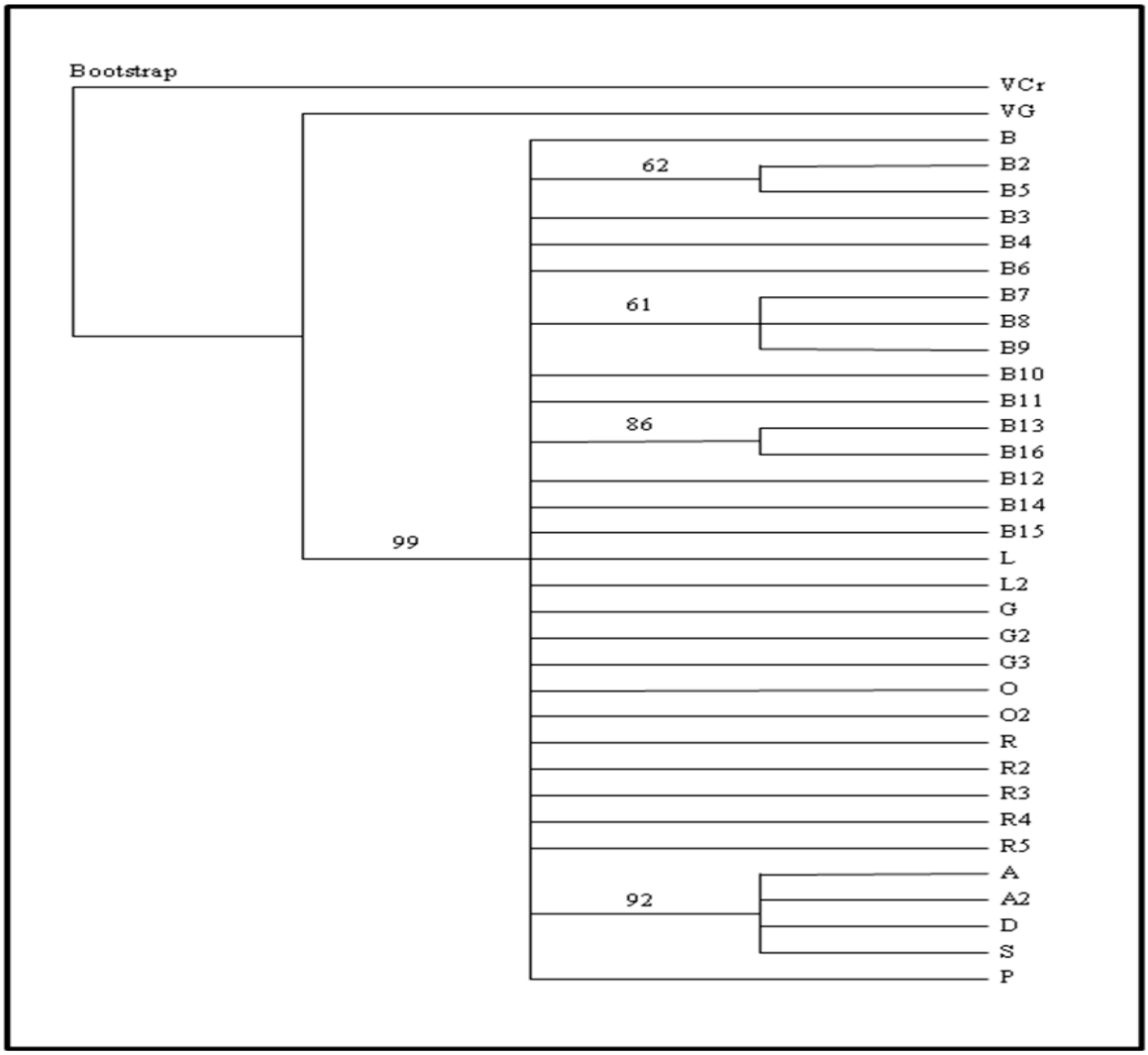

Figure 4. Bootstrap consensus tree of partial sequence of 16S rDNA produced using a fast-heuristic search and parsimony criteria. 1000 replicated bootstrap values present at each node. VCr *Vaejovis crassimanus*; VG *Vaejovis globosus*; B Big Bend National Park, TX; G Guadalupe Mountains National Park, TX; R Roswell, NM; P PaloDuro Creek Canyon, TX; L Los Palamos ; S San Antonio, NM; D Deming NM; A Albuquerque, NM; O Oliver Lee State Memorial Park, NM.

Village and one (B13) from Hot Springs, both from Big Bend National Park (BBNP). This subgroup is supported by a bootstrap of 86%. Individuals from two separate populations in BBNP including one from Grapevine Hills (B2) trail and one from Dugout Wells (B5) form another monophyletic subclade with a bootstrap of 62%. Four of specimens from the most northeastern populations including representatives from San Antonio (S), Deming (D), and Albuquerque (A and A2) form a fourth monophyletic subclade strongly supported by a bootstrap of 92%.

Cytochrome c Oxidase Subunit I (COI)

Isolated DNA from 16 specimens was PCR amplified to produce an approximately 710 base pair section of the cytochrome c oxidase subunit I (COI) mitochondrial DNA gene. The PCR products were analyzed by gel electrophoresis using *PhiX* 174 *Hae* size markers (Figure 5).

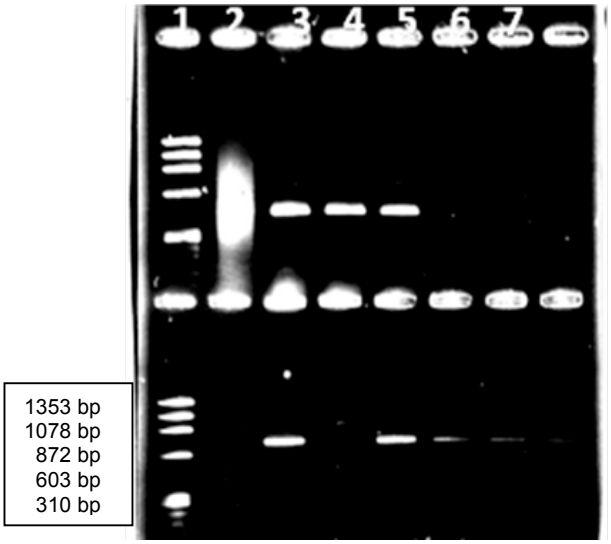

Figure 5. Cytochrome c oxidase subunit I. 710 bp PCR amplification product of partial sequence of COI gene (bottom: lane 3 and lanes 5-7) with *PhiX 174 Hae* III size marker (lane1).

The COI amplified sequences are shown on the bottom half of the pictured gel in lanes 3, 5, 6 and 7. All sequences were aligned and trimmed to a length of 696 base pairs. There were 532 constant characters, 91 variable characters, and 73 parsimony-informative characters (Appendix B).

The partial sequence of the COI was generated with a bootstrap analysis using a fast-heuristic search and parsimony criteria with 1,000 bootstrap replicates present at each node (Figure 6). The bootstrap consensus tree places all populations of *V. coahuilae* as a monophyletic clade separating the species from the outgroups *V. chisos* (VCh) and *V. crassimanus* (VCr). The *V. coahuilae* clade contains one unresolved population (G3) from the GMNP locality. This topology was supported in 100% of the bootstrap replicates. The remaining scorpion sequences form a large polytomy containing three strongly supported monophyletic subclades. One group contains the Dagger Flats population (B8 and B9) from BBNP. This subgroup, also used in the analysis of the16S rDNA, is strongly supported with bootstrap support of 94%. Three new monophyletic subclades were produced representing three collection localities in BBNP. One subclade supported with a bootstrap of 54% represents scorpions from Dugout Wells (B5 and B6). The other monophyletic subclade supported by a bootstrap value of 100 contains individuals from Sam Nail Ranch (B11) and Green Gulch (B17). Scorpion sequences from the Roswell (R4 and R5) and GMNP (G) populations were resolved into a monophyletic subclade in 98% of the bootstrap replicates. The only Albuquerque population (A2) in this analysis remained unresolved, as did sequences from BBNP at Rio Grande Village (B16) and Hot Springs (B13) collection sites.

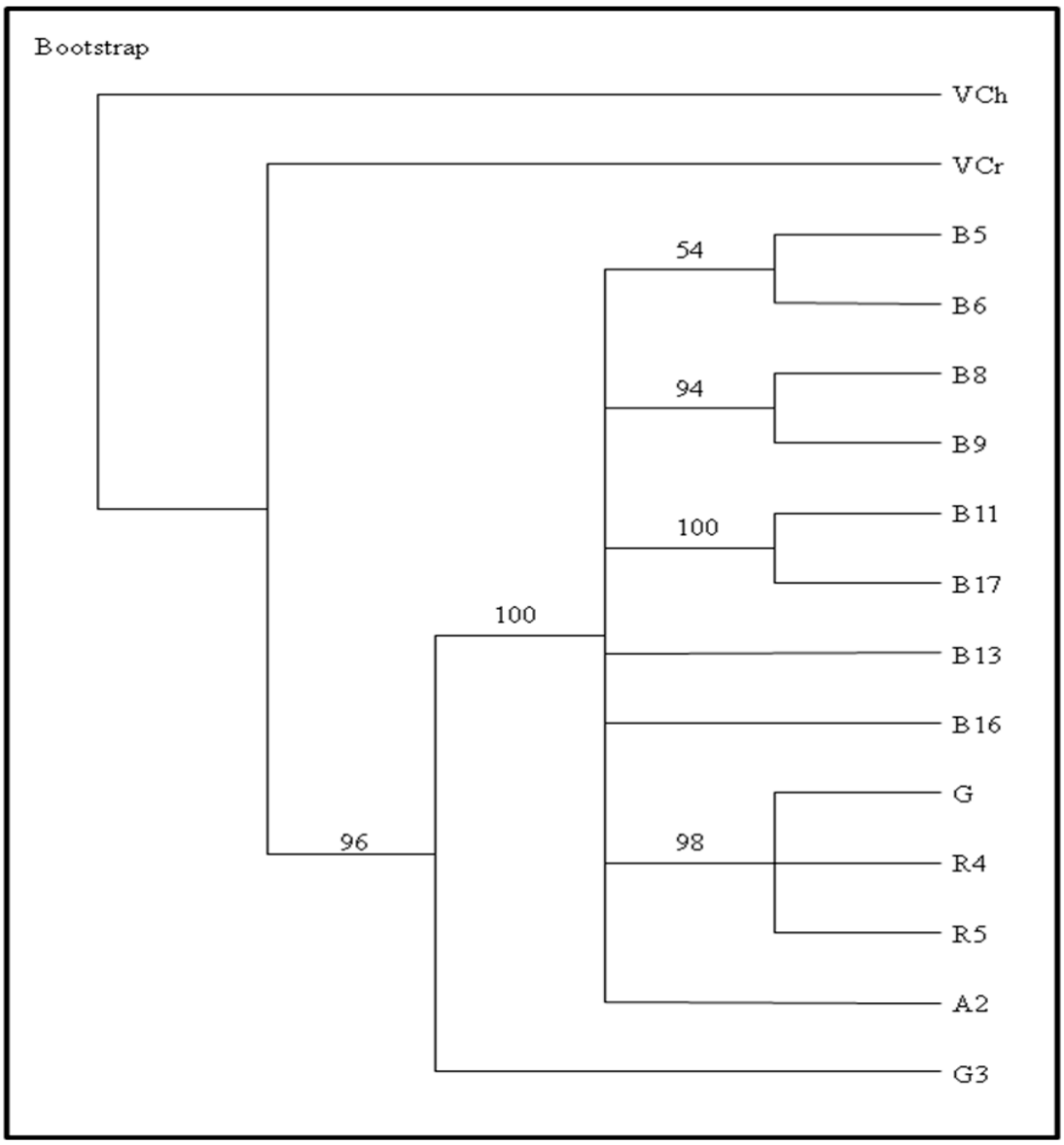

Figure 6. Bootstrap consensus tree of partial sequence of COI produced using a fast-heuristic search and parsimony criteria. 1000 replicated bootstrap values present at each node. VCh *Vaejovis chisos*; VCr *Vaejovis crassimanus*; B Big Bend National Park, TX; R Roswell, NM; A Albuquerque, NM; G Guadalupe Mountains National Park, TX.

Mitochondrial 16S rDNA and Cytochrome c Oxidase subunit I (COI)

Sequences of both the 16S rDNA and the COI were combined to create a larger sequence for each individual studied and the resulting sequences reanalyzed. Sequences

were aligned and trimmed to the same length of 1033 base pairs. Fifty-six of these characters were found to be parsimony-informative, 828 characters were constant, and 162 characters were variable (Appendix C). The bootstrap consensus tree of the combined 16S/COI sequences was produced using a fast-heuristic search and parsimony criteria (Figure 7). The *V. coahuilae* clade in this combined analysis shows greater resolution of some of the populations in the large polytomy presented in the tree topologies produced by the separate analyses of the 16s rDNA and COI sequence data alone. The majority of specimens of *V. coahuilae* form a monophyletic clade supported by 98% of the bootstrap replicates which is divided into two groups**.** One group supported by 51% of the bootstrap replicates contains all individuals from BBNP and the only specimen from Albuquerque (A2). Within this group, the Albuquerque and Sam Nail Ranch (B11) sequences remain unresolved. The remaining BBNP populations are separated into a monophyletic clade in 52% of the bootstrap replicates. This subclade contains two additional branches. One monophyletic group supported in 51% of the bootstrap replicates is further divided into two sister clades representing the population from Dugout Wells (B5 and B6) and one containing individuals from Rio Grande Village (B16) and Hotsprings (B13). The other branch was strongly supported by 97% of the bootstrap replicates representing the only two individuals(B8 and B9) in the Dagger Flats population that have retained distinction in both the 16S rDNA and the COI analyses. The other monophyletic subclade in the *V. coahuilae* clade was separated

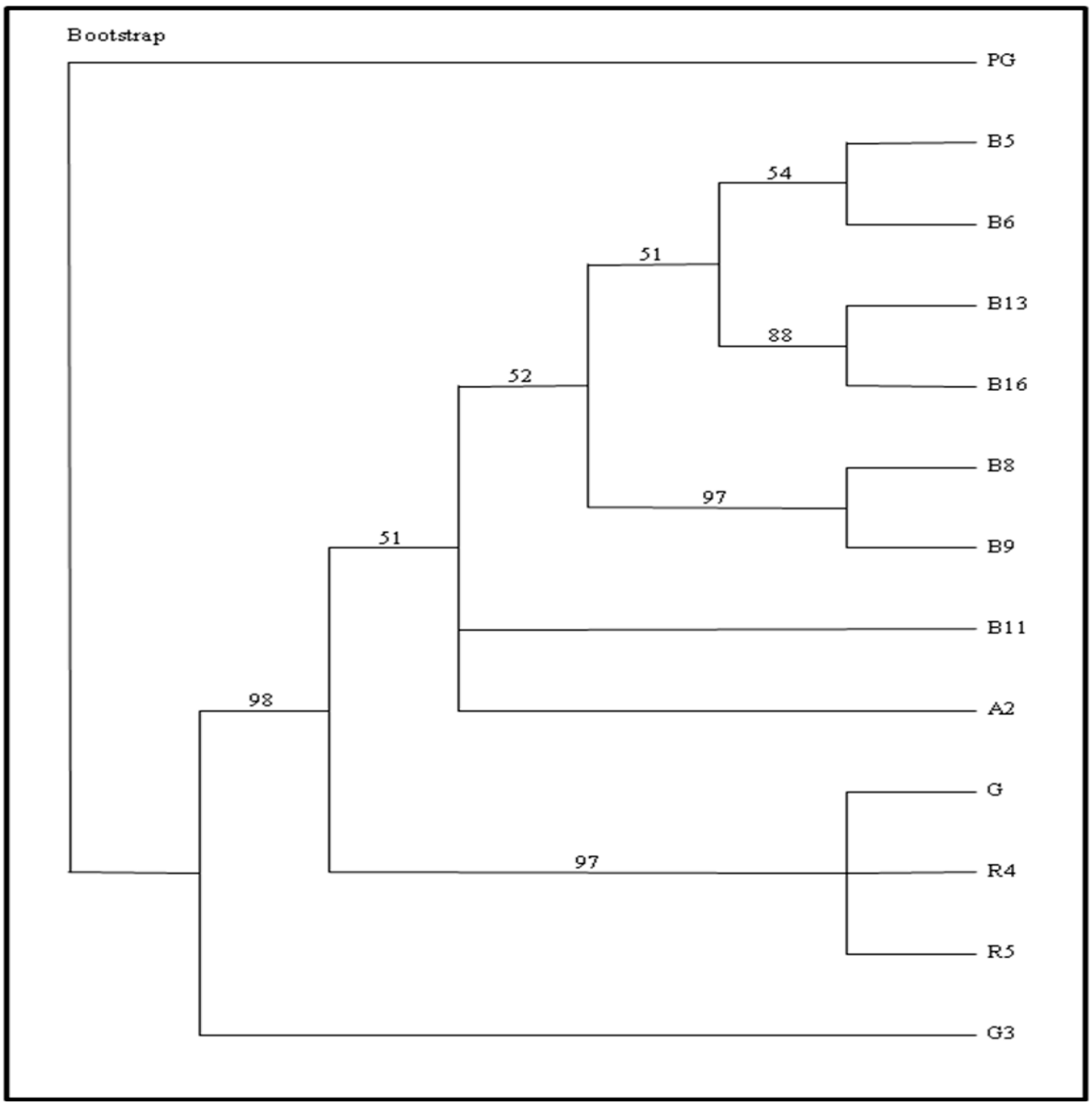

Figure 7. Maximum parsimony consensus tree of combined 16S/COI sequence data selected from equally parsimonious trees produced through a fast-heuristic search. 1000 replicated bootstrap values present at each node. PG *Paruroctonus gracilior;* G Guadalupe Mountains National Park, TX; R Roswell, NM; B Big Bend National Park, TX; Albuquerque, NM.

from all BBNP populations as well as Albuquerque in the maximum parsimony tree with strong support. In 97% of the bootstrap replicates two specimens from Roswell (R4 and

R5) and the only specimen in this analysis from GMNP (G) are suggested to be more closely related to each other than to any of the other populations.

Overall, analysis of the appended sequence data containing information from both the mitochondrial 16S rDNA and the COI gene provided greater resolution of included taxa than either analysis of the genes done separately. In addition, several more populations were represented in the analysis of the 16S rDNA than in the analysis of the just the COI data or the combined sequence set.

DISCUSSION

Experts are currently revising the family Vaejovidae and the relationship of the *V. coahuilae* to others members of this family even while the genus is being debated (R. Henson Personal Communication; Soleglad & Fet 2008). Soleglad & Fet (2008) propose that *V. coahuilae* should be placed in a new genus based on analysis of mitochondrial 16s rDNA and trichobothria character states. They also propose that *V. coahuilae* be placed in a new subfamily and tribe and be renamed *Hoffimannus coahuilae* Williams 1968. One goal of this study was to determine the phylogeograhical relationships that exist within and between populations of *V. coahuilae* collected throughout Texas and New Mexico to gain some insight into the present geographical distribution of the this species gene pool. Maximum parsimony analyses of the mitochondrial markers analyzed in this study simply determines the most likely explanation or the fewest number of evolutionary steps required to account for the amount of sequence variation; it cannot, however, take into account the amount of genetic variation based on the actual geographic distance between haplotypes. The nested clade analysis, however, can be used to identify geographical factors which influence the genetic variation based on the geographic distance from one haplotype to another (Templeton 1998). This type of analysis can also estimate how particular geographic factors have influenced the genetic variation among populations providing specific information about the direction and amount of gene flow. One study was able to explain the haplotype distribution of the sand-loving scorpion *Paruroctonus utahensis* Williams 1968 using the nested clade analysis (Estep et al.

2005). Combining specific GPS locality information and mitochondrial 16s rDNA sequence data Estep et al. (2005) showed that the biological factor influencing the observed differences in the sampled populations of *P.utahensis* on their respective dune systems was allopatric fragmentation due to the isolation by fragmentation of a larger habitat. It was also possible to postulate the migratory pathway of this scorpion by considering the historical and geological aspects of the Chihuahuan desert biome which throughout the geologic history of the area has variously expanded and receded based on periods of aridity (Estep et al. 2005).

Performing a nested clade analysis was one aim of this research because this method could provide more insight into the spatial distribution of sequences among populations of *V. coahuilae.* Due to the fact that there was not enough variability in the sequence data from the populations of *V. coahuilae* sampled for this study, invariably in part due to lack of enough sequenced samples represented from each locality it was not feasible to analyze the sequenced mitochondrial DNA markers using the nested clade analysis. Owing to the fact that this species is a generalist it can be reasonably suggested that the small amount of observed sequence variability could be the result of colonization via long distance dispersal which can be accelerated by the introduction of species into new populations by human activity (Doukakis et al. 2002). Although as Estep et al. (2005) point out, the psammophilic nature of many species in the family Vaejovidae, like *P. utahensis* could be due to multiple speciation events initiated by habitat fragmentation produced throughout the dynamic climatic history of the Chihuahuan desert. *Vaejovis coahuilae* was possibly advantaged during these fragmentation events because of their "errant" nature. While this species is considered to be a generalist, Williams (1968)

describes several populations collected on white gypsum noting that they seemed to prefer the sandy, dune-habitats over other suitable habitats. He noted that in 9 out of 10 localities *V. coahuilae* was numerically subdominate and that the base body color of these specimens was uncharacteristically light (almost white) and the dusky pattern characteristic of this species was greatly reduced.

Resolution of the populations sampled in the present study increases as more characters are added to the maximum parsimony analysis. This refinement suggests that if more of the COI sequence data were represented in the combined analysis with the 16S rDNA sequences, a clearer picture of the spatial distribution of haplotypes could be postulated. Similarities that exist in the parsimony-informative nucleotide sites in this sequence analyses suggest that some populations of *V. coahuilae* have more recently experienced gene flow with other populations in the sampled range. For example, populations in Big Bend National Park, could be suggested to be more closely related to each other genetically than with more geographically distant populations. This is more strongly supported by the topology of the tree in the combined sequence analysis by the sister group association between the Dugout Wells population and the monophyletic group containing the Boquillas Canyon and Hot Springs populations. The scorpions from Dagger Flats maintained their association throughout all three analyses with bootstrap support for their separation increasing as the number of characters in the analyses increased. The topology of the combined sequence data tree suggests the specimen from GMNP collected at an elevation high in the mountains (G3) has in the evolutionary history of this species experienced gene flow less recently with other populations sampled. The branching pattern of the tree produced from the COI sequence data

suggests the second specimen (G) from GMNP, which was collected at a much lower elevation on the eastern side of the mountain range has more recently experienced gene flow with populations in Roswell. In both the COI tree and the combined sequence data tree the Albuquerque population appears to be more genetically related to populations from BBNP which represents the most northern and southern reaches of the sampled range. This suggests an accessible route for gene flow between the populations. It could be reasonably suggested that *V. coahuilae* was able to use the shifting sands along the Rio Grande or the river itself which runs from Albuquerque to BBNP in Texas. The Rio Grande may be an accessible means for scorpions to travel from Albuquerque, NM to GMNP in TX. However, in the analysis of the 16S rDNA sequences, specimens from Albuquerque formed an association with the San Antonio and Deming populations which are all along the Rio Grande. These three populations represent the northwestern edge of the sampled range and all are near the path of the Rio Grande.

The mitochondrial markers used in this analysis evolve, in general, five to ten times slower than nuclear DNA and even less rapidly than microsatellites (Doukakis et al. 2002). In retrospect it may have been more appropriate to include other genetic markers to analyze the population structure of *V. coahuilae* owing in part to the generalist nature of this species. Additionally, including different types of data other than genetic markers like morphological, physiological, and behavioral aspects of the sampled populations could have shed more light on the true phylogeny of this species. Researchers are now combining all of these different types of data to show how changes can trigger speciation (Liu, et al. 2009). It is becoming increasingly evident that the availability of appropriate sequence data limits the probability of deciphering the phylogentics of chelicerates.

Scorpion sequence data in particular is of paramount importance to understanding the evolution of chelicerates. The phylogentic position of Scorpiones is highly disputed and experts believe that their unique genetic position in chelicerate phylogeny has not been adequately studies (Jones et al. 2007). In a recent review of model choice on phylogenetic inference utilizing mitochondrial DNA, Jones et al. (2007) point out several pressing issues concerning evolutionary model choice that arise when multiple sequences are analyzed. This review shows that in scorpions and others arthropods single gene analysis of mitochondrial DNA is limited to the amount of phylogenetic signal present in a relatively few number of characters which may be very conserved among these lineages; but the amount of phylogenetic signal present in a multigene analysis depends on substitution discrepancies in rates and patterns of substitution rates between lineages, as well as, other factors like compositional bias (Jones et al. 2007). Jones et al. (2007) sequenced the entire mitochondrial genome of the scorpion species *Mesobuthus gibbosus* Brullé 1832 and found that the gene organization is the same for all scorpion species and other arthropods. Jones et al. (2007) demonstrated how the true nature of phylogenies can be masked by multiple independent reversals of strand-bias observed in scorpions and related taxa. They suggest removing the effects of this strand-bias by recoding the sequence data with neutral transitions (the third nucleotide position in a codon) excluded using the NTE model.

The central evolutionary position of scorpions makes them a good model for evaluating questions concerning basic evolutionary questions and a compilation or database of scorpions sequences are needed (Dunlap & Braddy 2009). Such information

could be very useful in deciphering relationships where morphological data can be misleading but the choice of an appropriate evolutionary model is essential.

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APPENDIX A
Partial sequence of 16S rDNA

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                10        20        30        40        50
      ....|....|....|....|....|....|....|....|....|....|
VCr   TTCCTTCTTTCATTGTTGCACGAATAAGGGCTCTTAATCCAACATCGAGG
VG    ......AA......A..........T...AA.T..................
B     ......A.....C.A.....T....T...AA.T..................
B2    ......A.....C.A.....T....T...AA.T..................
B3    ......A.....C.A.....T....T...AA.T..................
B4    ......A.....C.A.....T....T...AA.T..................
B5    ......A.....C.A.....T....T...AA.T..................
B6    ......A.....C.A.....T....T...AA.T..................
B7    ......A.....C.A.....T....T...AA.T..................
B8    ......A.....C.A.....T....T...AA.T..................
B9    ......A.....C.A.....T....T...AA.T..................
B10   ......A.....C.A..........T...AA.T..................
B11   ......A.....C.A.....T....T...AA.T..................
B13   ......A.....C.A.....T....T...AA.T..................
B12   ......A.....C.A.....T....T...AA.T..................
B14   ......A.....C.A.....T....T...AA.T..................
B15   ......A.....C.A.....T....T...AA.T..................
B16   ......A.....C.A.....T....T...AA.T..................
L     ......A.....C.A.....T....T...AA.T..................
L2    ......A.....C.A.....T....T...AA.T..................
G     ......A.....C.A.....T....T...AA.T..................
G2    ......A.....C.A.....T....T...AA.T..................
G3    ......A.....C.A.....T....T...AA.T..................
O     ......A.....C.A.....T....T...AA.T..................
O2    ......A.....C.A.....T....T...AA.T..................
R     ......A.....C.A.....T....T...AA.T..................
R2    ......A.....C.A.....T....T...AA.T..................
R3    ......A.....C.A.....T....T...AA.T..................
R4    ......A.....C.A.....T....T...AA.T..................
R5    ......A.....C.A.....T....T...AA.T..................
A     ......A.....C.A.....T....T...AG.T..................
A2    ......A.....C.A.....T....T...AG.T..................
D     ......A.....C.A.....T....T...AG.T..................
S     ......A.....C.A.....T....T...AG.T..................
P     ......A.....C.A.....T....T...AA.T..................
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```
                60        70        80        90       100
      ....|....|....|....|....|....|....|....|....|....|
VCr   TCACAAACTTTTTTGATGATAAGAACTCTCTAAAAAAATTATGCTGTTAT
VG    ..............................T.....................
B     ..............................T.....................
B2    ..............................TA....................
B3    ..............................T.....................
B4    ..............................T.....................
B5    ..............................TA....................
B6    ..............................TA....................
B7    ..............................TA....................
B8    ..............................TA....................
B9    ..............................TG....................
B10   ..............................T.....................
B11   ..............................T.....................
B13   ..............................TA....................
B12   ..............................T.....................
B14   ..............................TA....................
B15   ..............................T.....................
B16   ..............................TA....................
L     ..............................T.....................
L2    ..............................T.....................
G     ..............................T.....................
G2    ..............................T.....................
G3    ..............................T.....................
O     ..............................T.....................
O2    ..............................T.....................
R     ..............................T.....................
R2    ..............................T.....................
R3    ..............................T.....................
R4    ..............................T.....................
R5    ..............................T.....................
A     ..............................T.....................
A2    ..............................T.....................
D     ..............................T.....................
S     ..............................T.....................
P     ..............................T.....................
```

```
             110       120       130       140       150
      ....|....|....|....|....|....|....|....|....|....|
VCr   CCCTACAGTAACTTATTTTTTATTAAATGATTTT-GATTTTTCAAGAATT
VG    ..........T.....C..CT.....AAT....T........T...T..
B     ..........T........A......AAT....T.G..........T..
B2    ..........T........A......AAT....T.G..........T..
B3    ..........T........A......AAT....T.G..........T..
B4    ..........T........A......AAT..C.T.G..........T..
B5    ..........T........A......AAT....T.G..........T..
B6    ..........T........A......AAT....T.G..........T..
B7    ..........T........A......AAT....T.G..........T..
B8    ..........T........A......AAT....T.G..........T..
B9    ..........T........A......AAT....T.G..........T..
B10   ..........T........A......AAT....T.G..........T..
B11   ..........T........A......AAT....T.G..........T..
B13   ..........T........A......AAT...AA.G..........T..
B12   ..........T........A......AAT....T.G..........T..
B14   ..........T........A......AAT....T.G..........T..
B15   ..........T........A......AAT....T.G..........T..
B16   ..........T........A......AAT....T.G..........T..
L     ..........T........A......AAT....T.G..........T..
L2    ..........T........A......AAT....T.G..........T..
G     ..........T........A......AAT....T.G..........T..
G2    ..........T........A......AAT....T.G..........T..
G3    ..........T........A......AAT....T.G..........T..
O     ..........T........A......AAT....T.G..........T..
O2    ..........T........A......AAT....T.G..........T..
R     ..........T........A......AAT....T.G..........T..
R2    ..........T........A......AAT....T.G..........T..
R3    ..........T........A......AAT....T.G..........T..
R4    ..........T........A......AAT....T.G..........T..
R5    ..........T........A......AAT....T.G..........T..
A     ..........T........A......AAT....T.G..........T..
A2    ..........T........A......AAT....T.G..........T..
D     ..........T........A......AAT....T.G..........T..
S     ..........T........A......AAT....T.G..........T..
P     ..........T........A......AAT....T.G..........T..
```

```
              160       170       180       190       200
     ....|....|....|....|....|....|....|....|....|....|
VCr  ATATTTCACAATAATTAAATAAAAAGTTTTTTATTTGCTACCCCAGCGAA
VG   ..GAA.......TTAA.........-........C....G.......A..
B    ...AA...T...TT.G.........-.....-..C....G.......A..
B2   ...AA...T...TT.G.........-........C....G.......A..
B3   ...AA...T...TT.G......----........C....G.......A..
B4   ...AA...T...TT.G.........-........C....G.......A..
B5   ...AA...T...TT.G.........-........C....G.......A..
B6   ...AA...T...TT.G.........-........C....G.......A..
B7   ...AA...T...TT.G.........-........C....G.......A..
B8   ...AA...T...TT.G.........-........C....G.......A..
B9   ...AA...T...TT.G.........-........C....G.......A..
B10  ...AA...T...TT.G.......---........C....G.......A..
B11  ...AA...T...TT.G.......---........C....G.......A..
B13  ...AA...T...TT.G.......---.....A..C....G.......A..
B12  ...AA...T...TT.G.......---........C....G.......A..
B14  ...AA...T...TT.G.......---........C....G.......A..
B15  ...AA...T...TT.G.........-........C....G.......A..
B16  ...AA...T...TT.G.......---.....A-.C....G.......A..
L    ...AA...T...TT.G......----........C....G.......A..
L2   ...AA...T...TT.G......----........C....G.......A..
G    ...AA...T...TT.G.........-........C....G.......A..
G2   ...AA...T...TT.G.........-........C....G.......A..
G3   ...AA...T...TT.G.........-.....-..C....G.......A..
O    ...AA...T...TT.G.........-........C....G.......A..
O2   ...AA...T...TT.G.........-........C....G.......A..
R    ...AA...T...TT.G.......---........C....G.......A..
R2   ...AA...T...TT.G.......---........C....G.......A..
R3   ...AA...T...TT.G.........-........C....G.......A..
R4   ...AA...T...TT.G.........-........C....G.......A..
R5   ...AA...T...TT.G.........-........C....G.......A..
A    ...AA...T...TT.G.........-........C....G.......A..
A2   ...AA...T...TT.G.........-........C....G.......A..
D    ...AA...T...TT.G.........-........C....G.......A..
S    ...AA...T...TT.G.........-.....-..C....G.......A..
P    ...AA...T...TT.G.........-........C....G.......A..
```

```
             210       220       230       240       250
     ....|....|....|....|....|....|....|....|....|....|
VCr  ACAATTTTTAATTTAATTTGAAAATAAACTTTAATTGTAAAGCTTGATAG
VG   ........AT..................T...T........A........
B    .T......CC.....G............T...T...A....A........
B2   .T......CC.....G............T...T...A....A.....C..
B3   .T......CC.C...G............T...T...A....A........
B4   .T......CC.....G............T...T...A....A........
B5   .T......CC.....G............T...T...A....A.....C..
B6   .T......CC.....G............T...T...A....A........
B7   .T......CC.....G............T...T...A....A........
B8   .T......CC.....G............T...T...A....A........
B9   .T......CC.....G............T...T...A....A........
B10  ........CC.....G............T...T........A........
B11  .T......CC.....G............T...T...A....A........
B13  .T......CC.....G............T...T...A....A........
B12  .T......CC.....G............T...T...A....A........
B14  .T......CC.....G............T...T...A....A........
B15  .T......CC.....G............T...T...A....A........
B16  .T......CC.....G............T...T...A....A........
L    .T......CC.....G............T...T...A....A........
L2   .T......CC.....G............T...T...A....A........
G    .T......CT.....G............T...T...A....A........
G2   .T......CT.....G............T...T...A....A........
G3   .T......CT.....G............T...T...A....A........
O    .T......CT.....G............T...T...A....A........
O2   .T......CT.....G............T...T...A....A........
R    .T......CT.....G............T...T...A....A........
R2   .T......CT.....G............T...T...A....A........
R3   .T......CT.....G............T...T...A....A........
R4   .T......CT.....G............T...T...A....A........
R5   .T......CT.....G............T...T...A....A........
A    .T.......C..................T...T...A....A........
A2   .T.......C..................T...T...A....A........
D    .T.......C..................T...T...A....A........
S    .T.......C..................T...T...A....A........
P    .T......CC.....G............T...T...A....A........
```

```
              260       270       280       290       300
     ....|....|....|....|....|....|....|....|....|....|
VCr  GGTCTTCTCGTCTTTAAAA-TTTATTTTAGCTTTTTTACTAAAAAATAAA
VG   ........T...CCA.T..A.AA-.........................
B    ........T.....A.T..-.AA.......................T.....
B2   ........T.....A.T..-.AA.......................T.....
B3   ........T.....A.T.G-.AA.......................G.....
B4   ........T...C.A.T.G-.AA.......................G.....
B5   ........T.....A.T..-.AA.......................T.....
B6   ........T.....A.T..-.AA.......................T.....
B7   ........T.....A.T..-.AA.......................T.....
B8   ........T.....A.T..-.AA.......................T.....
B9   ........T.....A.T..-.AA.......................T.....
B10  ........T...C.A.T.GG.AA.......................G.....
B11  ........T...C.A.T.G-.AA.......................G.....
B13  ........T.....A.T..-.AA.......................T.....
B12  ........T...C.A.T.G-.AA.......................G.....
B14  ........T.....A.T..-.AA.......................T.....
B15  ........T...C.A.T.G-.AA.......................G.....
B16  ........T.....A.T..-.AA.......................T.....
L    ........T...C.A.T..-.AA.......................G.....
L2   ........T...C.A.T..-.AA.......................G.....
G    ........T...C.A.T..-.AA.......................G.....
G2   ........T...C.A.T..-.AA.......................G.....
G3   ........T...C.A.T..-.AA.......................G.....
O    ........T...C.A.T..-.AA.......................G.....
O2   ........T...C.G.T..-.AA.......................G.....
R    ........T...C.A.T..-.AA.......................G.....
R2   ........T...C.A.T..-.AA.......................G.....
R3   ........T...C.A.T..-.AA.......................G.....
R4   ........T...C.A.T..-.AA.......................G.....
R5   ........T...C.A.T..-.AA.......................G.....
A    ........T...CCA.T.G-.AA.......................T.....
A2   ........T...CCA.T.G-.AA.......................T.....
D    ........T...CCA.T.G-.AA.......................T.....
S    ........T...CCA.T.G-.AA.......................T.....
P    ........T...C.A.T..-.AA.........................
```

```
        310        320        330        340        350
     ....|....|....|....|....|....|....|....|....|....|
VCr  ATTCAAGATATTATTATAAGACAACATTTCATAGTCTAACCTTTCATTCC
VG   T.....A.........C......TA.A.AT......A.G...........
B    T.....A......AA.........A.A.A.......A............T
B2   T.....A......AA.........A.A.A.......A............T
B3   T.....A......AA.........A.A.A.......A............T
B4   T.....A......AA.........A.A.A.......A............T
B5   T.....A......AA.........A.A.A.......A............T
B6   T.....A......AA.........A.A.A.......A............T
B7   T.....A......AA.........A.ACA.......A............T
B8   T.....A......AA.........A.ACA.......A............T
B9   T.....A......AA.........A.ACA.......A............T
B10  T.....A......AA.........A.A.A.......G............T
B11  T.....A......AA.........A.A.A.......A............T
B13  T.....A......AA.........A.A.A.......A............T
B12  T.....A......AA.........A.A.A.......A............T
B14  T.....A......AA.........A.A.A.......A............T
B15  T.....A......AA.........A.A.A.......A............T
B16  T.....A......AA.........A.A.A.......A............T
L    T.....A......AA.........A.A.A.......A............T
L2   T.....A......AA.........A.A.A.......A............T
G    T.....A......AA.........A.A.A.......A............T
G2   T.....A......AA.........A.A.A.......A............T
G3   T.....A......AA.........A.A.A.......A............T
O    T.....A......AA.........A.A.A.......A............T
O2   T.....A......AA.........A.A.A.......A............T
R    T.....A......AA.........A.A.A.......A............T
R2   T.....A......AA.........A.A.A.......A............T
R3   T.....A......AA.........A.A.A.......A............T
R4   T.....A......AA.........A.A.A.......A............T
R5   T.....A......AA.........A.A.A.......A............T
A    T.....A......AA.........A.A.A.......A............T
A2   T.....A......AA.........A.A.A.......A............T
D    T.....A......AA.........A.A.A.......A............T
S    T.....A......AA.........A.A.A.......A............T
P    T.....A......AA.........GA.A.A.......AG...........T
```

```
            360
     ....|....|
VCr  AGTCCTAAAT
VG   ....T.C...
B    ....T.T...
B2   ....T.T...
B3   ....T.T...
B4   ....T.T...
B5   ....T.T...
B6   ....T.T...
B7   ....T.T...
B8   ....T.T...
B9   ....T.T...
B10  ....T.T...
B11  ....T.T...
B13  .T..T.T...
B12  ....T.T...
B14  ....T.T...
B15  ....T.T...
B16  .T..T.T...
L    ....T.T...
L2   ....T.T...
G    ....T.T...
G2   ....T.T...
G3   ....T.T...
O    ....T.T...
O2   ....T.T...
R    ....T.T...
R2   ....T.T...
R3   ....T.T...
R4   ....T.T...
R5   ....T.T...
A    ....T.G...
A2   ....T.G...
D    ....T.G...
S    ....T.G...
P    ....T.T...
```

APPENDIX B
Partial sequence of Cytochrome c Oxidase subunit I

```
              10        20        30        40        50
     ....|....|....|....|....|....|....|....|....|....|
VCh  AAACCATAAAGATATTGGGACTATGT--ATTAATTTTGGGAGGTTGAGCT
VCr  ..T.............A.....A.-AT........A.............
B5   ..T.............A..A....-AT........A.............
B6   ..T.............A..A....-AT........A.............
B8   ..T.................A....-ATC.......A.............
B9   ..T.................A....-ATC.......A.............
B11  ..T.................A....-AT........A.............
B13  ..T.................A....-AT........A.............
B16  ..T.................A....-AC........A.............
B17  ..T.................A....-AT........A.............
G    ..T.................A....-AT........A.............
G3   ..T.............A..A..AC-ATC.......A.............
R4   ..T.................A....-AT........A.............
R5   ..T.................A....CAT........A.............
A2   ..T.................A..A.-AT..G....A.............

              60        70        80        90       100
     ....|....|....|....|....|....|....|....|....|....|
VCh  TCTATAGTTGGAGCTGCTCTTAGTTTAATGATTCGTGCTGAAATTGGAAG
VCr  ........G..GA.G...T.A..A...T.A.....A.............
B5   ..A.........A.........A.....A.....A.............
B6   ..A.........A.........A.....A.....A.............
B8   ..A.........A.........A.....A.....A.............
B9   ..A.........A.........A.....A.....A.............
B11  ..A..G......A.........A.....A.....A...........G..
B13  ..A.........A.........A.....A.....A.............
B16  ..A.........A.........A.....A.....A.............
B17  ..A..G......A.........A.....A.....A...........G..
G    ..A.........A.........A.....A.....A.............
G3   ..A........GA...A.....TA........C..G.............
R4   ..A.........A.........A.....A.....A.............
R5   ..A.........A.........A.....A.....A.............
A2   ..A..G..A...A.........A.....A.....A..G..........
```

```
             110       120       130       140       150
     ....|....|....|....|....|....|....|....|....|....|
VCh  ACCGGGTTCTTTTATTGGTGATGATCAAATTTATAATGTTGTAGTGACGG
VCr  T..A..A......G....G........G..............G.......
B5   ...T..A..A........A..C.......................A..A.
B6   ...T..A..A........A...........................A..A.
B8   ...T..A..A........A.....................C.....A..A.
B9   ...T..A..A........A.....................C.....A..A.
B11  ...T..A..A........A...........................A..A.
B13  ...T..A..A........A...........................A..A.
B16  ...T..A..A........A...........................A..A.
B17  ...T..A..A........A...........................A..A.
G    ...T..A..A........G...........................A..A.
G3   C..A..A..A..C.....G........G..........C......T..A.
R4   ...T..A..A........G...........................A..A.
R5   ...T..A..A..C.....G...........................A..A.
A2   ...C..A..A........A.....C..G.................A..A.


             160       170       180       190       200
     ....|....|....|....|....|....|....|....|....|....|
VCh  CTCATGC-TTTTGTAATAATTTTTTTTATAGTTATGCCTATTATGATTGG
VCr  .......-.............................A..C.....A.....
B5   .......-......T..............G..............A.....
B6   .......-......T..............G..............A.....
B8   .......-......T..............G..............A.....
B9   .......-......T..............G..............A.....
B11  .......-......T..............G.....A........A.....
B13  .......-......T..............G..............A.....
B16  .......-......T..............G..............A.....
B17  .......-......T..............G.....A........A.....
G    .......-......T............C..G..A..A..............
G3   T......-G........G........-........A........A.....
R4   .......-......T............C..G..A..A..............
R5   .......-......-............C..G..A..A..............
A2   .......A......T..............G..............A.....
```

```
             210       220       230       240       250
      ....|....|....|....|....|....|....|....|....|....|
VCh   TGGATTTGGAAATTGGTTAGTACCTTTAATGTTGGGGGCTCCTGATATAG
VCr   ...G.....T........G..C...........A..T.............
B5    A..G...........A.....T...........A..A.............
B6    A..G...........A.....T...........A..A.............
B8    A..G...........A.....T...........A..A.............
B9    A..G...........A.....T...........A..A.............
B11   A..............A.....T..............A.............
B13   A..G...........A.....T...........A..A.............
B16   A..G...........A.....T...........A..A.............
B17   A..............A.....T..............A.............
G     A..............AC....T...........A..A.............
G3    G...G..........A..G.................A.............
R4    A..............AC....T...........A..A.............
R5    A..............AC....T...........A..A.............
A2    G..G...........A.....T........A..A..A...........G.

             260       270       280       290       300
      ....|....|....|....|....|....|....|....|....|....|
VCh   CTTTTCCTCGATTGAATAATATAAGTTTTTGAT-TATTACCTCCAGCATT
VCr   ..........T..A...........A.......-.G........TT.T..
B5    ..........T..A........G..........-....G...........
B6    ..........T..A........G..........-....G...........
B8    ..........T..A........G........G.-....G...........
B9    ..........T..A........G........G.-....G...........
B11   ..........T..A..................G.-....G...........
B13   ..........T..A........G..........-....G...........
B16   ..........T..A........G..........-....G...........
B17   ..........T..A..................G.-....G...........
G     ..........T..A........G........G.-....G...........
G3    .........AT..A....................A..C.G.....T..T..
R4    ..........T..A........G........G.-....G...........
R5    ..........T..A........G........G.-....G...........
A2    ..........T..A....................-....G...........
```

```
             310       320       330       340       350
     ....|....|....|....|....|....|....|....|....|....|
VCh  TTTTCTTTTATTAGGTTCAGCATCATTGGAAAGAGGTGCAGGTACAGGCT
VCr  ....T.A..G.............................A..G.........
B5   ....T.A..G..G........T..............G........T..G.
B6   ....T.A..G...........T..............G........T..G.
B8   ....T.A..G..G........T..............G........T..G.
B9   ....T.A.....G..A.....T..............G........T..G.
B11  ....T.A..G..G........T..............G........T..G.
B13  ....T.A..G..G........T..............G........T..G.
B16  ....T.A..G..G........T..............G........T..G.
B17  ....T.A..G..G........T..............G........T..G.
G    ....T.A.....G........T..............G........T..G.
G3   ................A.....T..............G........T..G.
R4   ....T.A..G..G........T..............G........T..G.
R5   ....T.A..G..G........T..............G........T..G.
A2   ....T.A.....G........T..............G........T..G.

             360       370       380       390       400
     ....|....|....|....|....|....|....|....|....|....|
VCh  GAACTGTATACCCACCTCTTTCCTCATATATGTTTCATTCTGGAGGGTCT
VCr  .......G.....T.............................G..T...
B5   .G..A..G.....C...........T.....................C..A
B6   .G..A..G.....C...........T.....................C..A
B8   .G..A..G.....C...........T.....................C..A
B9   .G..A..G.....C...........T.....................C..A
B11  .G..A..G.....C...........T.....................C..A
B13  .G..A..G.....C...........T.....................C..A
B16  .G..A..G.....C...........T.....................C..A
B17  .G..A..G.....C...........T.....................C..A
G    .G..A..G.....C...........T.....................C..A
G3   ....A..G..A..C...........T..........C........C.A.
R4   .G..A..G.....C...........T.....................C..A
R5   .G..A..G.....C...........T.....................C..A
A2   .G..A..G.....C...........T.....................C..A
```

```
            410       420       430       440       450
     ....|....|....|....|....|....|....|....|....|....|
VCh  GTTGATATAACTATTTTTTCTTTACATTTAGCTGGTGTTTCTTCTATTTT
VCr  ......................G........A..G........A.....
B5   ..G.............................A..GA.......A.....
B6   ..G.............................A..GA.......A.....
B8   ..G.............................A..AA.......A.....
B9   ..G.............................A..AA.......A.....
B11  ..G.............................A..AA.......A.....
B13  ..G.............................A..GA.......A.....
B16  ..G.............................A..GA.......A.....
B17  ..G.............................A..AA.......A.....
G    ..G.............................A..GA.......A.....
G3   ..A..C..................G......T.A..AA.......A....A
R4   ..G.............................A..GA.......A.....
R5   ..G.............................A..GA.......A.....
A2   ..A.........................C....A..GA.......A.....

            460       470       480       490       500
     ....|....|....|....|....|....|....|....|....|....|
VCh  AGGGGCTATTAATTTTATTACTACTATTTTAAATATACGTATGAGAGGAA
VCr  ...A............................G.....G....GA.....T.
B5   ...A.........................................A..T....
B6   ...A.........................................A..T....
B8   ...A.........................................A..T....
B9   ...A.........................................A..T....
B11  ...A...............................C........A..T....
B13  ...A.........................................A..T..G.
B16  ...A.........................................A..T....
B17  ...A...............................C........A..T....
G    ...A.........................................A..T....
G3   ...A.........................C..........G..A.....G.
R4   ...A.........................................A..T....
R5   ...A.........................................A..T....
A2   ...A.................................G.....A..T....
```

```
            510       520       530       540       550
     ....|....|....|....|....|....|....|....|....|....|
VCh  TATTGTTGGAACGTATTCCTTTGTTTGTTTGATCTGTAAAGATTACTGCT
VCr  ....A.....G...G.C.....A.................G.........A
B5   ....A..A..G...........A........G.....G..A.........
B6   ....A..A..G...........A........G.....G..A.........
B8   ....A..A..G...........A........G.....G..A.........
B9   ....A..A..G...........A........G.....G..A.........
B11  ....A..A..G...........A........G........A.........
B13  ....A..A..G...........A..C.....G.....G..A.........
B16  ....A..A..G...........A........G.....G..A.........
B17  ....A..A..G...........A........G........A.........
G    ....A..A..G...........A........G........A.........
G3   ..........G.AA........A........G.....TC.A.........
R4   ....A..A..G...........A........G........A.........
R5   ....A..A..G...........A........G........A.........
A2   ....A..A..G...........A........G.....G..A.........

            560       570       580       590       600
     ....|....|....|....|....|....|....|....|....|....|
VCh  GTTTTATTATTATTGTCTCTTCCTGTTCTTGCAGGTGCTATTACAATATT
VCr  A........C.TC.T...T.A......T.A..G..A........T..GC.
B5   .........C.TC.T........A........T..G..A.....T.....
B6   .........C.TC.T........A........T..G..A.....T.....
B8   .........C.TC.T........A........T..G..A.....T.....
B9   .........C.TC.T........A........T..G..A.....T.....
B11  ..C......C.TC.T........A........T..A..A.....T.....
B13  .........C.TC.T........A........T..G..A.....T.....
B16  .........C.TC.T........A........T..G..A.....T.....
B17  ..C......C.TC.T........A........T..A..A.....T.....
G    .........C.TC.A........A........T..G..A.....T.....
G3   .....G...C.TC.T..A.....A........T..G..A.....T.....
R4   .........C.TC.A........A........T..G..A.....T.....
R5   .........C.TC.A........A........T..G..A.....T.....
A2   .........C.TC.T........A........T.AG..A.....T.....
```

```
             610       620       630       640       650
     ....|....|....|....|....|....|....|....|....|....|
VCh  ATTGACTGATCGTAATTTTAATACTTCATTTTTTGATCCTGCTGGAGGAG
VCr  TC.T........G..............T..................T....
B5   G..A..G.....G..............T.......................
B6   ...A..G.....A..............T.......................
B8   ......G.....G..............T.......................
B9   ......G.....G..............T.......................
B11  ...A..G.....G..............T.......................
B13  ...A..G.....G..............T.......................
B16  ...A..G.....G..............T.......................
B17  ...A..G.....G..............T.......................
G    ...A..G.....G..............T.......................
G3   G..A........A..............T.......................
R4   ...A..G.....G..............T.......................
R5   ...A..G.....G..............T.......................
A2   ...A..G.....G..............T.......................

             660       670       680       690
     ....|....|....|....|....|....|....|....|....|.
VCh  GAGATCCTATTTKRTCACTCAACATTTATTTTGATTTTTTGGTCAC
VCr  ............TA.A--.............................
B5   .T..C.....C.TA.A--.............................
B6   .T..C.....C.TA.A--.............................
B8   .T..C.......TA.A--C............................
B9   .T..C.......TA.A--C............................
B11  .T..C.......TA.A--C............................
B13  .T..C.....C.TA.A--.............................
B16  .T..C.....C.TA.A--.............................
B17  .T..C.......TA.A--C............................
G    .T..........TA.A--.............................
G3   .........C..TA.A--.............................
R4   .T..........TA.A--.............................
R5   .T..........TA.A--.............................
A2   .T........C.TA.A--.............................
```

APPENDIX C
Combined partial sequences of 16S rDNA and Cytochrome c Oxidase Subunit I

```
          10        20        30        40        50
     ....|....|....|....|....|....|....|....|....|....|
PG   TTCCTTTTTTTACTACTGCGTAAAATAGGTTTCTTAATCCAACATCGAGG
B5   ......A...C....T...A.G..T....AA.T..................
B6   ......A...C....T...A.G..T....AA.T..................
B8   ......A...C....T...A.G..T....AA.T..................
B9   ......A...C....T...A.G..T....AA.T..................
B11  ......A...C....T...A.G..T....AA.T..................
B13  ......A...C....T...A.G..T....AA.T..................
B16  ......A...C....T...A.G..T....AA.T..................
G    ......A...C....T...A.G..T....AA.T..................
G3   ......A...C....T...A.G..T....AA.T..................
R4   ......A...C....T...A.G..T....AA.T..................
R5   ......A...C....T...A.G..T....AA.T..................
A2   ......A...C....T...A.G..T....AG.T..................

          60        70        80        90       100
     ....|....|....|....|....|....|....|....|....|....|
PG   TCACAAACTTTTTTGTTGATGAGAACTCTTTAAAAAAATTATGCTGTTAT
B5   ..............A....A.........A....................
B6   ..............A....A.........A....................
B8   ..............A....A.........A....................
B9   ..............A....A.........G....................
B11  ..............A....A..............................
B13  ..............A....A.........A....................
B16  ..............A....A.........A....................
G    ..............A....A..............................
G3   ..............A....A..............................
R4   ..............A....A..............................
R5   ..............A....A..............................
A2   ..............A....A..............................
```

```
            110       120       130       140       150
     ....|....|....|....|....|....|....|....|....|....|
PG   CCCTATAGTAACTTGTTATTATTTAGTAAATTATTGATTTTTCTAGATTT
B5   .....C.....T..A..T...A...AA..T..T...G......A......
B6   .....C.....T..A..T...A...AA..T..T...G......A......
B8   .....C.....T..A..T...A...AA..T..T...G......A......
B9   .....C.....T..A..T...A...AA..T..T...G......A......
B11  .....C.....T..A..T...A...AA..T..T...G......A......
B13  .....C.....T..A..T...A...AA..T..TAA.G......A......
B16  .....C.....T..A..T...A...AA..T..T...G......A......
G    .....C.....T..A..T...A...AA..T..T...G......A......
G3   .....C.....T..A..T...A...AA..T..T...G......A......
R4   .....C.....T..A..T...A...AA..T..T...G......A......
R5   .....C.....T..A..T...A...AA..T..T...G......A......
A2   .....C.....T..A..T...A...AA..T..T...G......A......

            160       170       180       190       200
     ....|....|....|....|....|....|....|....|....|....|
PG   ATTTATCACAATATATAAATAATATTTATTTATTTGCTGCCCCAGCAGAA
B5   ..AA....T...T.TG......A.A..T.....C.............A..
B6   ..AA....T...T.TG......A.A..T.....C.............A..
B8   ..AA....T...T.TG......A.A..T.....C.............A..
B9   ..AA....T...T.TG......A.A..T.....C.............A..
B11  ..AA....T...T.TG......--A..T.....C.............A..
B13  ..AA....T...T.TG......A--..T..A..C.............A..
B16  ..AA....T...T.TG......A--..T..A-.C.............A..
G    ..AA....T...T.TG......A.A..T.....C.............A..
G3   ..AA....T...T.TG......A.A..T..-..C.............A..
R4   ..AA....T...T.TG......A.A..T.....C.............A..
R5   ..AA....T...T.TG......A.A..T.....C.............A..
A2   ..AA....T...T.TG......A.A..T.....C.............A..
```

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          210       220       230       240       250
     ....|....|....|....|....|....|....|....|....|....|
PG   CAATTGTTTTATTAGTTTGAAAATAATGTGGAATTGTAAAGCTTAATAGG
B5   T....T.CCAT..............AT.TTT...A....A...G.C...
B6   T....T.CCAT..............AT.TTT...A....A...G.....
B8   T....T.CCAT..............AT.TTT...A....A...G.....
B9   T....T.CCAT..............AT.TTT...A....A...G.....
B11  T....T.CCAT..............AT.TTT...A....A...G.....
B13  T....T.CCAT..............AT.TTT...A....A...G.....
B16  T....T.CCAT..............AT.TTT...A....A...G.....
G    T....T.C.AT..............AT.TTT...A....A...G.....
G3   T....T.C.AT..............AT.TTT...A....A...G.....
R4   T....T.C.AT..............AT.TTT...A....A...G.....
R5   T....T.C.AT..............AT.TTT...A....A...G.....
A2   T....T..CAT...A..........AT.TTT...A....A...G.....

          260       270       280       290       300
     ....|....|....|....|....|....|....|....|....|....|
PG   GTCTTCTTGTCTTTAAGATAGATTTTAGCTTTTTAACTAAAAGATTAATT
B5   ............A.TA...A............T.......T..A....
B6   ............A.TA...A............T.......T..A....
B8   ............A.TA...A............T.......T..A....
B9   ............A.TA...A............T.......T..A....
B11  ...........C.A.TAG..A............T.........A....
B13  ............A.TA...A............T.......T..A....
B16  ............A.TA...A............T.......T..A....
G    ...........C.A.TA...A............T.........A....
G3   ...........C.A.TA...A............T.........A....
R4   ...........C.A.TA...A............T.........A....
R5   ...........C.A.TA...A............T.........A....
A2   ...........CCA.TAG..A............T.......T..A....
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```
            310       320       330       340       350
     ....|....|....|....|....|....|....|....|....|....|
PG   TCGAAGTATTACTATAAGACAAAATTATCATAGTCAAACCTTTCATTCTA
B5   ..A..A.....AA..........A..-....................
B6   ..A..A.....AA..........A..-....................
B8   ..A..A.....AA..........AC.-....................
B9   ..A..A.....AA..........AC.-....................
B11  ..A..A.....AA..........A..-....................
B13  ..A..A.....AA..........A..-....................
B16  ..A..A.....AA..........A..-....................
G    ..A..A.....AA..........A..-....................
G3   ..A..A.....AA..........A..-....................
R4   ..A..A.....AA..........A..-....................
R5   ..A..A.....AA..........A..-....................
A2   ..A..A.....AA..........A..-....................

            360       370       380       390       400
     ....|....|....|....|....|....|....|....|....|....|
PG   GTCCTCCATAAAGATATTGGAACTATGT-ACTTGATTTTAGGGGGTTGGG
B5   ...T.................A....-.T..A........A.....A.
B6   ...T.................A....-.T..A........A.....A.
B8   ...T..............G..A....-.TC.A........A.....A.
B9   ...T..............G..A....-.TC.A........A.....A.
B11  ...T..............G..A....-.T..A........A.....A.
B13  T..T..............G..A....-.T..A........A.....A.
B16  T..T..............G..A....-....A........A.....A.
G    ...T..............G..A....-.T..A........A.....A.
G3   ...T.................A..AC-.TC.A........A.....A.
R4   ...T..............G..A....-.T..A........A.....A.
R5   ...T..............G..A....C.T..A........A.....A.
A2   ...T..............G..A..A.-.T..........A.....A.
```

```
            410       420       430       440       450
     ....|....|....|....|....|....|....|....|....|....|
PG   CTTCTATGGTAGGAACTGCTTTGAGATTGTTAATTCGAGCTGAAATTGGA
B5   ....A..A..T.........C.T.....AA....................
B6   ....A..A..T.........C.T.....AA....................
B8   ....A..A..T.........C.T.....AA....................
B9   ....A..A..T.........C.T.....AA....................
B11  ....A.....T.........C.T.....AA....................G
B13  ....A..A..T.........C.T.....AA....................
B16  ....A..A..T.........C.T.....AA....................
G    ....A..A..T.........C.T.....AA....................
G3   ....A..A..T..G....A.C.T.T...AA.G..C..G............
R4   ....A..A..T.........C.T.....AA....................
R5   ....A..A..T.........C.T.....AA....................
A2   ....A...............C.T.....AA..........G.........

            460       470       480       490       500
     ....|....|....|....|....|....|....|....|....|....|
PG   AGTCCAGGATCTTTTGTTGGGGATGATCAAATTTATAACGTTGTGGTAAC
B5   ..A..T.....A...A....A..C..............T.....A.....
B6   ..A..T.....A...A....A.................T.....A.....
B8   ..A..T.....A...A....A.................T..C..A.....
B9   ..A..T.....A...A....A.................T..C..A.....
B11  ..A..T.....A...A....A.................T.....A.....
B13  ..A..T.....A...A....A.................T.....A.....
B16  ..A..T.....A...A....A.................T.....A.....
G    ..A..T.....A...A.......................T.....A.....
G3   ..C........A..CA.............G........T.C...A..T..
R4   ..A..T.....A...A.......................T.....A.....
R5   ..A..T.....A..CA.......................T.....A.....
A2   ..A..C.....A...A....A.....C..G........T.....A.....
```

```
            510       520       530       540       550
     ....|....|....|....|....|....|....|....|....|....|
PG   GGCTCATGC-TTTTGTGATAATTTTTTTCATAGTTATGCCTATTATAATT
B5   A........-......T...........T..G..................
B6   A........-......T...........T..G..................
B8   A........-......T...........T..G..................
B9   A........-......T...........T..G..................
B11  A........-......T...........T..G.....A............
B13  A........-......T...........T..G..................
B16  A........-......T...........T..G..................
G    A........-......T..............G..A..A........G...
G3   A.T......-G.....A..G.........-........A............
R4   A........-......T..............G..A..A........G...
R5   A........-......-..............G..A..A........G...
A2   A........A......T...........T..G..................

            560       570       580       590       600
     ....|....|....|....|....|....|....|....|....|....|
PG   GGTGGATTTGGTAATTGATTGGTTCCCTTAATGTTGGGTGCTCCTGATAT
B5   ..A..G.....A........A.....T........A..A...........
B6   ..A..G.....A........A.....T........A..A...........
B8   ..A..G.....A........A.....T........A..A...........
B9   ..A..G.....A........A.....T........A..A...........
B11  ..A........A........A.....T...........A...........
B13  ..A..G.....A........A.....T........A..A...........
B16  ..A..G.....A........A.....T........A..A...........
G    ..A........A......C.A.....T........A..A...........
G3   ..G...G....A...........A..T...........A...........
R4   ..A........A......C.A.....T........A..A...........
R5   ..A........A......C.A.....T........A..A...........
A2   ..G..G.....A........A.....T.....A..A..A...........
```

```
            610       620       630       640       650
     ....|....|....|....|....|....|....|....|....|....|
PG   GGCTTTTCCTCGATTAAATAACATGAGATTTTGAT-TGTTACCTCCTTCT
B5   A..........T.......T.....T.......-.A..G.....AG.A
B6   A..........T.......T.....T.......-.A..G.....AG.A
B8   A..........T.......T.....T.....G.-.A..G.....AG.A
B9   A..........T.......T.....T.....G.-.A..G.....AG.A
B11  A..........T.......T..A..T.....G.-.A..G.....AG.A
B13  A..........T.......T.....T.......-.A..G.....AG.A
B16  A..........T.......T.....T.......-.A..G.....AG.A
G    A..........T.......T.....T.....G.-.A..G.....AG.A
G3   A.........AT.......T..A..T.......A.AC.G......G..
R4   A..........T.......T.....T.....G.-.A..G.....AG.A
R5   A..........T.......T.....T.....G.-.A..G.....AG.A
A2   ...........T.......T..A..T.......-.A..G.....AG.A

            660       670       680       690       700
     ....|....|....|....|....|....|....|....|....|....|
PG   TTTTTTTTATTGTTAGGTTCAGCATCATTGGAAAGAGGAGCGGGTACAGG
B5   ............G........T.............G..A.....T..
B6   ......................T.............G..A.....T..
B8   ............G........T.............G..A.....T..
B9   ..........A..G..A.....T.............G..A.....T..
B11  ............G........T.............G..A.....T..
B13  ............G........T.............G..A.....T..
B16  ............G........T.............G..A.....T..
G    ..........A..G........T.............G..A.....T..
G3   ......C.T..A.....A.....T.............G..A.....T..
R4   ............G........T.............G..A.....T..
R5   ............G........T.............G..A.....T..
A2   ..........A..G........T.............G..A.....T..
```

```
          710       720       730       740       750
     ....|....|....|....|....|....|....|....|....|....|
PG   CTGAACTGTGTACCCTCCTCTTTCCTCATATATGTTTCATTCTGGAGGTT
B5   G..G..A........C...........T.....................C.
B6   G..G..A........C...........T.....................C.
B8   G..G..A........C...........T.....................C.
B9   G..G..A........C...........T.....................C.
B11  G..G..A........C...........T.....................C.
B13  G..G..A........C...........T.....................C.
B16  G..G..A........C...........T.....................C.
G    G..G..A........C...........T.....................C.
G3   G.....A.....A..C...........T...........C........C.
R4   G..G..A........C...........T.....................C.
R5   G..G..A........C...........T.....................C.
A2   G..G..A........C...........T.....................C.

          760       770       780       790       800
     ....|....|....|....|....|....|....|....|....|....|
PG   CTGTTGATATAACTATTTTTTCTTTACATTTAGCTGGGGTTTCTTCAATT
B5   .A..G.............................A...A...........
B6   .A..G.............................A...A...........
B8   .A..G.............................A..AA...........
B9   .A..G.............................A..AA...........
B11  .A..G.............................A..AA...........
B13  .A..G.............................A...A...........
B16  .A..G.............................A...A...........
G    .A..G.............................A...A...........
G3   A...A..C.................G......T.A..AA...........
R4   .A..G.............................A...A...........
R5   .A..G.............................A...A...........
A2   .A..A........................C....A...A...........
```

```
            810       820       830       840       850
     ....|....|....|....|....|....|....|....|....|....|
PG   TTAGGAGCTATTAATTTTATTACTACTATTTTGAATATACGTAGAAGAGG
B5   ..............................A.........T...T..
B6   ..............................A.........T...T..
B8   ..............................A.........T...T..
B9   ..............................A.........T...T..
B11  ..............................A..C.......T...T..
B13  ..............................A.........T...T..
B16  ..............................A.........T...T..
G    ..............................A.........T...T..
G3   .A............................C.A........G.T......
R4   ..............................A.........T...T..
R5   ..............................A.........T...T..
A2   ..............................A.....G....T...T..

            860       870       880       890       900
     ....|....|....|....|....|....|....|....|....|....|
PG   AATACTTTTAGAGCGTGTTCCGTTGTTTGTTTGGTCTGTAAAAATTACTG
B5   ....T.A.........A....T..A.............G..........
B6   ....T.A.........A....T..A.............G..........
B8   ....T.A.........A....T..A.............G..........
B9   ....T.A.........A....T..A.............G..........
B11  ....T.A.........A....T..A........................
B13  G...T.A.........A....T..A..C..........G..........
B16  ....T.A.........A....T..A.............G..........
G    ....T.A.........A....T..A........................
G3   G...T.G..G....AAA....T..A.............TC.........
R4   ....T.A.........A....T..A........................
R5   ....T.A.........A....T..A........................
A2   ....T.A.........A....T..A.............G..........
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```
            910       920       930       940       950
     ....|....|....|....|....|....|....|....|....|....|
PG   CAGTTTTGTTACTTCTTTCATTACCTGTTTTAGCGGGGGCTATTACTATG
B5   .T.....A..........TC.T..A...C.T..T.....A........A
B6   .T.....A..........TC.T..A...C.T..T.....A........A
B8   .T.....A..........TC.T..A...C.T..T.....A........A
B9   .T.....A..........TC.T..A...C.T..T.....A........A
B11  .T..C..A..........TC.T..A...C.T..T..A..A........A
B13  .T.....A..........TC.T..A...C.T..T.....A........A
B16  .T.....A..........TC.T..A...C.T..T.....A........A
G    .T.....A........A..TC.T..A...C.T..T.....A........A
G3   .T.................C.T..A...C.T..T.....A........A
R4   .T.....A........A..TC.T..A...C.T..T.....A........A
R5   .T.....A........A..TC.T..A...C.T..T.....A........A
A2   .T.....A..........TC.T..A...C.T..T.A...A........A

            960       970       980       990      1000
     ....|....|....|....|....|....|....|....|....|....|
PG   CTTCTTACTGATCGGAATTTTAATACTTCTTTTTTTGATCCTGCTGGTGG
B5   T.GT.A..G.....................................A..
B6   T.AT.A..G.....A...............................A..
B8   T.AT.G..G.....................................A..
B9   T.AT.G..G.....................................A..
B11  T.AT.A..G.....................................A..
B13  T.AT.A..G.....................................A..
B16  T.AT.A..G.....................................A..
G    T.AT.A..G.....................................A..
G3   T.GT.A........A...............................A..
R4   T.AT.A..G.....................................A..
R5   T.AT.A..G.....................................A..
A2   T.AT.A..G.....................................A..
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```
            1010      1020      1030      1040
     ....|....|....|....|....|....|....|....|....|.
PG   TGGGGATCCTATTTTATACCAACATTTATTTTGATTTTTTGGTCAC
B5   A..T..C.....C.....T.........................
B6   A..T..C.....C.....T.........................
B8   A..T..C.....................................
B9   A..T..C.....................................
B11  A..T..C.....................................
B13  A..T..C.....C.....T.........................
B16  A..T..C.....C.....T.........................
G    A..T............T.........................
G3   A..A.......C......T.........................
R4   A..T............T.........................
R5   A..T............T.........................
A2   A..T........C.....T.........................
```

BIOGRAPHICAL SKETCH

Ramona Nichole Lopez was born April 24, 1978 in Danville, Kentucky. As a young child she lived in Tennessee, Texas, Florida, Louisiana, and South Carolina with her family settling in Charlotte, North Carolina by 1985. She graduated in Charlotte from Midwood High School in 1996 and attended Central Piedmont Community College during her senior year of high school where, she decided to pursue a career in veterinary medicine. During her undergraduate studies in the biology department, while in the pre-professional program at Appalachian State University, she discovered her passion for molecular and evolutionary biology and applied to the Masters program. After receiving her Bachelor of Science degree in 2002 with a major in Biology and a minor in Chemistry, she began studying for a Master of Science degree with a focus in molecular biology. She currently lives in Boone, North Carolina and is planning on starting an organic, sustainable gardening business.