

Phylogeography of Bark Scorpions (*Centruroides exilicauda*) from the Baja California
peninsula

By

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A Thesis Submitted in partial fulfillment of the requirements for the degree
Master of Science in Integrative Biological Diversity

At

Western Connecticut State University

2025

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ABSTRACT

Scorpions are an arthropod order known for their potent venom and stinger. We currently recognize over 2,800 species, which are mainly differentiated using morphology. Bark Scorpions (Buthidae: *Centruroides*) occur across the southern and western United States and western and northern Mexico. They have also been recorded in Central America and northern South America. The low vagility of these animals makes them ideal candidates to study biogeographic and phylogeographic patterns, especially in areas with great biogeographical complexity. This thesis investigates the phylogeographic patterns of *Centruroides exilicauda*, a species distributed across the Baja California peninsula. Phylogenetic analysis of the cytochrome oxidase subunit I (COI) sequences from 24 sequences (21 sequenced here) plus seven outgroup samples revealed the presence of two distinct clades within this species, one restricted to the south (the Cape), and another one distributed across the peninsula. The divergence of these two clades predates the split of the peninsula from mainland Mexico. While the focus on this work is not new, as it builds on previously published studies, it aims to provide further insights into the biogeographic process influencing these patterns. A key framework for understanding these divergences is the mid-peninsular seaway hypothesis, which proposed that a historical water barrier may have influenced the genetic isolation of *Centruroides* populations along the Baja California peninsula. Further studies are needed to clarify the taxonomic status of these clades and to determine whether the Northern and Southern clades represent distinct species.

ACKNOWLEDGEMENTS

I would like to express my gratitude to those who have supported me during the course of this research. First and foremost, I wish to thank Dr. Carlos Santibáñez-López. Without his persistent guidance and encouragement, I wouldn't have been able to complete this project. I would also like to thank my thesis committee, Dr. Matthew Graham and Dr. Edwin Wong. I am grateful to Dr. Matthew Graham for his recommendation of this project and the extensive efforts and professional wisdom he has provided me throughout my education. I would not be achieving this milestone in my life without his support and belief in me. I am grateful to Dr. Wong for his participation as a thesis committee member and for his helpful advice.

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CHAPTER I

INTRODUCTION

1.1 Phylogeography and its relevance in studying species distributions

Phylogeography is the study of the historical processes responsible for the past and present geographic distributions of genealogical lineages (Avice, 2000). By analyzing genetic variation across populations and correlating these patterns with geographical features, researchers can infer patterns of historical events such as migration, vicariance, and population isolation. This is especially pertinent in regions shaped by complex topography or historical geographic events, such as glaciation or tectonic shifts. The formation of a seaway or the lifting of a mountain range, for example, can divide species populations, preventing gene flow which results in genetic divergence that represents a vicariant event (Chamberlin et al., 1890; reprinted in 1965). A case in point is, *Paruroctonus becki*, a scorpion species exhibiting distinct mitochondrial clades within the Mojave and Great Basin deserts that have been attributed to Pliocene and Pleistocene vicariance events associated with geographical and climate shifts (Graham et al., 2013). Similarly, a study on the Black Flat-tailed Scorpion (*Androctonus crassicauda*) used mitochondrial genetic markers to assess population structures across different regions. This revealed significant genetic diversity among populations, highlighting how geographic isolation shapes genetic diversity within a scorpion species (Ghavami et al., 2022).

The relevance of phylogeography extends beyond understanding distribution patterns; it also contributes to our understanding of community dynamics and species interactions. Phylogeographic studies help reconstruct evolutionary relationships and uncover how historical processes influenced current biodiversity patterns. For example, the occurrence of closely related species within distinct habitats can reflect historical processes of speciation and the differentiation of ecological niches (Pyron et al., 2015). In scorpions, one well-documented example involves species within the *Vaejovis vorhiesi* group, which occupy isolated montane habitats in the southwestern United States. Genetic evidence suggests that most of their lineages diverged during the Neogene and early Pleistocene, likely the result of vicariance driving by geological processes of the Basin and Range along the Mogollon Rim, illustrating how historical landscape changes have come to shape present-day patterns of diversity (Bryson et al., 2013).

In fragmented habitats, where isolated populations are prone to inbreeding and reduced genetic diversity, phylogeographic insights are essential for conservation planning, such as habitat restoration and the design of protected areas, ensuring the preservation of both species and their evolutionary potential in a rapidly changing world. For example, a study of White Pines (*Pinus* subsect. *Stobus*) in China used cytoplasmic genetic markers to uncover population structure and stress the urgency of targeted strategies (Lin et al., 2021).

The phylogeographic study of species in peninsular regions, such as Baja California in Mexico, can help understand how tectonic and climatic events have shaped the genetic diversity and distribution of organisms. As mentioned above,

vicariance is a process by which a population is geographically divide by a physical barrier, often leading to genetic divergence and the formation of distinct lineages over time. Characterized by its gradual separation from mainland Mexico and the potential influence of the hypothesized historical events like transpeninsular seaways, Baja California geological history provides a distinct context for understanding the role of vicariance and isolation in driving biodiversity. These processes are thought to have significantly contributed to the genetic divergence observed across the region (Grismer et al., 2000), making Baja California an ideal area for exploring the impacts of these evolutionary forces.

1.2 The Baja California Peninsula and Its Geological Context

Roughly 12.3 million years ago (Ma), the Pacific Plate, encompassing the majority of the Pacific Ocean, began moving approximately 250 kilometers (Km) northwest across the San Andreas Fault (Atwater and Stock, 1998). This movement initiated the separation of the Baja California peninsula from mainland Mexico, causing the formation of small valleys in Sonora, Sinaloa, and Nayarit (Grismer et al., 2000). The Gulf of California began its formation in the southern portion of the peninsula (modern-day Cape region) around 10 Ma (Gastil and Krummenacher, 1978), while the northern segment emerged around 6 Ma (Oskin and Stock, 2003; Bennett, 2015).

Flooding accompanied this tectonic activity, with some areas flooding almost immediately, such as Punta Mita at the southern tip of modern-day Nayarit. Other regions flooded slowly as the water moved farther northwest, such as the Islas Tres Marias, off the coast of Nayarit, which flooded approximately 8 Ma (Umhoefer et al., 2018). A major

marine incursion around 6 Ma rapidly filled the proto-Gulf of California, creating a continuous seaway that separated southern Baja California from the mainland and submerged low-lying regions as far north as Isla Tiburon, establishing a marine connection between them (Bennett et al., 2015). Although the peninsula was tectonically active before this flooring, by approximately 5.2 Ma it had shifted northwest from mainland Mexico and established the modern Gulf, reaching its current location; since then Baja has continued to move northwest along the Pacific Plate at a rate to roughly 50 km per million years (Antonelis et al., 1999).

Today, the Baja California peninsula is a rugged, arid landmass extending into the Pacific Ocean off the western coast of Mexico. It is bounded by California to the north, the Gulf of California to the east, and the Pacific Ocean to the west, and it is politically divided into the states of Baja California and Baja California Sur. The peninsula's geology is defined by its complex tectonic volcanic history, with features such as the Las Tres Virgenes volcanic complex helping reconstruct its geological past (20.6061° N, 90.0886° W) (Figure 1). Due to their age and geographic structure, these volcanoes help provide a record of past tectonic processes, offering clues about the timeline and character of geological events that have shaped the region over time (Avellán et al., 2018).

The oldest of these volcanoes, "El Viejo," formed approximately 300,000 years ago, followed by El Azufre which formed between approximately 173 and 128 Ma. And La Virgen approximately 128-22 million years ago, all during the Pleistocene, (Avellán et al., 2018; 2019). These formations, along with elevation gradients, have played a major role in shaping regional ecosystems and biodiversity patterns (Riginos, 2005). Tectonic

shifts also reconfigured coastal geography, influencing seaway routes and marine ecosystems over time (Bernardi et al., 2003).

Some hypotheses suggest seaways have acted as both connectors and dividers of ecosystems throughout the history of Baja California. A seaway is mainly identified as a water passage between two large bodies of water, whereas the depth and width of the connection closely resemble a large body of water (Rossi et al., 2023). In contrast, a strait resembles a river or stream where it is shallower and narrower (Rossi et al., 2023).

Seaways are important because of the obstacles they provide for terrestrial organisms and their habitats, as they might serve as physical barriers and promote allopatric speciation events. These bodies of water not only provide habitats for marine life but also present geographic barriers that influence the evolutionary trajectories of terrestrial organisms.

Geological evidence suggests that the formation of the Gulf of California may have functioned as a vicariance event, potentially acting as a barrier between the Baja California peninsula and mainland Mexico. This isolation could have driven distinct evolutionary trajectories in populations on either side of the Gulf.

One similar hypothesis suggests that a mid-peninsular seaway formed as sea levels fluctuated during the Quaternary period, dividing the peninsular into northern and southern populations, limiting gene flow and leading to genetic differentiation (Dolby et al., 2015).

Another potential biogeographic barrier consistent with the seaway hypothesis is found in the La Paz Break, a genetic discontinuity observed in multiple taxa near the city of La Paz in southern Baja California Sur. This break, although not associated with any obvious topographic features, has been repeatedly identified through mitochondrial and

nuclear DNA analyses across a range of organisms, including lizards, birds, and arthropods (Riddle et al., 2000; Aguirre-Leon & Murphy, 2002). Although the specifics are still unclear, its consistent presence across the phylogenies indicates a shared evolutionary response to an isolating mechanism.

Similarly, the Cabo Break refers to the genetic differentiation further south, near the Cape region of Baja California Sur. This break outlines lineages confined to the farthest southern tip of the peninsula, which is geographically and ecologically distinct from the rest of Baja. The Cape Region, which is characterized by a tropical dry forest, is thought to have remained isolated throughout the Quaternary Era due to its climate and topography. Genetic studies have revealed that several southern clades of various terrestrial organisms that are restricted to the Cape have unique southern clades that are strictly located in this area (Graham et al., 2014). This suggests that the Cabo Break might represent a biogeographical barrier such as a potential seaway. This break, together with the La Paz break, highlights how both climatic and geological processes may have interacted to drive lineage diversification across the Baja California peninsula. Over geological time, these hypothetical transpeninsular seaways would have risen and fallen, influencing the connectivity and isolation of landmasses. The fluctuation of sea levels, a consequence of glacial-interglacial cycles, has intermittently exposed and submerged land bridges, affecting the gene flow and dispersal of organisms (Hafner et al., 2011). These transformations in landscape configuration are thought to have left a lasting mark on the distribution and adaptation of species on the Baja California peninsula.

The Baja California peninsula complex geographic history allows for testing the possibility of seaways, in the role of genetic disconnection of species in that area. However, geological evidence, such as physical objects or structures along the Earth's surface, is required to confirm these hypothetical seaways. Geologists have yet to discover significant evidence to help prove or disprove this hypothesis.

One factor adding to the lack of geographical evidence is the geographic changes to the peninsula that have been made larger by wide-scale fluctuations in sea levels that have been known to be associated with the glacial cycles during the Quaternary period (Ortlieb, 1991). The Quaternary period is the current and most recent time era in the geological time scale and spans from 2.58 million years ago to the present day (Cohen et al, 2013). It is also suggested that these glacial cycles affected the range of species, which in turn affected speciation (FAUNMAP Working Group 1996; Comes & Kadereit, 1998; Hewitt, 2000). In addition, it also suggested that after the changes in these ranges, there was a development of a second area of contact where populations reunited during the interglacial periods (Schneider et al., 1998; Hugall et al., 2002).

There also seems to be a lack of marine sediments being found across the entirety of the region of the proposed Mid-peninsula seaway, adding to the doubts about its potential (Hafner & Riddle, 2011). It is also suggested that the molecular evidence previously found by Riddle et al, (2000) wasn't pointing to only one possible solution (the seaway) but instead that its results could also be interpreted as having other potential solutions – he proposed the molecular changes were because of geology and climatic patterns which led to the genetic divergences (Grismer, 2002).

Several studies identified various taxa, such as lizards, snakes, mammals, birds, and spiders, as highlighting a recurring biogeographic pattern in the mid-peninsula region by using mtDNA divergences (Zink et al., 1997; Riddle et al., 2000; Rodríguez-Robles & De Jesús-Escobar; Aguirre-León & Murphy, 2002; Crews & Hedin, 2006). Molecular studies, like those above, with similar results, suggest a potential barrier in the biogeography of the Baja California peninsula: that seaways isolated the northern and southern populations (Dolby et al., 2015). One study in particular that focused on the Red Diamond Rattlesnake (*Crotalus ruber*), whose range covers the entire peninsula (Harrington et al., 2018). In their study, they used RAD sequencing and coalescent-based modeling to determine that *C. ruber* was originally isolated during the Last Glacial Maximum (LGM) approximately 19-26 Ma, also known as the Pleistocene. The authors suggest climate fluctuations caused by the LGM initiated *C. ruber*'s population isolation. However, the seaway highlighted in this study does not align with the one outlined in Dolby's work.

Studying groups like arachnids can provide more information about the impact of potential seaways in this area since most of these organisms have limited mobility. Scorpions are organisms with deep lineages dating as far back as 434 million years ago, making them one of the first animals to live on land full-time (Wendruff et al, 2020). Their persistence allows for research to explore older phylogeographic patterns and to make inferences on vicariance events over millions of years. One factor of these organisms that adds to their effectiveness in phylogeographical studies is their low vagility. This refers to their limited ability to disperse over long distance, which in turn means that even small geographic barriers can lead to long-term population isolation.

This means their populations become geographically and genetically isolated easily, which allows for clear identification of historical biogeographic events within their genomes.

Scorpions often display cryptic speciation, which is when populations that are morphologically similar but are genetically distinct (Riddle et al., 2000). Although studies using arachnids to test the seaway hypotheses in Baja are lacking, Graham et al. (2014) investigated how 13 scorpion species in the Baja California Peninsula responded to historical climate change. Highlighting the Pleistocene to Holocene, they suggested that most species' distributions remained stable over time. They underlined three regions with consistent climate suitability that aligned with known genetic breaks, the previously mentioned transpeninsular seaways. They stated that long-standing climate boundaries, along with these seaways, may have played a key role in shaping biodiversity across the peninsula. To that end, in the present study, we explored the phylogeography of the Baja California Bark Scorpion (*Centruroides exilicauda*) in the area and tested the hypothesis that transpeninsular seaways influenced genetic patterns within the species.

1.3. *Centruroides exilicauda* (Wood, 1863)

In this study, I am focusing on the phylogeography of Bark Scorpions, *Centruroides exilicauda* (Figure 2). The genus *Centruroides* consists of approximately 50 species that are currently recognized and distributed across the Southern United States of America, Mexico, and Central America, with the majority of its diversity found in Mexico (Santibáñez-López et al., 2016; Ove-Rein, 2017). Scorpions in this genus are known for several highly venomous species like *Centruroides limpidus*, which has caused

fatal envenomation in humans via neurotoxins such as CIII and CIII which modify sodium channels (Dehesa-Dávila et al., 1996). The venom of these organisms and many others within the same genus is venomous to vertebrates. Within the last 20 years, their venom has become medically relevant, with many uses for the venom, like the production of antibody fragments that are capable of neutralizing several of the toxins of several venom scorpion species. Current antivenoms are created from fragments using hyperimmunization of horses. By changing to fragments created from scorpions, scientists aim to create recombinant antivenoms that they could potentially use to neutralize the venom of all Mexican species of scorpions (Riano-Umbarila et al., 2017).

Centruroides exilicauda is the type species for the genus *Centruroides* (described originally as *Buthus exilicauda* by Wood in 1863 from Lower California, now Baja California, Mexico) and transferred by Marx (1890). Gantenbeini et al. (2001), using COI sequences from mainland Mexico and the Baja California Peninsula, recovered the presence of three genetically distinct clades across the peninsula: a Southern clade mainly in the Cape of Baja California Sur, a northern clade spread across the middle and upper portions of in Baja California Sur and parts of Baja California, and a mainland clade spanning the Sonoran Desert into both Arizona, USA and Sonora, Mexico. They also propose that of these three clades, the Southern Clade and the mainland clade are more genetically similar to each other than either one is to the northern clade. This brings into question what sort of geographic history would allow for this distinct genetic divergence. Later, in 2004, biochemical analyses were conducted on the two sister taxa by Valdez-Crus et al. (2004), *C. exilicauda* and *C. sculpturatus*, where it was concluded that the chromatographic profile of the two species differed.

My goal was to determine whether the observed genetic breaks align with the hypothesized location of the Midpeninsular Seaway, providing evidence that such a seaway played a role in shaping the evolutionary history of *C. exilicauda* on the Baja Peninsula. To support the existence of transpeninsular seaways, I will be looking for a genetic ‘signature’ left in the phylogeographic patterns of these scorpion species. We did this by sequencing the mitochondrial CO1 marker from samples at different localities spanning the distribution of the species, in combination with the samples from Gantenbein et al. (2001). Due to the elevated mutation rate, lack of recombination, and maternal inheritance, mitochondrial DNA (mtDNA) serves as a tool for scientists to piece together the intricate relationships within species (Gupta et al., 2015), including the study of intraspecific patterns of genetic diversity in scorpions (i.e., Graham et al., 2013). Through the lens of mitochondrial genetic diversity and population structure across various localities, I aim to test the hypothesis that transpeninsular seaways impacted the phylogeography of *C. exilicauda*. In addition, data from this study will help illuminate the evolutionary history of Bark Scorpions in arid environments.

CHAPTER II

METHODS AND MATERIALS

2.1 Sample Collection

Samples were collected by Drs. Matthew Graham, Carlos Santibáñez-López, and Joshua Idjadi between the years of 2019 and 2021 from field expeditions. Other samples of *C. exilicauda* and other *Centruroides* species; *C. elegans*, *C. infamatus*, *C. insularis*, *C. sculpturatus*, and *C. suffusus*, across Baja California, Mexico, were donated by Victor Fet and Carlos A. López González, or retrieved from GenBank (Table 1). Samples were collected using ultraviolet light detection (Stahnke, 1972), placed in a vial containing 95% EtOH, and labeled with location, dates, and other relevant information. Samples were stored at -80°C at the Department of Biology, Eastern Connecticut State University, and the Department of Biology, Western Connecticut State University. A database of the collected samples and other donations is shown in Table 2. A map was then created using ArcGIS Online to display our samples (Figure 3).

2.2 DNA extraction, amplification, sequencing and editing

Muscle tissue was harvested by removing the walking legs from the left side of each specimen. Genomic DNA was then extracted from the muscle tissue of the legs of specimens using a DNEasy Blood and Tissue Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. Gene fragments were amplified

using a forward primer COI_{modF} and a reverse primer LEI_r (Table 3) (Bryson *et al.*, 2013; Esposito, 2011). PCR program was run in Eppendorf[®] Mastercycler[®] Nexus Thermal Cyclers (Eppendorf, Framingham, MA, USA). The PCR program consisted of an initial denaturation step at 94°C for 5 minutes, 38 cycles (94°C for 60 s, 54 °C for the 60s, 72 °C for 75), and a final extension at 72 °C for 5 min. PCR products were purified using ExoSAP-IT (GE Healthcare, Piscataway, NJ, USA) PCR products were verified by electrophoresis at 100 V for 30 minutes in a 1% (w/v) agarose gel in 1 x TAE buffer using GELATO[™] electrophoresis and visualization system (miniPCR bio[™]). Products were then sent to Eton Bioscience, Inc. for bidirectional sequencing.

Sequences were assembled and edited manually in Geneious 2023.2.1 (Biomatters Ltd, Auckland, New Zealand). Each site of the generated sequenced data was evaluated by the integrity of the chromatogram signal. Outgroup sequences were retrieved from GenBank (see Table 4).

2.3 Phylogenetic Analyses

Our sequences were aligned using MAFFT 7 (Kato and Standley, 2013) using the default parameters. Phylogenetic analysis was conducted using the Maximum Likelihood principle as implemented in IQTREE v 2.0 (Nguyen *et al.*, 2015) with ModelFinder Plus (Kalyaanamoorthy *et al.*, 2017) to search for the best-fit model and 1000 ultrafast bootstrap replicates (Hoang *et al.*, 2021) [iqtree -s MATRIX -m MFP -B 1000]. In addition to this analysis, Bayesian inference (BI) was performed using MrBayes v. 3.2.2. (Ronquist *et al.*, 2012) using the GTR + Γ + I model with four runs, each with

four Markov chains implemented for 1×10^7 using default priors and discarding 5×10^6 generations (25%) as burnin.

2.4 Divergence Dating and Demographic History

Estimation of divergence times was assessed using BEAST v. 1.10 (Drummond et al., 2012) with the GTR + Γ + I model, a Yule process speciation, and an uncorrelated relaxed clock (Drummond et al., 2006) with a rate calibration commonly used for COI in buthid scorpions (0.007 substitution/site/my (Gantenbein et al., 2005) and a SD of 0.00146). This analysis was split into four mcmc independent runs of 100 million generations each (sampling every 10,000). In addition, the molecular diversity was calculated (e.g., nucleotide diversity) and tested for evidence of recent demographic changes using Tajima's D (Tajima, 1989) and Fu's FX (Fu, 1997) using DNAsap v.5. A network to visualize the relatedness of COI haplotypes was produced using the median-joining network with the epsilon value = 0 with the software PopART (Population Analysis with Reticulate Trees) version 1.7.2. (Leigh and Bryant, 2015). Changes in effective population size over time were assessed with Bayesian skyline plots (Minin et al. 2008) implemented in BEAST v 2.5.2 (Bouckaert et al. 2019) for 100 million generations with the number of population size changed to four and the same COI buthid rates as above for the two clades recovered in our phylogenetic analyses. Lastly, a Discriminant Analysis of Principal Components (DAPC) using the COT sequences and the R package ADEGENET was conducted. This multivariate method is designed to identify and describe clusters of individuals that are genetically related by using sequential K-means and model selection to infer these clusters (Jombart et al., 2010). The

value of clusters (K) was constrained to “4” as suggested by the number of clades we recovered in the ML analysis and by the BIC values using the first 9 PCs, retaining three axes in the Discriminant Analysis (DA).

CHAPTER III

RESULTS

1.1 Phylogenetic analysis

We sequenced COI sequences from 21 collected specimens determined as *Centruroides exilicauda*. Additionally, we incorporated ten samples, three of them determined as *C. exilicauda*, and seven outgroups obtained from GenBank (Table 1). As a result, 31 sequences (24 putatively assigned as *C. exilicauda*, plus seven outgroups) were concatenated in our matrix (mtDNA dataset).

Maximum-likelihood analysis of the mtDNA dataset (31 sequences, 822 nucleotides, using the TN+F+4 model of substitution) did not support the monophyly of *Centruroides exilicauda*, as one specimen of this species was recovered nested within the *Centruroides sculpturatus* clade. The rest of the specimens determined as *C. exilicauda* were recovered in two clades (sister to each other, albeit no nodal support). These two clades (referred to here as the Northern clade and Southern clade) contain the 21 individuals sequenced here, plus only two samples retrieved from GenBank (Figure 4). Four of my samples (MRG2030, MRG2053, MRG2054, and MRG2029) were clustered with a specimen of *C. exilicauda* (100% ultrafast bootstrap nodal support) retrieved from GenBank (KY982179, collected from Cabo San Lucas, Baja California Sur, Mexico). This clade is hereafter named the Southern clade. The Northern clade comprises 17 MRG samples (sequenced here) and another specimen of *C. exilicauda* retrieved from GenBank (AY649858, collected from Punta Banda, Baja California, Mexico) with the highest

nodal support (100%, Figure 4). Unfortunately, the relationship between the Southern and Northern clades was not supported by ultrafast bootstrap (57%). The *C. exilicauda* clade (containing the Northern and Southern clades) was recovered as sister taxa to a clade (with the highest nodal support) containing three specimens of *C. sculpturatus* and a specimen of *C. exilicauda* (AY995833) (possibly a misidentification, hereafter re-determined as *C. sculpturatus*). However, the relationship between *C. exilicauda* and *C. sculpturatus* has no nodal support (Figure 4). Finally, the clade containing *C. exilicauda* and *C. sculpturatus* was recovered as sister to *C. suffusus* with 95% ultrafast bootstrap nodal support.

The Bayesian Inference (BI) topology is highly congruent with the ML tree. Figure 5 shows a collapsed topology with the two clades including our samples (the Northern and Southern clades), and the clade representing *C. sculpturatus*. However, this topology does not support the monophyly of *C. exilicauda*, as the relationships between clades were not supported by high posterior probabilities (Figure 5). Similarly, the relationships between the different species are also not supported (e.g., *C. suffusus* is not the sister taxa to *C. Exilicauda* and *C. sculpturatus*).

1.2 Divergence dating, Population structure, and demographic history

Divergence times were estimated using BEAST v.1.10 with a relaxed clock and the buthid COI substitution rate. This dated topology (Figure 6) is congruent with the ML tree. The clade containing the species *C. suffusus*, *C. sculpturatus*, and *C. exilicauda* diverged ca. 13.14 million years ago (Ma). Unlike in the ML topology, *C. suffusus* was recovered as the sister taxon to *C. sculpturatus*, with a mean divergence time between

these taxa around 11.05 Ma. More importantly, the monophyly of *C. exilicauda* was recovered with this analysis, with the Southern clade recovered as sister taxa to the Northern clade with a mean divergence time between them around 10.51 Ma (during the Late Miocene). This clade then diversified around 5.27 Ma. Similarly, the Northern clade diversified around 5.64 Ma.

Haplotype and haplotype diversity were higher for the Northern clade than for the Southern clade (Table 4). Tajima's D was negative for the Northern clade but positive for the Southern clade, with positive Fu's Fs for both clades. While the Northern clade possessed ten haplotypes, the Southern clade possessed only five (Figure 7). The Bayesian skyline for the species (*C. exilicauda*, both clades combined) showed a decrease in effective population size in the late Pleistocene, but no recent increase is visible (Figure 8). Lastly, the DAPC recovered four distinct clusters (Figure 9; Table 4). Cluster 1 contained nine *C. exilicauda* samples recovered within the Northern clade in our ML topology (however, these samples do not form a monophyletic group within this clade). Cluster 2 contained all samples from the Southern clade in agreement with our topologies (i.e., ML, BI, and dated topology; Figures 4-6). Cluster 3 contained six samples from the Northern clade (five samples sequenced here, plus one retrieved from GenBank, all species distributed in Baja California, the northern limit in our sampling), which were recovered as a monophyletic group (in agreement with our ML topology; Figure 4). Finally, Cluster 4 contained three samples also from the Northern clade, which were recovered as sister taxa to samples in Cluster 3 with a high ultrafast bootstrap (95%) in our ML topology (Figure 4).

CHAPTER IV

DISCUSSION

Maximum likelihood phylogenetic analysis of an alignment comprising 31 sequences and the mtDNA sequences of the COI recovered the monophyly of species *Centruroides exilicauda*, *Centruroides sculpturatus*, and *Centruroides suffusus* as established elsewhere (Esposito et al., 2017). First, *C. suffusus* was recovered as the sister taxa to a clade containing *C. sculpturatus* and *C. exilicauda*. The clade comprising samples from *C. sculpturatus* included a specimen previously determined as *C. exilicauda* (GenBank accession number AY995833), suggesting a possible misdetermination by the authors. Notably, we were unable to determine the precise geographic origin of specimen AY995833, limiting our ability to interpret its phylogenetic placement in the context of our study.

Here, we propose to revise this species determination and change it to *C. sculpturatus* based on our phylogenetic and phylogeographic analyses. With the identity of this specimen solved, we observed that *C. exilicauda* and *C. sculpturatus* are sister to each other in our ML topology (as suggested earlier by Valdez-Cruz et al., 2004), albeit with poor nodal support. Unlike the Maximum-likelihood analysis, which recovered two clades of *C. exilicauda*, the Bayesian Inference analysis recovered a polytomy between *C. suffusus*, *C. sculpturatus*, and the two clades within *C. exilicauda* (Figure 5). This indicates no clear relationship between the species and the clades within *C. exilicauda*

(Figure 4). However, the dated topology recovered a monophyletic *C. exilicauda* (Figure 5).

The divergence time for the clade comprising *C. suffusus*, *C. sculpturatus*, and *C. exilicauda* was estimated to share a common ancestor between about 5 to 18 Ma, with a mean estimate of 13Ma (Figure 5). This timeframe, particularly considering the large error margins, includes the period associated with the early formation and separation of the Baja California Peninsula from mainland Mexico. (Atwater and Stock, 1998; Reisewitz, 2009). This topology suggests a subsequent divergence of *C. exilicauda* from their most recent common ancestor is shared with the clade that includes *C. suffusus* and *C. sculpturatus*. According to our divergence time estimates, this occurred roughly 13 million years ago, prior to the Peninsula's detachment from mainland Mexico (Gastil and Krummenacher, 1978). Then, the two clades (Northern and Southern clades) diverged about 10 Ma. Both clades exhibit similar times to their most recent common ancestors (ca 5 Ma). Divergence within these clades is estimated to have occurred during the late-Miocene, Pliocene, and Pleistocene. The southern clade, as sister taxa to the Northern clade, includes samples found on both sides of the hypothesized Midpeninsular Seaway, but the mean divergence date for this group predates the proposed Quaternary age of the seaway, While the 95% highest posterior density interval does extend slightly into the Quaternary, the overall timing and spatial distribution of the lineages are not consistent with a mid-peninsula vicariant event. With this, the Midpeninsular seaway hypothesis is not supported by our data.

Geographically, our results reveal two well-supported clades of *C. exilicauda*, distributed along the north-south axis of the Baja California peninsula. The Northern

clade includes samples from the mid-peninsula, while the Southern clade consists of specimens collected from the southernmost regions of the peninsula. These distributions align most closely with the proposed Isthmus of La Paz, or the La Paz Break, rather than the more northern Agua Armada, mid-peninsular, and Loreto breaks, or the southern Cabo break.

Unfortunately, the low nodal support for these clades clustering both populations in ML and BI topologies suggested that additional data may be necessary to definitively support the presence of two distinct species. If confirmed, the southern clade, which includes specimens closer to the putative type locality for *C. exilicauda* (sensu Esposito et al., 2017), would represent the true *C. exilicauda*. The northern clade could then be reconciled with *Centruroides zweifeli*, a synonym of *C. exilicauda* described from Baja California (Williams, 1980). Gertsch (1958) described the species *Centruroides zweifeli* from a female (deposited at the AMNH) from San Martin Island, Baja California, Mexico, but later synonymized it to *C. exilicauda* by Williams, 1980. Here, our ML and BI analyses support a clade containing samples from the mid-Peninsula and the northern parts [including a sample used in the Valdez-Cruz et al.'s (2004) study (AY649858)].

The patterns recovered in our phylogeny are consistent with the phylogeographic structure observed in other Baja California taxa, including *C. exilicauda* (Gantenbein et al., 2001), zebra-tailed lizards (*Callisaurus draconoides*) (Lindell et al., 2005), and *Urosaurus* (tree lizards) species (Aguirre et al., 1999). These similarities suggest that historical fragmentation events, such as the formation of temporary or intermittent seaways, may have frequently occurred, influencing terrestrial species diversification in this region. Alternatively, patterns of divergence might also explain the responses of

organisms to environmental differences across the peninsula, such as adaptation to distinct climate or habitat conditions, suggesting that multiple factors likely contributed to the forming of the region's biodiversity.

Further, our haplotype and nucleotide diversity analysis aimed to explore if populations of *C. exilicauda*'s populations were affected by the transpeninsular seaways. Our study supports the separation of *C. exilicauda* and the MRCA of *C. sculpturatus* and *C. suffusus* based on the proposed timing of the Pilo-Pleistocene transpeninsular seaway, also known as the Isthmus of La Paz or the La Paz Break. This break is much like the Midpeninsular seaway in that it contains no consistent geological evidence for its existence, such as marine sediments across the entire proposed locality (Hausback, 1984). It is also similar in that previous studies on terrestrial organisms such as zebra-tailed lizards (*Callisaurus draconoides*) (Lindell et al., 2005) and various other lizard species, *Urosaurus* (Aguirre et al., 1999) have provided a foundation to continue to study the effects of proposed transpeninsular seaways across the Baja California peninsula.

Demographic analyses using the mtDNA data indicate that *C. exilicauda* underwent a Pleistocene decrease in the population size. Molecular diversity statistics support that the Northern clade possesses high genetic diversity. The Isthmus of La Paz break is estimated to have been during the Plio-Quaternary period, approximately 2.58 Ma. This, however, is consistent with the split of the *C. exilicauda* Southern clade from the Northern one, approximately 2.59 Ma (Figure 6). This, alongside acknowledging the differences in our collected sample size for each clade, could account for the difference in haplotype diversity between the Northern and Southern clades (Table 6). This is also further supported by DACP analysis. The clusters within our DACP analysis mirror the

groups within our ML tree and our dated topology, as seen in Figure 3. Clusters 1, 3, and 4 are all the same species that are contained within the Northern clade. Cluster 2 contains all the specimens within the Southern clade. The placement of the clusters along the discriminant function 1 axis indicates that clusters 1 and 3 are more closely related than either one is to cluster 4, and even more so to cluster 2. The placement of clusters 2 and 4 is also more closely related along the discriminant function 2 axis than they are to clusters 1 and 3. This means that cluster 2 is separated from the other three, which correlates with our earlier statement that the Southern clade samples were more recently isolated from the Northern clade via the La Paz break.

Ecologically, the genetic divergence between the two clades might reflect differences in habitat stability and historical connections across the peninsula, potentially driven by changes in sea levels or geological uplift. These could have facilitated a restriction in gene flow. Despite our findings, several questions remain. Our reliance on mitochondrial DNA limits our capacity to detect nuclear gene flow or incomplete lineage sorting (Shaw et al., 2002). Further studies incorporating nuclear markers and ecological niche modeling will be necessary for a more rounded understanding of the evolutionary process underlying the diversification in *Centruroides* and other related taxa. We also propose that additional genetic sampling from a broader range of *Centruroides* populations within the Baja California peninsula, along with the use of inclusion of other mtDNA loci such as 16S, which has been used to infer high-order phylogenetic relationships in scorpions (Fet et al., 1999; Guo et al., 2011), will be important for evaluating the strength of our hypothesis. Including 16S data in our study would also allow for meaningful comparisons with previously published datasets, particularly those

generated by Gantenbein et al., helping to place our findings within a broader phylogenetic context.

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Table 1. Collected and sequenced *Centruroides exilicauda* MRG samples

<i>ID</i>	<i>Date</i>	<i>Genus</i>	<i>Species</i>	<i>Location</i>	<i>Coordinates</i>	<i>Comments</i>	<i>Tissue?</i>	<i>EXT. Qubit</i> (ng/u)
MRG2028	8/20/19	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: N Cd Insurgents	25.590556, -111.949444	1 male; Lid of tube says "OI"; Coll. MRG, JAJ, CESL, RRL	Yes	4.16
MRG2029	8/12/19-8/13/19	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: 19.2 Km S of El Pescadero	23.185000, -110.129444	Lid says "H"; Coll. MRG, CESL, RRj	Yes	8.78
MRG2030	8/12/19-8/13/19	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: 19.2 Km S of El Pescadero	23.185000, -110.129444	Lid says "H"	Yes	0.5
MRG2031	09/2019			Mexico: Baja California Sur: Puerto San Carlos	24.863056, -112.03861	Coll. MRG, JAJ, CESL, RRJ	Yes	0.748
MRG2032	8/20/19-8/21/19			Mexico: Baja California Sur: El Conejo	24.863056, -112.03861	Lid says "J"; Coll. MRG, JAJ, CESL, RRJ	Yes	7.28
MRG2039	8/16/2019-8/17/2019	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: Puerto San Carlos	24.863056, -112.038611	Lid "O"; Coll. MRG, JAJ, CESL, RRJ	Yes	1.09
MRG2040	8/16/2019-8/17/2019	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: Puerto San Carlos	24.863056, -112.038611	Lid "O"; Coll. MRG, JAJ, CESL, RRJ	Yes	5.32
MRG2041	8/16/2019-8/17/2019	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: Puerto San Carlos	24.863056, -112.038611	Lid "O"; Coll. MRG, JAJ, CESL, RRJ	Yes	4.56
MRG2042	8/16/2019-8/17/2019	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: Puerto San Carlos	24.863056, -112.038611	Lid "O"; Coll. MRG, JAJ, CESL, RRJ	Yes	3.31

MRG2044	8/16/2019- 8/17/2019	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: Puerto San Carlos	24.863056, -112.038611	Lid "O"; Coll. MRG, JAJ, CESL, RRJ	Yes	3.27
MRG2046	7/20/2019- 7/21/2019	<i>Centruroides</i>		Mexico: Baja California: Ensenada: Catalina: Santa Inez	29.729536, -114.697653	Tube "H"; 2 Hand Colelcting; Coll. MRG, CESL, RRJ, MYPC	Yes	0.387
MRG4048	7/20/2019- 7/21/2019	<i>Centruroides</i>		Mexico: Baja California: Ensenada: Catalina: Santa Inez	29.729536, -114.697653	Tube "H"; 2 Hand Colelcting; Coll. MRG, CESL, RRJ, MYPC	Yes	3.12
MRG2049	7/20/2019- 7/21/2019	<i>Centruroides</i>		Mexico: Baja California: Ensenada: Catalina: Santa Inez	29.729536, -114.697653	Tube "H"; 2 Hand Colelcting; Coll. MRG, CESL, RRJ, MYPC	Yes	7.52
MRG2053	8/14/2019- 8/15/2019	<i>Centruroides</i>		Mexico: Baja California Sur: East Cape Beach	23.097500, -109.658889	Lid "E"; Tube: "East Cape Beach, 14 Aug 19"; Coll. MRG, CESL, RRJ	Yes	7.64
MRG2054	8/14/2019- 8/15/2020	<i>Centruroides</i>		Mexico: Baja California Sur: East Cape Beach	23.097500, -109.658889	Lid "E"; Tube: "East Cape Beach, 14 Aug 19"; Coll. MRG, CESL, RRJ	Yes	4.48
MRG2057	8/16/2019- 8/17/19	<i>Centruroides</i>		Mexico: Baja California Sur: East Puerto San Carlos	24.863056, -112.038611	Coll. MRG, CESL, RRj, IJ	Yes	17.5
MRG2058	8/16/2019- 8/17/19	<i>Centruroides</i>		Mexico: Baja California Sur: East Puerto San Carlos	24.863056, -112.038611	Coll. MRG, CESL, RRj, IJ	Yes	19
MRG2060	7/19/2019- 7/20/2019	<i>Centruroides</i>		Mexico: Baja California Sur: San Quintin: Don Eddie's Camping Side	30.486800, -115.977242	Lid "L Cexi"; Hand Collecting 1; Coll. CESL, RRJ, MYPC	Yes	20.4

MRG2062	7/19/2019- 7/20/2021	<i>Centruroides</i>		Mexico: Baja California Sur: San Quintin: Don Eddie's Camping Side	30.486800, -115.977242	Lid "L Cexi"; Hand Collecting 1; Coll. CESL, RRJ, MYPC	Yes	6.88
MRG2065	8/16/2019- 8/17/2019	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: Puerto Escondido: Playa Quemada	25.79657, -111.311728	Tube: "Playa Quemado Pto... Aug 16-17 Cexi"	Yes	10.2
MRG2069	8/16/2019- 8/17/2019	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: Puerto Escondido: Playa Quemada	25.79657, -111.311728	Tube: "Playa Quemado Pto... Aug 16-17 Cexi"	Yes	6.88
MRG2102	2001	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California: San Quintin	30.571710, - 115.944956			
MRG2102	2001	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California: Bahia de Los Angeles	28.954411, -113.56546			
MRG2103	2001	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: San Igancio	27.281662, -112.895289			
MRG2104	2001	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: Juncalito	25.771363, -111.31504			
MRG2105	2001	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur: Cabo San Lucas	22.902516, -109.911205			
MRG2106	2001	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Baja California Sur:Miraflores	23.367066, -109.777486			
MRG2107	2001	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Sonora:Guaymas	27.910914, -110.911844			
MRG2108	2001	<i>Centruroides</i>	<i>exilicauda</i>	Mexico: Sonora:Puerto Penasco	31.317843, -113.531582			

<i>MRG2109</i>	2001	<i>Centruroides</i>	<i>exilicauda</i>	United State of America: Arizona: Yuma	32.651257, -114.555594
<i>MRG2110</i>	2001	<i>Centruroides</i>	<i>exilicauda</i>	United State of America: Arizona: Tempe	33.378672, -111.930653
<i>MRG2111</i>	2001	<i>Centruroides</i>	<i>exilicauda</i>	United State of America: Arizona: Black Canyon City	32.015962, -110.747853
<i>MRG2112</i>	2001	<i>Centruroides</i>	<i>exilicauda</i>	United State of America: Arizona: Tuscon	32.067079, -110.826191

Table 2. Forward and reverse primers used for COI amplification during Polymerase Chain Reaction

Primer	Sequence 5' → 3'	Source
COI mod Forward	5'-ATCATAAGGATATTGGGACTATGT-3'	Bryson et al. (2013)
LE1 Reverse	5'- GTAGCAGCAGTAAARTARGCYCGAGTATC- 3'	Esposito (2011)

Table 3. Samples from six *Centruroides* species retrieved from GenBank

<i>Access Number</i>	<i>Genus</i>	<i>Species</i>	<i>Locality (if known)</i>
AY649858	<i>Centruroides</i>	<i>exilicauda</i>	Punta Banda, Baja California, Mexico
KY982179	<i>Centruroides</i>	<i>exilicauda</i>	Cabo San Carlos, Baja California Sur, Mexico
AY995833	<i>Centruroides</i>	<i>exilicauda</i>	
EU381065	<i>Centruroides</i>	<i>sculpturatus</i>	Tempe, Arizona, USA
AY649857	<i>Centruroides</i>	<i>sculpturatus</i>	Tucson, Arizona, USA
MK479199	<i>Centruroides</i>	<i>sculpturatus</i>	Sonora, Mexico
MK479203	<i>Centruroides</i>	<i>suffusus</i>	Durango, Mexico
MK479181	<i>Centruroides</i>	<i>insularis</i>	Nayarit, Mexico
MK479170	<i>Centruroides</i>	<i>elegans</i>	Jalisco, Mexico
MK479179	<i>Centruroides</i>	<i>infamatus</i>	Jalisco, Mexico

Table 4. Summary statistics for COI data for the two clades recovered within *Centruroides exilicauda*. Standard deviations are in parentheses. Abbreviations: *D*, Tajima's D; *F*, Fu's *FS*; *h*, haplotype diversity; *H*, number of different haplotypes; *n*, number of individuals; π , nucleotide diversity.

Clade	n	H	h	D	π	F
Southern	5	5	1.0 (+- 0.016)	0.53394	0.036 (+- 0.0005)	0.938
Northern	17	10	0.902 (+- 0.00247)	-0.89111	0.024 (+- 0.0002)	1.325



Figure 1. View of La Tres Virgines from Santa Rosalia, Baja California Sur, Mexico

27.40793° N, 112.54916° W. Photo by Dr. Matthew Graham



Figure 2. Photograph of a juvenile of the species *C. exilicauda*. Photo by raphetnature
retrieved from iNaturalist (<https://www.inaturalist.org/observations/146188838>)

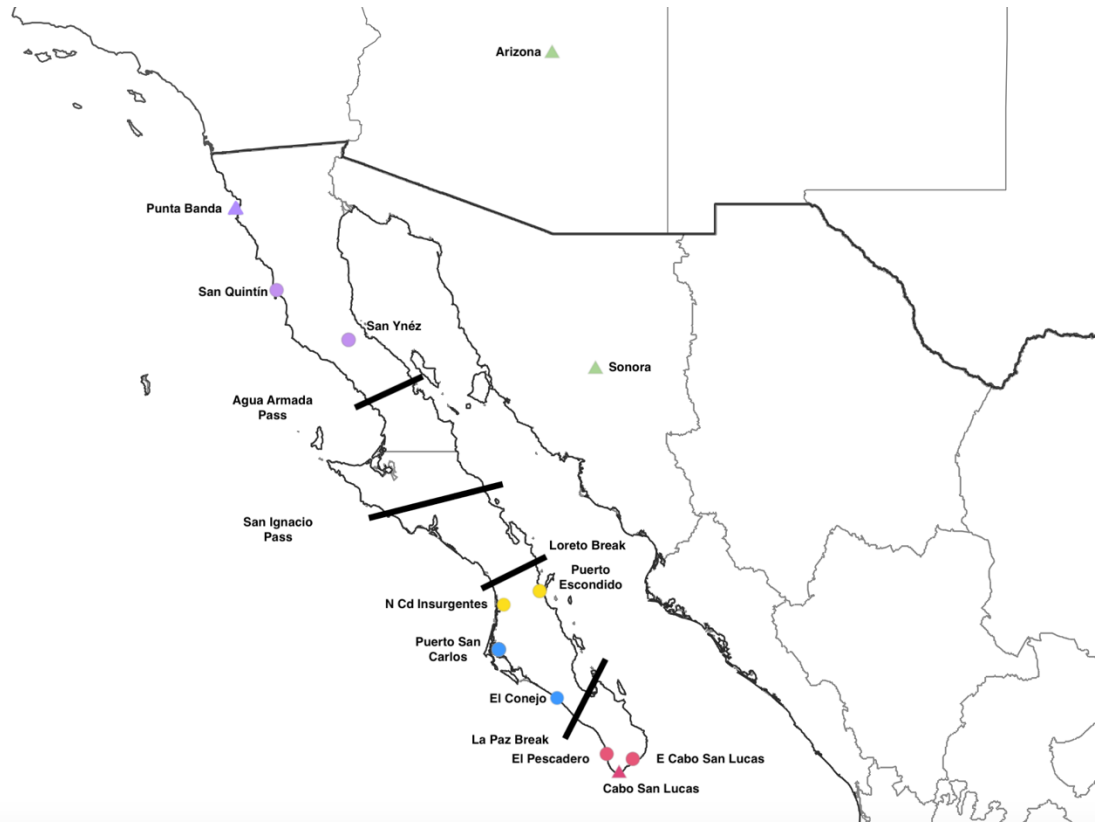


Figure 3. Map of *Centruroides* sample locations collected across the Baja California Peninsula used for this study. Points represent MRG samples collected in this study. Triangles represent samples retrieved from GenBank. Locations and Seaways are labeled accordingly. Each sample correlates with the clade colored in the Maximum likelihood tree (Figure 5) and the DACP analysis (Figure 10). Map created using ArcGIS Online.

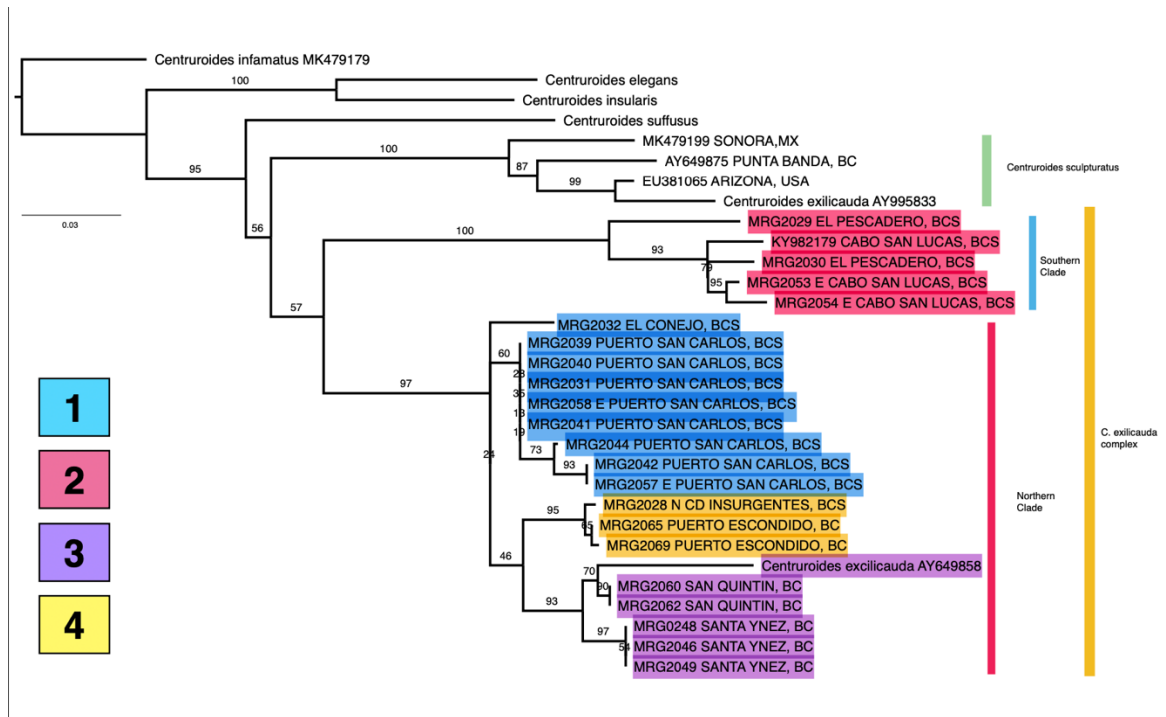


Figure 4. Maximum likelihood topology (lnl = -3550.0562) inferred from partial COI mtDNA sequences from 31 samples of genus *Centruroides* and 822 nucleotides (of those 600 were constant sites, and 160 were parsimony informative).

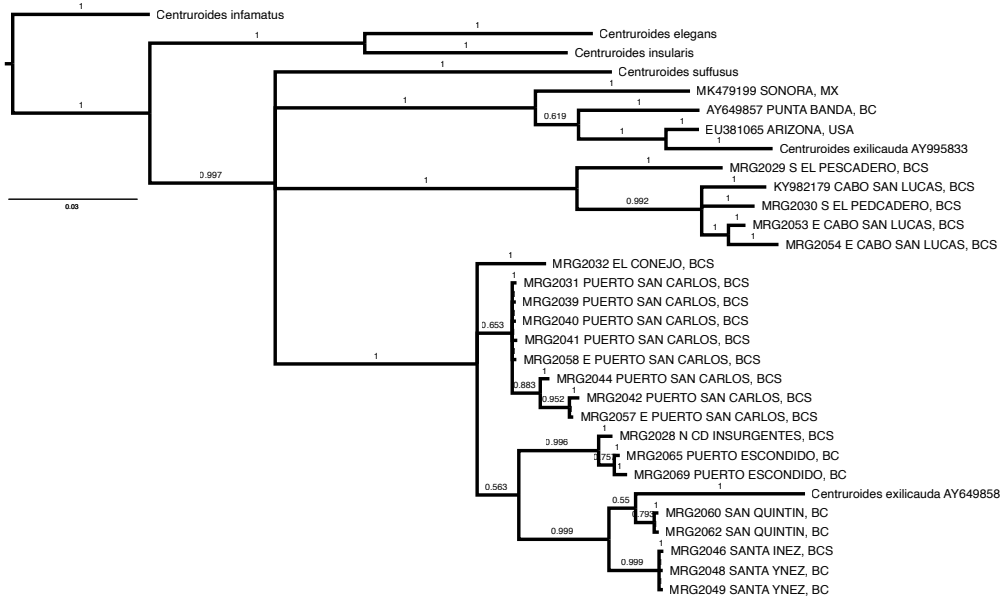


Figure 5. Bayesian Inference phylogenetic tree inferred from partial COI mtDNA (31 samples, 822 nucleotides) using Mr.Bayes and the GTR + Γ + I model implemented in four independent chains. Nodes with posterior probabilities lower than 0.50 were collapsed.

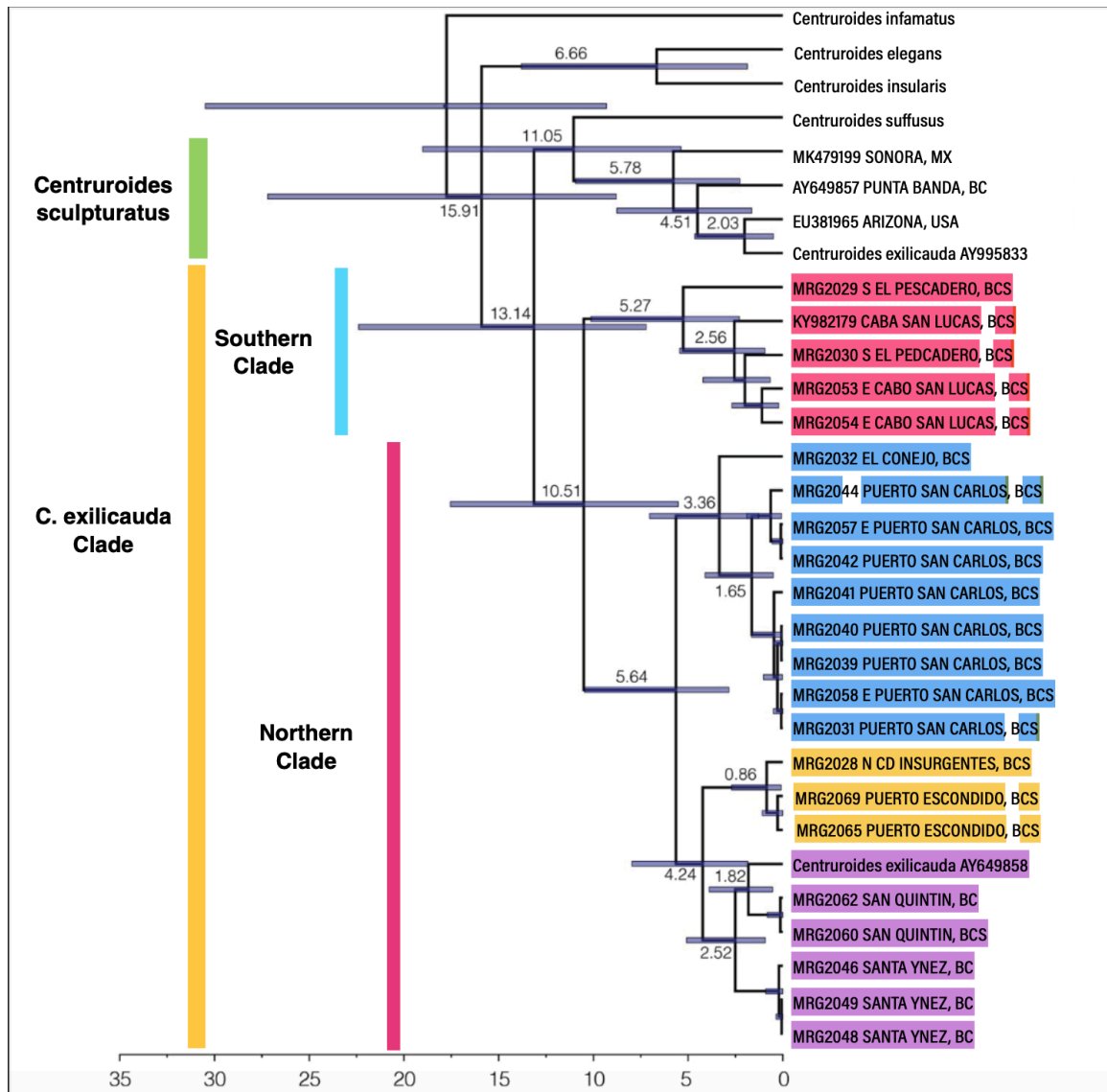


Figure 6. Divergence time topology recovered by the analysis of 31 partial COI sequences with 822 nucleotides using Beast 1.10.4, and a GTR + Γ + I model, a Yule process speciation, and an uncorrelated relaxed clock with a rate calibration of 0.007 substitution/site/my and a SD of 0.00146).

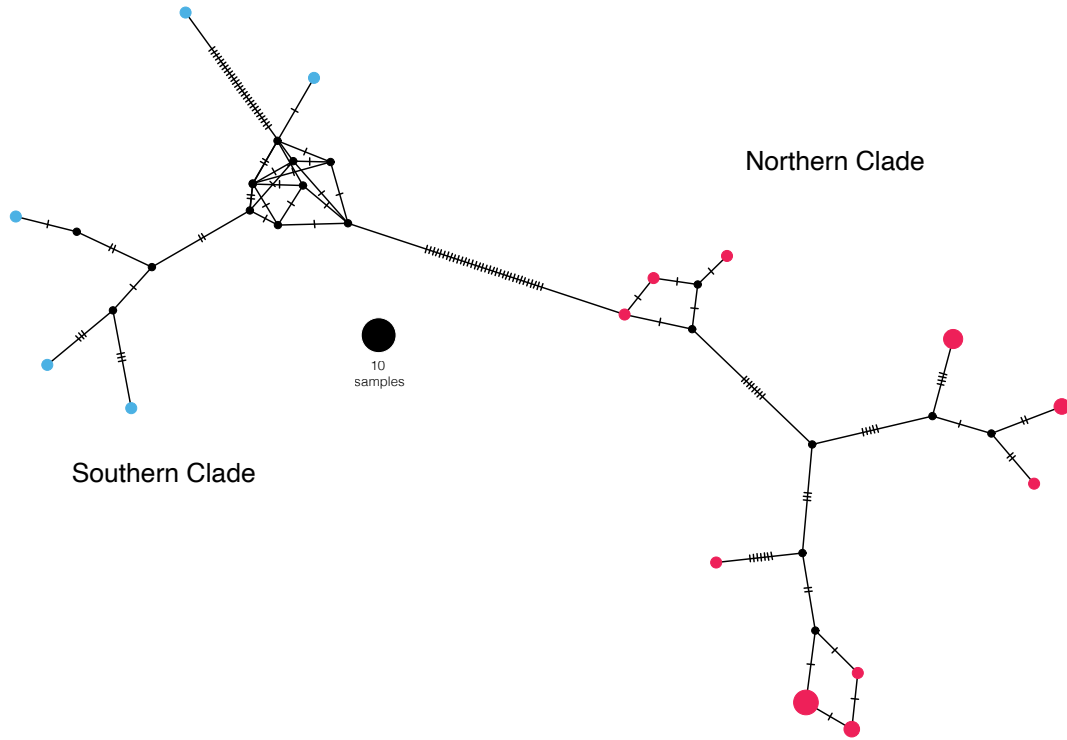


Figure 7. Median-joined haplotype network of COI sequences, colors correspond to the Northern and Southern clades outlined in the ML phylogeny (above). Circle sizes are proportional to sample sizes (see circle in figure).

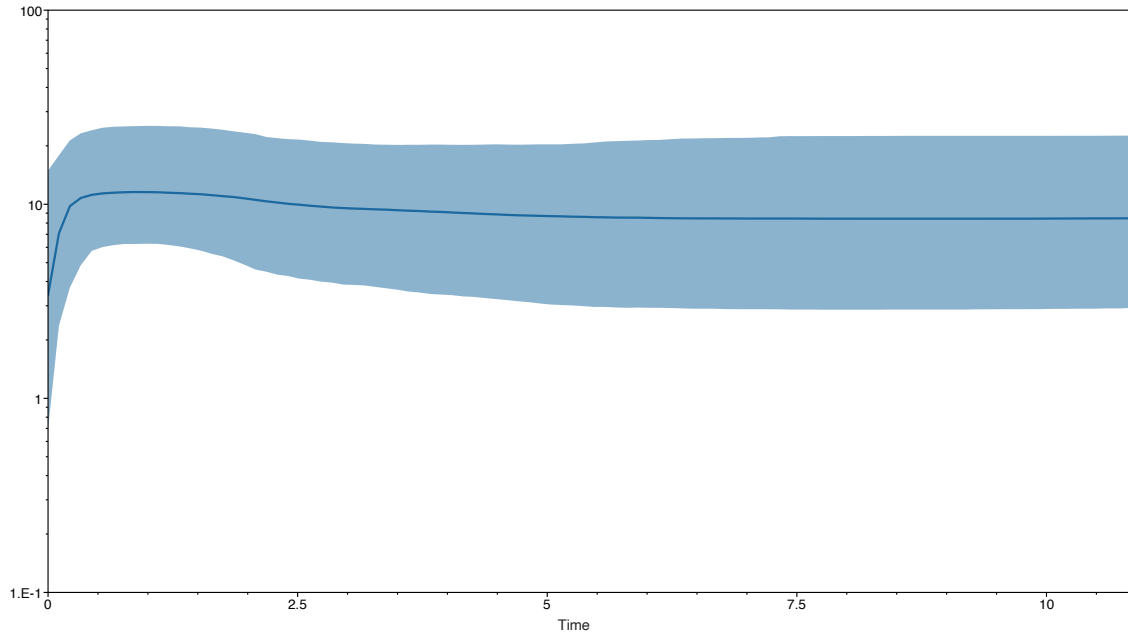


Figure 8. Bayesian Skyline Plot generated for *Centruroides exilicauda* using the partial COI alignment. Time is in millions of years.

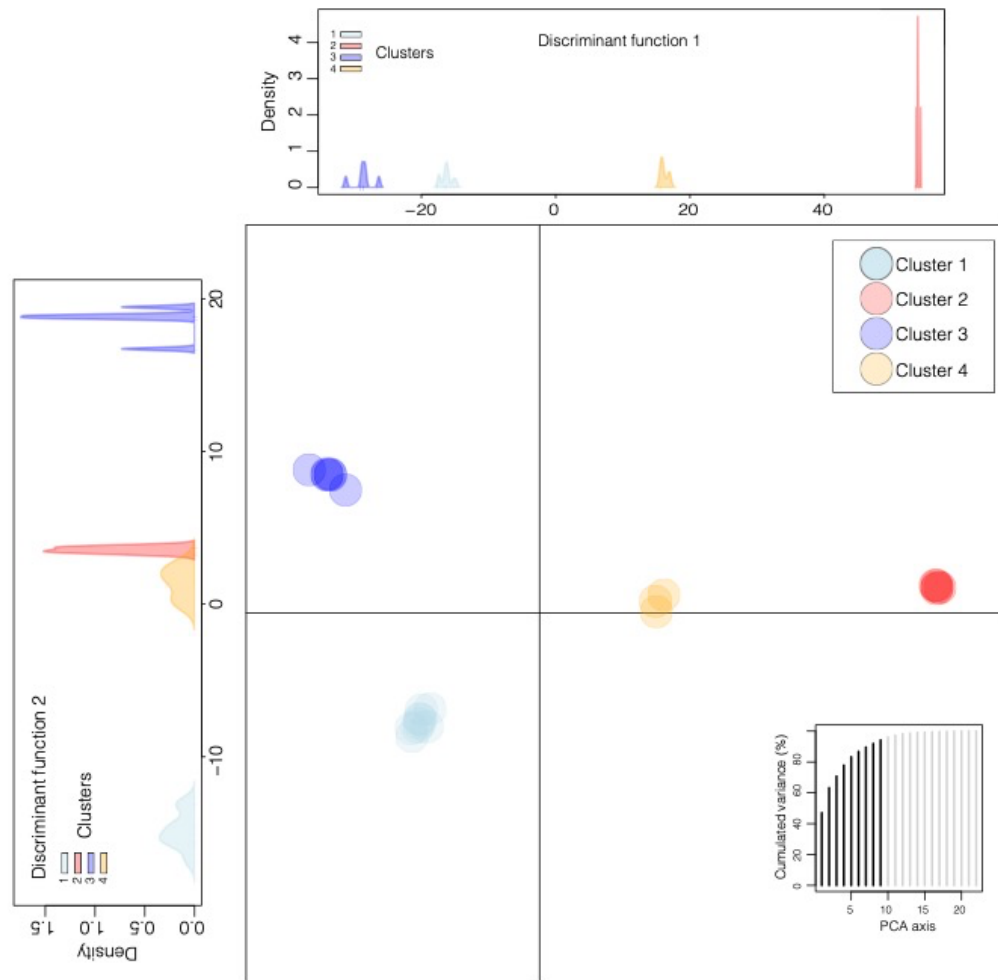


Figure 9. Discriminant Analysis Principal Components (DACP) for 23 COI sequences from *Centruroides exilicauda* showing the presence of four distinct genetic clusters.