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An Integrative Approach to Species Delimitation in the Massasauga Rattlesnake (Sistrurus Catenatus) with an Emphasis on the Western Massasauga, S.C. Tergeminus, and Desert Massasauga, S.C. Edwardsii in Texas

Steven R. Hein

AN INTEGRATIVE APPROACH TO SPECIES DELIMITATION IN THE MASSASAUGA RATTLESNAKE (*SISTRURUS CATENATUS*) WITH AN EMPHASIS ON THE WESTERN MASSASAUGA, *S. C. TERGEMINUS*, AND DESERT MASSASAUGA, *S. C. EDWARDSII* IN TEXAS

by

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Table of Contents

List of Tables

List of Figures

Abstract

AN INTEGRATIVE APPROACH TO SPECIES DELIMITATION IN THE MASSASAUGA RATTLESNAKE (*SISTRURUS CATENATUS*) WITH AN EMPHASIS ON THE WESTERN MASSASAUGA, *S. C. TERGEMINUS*, AND DESERT MASSASAUGA, *S. C. EDWARDSII* IN TEXAS

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The subspecies concept was originally introduced as a means to explain geographic variation in species with subspecific boundaries normally being designated by morphological variation. Because a growing wealth of studies have shown that these morphologically defined subspecies are often not reflective of true evolutionary history, it is important to reassess subspecific boundaries. Subspecific designations have conservational consequence with regards to management practices. I reassessed the subspecific designations of the massasauga rattlesnake, *S. catenatus*, using both ecological niche modeling and molecular phylogenetic techniques. The ecological niche modeling determined the western and desert massasauga, *S. c. tergeminus* and *S. c. edwardsii* occupy completely distinct niches. This is evidence that these two subspecies represent evolutionary divergent lineages. There is no obvious isolating geographical boundary, but other studies have shown that strong local adaptation to environmental gradients can cause ecological divergence in parapatric populations in ectotherms. My genetic data provide

differential results dependent upon type of DNA, mitochondrial vs nuclearThe mitchondial DNA sequences showed an eastern clade consisting entire of the eastern massasauga, *S. c. catenatus*, and western clade consisting of both the western and desert massasauga, *S. c. tergeminus* and *S. c. edwardsii*. Mitochondrial DNA also show strong evidence that the eastern massasauga should be elevated to its own species, which is consistent with previous studies (Kubatko et al. 2011; Ryberg et al. 2014). Within the western clade using mtDNA there is only slight differentiation between *S. c. tergeminu*s and *S. c. edwardsii*. The nuclear DNA showed only very little differentiation between all three subspecies. I feel this is an artifact of recent divergence within *S. catenatus* and that the mtDNA, which has much higher mutation rates, is a better matric for assessing the phylogenetic relationship within this species. This study provides evidence that *S. c. catenatus* should be elevated to the sole member of the species of *S. catenatus*. The other two subspecies, *S. c. tergeminus* and *S. c. edwardsii*, reflect divergent evolutionary lineages however should be separated into their own species, *S. tergeminus*, and renamed *S. t. tergeminus* and *S. t. edwardsii* respectively. Keeping the western and desert massasaugas as separate subspecies has conservational impacts as they need to be treated as biological separate management units.

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Chapter 1

Introduction and Background Information

What constitutes a distinct species? This question is the catalyst for one the most debated topics in taxonomy fueled by a fundamental disagreement in species concepts (De Queiroz 2007; Padial et al. 2010; Frankham et al. 2012). While it is generally agreed upon that a species represents a separately evolving metapopulation lineage, the debate lies at what point in the evolutionary history of the linage is the species delimiting boundary drawn (De Queiroz 2007; Padial et al. 2010; Frankham et al. 2012; Torstrom et al. 2014). Depending on the species concept used boundaries can be drawn based on haplotype variation, reproductive isolation, ecological divergence, or morphological distinctiveness (De Queiroz 2007; Leaché et al. 2009; Padial et al. 2010; Frankham et al. 2012). However, reliance on only one of these criteria is problematic as they do not arise in any set order or time. The order that each of the given criteria arise is set by the primary mode of speciation that is driving the evolutionary trajectory of a given lineage (De Queiroz 2007; Leaché et al. 2009). In recent years, species delimiting studies have begun to take into account multiple lines of evidence when determining whether conspecific lineages are separately evolving units. This integrative approach to taxonomy mitigates the need for any one specific species concept and incorporates multiple concepts when setting species boundaries (De Queiroz 2007; Padial et al. 2010; Torstrom et al. 2014), although it has yet to bring about a universally accepted species definition.

Compounded by this lack of agreement in defining what constitutes a species, the subspecies concept also remains a subject of debate. Systematists continue to argue over the subspecies definition, usefulness and even validity as a taxonomic designation (Wilson and Brown 1953; Haig et al. 2006; Sackett et al. 2014; Torstrom et al. 2014). The subspecies concept

was originally introduced to explain geographical variation amongst populations of the same species (Wilson and Brown 1953; Phillimore and Owens 2006; Torstrom et al. 2014). However, since its inclusion into taxonomy in the late 19th century the idea of a subspecies has been fraught with controversy, embraced by some systematists and resisted by others (Haig et al. 2006; Phillimore and Owens 2006; Torstrom et al. 2014). Wilson and Brown (1953) argue that a subspecies is not a real taxon, and therefore the formal trinomial naming system should be rejected. This school of thought remains among some systematists today, who argue subspecies continue to persist solely out of our need to classify and do not represent real taxonomic separation (Torstrom et al. 2014). Others argue that subspecies are a "true taxa" representing geographically separated, evolutionarily diverging populations (Phillimore and Owens 2006; Sackett et al. 2014; Torstrom et al. 2014).

Differences in biology of subspecies, such as intraspecific differences in physiology and reproductive viability, have real world consequences when making informative species management decisions (Phillimore and Owens 2006; Sackett et al. 2014). Government agencies, such as the United States Fish and Wildlife Service, use currently assigned subspecies designations when making fiscal, legal, and conservation decisions (Haig et al. 2006; Funk et al. 2007; Gibbs et al. 2011; Sackett et al. 2014). Therefore, it is important that subspecies be correctly assigned so those biological groups in need of protection receive the attention needed and effort is not misused on groups not in need (Gibbs et al. 2011). Traditionally subspecies were designated based on geographically distinctive morphological differences. However, as science entered the "genetic revolution," reevaluation of many morphologically designated subspecies have shown morphology is not always an accurate representation of evolutionary history (Burbrink et al. 2000; Phillimore and Owens 2006; Leaché et al. 2009; Makowsky et al. 2010;

Torstrom et al. 2014). As genetic information became easier to obtain in the late 20th century, incorporation of molecular techniques into species and subspecies delimitating studies became the *status quo*. Yet, even using such quantitative methods as genetic divergence debate still persists and where to draw the delimiting boundary continues to be an issue (Torstrom et al. 2014). It has also been argued that a reliance purely on genetic information may confound true phylogenetic relationships and give inaccurate evolutionary histories (Losos et al. 2012).

Lineage divergence driven by ecological speciation will cause a species or subspecies to develop ecological dissimilarity prior to pronounced genetic or morphological differentiation (Schluter 2009). Under ecological speciation theory, two lineages of a species will develop local adaptation to environmental conditions causing a divergence in ecological niches early in the speciation process (Van Valen 1976). These divergent niches drive geographic isolation, eventually leading to more stark morphological and genetic differentiation (Pyron and Burbrink 2009; Leaché et al. 2009; Khimoun et al. 2013; Soto-Centeno et al. 2013; Wooten and Gibbs 2012; Zhang et al. 2014). Therefore, niche differentiation can provide viable evolutionary evidence for lineage divergence within a species before the development of genetic or morphological discontinuities.

In the past decade, the taxonomic literature has surged with studies seeking to reassess traditionally defined species and subspecies. Among these studies, there has been a growing trend to incorporate an integrative taxonomic approach. In integrative delimitation the investigators take into account multiple line of evidence, including genetic, ecological, and morphological data, in making decisions (Raxworthy et al. 2007; Rissler and Apodaca 2007; Leaché et al. 2009; Makowsky et al. 2010; Soto-Centeno et al. 2013; Sackett et al. 2014; Zhang

et al. 2014). The integrative taxonomic approach was used in the current study to evaluate the current systematics of *Sistrurus catenatus*, the massasauga rattlesnake.

Sistrurus catenatus is one of two species of rattlesnake found within the genus *Sistrurus*, which is considered a basal group to the other genus of rattlesnake, *Crotalus* (Murphy et al. 2002; Kubatko et al. 2011). *Sistrurus catenatus* is a wide-ranging species distributed in a series of patchy populations from the Great Lakes region of the United States and Canada across the Great Plains as far south as South Texas and as far west as Eastern Arizona (Mackessy 2005; Kubatko et al. 2011; Wooten and Gibbs 2012; Figure 1). Across that range the species is divided into three morphologically-based subspecies (Gloyd 1955; Mackessy 2005; Kubatko et al. 2011; Wooten and Gibbs 2012; figure 1). *Sistrurus c. catenatus*, the eastern massasauga, inhabits the northeastern area of the range found throughout the Great Lakes region in Ontario, New York, Pennsylvania, Michigan, Ohio, Illinois, Indiana, and Wisconsin. *Sistrurus c. catenatus* is distinguished by a lower number of ventral scales and dorsal blotches, as well as, its overall darker coloration. *Sistrurus c. tergeminus*, the western massasauga, is marked by a larger number of ventral scale and dorsal blotches is found throughout the Central United States in Missouri, Kansas, Oklahoma, Nebraska, and North Texas. *Sistrurus c. edwardsii*, the desert massasauga, is lighter in color and the smallest subspecies in terms of overall size, as well as, having a fewer number of dorsal blotches, mid-body dorsal scales, and ventral scales. *Sistrurus c. edwardsii* is the most westerly subspecies and found in West and South Texas, Colorado, New Mexico, and Arizona. Throughout its entire range, *S. c. edwardsii* is in decline, a decline attributed to habitat fragmentation and other anthropogenic disturbances, which has raised concerns by scientists and conservations.

Protective statuses of *S. catenatus* vary by both subspecies and state. The Eastern subspecies, *S. c. catenatus*, is provided the greatest level of protection. Currently *S. c. catenatus* is listed as state endangered in every state in which it occurs (Durbian 2006; Ray et al. 2013). It is also a candidate for federal protection under the Endangered Species Act (US Federal Register 1999; Gibbs et al. 2011). However, the western subspecies, *S. c. tergeminus* and *S. c. edwardsii*, are not afforded the same level of protection. Only two states give protective statues to *S. c. tergeminus*; Nebraska lists this subspecies as threatened (Panella and Johnson 2014) and Missouri lists it as endangered (MO Dept. Conservation). In the other three states *S. c. tergeminus* occurs, Oklahoma, Kansas, and Texas, this species can be legally collected or killed with a hunting permit (Ryberg et al. 2014). There is no current push to provide *S. c. tergeminus* with any Federal protection. The desert massasauga, *S. c. edwardsii*, is also only provided protection in part of its range. Arizona lists *S. c. edwardsii* as protected and Colorado as a species of special concern while Texas and New Mexico do not give it any form of protection (Ryberg et al. 2014). A petition to the United States Fish and Wildlife Service has been filed to list *S. c. edwardsii* as a candidate for protection under the Endangered Species Act and this petition is currently under review (US Federal Register August 9, 2012). Because subspecific designations are based on morphological data, an often poor predictor of evolutionary history (Burbrink et al. 2000; Phillimore and Owens 2006; Makowsky et al. 2010), coupled with variable protection statutes it is important that the subspecies designations be revaluated in order to provide appropriate levels of protection to different populations of *S. catenatus*.

Sistrurus catenatus has been the subject of a variety of comparative studies investigating phylogenetic difference, as well as, basic ecological variation (Gloyd 1955; Holycross and Mackessy 2002; Kubatko et al. 2011; Wooten and Gibbs 2012; Ray et al. 2013). Kubatko et al.

(2011) used a large genetic data set consisting of 19 loci (a combination of nuclear and mitochondrial gene sequences) in an attempt to delineate between the three subspecies of *S. c. catenatus*. This study provided strong evidence for two divergent clades, one clade consisting of the eastern massasauga, *S. c. catenatus* and another clade consisting of a complex of the two western subspecies, *S. c. tergeminus* and *S. c. edwardsii*. There was enough differentiation between the eastern and western clades that Kubatko et al. (2011) suggested *S. c. catenatus* warranted elevation to its own species. Within the western complex, however, there was a smaller degree of genetic variation, a finding corroborated by Ryberg et al. (2014). Kubatko et al. (2011) and Ryberg et al. (2014) suggested further investigation was required before making any formal decisions regarding reclassification of the two western subspecies. In addition to determining the genetic phylogeny, Kubatko et al. (2011) also determined an estimated time of divergence between subspecies. Their study placed the split of the eastern clade from the western around 1 mya and the divergence between *S. c. tergeminus* and *S. c. edwardsii* around 0.5 mya (Kubatko et al. 2011). It is important to take into consideration this recent split between the western subspecies when investigating their taxonomy because at this earlier stage of divergence ecological speciation may be the primary driving mechanism(Schluter 2009; Wooten and Gibbs 2012). Therefore, ecological differences are likely to accumulate prior to large genetic difference and in fact ecological speciation has been shown to be an important mechanism of lineage divergence in this genus (Schluter 2009; Wooten and Gibbs 2012).

The goal of this study was to further investigate the evolutionary history and taxonomic status of the massasauga rattlesnake, *Sistrurus catenatus* sp. Specifically, I focused the majority of my efforts on the western subspecies complex, given the somewhat unresolved evolutionary history of that complex. Using an integrative approach I attempted to determine whether the

current subspecies designations of *S. c. tergeminus* and *S. c. edwardsii* should be maintained, combined into one subspecies, or elevated to two separate species.

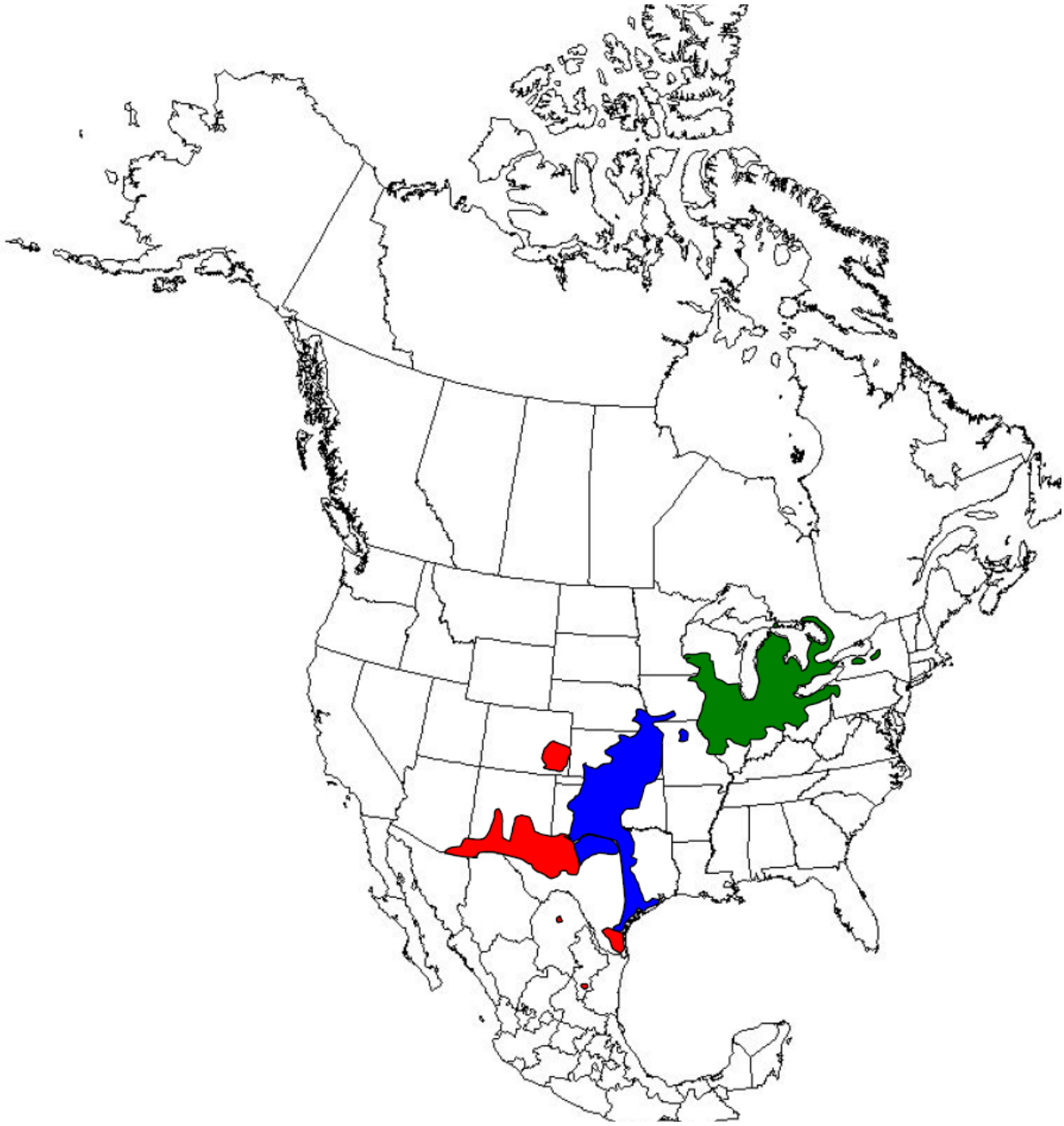

Figure 1: Approximate range of *Sistrurus catenatus* from Mackessy 2005. Green represents the eastern massasauga, *S. c. catenatus*, blue represents the western massasauga, *S. c. tergeminus*, and red represents the desert massasauga, *S. c. edwardsii*. Note: within respective ranges populations are not considered to be as widely distributed as displayed.

Chapter 2

Molecular phylogenetic of the massasauga rattlesnake, *Sistrurus catenatus*

Introduction

The "genetic revolution" has brought with it a wealth of studies seeking to incorporate molecular techniques into phylogenetic studies. Many of these studies have indicated that morphology is often a poor proxy for establishing the evolutionary heritage within a lineage at the lower taxonomic levels. This is problematic because most species and subspecies boundaries are were originally drawn based on morphological distinctions (Burbrink et al. 2000; Makowsky et al. 2010; Torstrom et al. 2014). Therefore it is important that the phylogeny of morphologically designated species and subspecies be reevaluated in order to make the decision whether current distinctions are merited or if a change to the species' taxonomy is warranted. Taxonomic reevaluations are particularly essential for those species of conservation concern because protection and management decisions are typically made based on the most currently recognized taxonomy (Haig et al. 2006).

The massasauga rattlesnake, *Sistrurus catenatus,* is a species currently divided into three subspecies based on geographic variation in morphological features (as described in Chapter 1). Specifically, color and pattern variation are characteristics used to distinguish between *S. catenatus* subspecies. However, these two characteristics can be highly variable and often may not be useful indicators of monophyletic lineages in snakes (Burbrink et al. 2000; Makowsky et al. 2010). Consequently, it is essential that the subspecific distinctions within *S. catenatus* be reevaluated using modern genetic techniques.

A number of studies have assessed the phylogeny of *S. catenatus*. However the majority focused on differentiating between the eastern and western massasaugas, *S. c. catenatus* and *S. c. tergeminus* (Gibbs and Mackessy 2009; Chiucchi and Gibbs 2010; Gibbs et al. 2011; Kubatko et al. 2011; Ray et al. 2013; Ryberg et al. 2014). Kubatko et al. (2011) incorporated samples from all three subspecies and using a combination of 19 mitochondrial and nuclear genes created a phylogeny of the species. They found strong evidence for two distinctive clades within *S. catenatus*, an eastern and a western clade. The eastern clade is comprised entirely of *S. c. catenatus* and the western clade is comprised of the western, *S. c. tergeminus*, and the desert massasauga, *S. c. edwardsii*. The eastern clade was genetically distinctive enough from the western for Kubatko et al. (2011) to suggest *S. c. catenatus* to be elevated to its own species. This is an important designation because full species are given higher priority than subspecies by the Endangered Species Act, and *S. c. catenatus* is a candidate under review (Ray et al. 2013). Within the western clade these authors found only weak evidence of genetic differentiation between *S. c. tergeminus* and *S. c. edwardsii* and suggested that further investigation is required before any taxonomic altering decisions are made. In a follow up study, Ryberg et al. (2014), conducted further investigation of the question by increasing the number of samples of *S. c. tergeminus* and *S. c. edwardsii* in their analysis. Using two mitochondrial genes Ryberg et al. (2014) agreed with the separation of *S. c. catenatus* as its own species, as well as, concluded *S. c. tergeminus* and *S. c. edwardsii* were genetically indistinguishable. The authors did, however, find some limited population level structuring and suggested that the western *S. catenatus* complex is comprised of a single species broken up into a number of large isolated populations.

In this study I sought to further investigate the phylogeny of the western *S. catenatus* complex by including another eight genes (~8000 base pairs) worth of information, as Ryberg et

al. (2014) based their conclusions on less than 1,500 base pairs (one mitochondrial gene and one nuclear gene), which may be insufficient in resolving the evolutionary history within the western clade (de Queiroz et al. 2002). The questions I looked to answer were 1) Does my data support separation of *S. c. catenatus* in to its own and species? 2) Does my data show any genetic distinctiveness between *S. c. tergeminus* and *S. c. edwardsii*?

Methods and Materials

Data collection

All three subspecies of *Sistrurus catenatus* (*S. c. catenatus, S. c. tergeminus*, and *S. c. edwardsii*) were included in my molecular analysis. Tissues samples were obtained from other researchers, museum collections, private parties, or collected during road surveys conducted by myself with the aid research assistants (Table 1). Tissue samples consisted of a combination of liver, muscle, scale clips or blood depending on the source. On the occasion that a sample was obtained without a subspecific designation the sample was tentatively assigned to a subspecies based on its collection locality (Dixon 2000; Tennant 2003; Werler and Dixon, 2008). Samples from a total of 69 individual *S. catenatus* were used in this study (i.e., 6 *S. c. catenatus*, 18 *S. c. edwardsii*, 45 *S. c. tergeminus*) from nine U.S. states and one Canadian province (Table 1). One individual *Agkistrodon contortrix* collected in Smith County, Texas was used as an outgroup in my phylogenetic analyses (Kubatko et al. 2011). Purified genomic and mitochondrial DNA was extracted from tissue samples using illustra™ tissue & cells genomicPrep Mini Spin Kit.

Polymerase chain reactions (PCR) were performed for eight genes in this study including 3 mitochondrial (mtDNA) and 5 nuclear (nDNA) genes. Genes ranges from 428 to 885 base pairs (bp) in length. The three mitochondrial loci included the large and small subunits of the

mitochondrial ribosome genes (*12S* and *16S*; 428 and 523bp) and cytochrome b (*cytb*; 687bp). The nuclear genes included brain-derived neutrophic factor (*bdnf*; 659bp), bone morphogenetic protein 2 (*bmp*2; 615bp), oocyte maturation factor (*c-mos*; 457bp), ornithine decarboxylase intron (*odc*; 585bp), and recombination-activating protein 1 (*rag1*; 885bp). All genes except *bmp2* were amplified in PCRs consisting of 4.0µl 5x Q-solution, 2.0µl 10X CoralLoad PCR buffer, 2.0µl 10X PCR buffer, 0.4µl dNTP's, 1.0µl forward primer, 1.0µl reverse primer, 0.1µl *Taq* DNA polymerase (Qiagen), 7.1µl sterile purified deionized H_2O, and 2.4µl DNA extract totaling 20µl PCR per sample. *bmp2* was amplified in a PCR consisting of 4.0µl 5x Q-solution, 2.0µl 10X CoralLoad PCR buffer, 2.0µl 10X PCR buffer, 0.4µl dNTP's, 0.4µl bovine serum albumin, 1.0µl forward primer, 1.0µl reverse primer, 0.1µl *Taq* DNA polymerase (Qiagen), 6.7 µl sterile purified deionized H_2O, and 2.4µl DNA extract also totaling 20µl PCR per sample. Forward and reverse primer sequences and reaction conditions are listed in tables 2 & 3. Polymerase chain reaction products were verified for amplification visually via gel electrophoresis on a 1% agarose gel including both positive and negative controls.

Verified PCR products were purified using E.Z.N.A. Cylce Pure kits (OMEGA biotek). Purified products were then concentrated to 20-40 ng x μl^{-1} and shipped to Eurofin MWG Operon to be sequenced using an automated DNA sequencer (ABI 3730XL). All eight loci were sequenced using the same forward and reverse primers used in amplification. Data sequences were initially edited using Sequencer (Version 5.2.4; Gene Codes Corporation, Ann Arbor, MI). Sequence alignments were performed using Clustal X (Thompson et al. 1997). Final sequence alignments and editing was performed in Mesquite 3.01 (Maddison and Maddison 2014).

Phylogenetic analysis

A best fit model of molecular evolution using Akaike's Information Criterion was determined for each individual locus, a concatenated matrix of all eight loci, a concatenated matrix of the five nuclear loci, and a concatenation of the three mitochondrial loci in jModelTest 2 (Darriba et al., 2012). Concatenated matrices were assembled using SequenceMatrix (Vaidya et al. 2011). Maximum likelihood (ML) gene trees were constructed based on suggested models (Table 4) using PhyML 3.1 (Guindon and Gascuel 2003). Node support was determined based on 100 non-parametric bootstrap replicate samples for each of the three concatenated trees also using PhyML 3.1. The generated trees were visualized and edited using Figtree.

Results

Gene sequences used in this study ranged from 428 to 883 base pairs (bp) per gene depending on specific locus, totaling 4837 bp (Table 5). However, I was not successful in sequencing all eight genes across all samples. The average bp sequenced for each sample was 3007 bp (range 457 – 4827bp) representing an average of 62.3% (9.4 – 100%) total bp data per sample. Total and specific genes sequenced for each gene are displayed in table 6.

Mitochondrial genes showed a greater degree of intersubspecific variation than nDNA genes (Table 7). For all three mtDNA loci, divergence estimates were greater for eastern X western massasauga ,*S. c. catenatus* X *S. c. tergeminus*, (range 2.49-11.1%; mean 5.85%; table 7) and eastern X desert massasauga, *S. c. catenatus* X *S. c. edwardsii* (range 3.06 – 9.61%; mean 5.55%; table 7) comparisons than for western X desert massasauga, S. c. tergeminus X *S. c. edwardsii* (range 1.16 – 2.62; mean 1.71; table 7). Divergence estimates for nDNA were overall much lower than for my mtDNA sequence data. Divergence estimates were lowest for the *S. c.*

tergeminus X *S. c. edwardsii* pairwise comparison in three of the five nDNA genes sampled (table 7). Interspecific nDNA diveregence estimates ranged from 0.45 – 1.71% with a mean value of 0.99% for *S. c. catenatus* X *S. c. edwardsii*, ranged from 0.45 – 1.75% with a mean 1.07% for *S. c. catenatus* X *S. c. tergeminus*, and ranged from 0.11 – 1.53% with a mean of 0.74% for *S. c. tergeminus* X *S. c. edwardsii* (Table 7). Intraspecific variation was variable depending on the gene and did not show any universal trends, however tended be higher in *S. c. tergeminus* (table 8). Ranges varied from 0 – 1.4% for *S. c. catenatus*, 0.34 – 1.02% for *S. c. edwardsii*, and 0.11 – 2.62% for *S. c. tergeminus* (table 8).

The vast majority of the differences observed between samples for nDNA loci consisted of ambiguous polymorphic sites within the gene. At every site of intraspecific variation for *S. c. tergeminus* and *S. c. edwardsii* at least one individual displayed an ambiguous designator.

Tree topology for all three individual and concatenated mtDNA ML trees recover an eastern clade consisting of *S. c. catenatus* and a western clade consisting of *S. c. tergeminus* and *S. c. edwardsii* (figures 2 - 5). This separation is supported by strong bootstrap values (96/96%; Figure 5). Within the western clade there is little separation between *S. c. tergeminus* and *S. c. edwardsii*. There is some evidence of population level separation within a few groups; however, bootstrap values for most of these population level groups are on average only low to moderate (Figure 5).

Nuclear DNA ML trees display a varying level of support for a separation of the eastern and western clades of *S. catenatus* (Figures 6 - 11). The concatenated nDNA ML tree separates the same eastern and the western clades as is in the mtDNA trees, however boot strap value do

not support this differentiation. Within the western clade a number of clades separate out again with no clear differentiation between *S. c. tergeminus* and *S. c. edwardsii*.

The total eight gene concatenated ML tree divides the samples into two major clades: the eastern, consisting of solely *S. c. catenatus*, and western, consisting of both *S. c. tergeminus* and *S. c. edwardsii* although with low boot strapping value support (Figure 12). Within the western clade there is a further divide into two large clades. However, within those two clades there is very little to differentiation between *S. c. tergeminus* and *S. c. edwardsii* (Figure 12).

Discussion

All three of the mitochondrial genes analyzed in this study corroborate the findings of both Kubatko et al. (2011) and Ryberg et al. (2014), in regards to the elevation of the eastern massasauga, *S. c. catenatus*, to its own species from the western, *S. c. tergeminus*, and the desert, *S. c. edwardsii*, massasauga. Topology of ML trees for all three mitochondrial genes (*12S*, *16S* and *cytb*; Figure 2 - 4) recovered *S. c. catenatus* as a separate clade from the western clade, consisting of *S. c. tergeminus* and *S. c. edwardsii*. The concatenated mtDNA tree had similar topology to the individual trees also recovering *S. c. catenatus* as a highly supported (96% ML bootstrap support; Figure 5) distinct clade from the western complex. While it might be a cause for concern that the concatenated tree does not include sequences from every individual in the study, accurate phylogenies can still be constructed via maximum likelihood techniques despite a large amount of missing data within the matrix (Pyron et al. 2011). The separation of *S. c. catenatus* from *S. c. tergeminus* and *S. c. edwardsii* is also supported by the mtDNA intersubspecific genetic distances. While there is no standardized level of genetic distance for elevation of a subspecies to species, the recommendation has been made at as low as 1.0%

divergence (Torstrom et al. 2014). In reevaluation of the ratsnake species, *Pantherophis (Elaphe) obsoletus,* using the mitochondrial gene *cytb,* Burbrink et al. (2000) recommended dividing one species with three subspecies into three distinct species based on 2.87-4.37%. In this study, all three of the mitochondrial genes fall either within or above the 2.87-4.37% range when comparing *S. c. catenatus* with either of the two western subspecies.

The mitochondrial gene results were also similar to those in Kubatko et al. (2011) in that I found only slight differentiation within the western clade consisting of *S. c. tergeminus* and *S. c. edwardsii*. I did however; find evidence of local population differentiation in a few instances. Specifically, the isolated South Texas population of *S. c. edwardsii* (SICA 60, 61, 66) clustered together in both *12S* and *cytb* (Figures 2 & 4) individual gene trees and was moderately supported (83% ML bootstrap value) in the concatenated tree (Figure 5). In the individual *16S* tree, a number of *S. c. edwardsii* grouped together from West Texas, New Mexico and Arizona; however, not all the West Texas and New Mexico samples were clustered within this grouping (Figure 3). This grouping was recovered in the concatenated gene tree, albeit with only very little bootstrap support (47%; Figure 5). An interesting finding from the *16S* tree is western clade is polyphyletic with the two Missouri *S. c. tergeminus* samples (SICA 41 & 43) separate from the rest of the *S. c. tergeminus* and *S. c. edwardsii* samples. This is particularly intriguing because there has been some debate whether the Missouri populations are *S. c. catenatus, S. c. tergeminus*, or possibly representative of an area of integradation (Gibbs et al. 2011). There were also separate populations of *S. c. tergeminus* distinctive from each other in the *16S* gene, one population from West Oklahoma and East Kansas and another population from North Texas. However, these same populations were not recovered in the concatenated mtDNA tree.

In addition to only slight differentiation displayed by tree topology, I found only minor differentiation in genetic divergences between *S. c. tergeminus* and *S. c. edwardsii*. In a review of species delimitation studies, Torstrom et al. (2014) determined the median genetic distance used to collapse a subspecies was 1.0%. The genetic distance between *S. c. tergeminus* and *S. c. edwardsii* for at three mtDNA gene fall above this 1.0% threshold (1.16 – 2.62%), so I do not recommend collapsing the subspecies into one based on these data. However, I also do not believe there is enough genetic differentiation to warrant elevating the subspecies to their own species.

Analysis of the five nuclear DNA genes (*cmos*, *odc*, *bdnf*, *bmp2*, and *rag1*: Figure 6 - 10) included in this study the results was not so clear. While tree topology for four of the five nuclear genes displayed at least some differentiation of *S. c. catenatus* from *S. c. tergeminus* and *S. c. edwardsii*, only one gene (*odc*; Figure 9) grouped *S. c. catenatus* as a separate monophyletic clade. For the other three nuclear genes, there is only minimal divergence of *S. c. catenatus*. The tree for gene *bdnf* (Figure 6) displays no genetic difference between *S. c. catenatus* and *S. c. tergeminus* or *S. c. edwardsii*. The concatenated tree for all five nuclear genes does recover *S. c. catenatus* as a separate polyphyletic clade from the western subspecies complex; however, there is no bootstrap support for this division (Figure 11). Included in the separate *S. c. catenatus* clade is one *S. c. tergeminus* individual from a population in North Texas. Intersubspecific divergence estimates also show only minimal differentiation (0.45 – 1.75%) between *S. c. catenatus* from either of the two western subspecies. According to nuclear data, there is not enough evidence to support elevating *S. c. catenatus* to its own species. In the western subspecies complex there is no evidence to warrant elevating *S. c. tergeminus* or *S. c. edwardsii* either. There is very little to no differentiation between *S. c. tergeminus* and *S. c. edwardsii* according the tree topology for all

five individual and concatenated trees. There is also very little genetic divergence between these two species with percentage estimates ranging from 0.11 to 1.56%. The concatenated ML tree of all eight mitochondrial and nuclear genes recovers *S. c. catenatus* as distinct monoplyletic clade and *S. c. tergeminus* and *S. c. edwardsii* as a separate complex; however, this distinction is not strongly supported by bootstrap values (Figure 12).

The discrepancy between the nuclear and mitochondrial data may be attributed to differences in mutation rates between the two types of DNA. Mitochondrial DNA mutational rates *Drosophila* models tend to be on average ten times higher than mutational rate in nuclear DNA (Haag-Liautard et al. 2008). The divergence between *S. c. tergeminus* and *S. c. edwardsii* in evolutionary time is a relatively recent occurrence (~0.5mya) (Kubatko et al. 2011). It is likely that genetic differences between subspecies have not had to time accumulate between *S. c. tergeminus* and *S. c. edwardsii*. Further evidence for this is the large number of polymorphisms present at variable sites between *S. c. tergeminus* and *S. c. edwardsii*. This retention of ancient polymorphisms occurs when a recently diverged lineage has not had time to achieve reciprocal monophyly. This is known as incomplete lineage sorting and is a common source of error in phylogenetic analysis, particularly when using nuclear data over mitochondrial because of the slower mutation rate (Kubatko et al. 2011). Due to the recent divergence within the western clade and because of the propensity of nuclear data to display incomplete lineage sorting, I believe that mitochondrial DNA is a much better metric for establishing an accurate phylogeny of *S. catenatus*.

Overall the results from the genetic analysis of *S. c. tergeminus* and *S. c. edwardsii* in this study leave room for further investigation. There were some observable differences between *S. c. tergeminus* and *S. c. edwardsii*, particularly within the mtDNA intersubspecific divergence

estimates, although the mtDNA ML gene trees did not fully support the separation of these two subspecies. The nDNA used in this study showed essentially no distinction between *S. c. tergeminus* and *S. c. edwardsii*. In conclusion I believe that my genetic analysis between *S. c. tergminus* and *S. c. edwardsii* is inconclusive. In the future I recommend any follow up studies incorporate more sensitive genetic marker such as microsatellites.

Appendix A

Molecular Phylogenetics Tables

Table 1. Tissue sample localities for *Sistrurus catenatus ssp.* and sources

Subspecies	ID	State	County	Source	Source ID
S. c. catenatus	SICA44	MI	Barry	J. Moore	27305637
S. c. catenatus	SICA45	MI	Barry	J. Moore	75539849
S. c. catenatus	SICA46	MI	Barry	J. Moore	27262123
S. c. catenatus	SICA57	ON	Dorcas bay	L. Gibbs lab	Sca 64
S. c. catenatus	SICA58	NY	Bergen	L. Gibbs lab	Sca 954
S. c. catenatus	SICA59	OH	Killdeer Plain	L. Gibbs lab	Sca 1006
S. c. edwardsii	SICA50	AZ	Cochise	L. Gibbs lab; A. Holycross	Sced036
S. c. edwardsii	SICA51	AZ	Cochise	L. Gibbs lab; A. Holycross	Sced041
S. c. edwardsii	SICA52	AZ	Cochise	L. Gibbs lab; A. Holycross	Sced051
S. c. edwardsii	SICA53	AZ	Cochise	L. Gibbs lab; A. Holycross	Sced053
S. c. edwardsii	SICA54	AZ	Cochise	L. Gibbs lab; A. Holycross	Sced057
S. c. edwardsii	SICA55	NM	Belen	L. Gibbs lab	Sced096
S. c. edwardsii	SICA56	NM	Belen	L. Gibbs lab	Sced029
S. c. edwardsii	SICA60	TX	Jim Hogg	R. Couvillian	
S. c. edwardsii	SICA61	TX	Jim Hogg	R. Couvillian	
S. c. edwardsii	SICA62	TX	Ward	S.Hein/S.Pitts	
S. c. edwardsii	SICA64	NM	Roosevelt	S. Pitts	
S. c. edwardsii	SICA66	TX	Nueces	NNTRC	838 S.c.e.
S. c. edwardsii	SICA67	NM	Otero	NNTRC	Alb.zoo Sce
S. c. edwardsii	SICA68	NM	Eddy	BRTC	H5143
S. c. edwardsii	SICA69	TX	Andrews	BRTC	CSA169
S. c. edwardsii	SICA71	TX	Borden	BRTC	TJH3503
S. c. edwardsii	SICA73	TX	Howard	BRTC	TJH2489
S. c. edwardsii	SICA75	TX	Shackelford	BRTC	WAR8
S. c. tergeminus	SICA1	TX	Parker	T. Becker	
S. c. tergeminus	SICA2	TX	Cottle	S.Hein/M.Barazowski	
S. c. tergeminus	SICA3	TX	Cottle	S.Hein/M.Barazowski	
S. c. tergeminus	SICA4	TX	Cottle	S.Hein/M.Barazowski	
S. c. tergeminus	SICA5	TX	Cottle	S.Hein/M.Barazowski	

S. c. tergeminus	SICA6	TX	Cottle	S.Hein/M.Barazowski	
S. c. tergeminus	SICA7	TX	Cottle	S.Hein/M.Barazowski	
S. c. tergeminus	SICA8	TX	Cottle	S.Hein/M.Barazowski	
S. c. tergeminus	SICA9	TX	Parker	T. Becker	
S. c. tergeminus	SICA10	TX	Parker	T. Becker	
S. c. tergeminus	SICA11	TX	Runnels	TNHC	TNHC55941
S. c. tergeminus	SICA12	TX	Motley	TNHC	TNHC66467
S. c. tergeminus	SICA13	TX	Dickens	TNHC	TNHC67573
S. c. tergeminus	SICA14	TX	Borden	TNHC	TNHC89754
S. c. tergeminus	SICA15	KS	Chase	KU	332081 / WPI 214
S. c. tergeminus	SICA16	KS	Chase	KU	332080 / WPI 213
S. c. tergeminus	SICA17	KS	Barber	KU	337105 / DSM 2020
S. c. tergeminus	SICA18	KS	Chase	KU	332078 / WPI 211
S. c. tergeminus	SICA19	KS	Chase	KU	332079 / WPI 212
S. c. tergeminus	SICA20	OK	Blaine	SNOMNH	2612
S. c. tergeminus	SICA21	KS	Butler	SNOMNH	2613
S. c. tergeminus	SICA22	OK	Roger Mills	SNOMNH	2615
S. c. tergeminus	SICA23	OK	Ellis	SNOMNH	2621
S. c. tergeminus	SICA24	OK	Dewey	SNOMNH	2682
S. c. tergeminus	SICA25	KS	Elk	SNOMNH	2683
S. c. tergeminus	SICA26	Ok	Beckham	SNOMNH	7045
S. c. tergeminus	SICA27	KS	Chautauque	SMNH	FHSM 10809
S. c. tergeminus	SICA28	KS	Comanche	SMNH	FHSM 10827
S. c. tergeminus	SICA29	KS	Allen	SMNH	FHSM 11020
S. c. tergeminus	SICA30	KS	Barber	SMNH	FHSM 11151
S. c. tergeminus	SICA31	KS	Reno	SMNH	FHSM 11546
S. c. tergeminus	SICA32	KS	Russell	SMNH	FHSM 11884
S. c. tergeminus	SICA33	KS	Kiowa	SMNH	FHSM 8631
S. c. tergeminus	SICA34	KS	Cowley	SMNH	FHSM 13031
S. c. tergeminus	SICA35	OK	Rogers	SMNH	FHSM 15714
S. c. tergeminus	SICA36	KS	Douglas	SMNH	FHSM 7900
S. c. tergeminus	SICA37	KS	Stafford	SMNH	FHSM 8424
S. c. tergeminus	SICA38	KS	Washington	SMNH	FHSM 8909
S. c. tergeminus	SICA39	KS	Meade	SMNH	FHSM 9539
S. c. tergeminus	SICA40	KS	Clark	SMNH	FHSM 9551
S. c. tergeminus	SICA41	MO	Chariton	SMNH	FHSM 9969
S. c. tergeminus	SICA42	MO	Linn	SMNH	FHSM 9970
S. c. tergeminus	SICA43	MO	Linn	SMNH	FHSM 9971
S. c. tergeminus	SICA47	TX	Cottle	S.Hein/M.Barazowski	
S. c. tergeminus	SICA48	TX	Cottle	S.Hein/M.Barazowski	
S. c. tergeminus	SICA49	TX	Cottle	S.Hein/M.Barazowski	
S. c. tergeminus	SICA63	TX	Parker	M. Smith	
S. c. tergeminus	SICA65	OK	Comanche	NNTRC	886 S.c.t.

S. c. tergeminus	SICA70	TX	Archer	BRTC	TJH3548
S. c. tergeminus	SICA72	TX	Clay	BRTC	TJH3506
S. c. tergeminus	SICA74	TX	Motley	BRTC	TJH3511
S. c. tergeminus	SICA76	TX	Hood	BRTC	CSA TX:Hood

(NNTRC- Nation Natural Toxin Research Center; BRTC- Texas A&M University's Biodiversity Research and Teaching Collection; TNHC- The University of Texas at Austin's Texas Natural History Collection; SNOMNH- Sam Noble Oklahoma Museum of Natural History; SMNH- Sternberg Museum of Natural History)

Table 2. List of primers used in polymerase chain reactions

Gene	Abbreviation	Primers (5´ - 3´)	Source
12S ribosomal RNA	*12S*	H1478 - TGACTGCAGAGGGTGACGGGCGGTGTGT L1091 - AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	Rawlings et al. 2008
16S ribosomal RNA	*16S*	16Sbr - CCGGTCTGAACTCAGATCACGT 16Sar - CGCCTGTTTATCAAAAACAT	Rawlings et al. 2008
Cytochrome b	*cytb*	cytbL - TCAAACATCTCAACCTGATGAAA cytbH - GGCAAATAGGAAGTATCATTCTG	Pook et al. 2000
Brain-derived neurotrophic factor	*bdnf*	bdnf_F - ACCATCCTTTTCCTKACTATGGTTATTTCATACTT bdnf_R - CTATCTTCCCCTTTTAATGGTCAGTGTACAAAC	Wiens et al. 2008
Bone morphogenetic protein 2	*bmp2*	bmp2_f6 - CAKCACCGWATTAATATTTATGAAA bmp2_r2 - ACYTTTTCGTTYTCRTCAAGGTA	Wiens et al. 2008
Oocyte maturation factor	*c-mos*	CMOS_Fsnk - GCTGTAAAACAGGTGAAGAGATGCAG CMOS-Rsnk - AGCACGATGGGTGTATGTTCCCCC	Noonan and Chippindale 2006
Ornithine decarboxylase intron	*odc*	ODC_F - GACTCCAAAGCAGTTTGTCGTCTCAGTGT ODC_R - TCTTCAGAGCCAGGGAAGCCACCACCAAT	Friesen et al. 1999
Recombination-activing protein 1	*rag1*	MartFL1 - AGCTGCAGYCARTAYCAYAARATGTA AmpR1 - AACTCAGCTGCATTKCCAATRTCA	Barlow et al. 2009

Table 3. Polymerase chain reaction condition used for each gene. After final extension all reaction were held indefinitely at 4°C

Gene	Number of cycles	Initial denature	Denature	Anneal	Extension	Final Extension
12S	35	95°C for 3min	95°C for 30sec	43°C for 45sec	72°C for 1.5min	72°C for 5min
16S	35	95°C for 3min	95°C for 30sec	43°C for 45sec	72°C for 1.5min	72°C for 5min
cytb	35	94°C for 4min	94°C for 1min	50°C for 1min	72°C for 2min	72°C for 3min
bdnf	30	95°C for 2min	95°C for 30sec	50°C for 15sec	72°C for 30sec	72°C for 10min
bmp2	40	94°C for 3min	94°C for 30sec	50°C for 40sec	72°C for 1min	72°C for 10min
c-mos	35	94°C for 3min	94°C for 45sec	55°C for 45sec	72°C for 1min	72°C for 6min
odc	35	95°C for 2min	95°C for 45sec	54°C for 30sec	72°C for 50sec	72°C for 10min
rag1	35	95°C for 3min	95°C for 30sec	55°C for 45sec	72°C for 1min	72°C for 5min

Table 4. Best-fit models of evolution as determined by JmodelTest 2

Gene	**Selected Model**
12S	HKY+I
16S	HKY+I
bdnf	HKY
Bmp2	K80+I
cmos	HKY
cytb	HKY+G
odc	HKY+I
rag-1	F81
Concatenated 8 genes	GTR+I+G
Concatenated Mitochondrial	GTR+I+G
Concatenated Nuclear	HKY+I+G

Table 5. Total base pairs sequenced per gene

Gene	Total base pairs
12S	428
16S	523
bdnf	659
bmp2	615
c-mos	457
cytb	687
odc	585
rag1	885

Table 6. Number of genes sequenced per samples and total number of base pairs sample. Sample "AGCO1" represents outgroup *Agkistrodon contortrix*

ID	Total length	Total genes	*12S*	*16S*	*bdnf*	*bmp2*	*cmos*	*cytb*	*odc*	*rag1*
AGCO1	4837 bp	8	X	x	x	x	x	x	x	x
SCA75	457 bp	1					x			
SICA1	3637 bp	6	x	x	x		x		x	
SICA2	4837 bp	8	x	x	x	x	x	x	x	x
SICA3	4409 bp	7		x	x	x	x	x	x	x
SICA4	2382 bp	4		x	x	x			x	
SICA5	2067 bp	4	x	x	x		x			
SICA6	2067 bp	4	x	x	x		x			
SICA7	3267 bp	6	x	x	x	x	x		x	
SICA8	3267 bp	6	x	x	x	x	x		x	
SICA9	1536 bp	3	x	x					x	
SICA10	1536 bp	3	x	x					x	
SICA11	4252 bp	7	x	x	x	x	x	x		x
SICA12	4837 bp	8	x	x	x	x	x	x	x	x
SICA13	4252 bp	7	x	x	x	x	x	x		x
SICA14	3954 bp	7	x	x	x	x	x	x	x	
SICA15	2682 bp	5	x	x	x	x	x			
SICA16	4837 bp	8	x	x	x	x	x	x	x	x
SICA17	4837 bp	8	x	x	x	x	x	x	x	x
SICA18	4252 bp	7	x	x	x	x	x	x		x
SICA19	2023 bp	4	x	x		x	x			
SICA20	523 bp	1		x						
SICA21	4837 bp	8	x	x	x	x	x	x	x	x
SICA22	4837 bp	8	x	x	x	x	x	x	x	x
SICA23	4837 bp	8	x	x	x	x	x	x	x	x
SICA24	4837 bp	8	x	x	x	x	x	x	x	x
SICA25	4837 bp	8	x	x	x	x	x	x	x	x
SICA26	4837 bp	8	x	x	x	x	x	x	x	x
SICA27	3369 bp	6	x	x	x	x	x	x		
SICA28	1087 bp	2	x		x					
SICA29	457 bp	1					x			
SICA30	457 bp	1					x			
SICA31	4252 bp	7	x	x	x	x	x	x		x
SICA32	457 bp	1					x			
SICA33	2067 bp	4	x	x	x		x			
SICA35	3295 bp	6	x	x		x	x	x	x	
SICA39	2225 bp	4	x	x	x	x				
SICA40	523 bp	1		x						
SICA41	951 bp	2	x	x						

SICA43	523 bp	1	x	x						
SICA44	4837 bp	8	x	x	x	x	x	x	x	x
SICA45	4837 bp	8	x	x	x	x	x	x	x	x
SICA46	4837 bp	8	x	x	x	x	x	x	x	x
SICA47	1340 bp	2					x			x
SICA48	1340 bp	2					x			x
SICA50	4252 bp	7	x	x	x	x	x	x		x
SICA51	951 bp	2	x	x						
SICA52	2682 bp	5	x	x	x	x	x			
SICA53	2254 bp	4		x	x	x	x			
SICA54	4252 bp	7	x	x	x	x	x	x		x
SICA55	2682 bp	5	x	x	x	x	x			
SICA56	2023 bp	4	x	x		x	x			
SICA57	4837 bp	8	x	x	x	x	x	x	x	x
SICA58	2682 bp	5	x	x	x	x	x			
SICA59	523 bp	1		x						
SICA60	1638 bp	3	x	x				x		
SICA61	4222 bp	7	x	x	x		x	x	x	x
SICA62	4222 bp	7	x	x	x		x	x	x	x
SICA63	1610 bp	3	x	x	x					
SICA64	1638 bp	3	x	x				x		
SICA65	4252 bp	7	x	x	x	x	x	x		x
SICA66	4837 bp	8	x	x	x	x	x	x	x	x
SICA67	4837 bp	8	x	x	x	x	x	x	x	x
SICA68	3637 bp	6	x	x	x		x	x		x
SICA69	3637 bp	6	x	x	x		x	x		x
SICA70	3637 bp	6	x	x	x		x	x		x
SICA72	1638 bp	3	x	x				x		
SICA73	1638 bp	3	x	x				x		
SICA75	3180 bp	5	x	x	x			x		x
SICA76	2095 bp	4	x	x			x	x		

Table 7. Intersubspecific divergence rates (%) for both mtDNA loci (16S, *12S*, *cytb*) and nDNA loci (*odc, bdnf, bmp2, cmos, rag1*)

Subspecies X Subspecies	*12S*	*16S*	*cytb*	*odc*	*bdnf*	*bmp2*	*cmos*	*rag1*
S. c. catenatus* X *S. c. edwardsii	3.97	3.06	9.61	1.71	0.46	0.81	1.53	0.45
S. c. catenatus* X *S. c. tergeminus	3.97	2.49	11.1	1.54	0.46	1.14	1.75	0.45
S. c. tergeminus* X *S. c. edwardsii	1.16	1.34	2.62	0.8	0.46	0.81	1.53	0.11

Table 8. Intrasubspecific divergence rates (%) for both mtDNA loci (*16S*, *12S*, *cytb*) and nDNA loci (*odc*, *bdnf*, *bmp2*, *cmos*, *rag1*)

Subspecies	*12S*	*16S*	*cytb*	*odc*	*bdnf*	*bmp2*	*cmos*	*rag1*
S. c. edwardsii	0.7	0.57	1.02	0.34	0.45	0.49	0.87	0.11
S. c. catenatus	1.4	0	0	0	0	0.33	0.66	0.34
S. c. tergeminus	0.93	0.76	2.62	1.2	0.45	0.81	1.1	0.11

Appendix B

Molecular Phylogenetics Figures

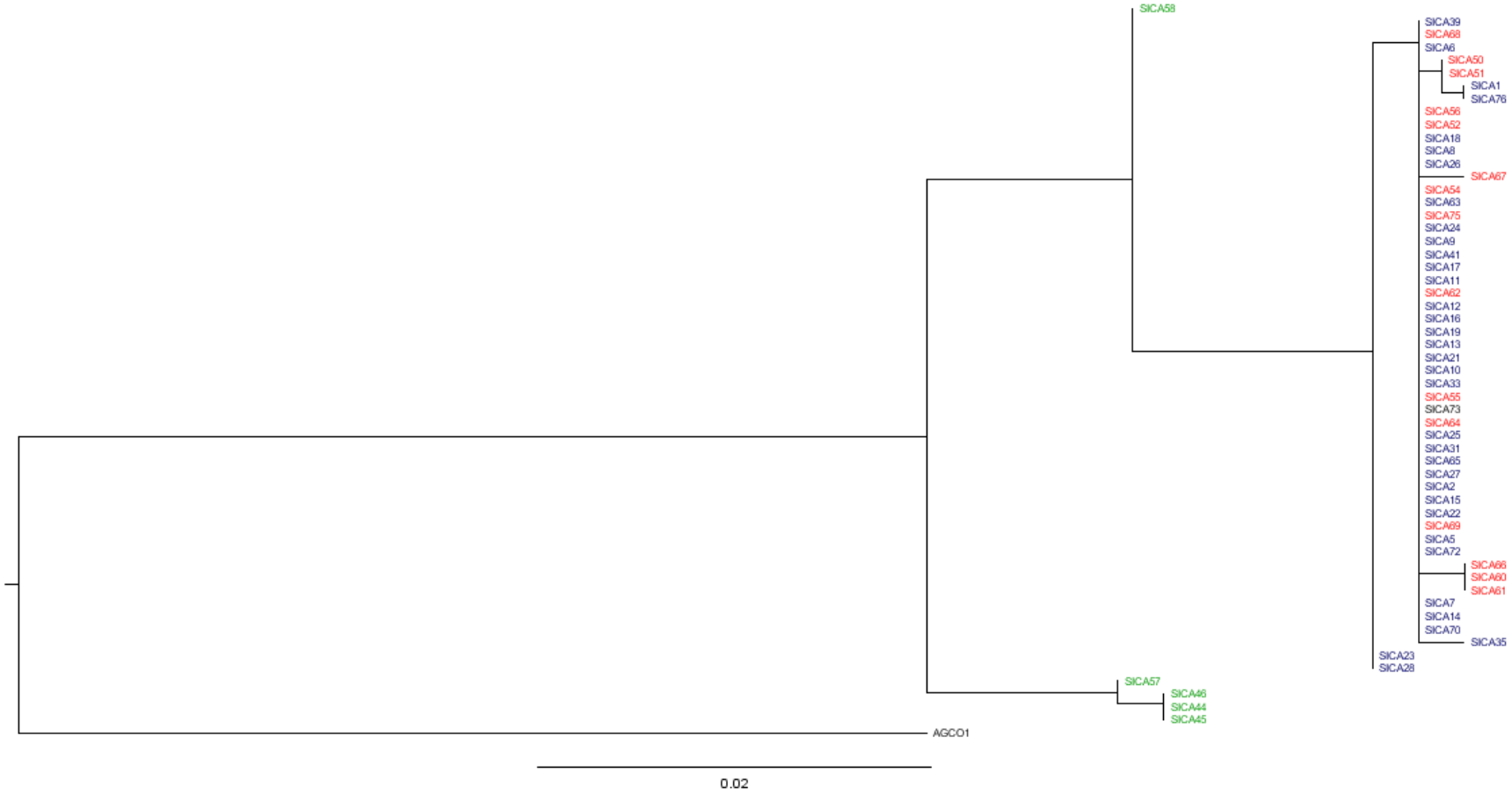

Figure 2. ML gene tree for *12S.* Green samples represent the eastern massasauga, *Sistrurus catenatus catenatus*, red samples represent the desert massasauga, *S. c. edwardsii*, blue samples represent the western massasauga, *S. c. tergeminus*. Sample "AGCO1" represents outgroup copperhead, *Agkistrodon contortrix*

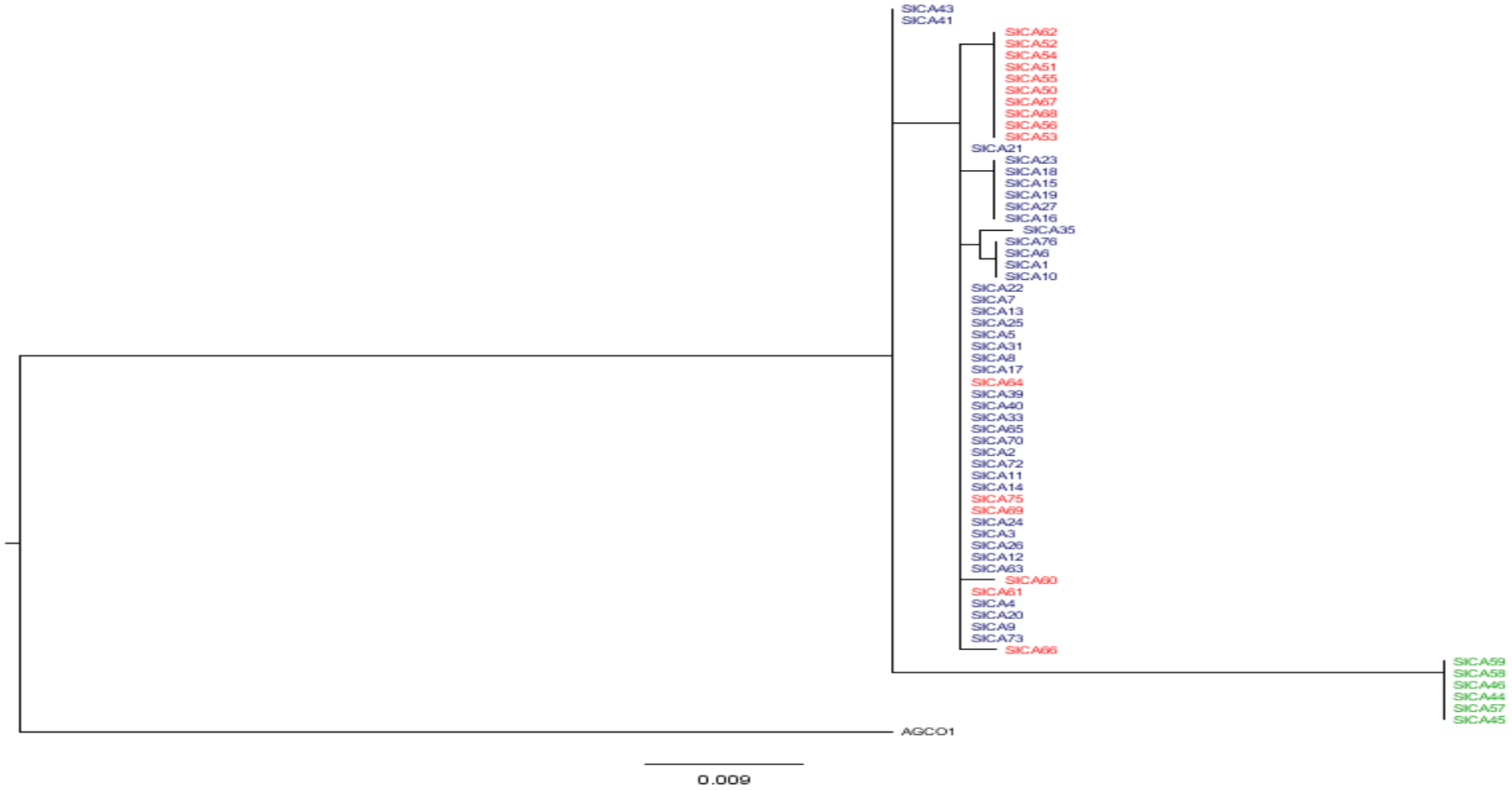

Figure 3. ML gene tree for *16S*. Green samples represent the eastern massasauga, *Sistrurus catenatus catenatus*, red samples represent the desert massasauga, *S. c. edwardsii*, blue samples represent the western massasauga, *S. c. tergeminus*. Sample "AGCO1" represents outgroup copperhead, *Agkistrodon contortrix*

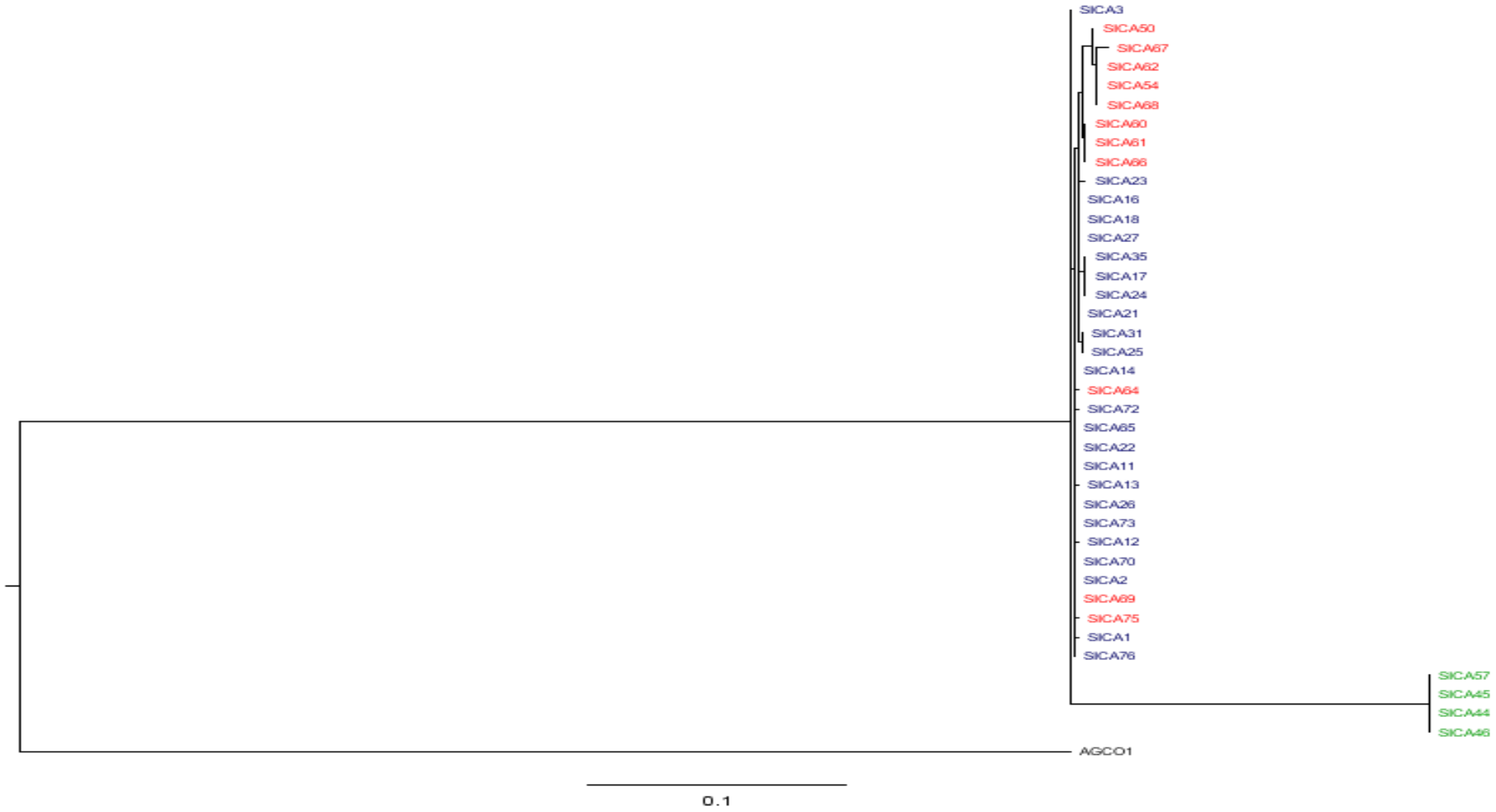

Figure 4. ML gene tree for *cytb*. Green samples represent the eastern massasauga, *Sistrurus catenatus catenatus*, red samples represent the desert massasauga, *S. c. edwardsii*, blue samples represent the western massasauga, *S. c. tergeminus*. Sample "AGCO1" represents outgroup copperhead, *Agkistrodon contortrix*

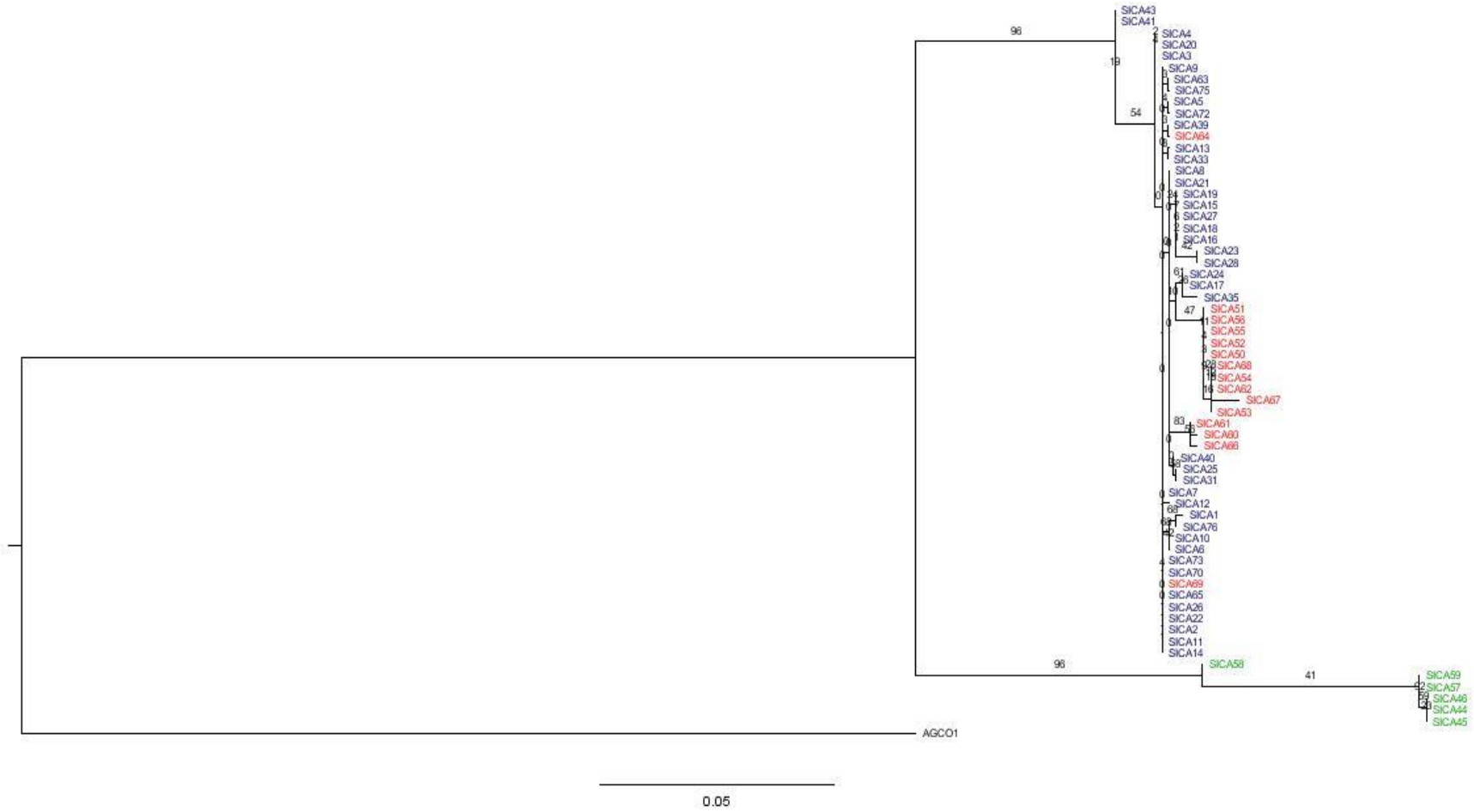

Figure 5. Concatenated mtDNA ML gene tree. Green samples represent the eastern massasauga, *Sistrurus catenatus catenatus*, red samples represent the desert massasauga, *S. c. edwardsii*, blue samples represent the western massasauga, *S. c. tergeminus*. Sample AGCO1" represents outgroup copperhead, *Agkistrodon contortrix*

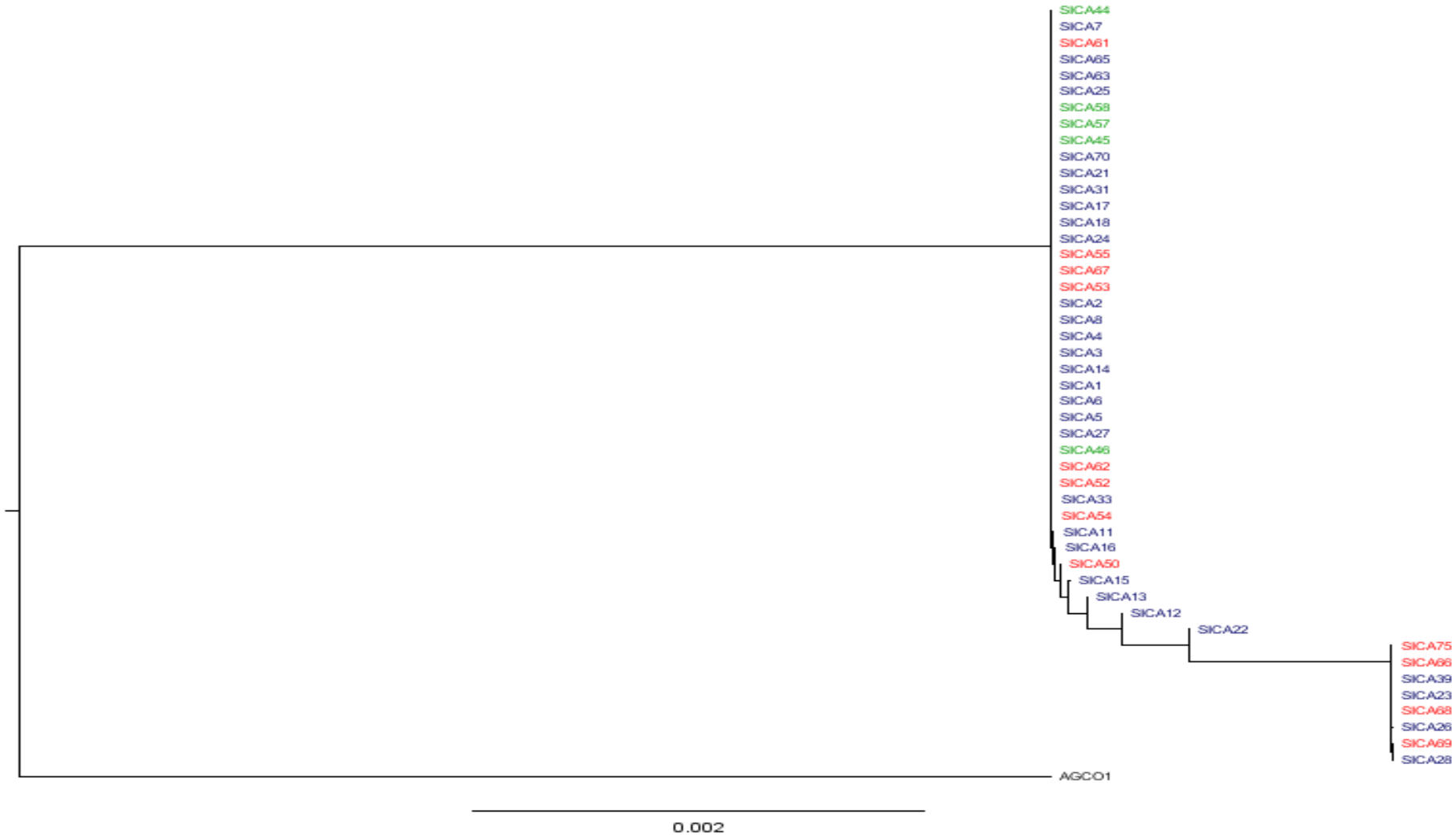

Figure 6. ML gene tree for *bdnf*. Green samples represent the eastern massasauga, *Sistrurus catenatus catenatus*, red samples represent the desert massasauga, *S. c. edwardsii*, blue samples represent the western massasauga, *S. c. tergeminus*. Sample "AGCO1" represents outgroup copperhead, *Agkistrodon contortrix*

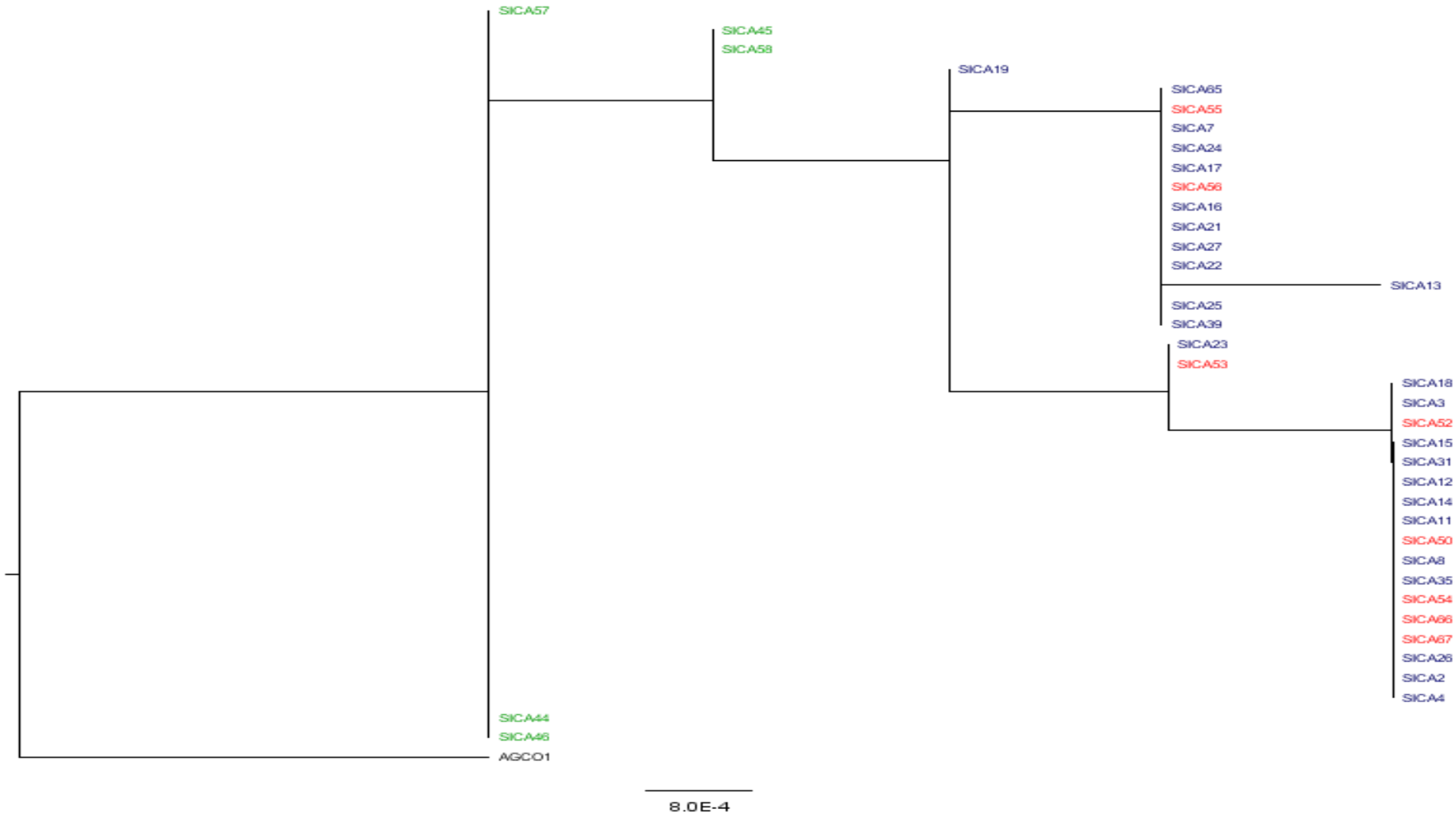

Figure 7. ML gene tree for *bmp2*. Green samples represent the eastern massasauga, *Sistrurus catenatus catenatus*, red samples represent the desert massasauga, *S. c. edwardsii*, blue samples represent the western massasauga, *S. c. tergeminus*. Sample "AGCO1" represents outgroup copperhead, *Agkistrodon contortrix*

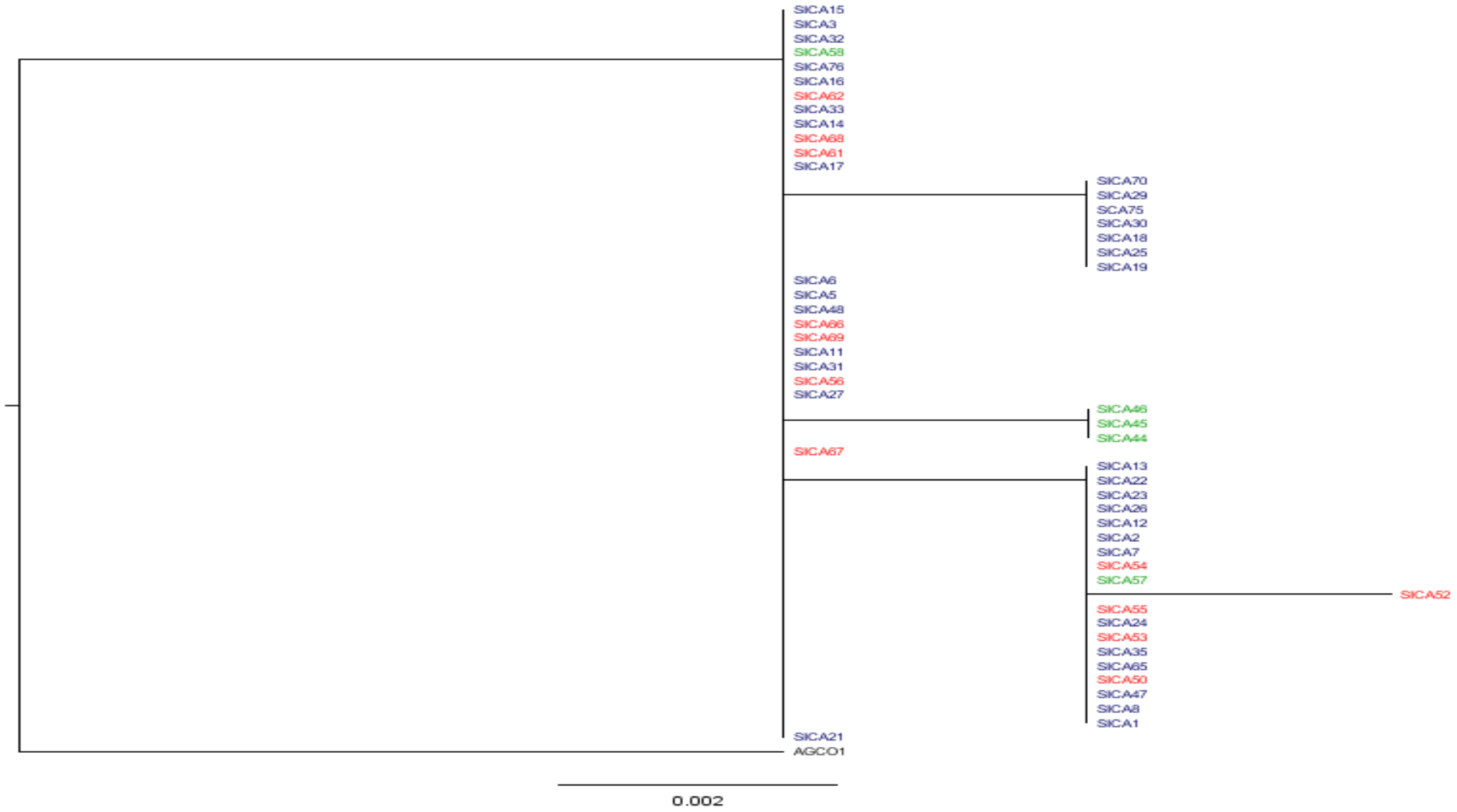

Figure 8. ML gene tree for *c-mos*. Green samples represent the eastern massasauga, *Sistrurus catenatus catenatus*, red samples represent the desert massasauga, *S. c. edwardsii*, blue samples represent the western massasauga, *S. c. tergeminus*. Sample "AGCO1" represents outgroup copperhead, *Agkistrodon contortrix*

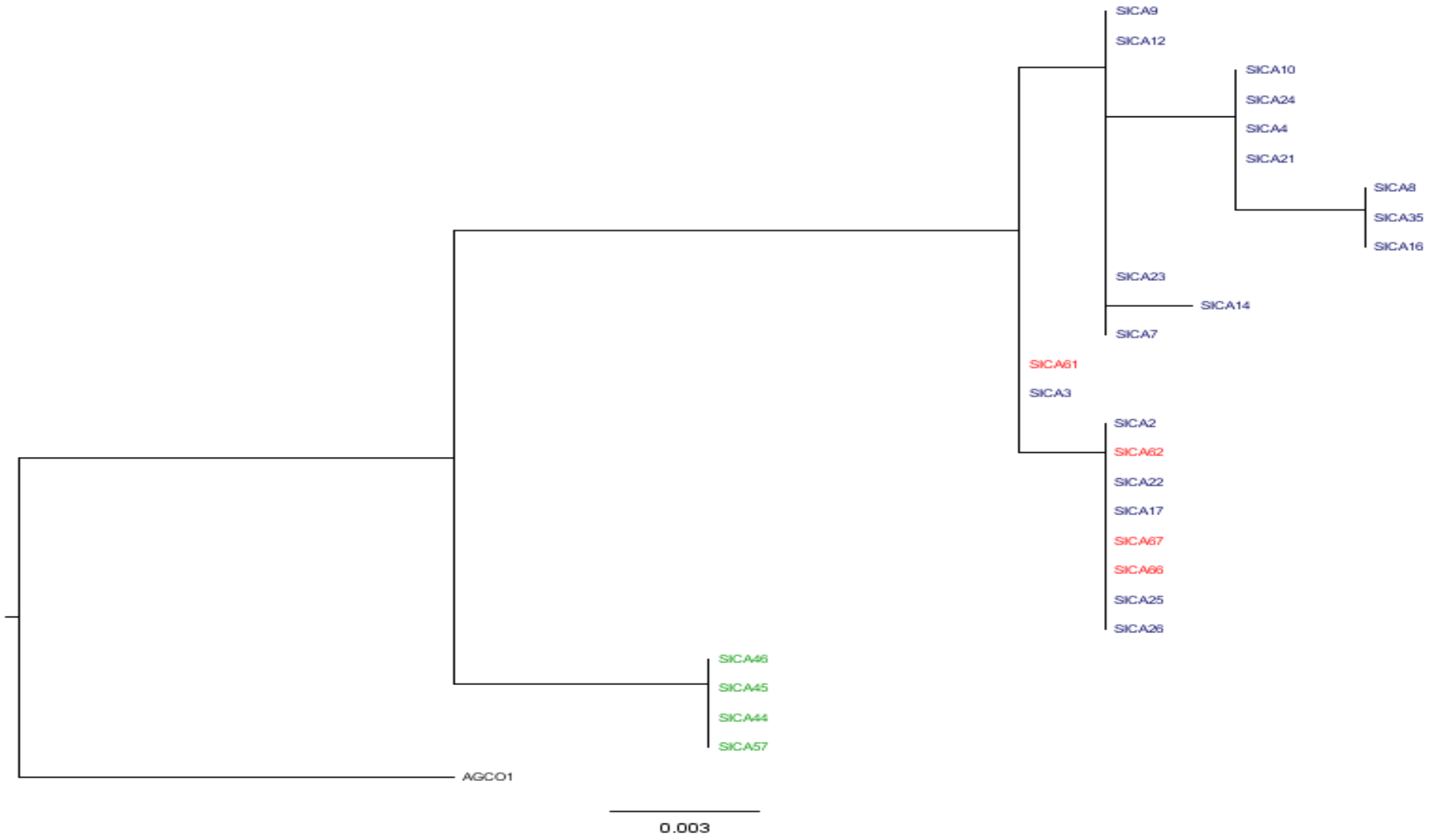

Figure 9. ML gene tree for *odc*. Green samples represent the eastern massasauga, *Sistrurus catenatus catenatus*, red samples represent the desert massasauga, *S. c. edwardsii*, blue samples represent the western massasauga, *S. c. tergeminus*. Sample "AGCO1"epresents outgroup copperhead, *Agkistrodon contortrix*

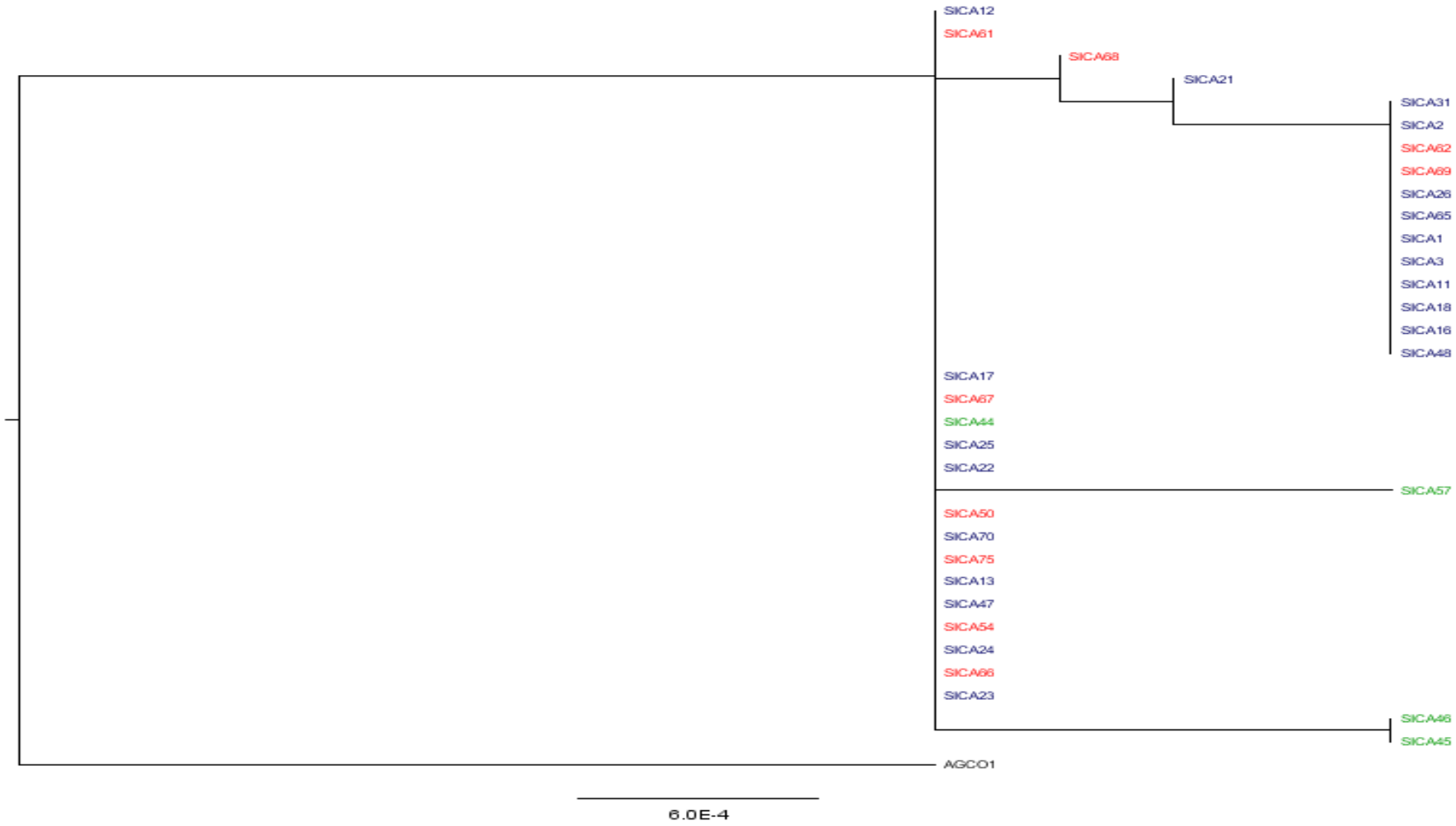

Figure 10. ML gene tree for *rag1*. Green samples represent the eastern massasauga, *Sistrurus catenatus catenatus*, red samples represent the desert massasauga, *S. c. edwardsii*, blue samples represent the western massasauga, *S. c. tergeminus*. Sample "AGCO1" represents outgroup copperhead, *Agkistrodon contortrix*

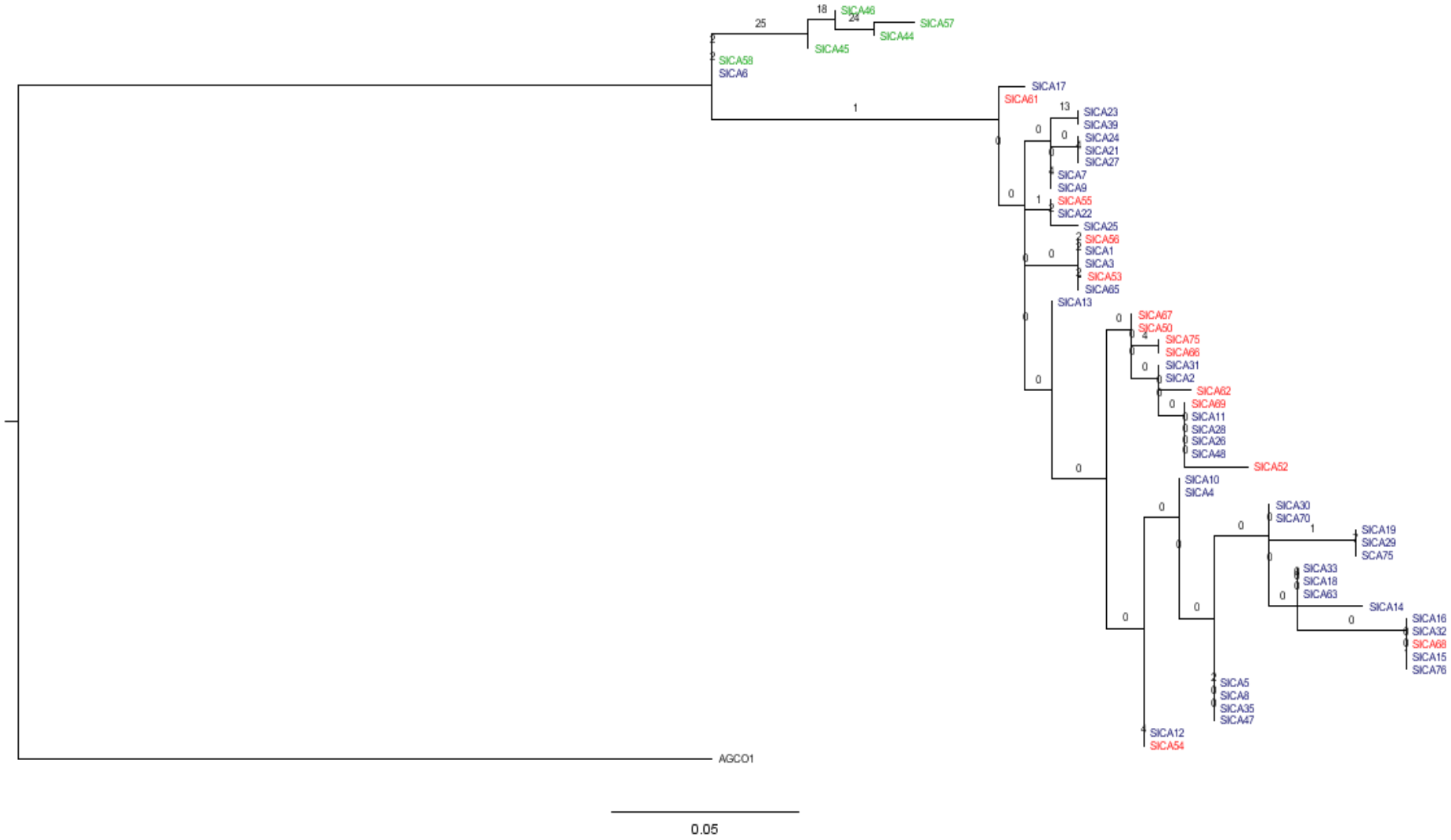

Figure 11. Concatenated nDNA ML gene tree. Green samples represent the eastern massasauga, *Sistrurus catenatus catenatus*, red samples represent the desert massasauga, *S. c. edwardsii*, blue samples represent the western massasauga, *S. c. tergeminus*. Sample "AGCO1" represents outgroup copperhead, *Agkistrodon contortrix*

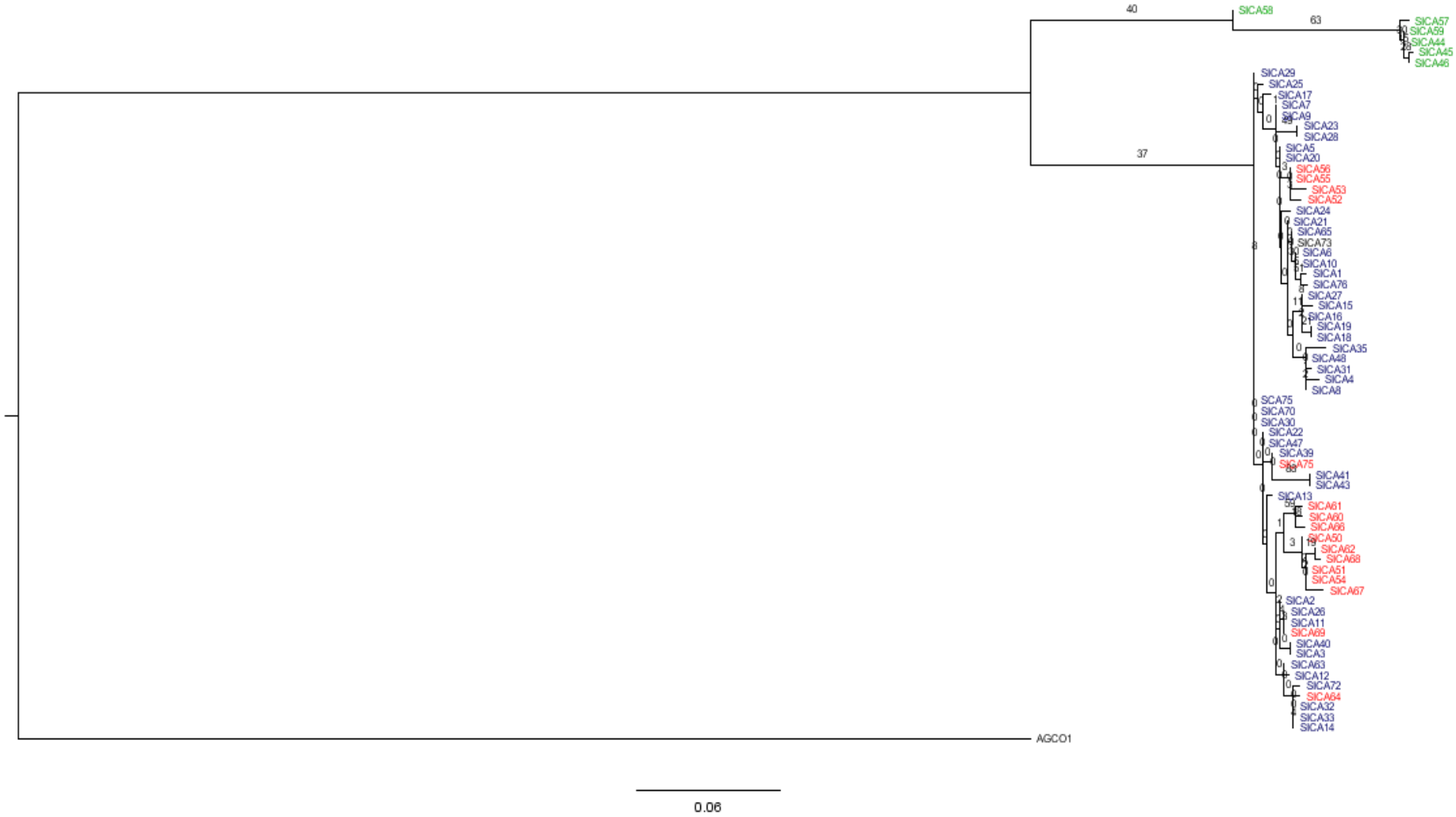

Figure 12. Concatenated 8 gene ML gene tree. Green samples represent the eastern massasauga, *Sistrurus catenatus catenatus*, red samples represent the desert massasauga, *S. c. edwardsii*, blue samples represent the western massasauga, *S. c. tergeminus*. Sample "AGCO1" represents outgroup copperhead, *Agkistrodon contortrix*

Chapter 3

Comparative Ecological Niche Modeling between *Sistrurus catenatus tergeminus* and *Sistrurus catenatus edwardsii* in Texas

Introduction

Ecological niche models (ENMs), also known as species distribution models (SDMs), are a quantitative form of ecological modeling that incorporates known species occurrence data along with environmental data to estimate a species' distribution across geographic space (Elith et al. 2011; Phillips et al. 2006; Warren and Seifert 2010). Ecological niche models have been used to address a variety of biological issues including, but not limited to, potential of invasive species invasions (Ward 2007; Rodder and Lotters 2010), climate change impacts (Wiens et al. 2009), species diversity (Graham et al. 2006), cryptozoological claims (Lozier et al. 2009), and species diversity at geographic boundaries (Escoriza 2010; Soto-Centeno et al. 2013). Specifically within evolutionary and conservation biology, ENMs have been used to understand different modes of speciation using comparative studies of niches between taxa (Anadón et al. 2015; Leaché et al. 2009; Pyron and Burbrink 2009; Wooten and Gibbs 2012; Khimoun et al. 2013). Information from these comparative studies can then be used in species delimitation, allowing the taxonomist to incorporate ecological data, which is particularly useful in recently diverged lineages that do not show high levels of molecular or morphological differentiation (Raxworthy et al. 2007; Rissler and Apodaca 2007; Leaché et al. 2009; Makowsky et al. 2010; Zhang et al. 2014).

The most commonly used form of ecological niche modeling is maximum entropy distributional modeling (MaxEnt; Phillips et al. 2006), which has been used in over 1000 published studies (Merow et al. 2013). MaxEnt requires "presence-only" data in order to develop

models and in general outperforms other modeling techniques such as genetic algorithm for rule set prediction (GARP), especially at low sample sizes (Pearson et al. 2007; Phillips and Dudík 2008). The ability of MaxEnt to produce high performing models using small sample sizes of presence-only data is particularly advantageous to studying snakes. Snakes are generally considered one of the most difficult taxa to study in nature due to their small size, patchy distributions, sporadic activity patterns, often inaccessible habitat, as well as, extremely cryptic and often subterranean nature (Durso et al. 2011). A number of recent studies have used MaxEnt developed ENMs for snakes and other reptile species to investigate niche conservation or divergence, and ecological speciation (Raxworthy et al. 2007; Leaché et al. 2009; Pyron and Burbrink 2009; Wooten and Gibbs 2012; Meik et al. 2015). Divergently evolving niches can then in turn lead to separate lineage formation by local adaptation (Leaché et al. 2009; Schluter 2009; Khimoun et al. 2013; Zhang et al. 2014).

The present study sought to develop ENMs using MaxEnt software for the Texas ranges of *S. c. catenatus* and *S. c. tergeminus*. In most studies using ENMs the entire range of a species is used; however, Gonzalez et al. (2011) and Soto-Centano et al. (2013) have emphasized that ENMs developed at smaller population levels can pick up more subtle environmental differences. The ENMs developed for this study were compared and used to answer two questions: 1) what environmental factors most affect the tolerances/preferences of *S. c. tergeminus* and *S. c. edwardsii*? 2) Are *S. c. tergeminus and S. c. edwardsii* taxonomically distinguishable based on ecology?

Methods and Materials

Ecological niche models (ENMs) were generated using MaxEnt (Version 3.3.3k; Phillips et al. 2006). MaxEnt works by projecting a list of GPS presence points across a GIS created user-defined landscape that is divided into cells of a pre-determined size (i.e. 0.5 km X 0.5 km in this case). The presence points are then compared to randomly generated background pseudo-absence points to determine if the cells occupied by the presence points are more similar to each other than these randomly generated background points (Phillips et al. 2006; Warren et al. 2010; Merow et al. 2013). The area under the receiver-operator curve (AUC) value generated by MaxEnt was used to evaluate the fit of the model to the data (Phillips et al. 2006; Merow et al. 2013). The higher the AUC value (ranked 0.0 – 1.0) the greater the ability of the model to distinguish between input presence locations and randomly generated pseudo-absence points (Merow et al. 2013). The level of impact of each variable on the overall construction of the model was assessed used the generated test gain values (Phillips et al. 2006)

Ecological niche models were generated for both *S. c. tergeminus* and *S. c. edwardsii*. Presence locations were compiled by data-mining VertNet and iNaturalist for locality information, taken from museum collection catalogues, provided by collaborators, or collected during road surveys conducted by myself with the aid of research assistants (Table 9 & 10). A few areas such as the samples collected in Parker, Hood, and Tarrent County from which GPS locations were obtained were much higher than other areas and believed to be because of sampling bias and not by greater population size. These three examples are from directly outside the Dallas-Fort Worth, TX metropolis and are very well known among both academic herpetologists and amateur reptile enthusiasts, and provided a very convenient area to collect this species. In order to reduce sampling bias, which can have a major impact on accurate modeling

efforts (Merow et al. 2013), an average number of GPS localities per county was generated. Only a maximum of the generated averages per county, five and four for *S. c. tergeminus* and *S. c. edwardsii* respectively, were used in the creation of ENMs (Tables 9 & 10). This resulted in a total of 60 presence points for *S. c. tergeminus* and 24 presence points for *S. c. edwardsii* being included in the models.

Environmental variable layers used by the ENMs were developed using ArcGIS (Version 10.3). A total of five environmental layers were included in the ENMs, three climatic and two landscape characteristics (Table 11), and the extent of each layer was restricted to Texas only. Prior to input into MaxEnt, environmental variable layers were set to a cell size of 500 m X 500 m, projected to NAD 1983 UTM zone 14, and converted to ASCII files. Presence data was also projected to NAD 1983 UTM zone 14. Test data were generated by setting run type in MaxEnt to the "leave-on-out" or *n*-1 crossvalidation method, where *n* is the number of observations. This methods was selected to accommodate the relatively low samples sizes used to generate the ENMs. Spatial autocorrelation along with further sampling bias was corrected for by only using one GPS point were grid cell. All other MaxEnt setting were set to default.

After ENMs were generated for both subspecies the degree of similarity between the two models was quantified using the program ENMtools (Version 1.4.1; Warren et al. 2010). First ENMtools was used to generate the "*I* statistic" described by Warren et al. (2010). The *I* statistic is a numerical value between 0 and 1 used to measure niche overlap (Warren et al. 2010). I then used ENMtools to create a null distribution of 100 randomly generated niche overlap values. The five percent quantile values of the null distribution were determined and used to access the statistical significance of the generated *I* statistic value. Ecological niche models for both subspecies were converted into binary average habitat suitability maps using the equal test

sensitivity and specificity logistic threshold value generated by MaxEnt (Phillips et al., 2006). These two binary maps were then combined onto a single map in order to better visualize any overlap in potential niches between the two subspecies.

Results

Ecological niche models for both *S. c. tergeminus* and *S. c. edwardsii* had AUC values above 0.9, 0.94 and 0.93 respectively, indicating they have strong predictive power (Phillips et al. 2006; Figures 13 & 14). However, test gains show the effect of each variable on model creation varied between subspecies (Figures 15 & 16). All three climatic variables contributed more than either landscape variable for *S. c. tergeminus* (Figure 13), whereas landform contributed the most followed by temperature seasonality and annual precipitation for *S. c. edwardsii* (Figure 16).

The most highly suitable habitats for *S. c. tergeminus* were correlated with an annual precipitation of approximately 650mm (figure 17). Highly suitable habitat *S. c. tergeminus* is also more likely to be found in areas with a low degree of daily temperature fluctuation and a moderate degree of temperature seasonality (Figure 18 & 19). Tablelands were the most predictive landform type and limestone/gravel the most predictive rock types to be associated with *S. c. tergeminus* (Table 12 & 13, Figure 20 & 21).

Sistrurus catenatus edwardsii was most associated with the landform type "plains with hills" (Figure 22; Table 14). In contrast to *S. c. tergeminus, S. c. edwardsii* is more likely to be found in habitats with a moderate degree of daily temperature fluctuation and a lower degree of temperature seasonality (Figure 23 & 24). Annual precipitation is also much lower in areas predicted to be highly suitable for *S. c. edwardsii* with the optimal values between 50.4 and

177.9 mm (Figure 25). Sand was the most commonly associated geology feature to be found in association with *S. c. edwardsii* (Figure 26, Table 15).

The *I* statistic (0.25) falls below the 95% permutation threshold (0.92). This indicates the ecological niche models for *S. c. tergeminus* and *S. c. edwardsii* are significantly different (Figure 27).

Discussion

The ENMs created for this study show a high degree of niche differentiation between *S. c. tergeminus* and *S. c. edwardsii*. This suggests there is niche divergence between these parapatric subspecies that may be an early stage of the speciation process (Raxworthy et al. 2007; Pyron and Burbrink 2009; Wooten and Gibbs 2012; Khimoun et al. 2013). Ecological speciation has been noted as a possible mechanism of diversification in recently diverged lineages, such as *S. c. tergeminus* and *S. c. edwardsii* (Kubatko et al. 2011), driving local adaptation and further niche divergence (Schluter 2009). Niche divergence creates ecological separation between species lineages leading to further reproductive isolation and higher levels of genetic differentiation (Wooten and Gibbs 2012; Khimoun et al. 2013). Divergently evolving niches are often associated with some type of geographical boundary created by ancient glacial events (Placyk et al. 2007; Pyron and Burbrink 2009; Soto-Centeno et al. 2013). However, Pyron and Burbrink (2009) indicated in the snake species *Lampropeltis getula*, parapatric subspecies lineages can evolve distinctive ecological niches without any distinctive isolating geographic barrier. Other studies have shown similar results in herpetofauna indicating that environmental gradients can act as boundaries causing lineages to diverge ecologically (Graham et al. 2004; Raxworthy et al. 2007; Leaché et al. 2009; Zhang et al. 2014). It is likely local environmental

differences are driving niche divergence between S. *c. tergeminus* and *S. c. edwardsii*, as there are no obvious or notable physically barrier dividing the two subspecies populations.

Ectotherms, such as snakes, can show strong physiological responses to local environmental factors (Raxworthy et al. 2007; Pyron and Burbrink 2009; Wooten and Gibbs 2012). The ENMs show evidence for this being the case between *S. c. tergeminus* and *S. c. edwardsii*. Annual precipitation played a differentiating role between the niche models. *Sistrurus c. tergeminus* preferred habitats with a much higher amount of precipitation than *S. c. edwardsii*. This finding supports previously known ecological differences between these two subspecies. Throughout its range *S. c. tergeminus* is associated with low laying wetter habitats, whereas *S. c. edwardsii* is more associated with dry xeric habitats (Seigel 1986; Holycross and Mackessy 2002; Wastell and Mackessy 2011). Differences in daily and seasonal temperature fluctuation preference between *S. c. tergeminus* and *S. c. edwardsii* also indicate local physiological adaptations to different environmental factors.

In addition to adaptations to climatic differences, the ENMs indicate *S. c. tergeminus* and *S. c. edwardsii* prefer different physical terrains as well, as indicated by their preference for different landform and geological features. Previous studies have shown have both *S. c. tergeminus* and *S. c. edwardsii* require multiple habitat types, where they utilize one type of habitat during brumation and another during their active season (Wastell and Mackessy 2011). Therefore, the right matrix of habitat types must exist in close proximity to one another in order for each subspecies to utilize an area, and these matrices of habitat types are different for the two subspecies. The specific habitats utilized by these two subspecies in Texas may only exist under certain landform, geological and environmental combinations. Furthermore differences in prey

preferences between *S. c. tergeminus* and *S. c. edwardsii* have been noted and are closely linked to habitat preferences (Holycross and Mackessy 2002).

My ecological niche modeling results support conclusions drawn by Wooten and Gibbs (2012). In a study of both species (which includes six total subspecies) of the genus *Sistrurus* Wooten and Gibbs (2012) concluded that niche divergence is acting a strong driving force in the ecological separation of all three subspecies of *S. catenatus*. However, their study used a much larger extent (The Continental United States and Lower Canada) in their ENMs than this study. The large extent could possibly have confounding effects by reducing modeling sensitivity. In order to increase model sensitivity and tease out more subtle environmental difference the ENM I used a smaller extent, but still found similar results. Finally, I conclude that based on the ENMs created in this study *S. c. tergeminus* and *S. c. edwardsii* lack ecological exchangeability indicating that they are separately evolving lineages within the species *S. catenatus*. This supports the current taxonomy.

Appendix C

Ecological Niche Modeling Tables

Table 9. Presence points and sources for *Sistrurus catenatus tergeminus* locale data used in ecological niche modeling

Subspecies	Latitude	Longitude	County	Source
S. c. tergeminus	33.5494	-98.946	Archer	UTEP
S. c. tergeminus	33.5722	-98.848	Archer	iNat
S. c. tergeminus	33.5415	-98.845	Archer	iNat
S. c. tergeminus	33.70028	-98.8745	Archer	iNat
S. c. tergeminus	33.71157	-98.7299	Archer	iNat
S. c. tergeminus	32.4968	-99.544	Callahan	UTEP
S. c. tergeminus	33.855	-98.347	Clay	UTAR
S. c. tergeminus	31.7167	-99.547	Coleman	TAMU
S. c. tergeminus	31.7095	-99.548	Coleman	TAMU
S. c. tergeminus	34.1118	-100.37	Cottle	S.Hein/M.Barazowski
S. c. tergeminus	34.1181	-100.34	Cottle	S.Hein/M.Barazowski
S. c. tergeminus	34.1379	-100.36	Cottle	S.Hein/M.Barazowski
S. c. tergeminus	34.1265	-100.35	Cottle	S.Hein/M.Barazowski
S. c. tergeminus	34.1096	-100.37	Cottle	S.Hein/M.Barazowski
S. c. tergeminus	33.8673	-101.11	Floyd	iNat
S. c. tergeminus	34.03224	-99.6593	Foard	iNat
S. c. tergeminus	34.7266	-101.44	Hall	UTAR
S. c. tergeminus	33.1815	-99.261	Haskell	UTAR
S. c. tergeminus	33.18758	-99.9678	Haskell	iNat
S. c. tergeminus	33.1713	-99.5004	Haskell	iNat
S. c. tergeminus	33.16181	-99.5716	Haskell	iNat
S. c. tergeminus	33.1404	-99.6968	Haskell	iNat
S. c. tergeminus	32.5331	-97.619	Hood	TAMU
S. c. tergeminus	32.5383	-97.63	Hood	UTAR
S. c. tergeminus	32.5546	-97.662	Hood	UTAR
S. c. tergeminus	32.5468	-97.647	Hood	UTAR
S. c. tergeminus	32.5548	-97.697	Hood	UTAR
S. c. tergeminus	32.5284	-97.614	Johnson	UTAR
S. c. tergeminus	32.2999	-97.564	Johnson	UTAR
S. c. tergeminus	32.3046	-97.554	Johnson	UTAR
S. c. tergeminus	32.306	-97.546	Johnson	UTAR
S. c. tergeminus	32.2789	-97.519	Johnson	UTAR
S. c. tergeminus	32.7447	-99.713	Jones	UTAR

S. c. tergeminus	33.58369	-99.8294	Knox	iNat
S. c. tergeminus	32.6328	-97.7	Parker	UTAR
S. c. tergeminus	32.585	-97.679	Parker	UTAR
S. c. tergeminus	32.6366	-97.559	Parker	UTAR
S. c. tergeminus	32.6112	-97.583	Parker	UTAR
S. c. tergeminus	32.6028	-97.686	Parker	iNat
S. c. tergeminus	35.7899	-100.74	Roberts	UTEP
S. c. tergeminus	32.5163	-99.561	Shackelford	UTEP
S. c. tergeminus	32.72345	-99.2973	Shackelford	iNat
S. c. tergeminus	33.1858	-99.972	Stonewall	UTAR
S. c. tergeminus	33.20927	-100.337	Stonewall	iNat
S. c. tergeminus	33.12342	-100.082	Stonewall	iNat
S. c. tergeminus	33.20967	-100.359	Stonewall	iNat
S. c. tergeminus	32.679	-97.51	Tarrant	UTAR
S. c. tergeminus	32.688	-97.499	Tarrant	UTAR
S. c. tergeminus	32.693	-97.499	Tarrant	UTAR
S. c. tergeminus	32.6772	-97.489	Tarrant	UTAR
S. c. tergeminus	32.6785	-97.499	Tarrant	iNat
S. c. tergeminus	33.0047	-99.158	Throckmorton	UTEP
S. c. tergeminus	32.9773	-99.186	Throckmorton	UTEP
S. c. tergeminus	33.17968	-99.2268	Throckmorton	iNat
S. c. tergeminus	32.9956	-99.148	Throckmorton	iNat
S. c. tergeminus	34.12499	-98.6741	Wichita	iNat
S. c. tergeminus	33.9664	-99.099	Wilbarger	UTAR
S. c. tergeminus	33.8712	-99.404	Wilbarger	iNat
S. c. tergeminus	33.1844	-98.502	Young	UTEP

(iNat- iNaturalist; UTEP – University of Texas at El Paso; TAMU – Texas A&M University; UTAR – University of Texas at Arlington)

Table 10. Presence points and sources for *Sistrurus catenatus edwardsii* used in ecological niche modeling

Subspecies	Latitude	Longitude	County	Source
S. c. edwardsii	32.129	-102.68	Andrews	iNat
S. c. edwardsii	32.3822	-102.42	Andrews	iNat
S. c. edwardsii	32.08822	-102.866	Andrews	iNat
S. c. edwardsii	32.6252	-101.39	Borden	iNat
S. c. edwardsii	32.5564	-101.26	Borden	TNHC
S. c. edwardsii	26.7348	-98.509	Brooks	TAMU
S. c. edwardsii	31.60731	-102.688	Crane	iNat
S. c. edwardsii	32.2929	-101.47	Howard	TAMU
S. c. edwardsii	32.5555	-101.23	Howard	iNat
S. c. edwardsii	32.5568	-101.28	Howard	iNat
S. c. edwardsii	31.0109	-101.17	Irion	iNat
S. c. edwardsii	30.5645	-104.47	Jeff Davis	UTAR
S. c. edwardsii	26.9137	-98.597	Jim Hogg	iNat
S. c. edwardsii	27.18716	-98.6205	Jim Hogg	R. Couvillon
S. c. edwardsii	27.12655	-98.5797	Jim Hogg	R. Couvillon
S. c. edwardsii	27.1269	-98.583	Jim Hogg	R. Couvillon
S. c. edwardsii	27.125	-98.59	Jim Hogg	R. Couvillon
S. c. edwardsii	26.95018	-98.5943	Jim Hogg	iNat
S. c. edwardsii	27.4162	-97.308	Kleberg	iNat
S. c. edwardsii	31.9478	-101.98	Midland	iNat
S. c. edwardsii	32.5182	-101.14	Mitchell	TAMU
S. c. edwardsii	31.4184	-102.95	Ward	S.Hein/S.Pitts
S. c. edwardsii	31.46451	-102.904	Ward	iNat
S. c. edwardsii	31.5368	-102.988	Ward	iNat
S. c. edwardsii	31.50866	-102.984	Ward	iNat

(iNat- iNaturalist; TNCH – Texas Natural History Collection; TAMU – Texas A&M University; UTAR – University of Texas at Arlington)

Table 11. Environmental layers used in ecological niche modeling

Environmental layer	Source
Bio3: Isothermality	WorldClim
Bio 4: Temperature seasonality	WorldClim
Bio 12: Annual precipitation	WorldClim
Geology	USGS
Landform	USGS

Table 12. Unique landform characteristics with corresponding ID value shown in response curves produced by Maxent for *Sistrurus catenatus tergeminus*

Maxent ID	Predictive score	CLASS ID	Attribute	Slope	Relief	profile type
1	0.640	B5a	Plains with low mountains	50-80% of area gently sloping	1000 - 3000 ft	More than 75% of gentle slope is in lowland
3	0.640	D5	Low mountains	Less the 20% of area gently sloping	1000 - 3000 ft	N/A
4	0.853	B3c	Tablelands moderate relief	50 -80% of area gently sloping	300 - 500 ft	50 - 75% of gentle slope is on upland
8	0.588	B3b	plains with hills	50 -80% of area gently sloping	300 - 500 ft	50 -75% of gentle slope is on lowland
11	0.674	B2c	irregular plains,50-75% gentle slope on upland	50 -80% of area gently sloping	100 - 300 ft	50 - 75% of gentle slope is on upland
13	0.640	B2b	irregular plains, 50-75% gentle slope on lowland	50 -80% of area gently sloping	100 - 300 ft	50 -75% of gentle slope is on lowland
14	0.640	A1	Flat Plains	More than 80% of area gently sloping	1 - 100 ft	N/A
16	0.640	B4b	Plains with high hills	50 -80% of area gently sloping	500 - 1000 ft	50 -75% of gentle slope is on lowland
17	0.628	A2c	Smooth Plains, 50 - 75% gentle slope on upland	More than 80% of area gently sloping	100 - 300 ft	50 - 75% of gentle slope is on upland
18	0.640	C4c	Open High Hills, 50 - 75% gentle slope on upland	20 - 50% of area gently sloping	500 - 1000 ft	50 - 75% of gentle slope is on upland
19	0.640	B3a	Plains with hills	50 -80% of area gently sloping	300 - 500 ft	More than 75% of gentle slope is in lowland
20	0.640	C4b	Open High Hills, 50 -75% gentle slope on lowland	20 - 50% of area gently sloping	500 - 1000 ft	50 -75% of gentle slope is on lowland
23	0.640	A2b	smooth plains, 50 - 75% of gentle slope on lowland	More than 80% of area gently sloping	100 - 300 ft	50 -75% of gentle slope is on lowland
25	0.640	B5b	plans with low mountains	50 -80% of area gently sloping	1000 - 3000 ft	50 -75% of gentle slope is on lowland
27	0.640	B6a	Plains with high mountains	50 -80% of area gently sloping	>3000 ft	More than 75% of gentle slope is in lowland
32	0.640	D4	High hills	Less the 20% of area gently sloping	500 - 1000 ft	N/A

Table 13. Unique geology characteristics with corresponding ID value shown in response curves produced by Maxent for *Sistrurus catenatus tergeminus*

Maxent ID number	Predictability Score	Rock Type
1	0.752	sand
2	0.701	evaporite
3	0.939	clay or mud
4	0.701	sandstone
5	0.849	shale
6	0.701	water
7	0.701	terrace
8	0.701	mixed clastic/carbonate
9	0.701	fine-grained mixed clastic
10	0.853	mudstone
11	0.958	limestone
12	0.701	silt
13	0.958	gravel
14	0.701	alluvial fan
15	0.701	dolostone (dolomite)
16	0.701	basalt
17	0.701	playa
18	0.701	landslide
20	0.701	granite
21	0.701	rhyolite
22	0.701	conglomerate
23	0.701	siltstone
24	0.701	indeterminate
25	0.701	trachyte
27	0.701	phyllite
28	0.701	paragneiss
29	0.701	amphibole schist
33	0.701	claystone
35	0.701	medium-grained mixed clastic
36	0.701	chert
37	0.701	tuff
39	0.701	ash-flow tuff

Table 14. Unique landform characteristics with corresponding ID value shown in response curves produced by Maxent for *Sistrurus catenatus edwardsii*

Maxent ID	Predictive score	CLASS ID	Attribute	Slope	Relief	profile type
1	0.099	B5a	Plains with low mountains	50-80% of area gently sloping	1000 - 3000 ft	More than 75% of gentle slope is in lowland
3	0.099	D5	Low mountains	Less the 20% of area gently sloping	1000 - 3000 ft	N/A
4	0.099	B3c	Tablelands moderate relief	50 -80% of area gently sloping	300 - 500 ft	50 - 75% of gentle slope is on upland
8	0.919	B3b	plains with hills	50 -80% of area gently sloping	300 - 500 ft	50 -75% of gentle slope is on lowland
11	0.857	B2c	irregular plains,50-75% gentle slope on upland	50 -80% of area gently sloping	100 - 300 ft	50 - 75% of gentle slope is on upland
13	0.099	B2b	irregular plains, 50-75% gentle slope on lowland	50 -80% of area gently sloping	100 - 300 ft	50 -75% of gentle slope is on lowland
14	0.099	A1	Flat Plains	More than 80% of area gently sloping	1 - 100 ft	N/A
16	0.099	B4b	Plains with high hills	50 -80% of area gently sloping	500 - 1000 ft	50 -75% of gentle slope is on lowland
17	0.659	A2c	Smooth Plains, 50 - 75% gentle slope on upland	More than 80% of area gently sloping	100 - 300 ft	50 - 75% of gentle slope is on upland
18	0.099	C4c	Open High Hills, 50 - 75% gentle slope on upland	20 - 50% of area gently sloping	500 - 1000 ft	50 - 75% of gentle slope is on upland
19	0.099	B3a	Plains with hills	50 -80% of area gently sloping	300 - 500 ft	More than 75% of gentle slope is in lowland
20	0.099	C4b	Open High Hills, 50 -75% gentle slope on lowland	20 - 50% of area gently sloping	500 - 1000 ft	50 -75% of gentle slope is on lowland
23	0.099	A2b	smooth plains, 50 - 75% of gentle slope on lowland	More than 80% of area gently sloping	100 - 300 ft	50 -75% of gentle slope is on lowland
25	0.099	B5b	plans with low mountains	50 -80% of area gently sloping	1000 - 3000 ft	50 -75% of gentle slope is on lowland
27	0.267	B6a	Plains with high mountains	50 -80% of area gently sloping	>3000 ft	More than 75% of gentle slope is in lowland
32	0.099	D4	High hills	Less the 20% of area gently sloping	500 - 1000 ft	N/A

Table 15. Unique geology characteristics with corresponding ID value shown in response curves produced by Maxent for *Sistrurus catenatus edwardsii*

MAXENT ID	Predictive score	Rock Type
1	0.652	sand
2	0.213	evaporite
3	0.213	clay or mud
4	0.213	sandstone
5	0.213	shale
6	0.213	water
7	0.213	terrace
8	0.213	mixed clastic/carbonate
9	0.620	fine-grained mixed clastic
10	0.213	mudstone
11	0.241	limestone
12	0.213	silt
13	0.213	gravel
14	0.213	alluvial fan
15	0.213	dolostone (dolomite)
16	0.213	basalt
17	0.213	playa
18	0.213	landslide
20	0.213	granite
21	0.213	rhyolite
22	0.213	conglomerate
23	0.213	siltstone
24	0.213	indeterminate
25	0.213	trachyte
28	0.213	paragneiss
29	0.213	amphibole schist
30	0.213	coarse-grained mixed clastic
32	0.213	diorite
33	0.213	claystone
35	0.213	medium-grained mixed clastic
36	0.213	chert
37	0.213	tuff

Appendix D

Ecological Niche Modeling Figures

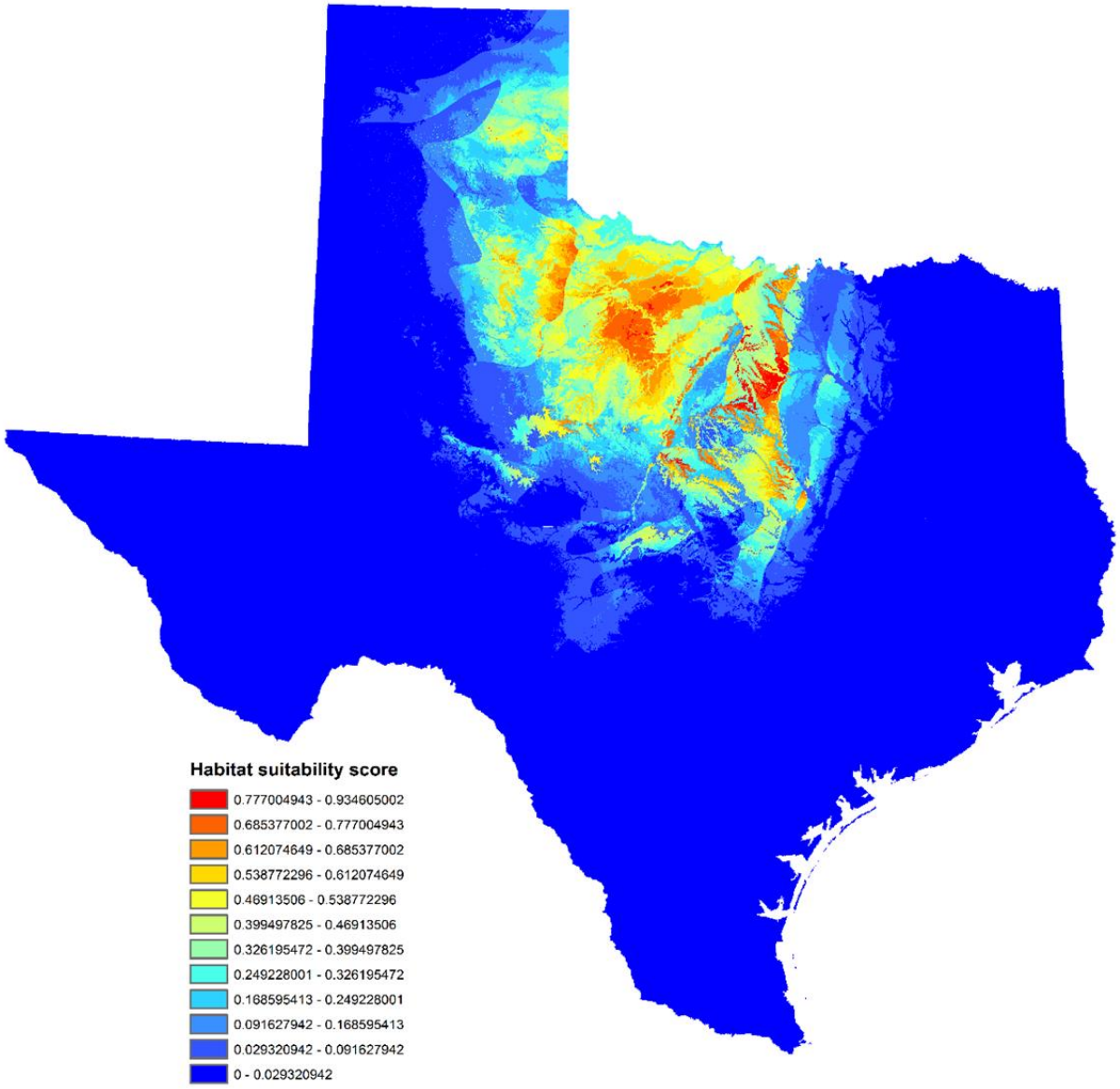

Figure 13. Ecological niche model for the western massasauga, *Sistrurus catenatus tergeminus*; AUC = 0.94

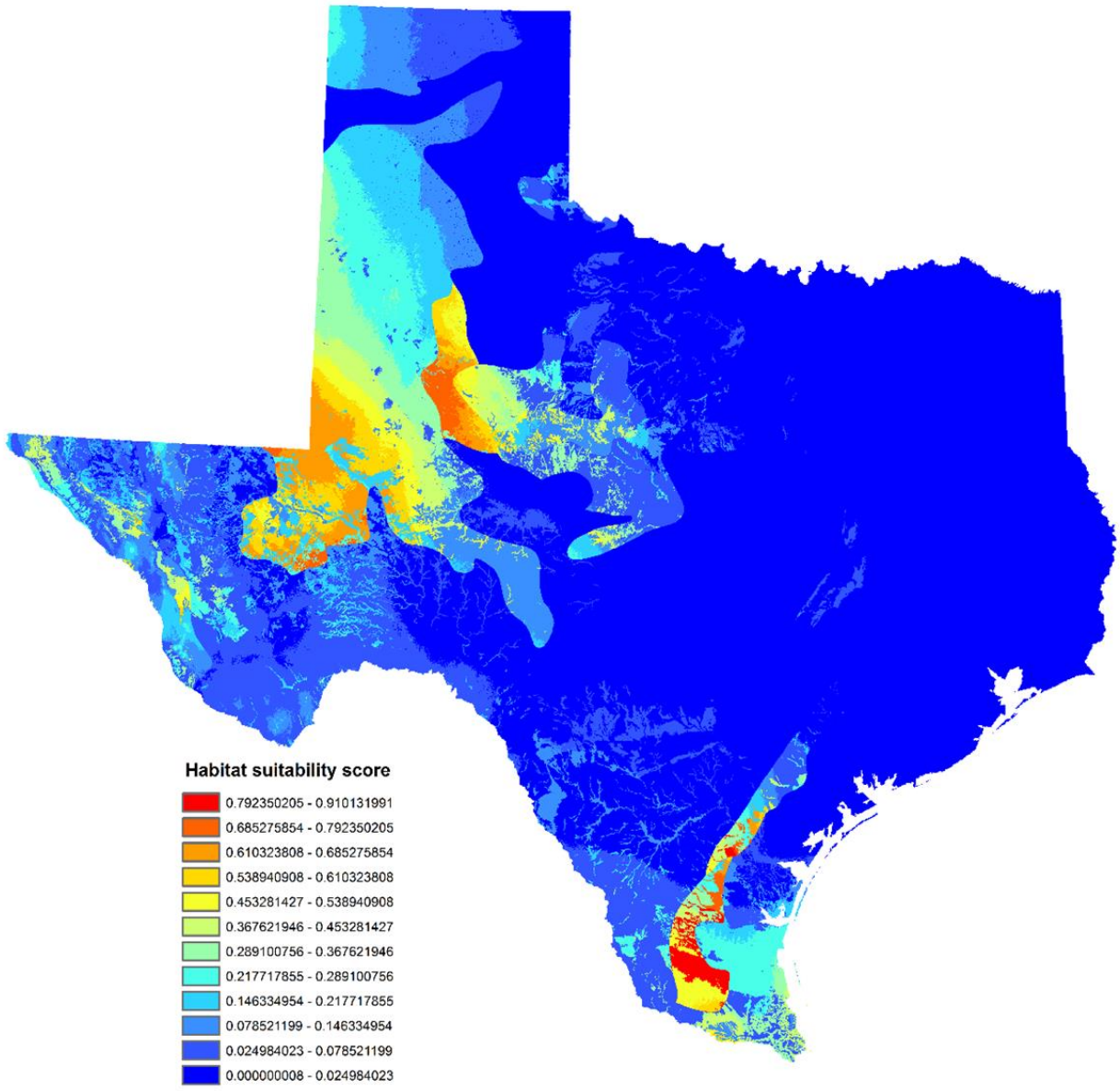

Figure 14. Ecological niche model for the desert massasauga, *Sistrurus catenatus edwardsii*; AUC = 0.93

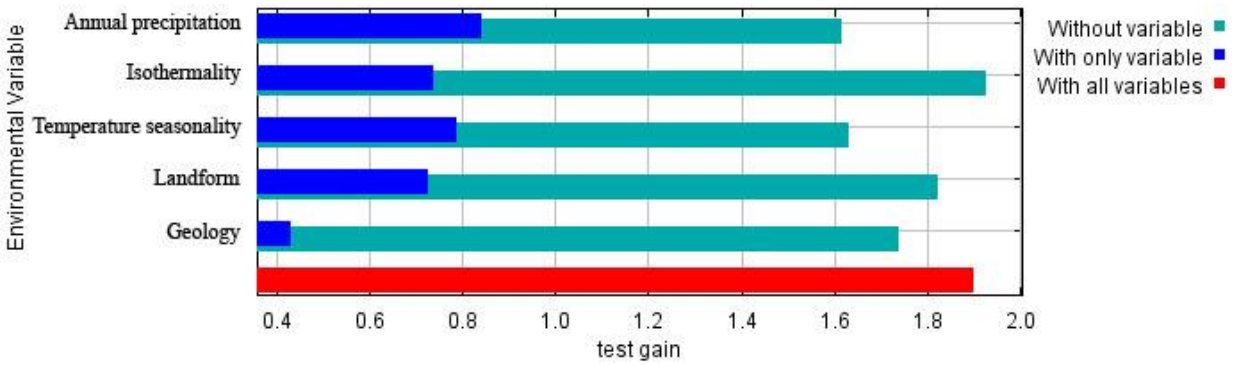

Figure 15. Test gains of each environmental variable for the western massasauga, *Sistrurus catenatus tergeminus*

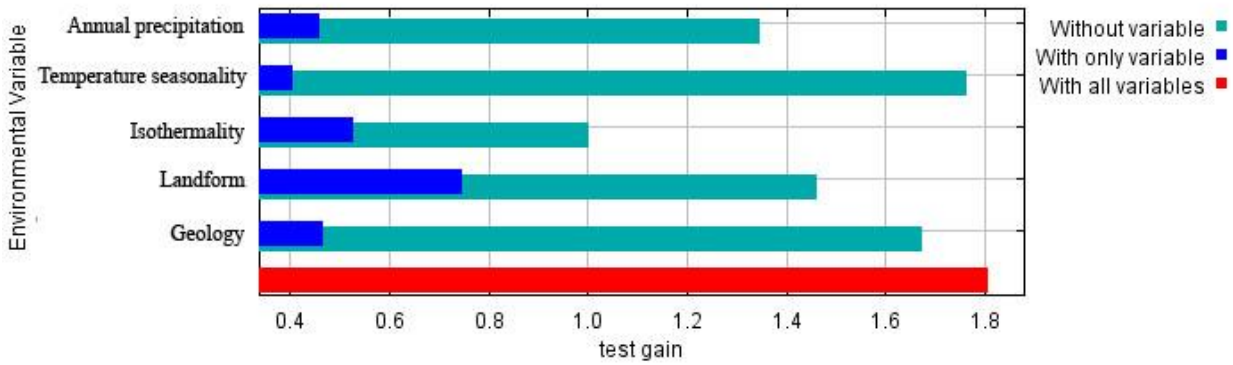

Figure 16. Test gains of each environmental variable for the desert massasauga, *Sistrurus catenatus edwardsii*

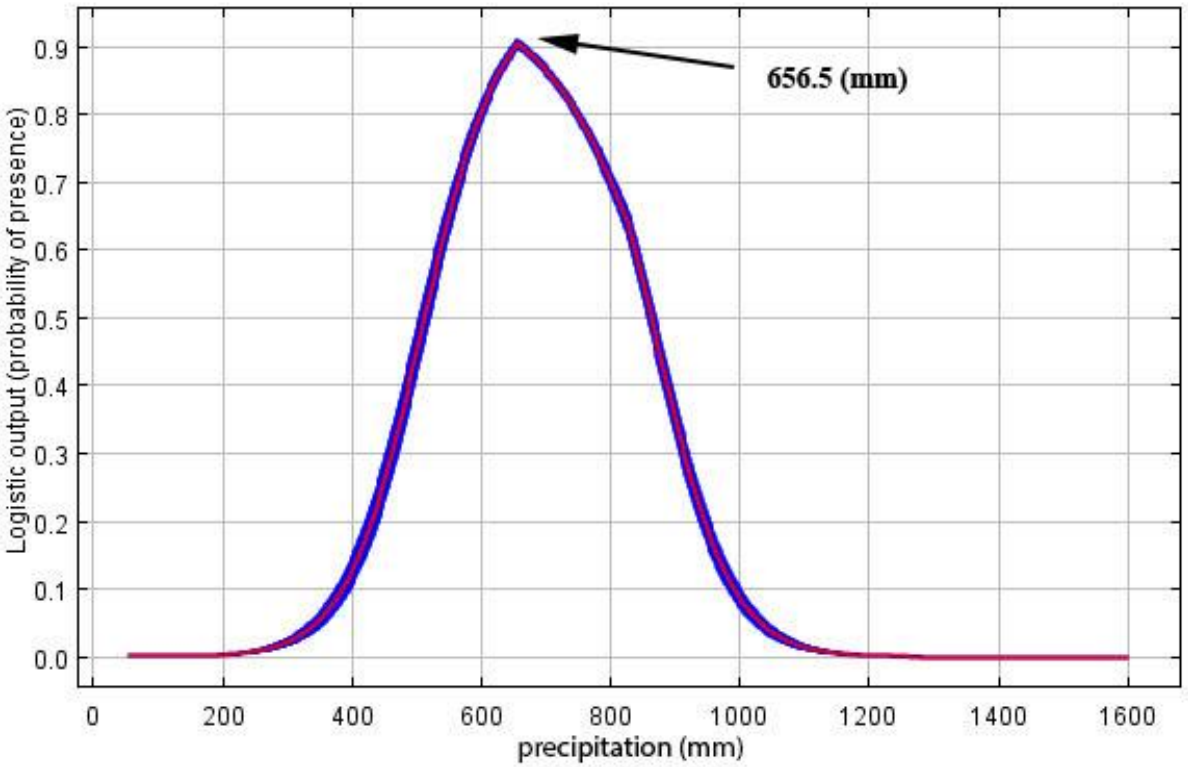

Figure 17. Mean response curve with 58 replicate Maxent runs (red) of environmental variable: annual precipitation for the western massasauga, *Sistrurus catenatus tergeminus*. Blue area represent +/- one standard deviation

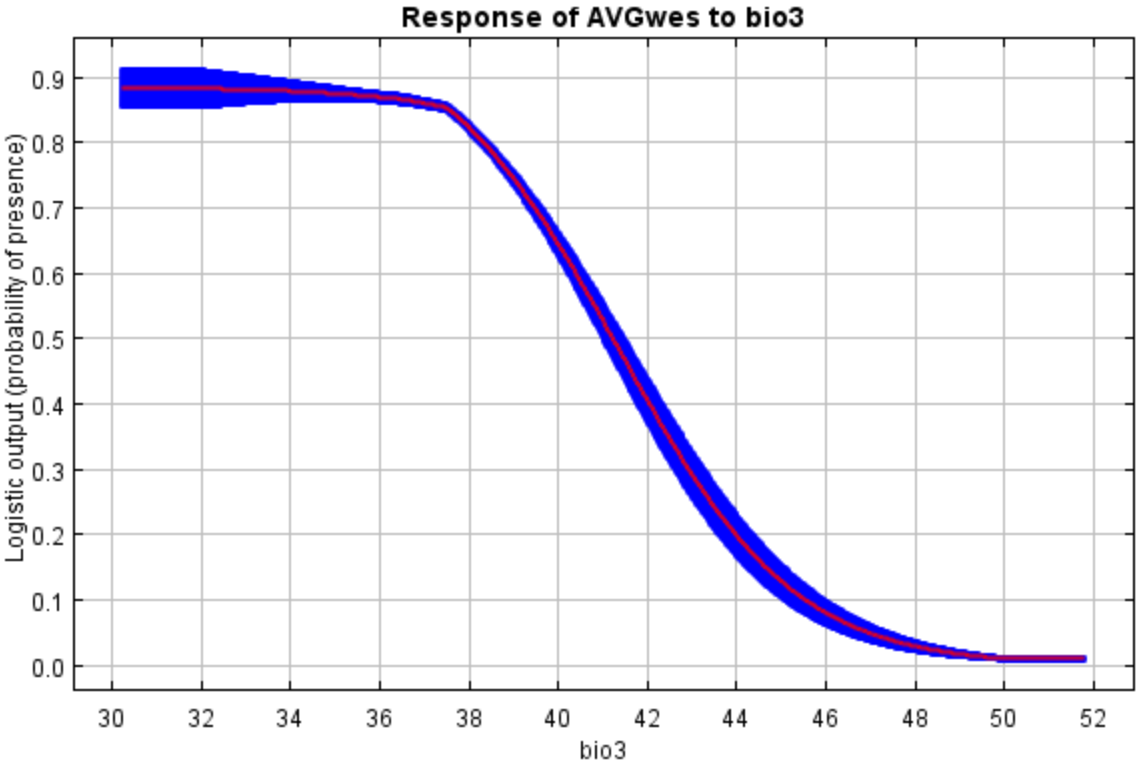

Figure 18. Mean response curve with 58 replicate Maxent runs (red) of environmental variable: isothermality (Mean diurnal temperature range/temperature annual range) for the western massasauga, *Sistrurus catenatus tergeminus*. Blue area represent +/- one standard deviation.

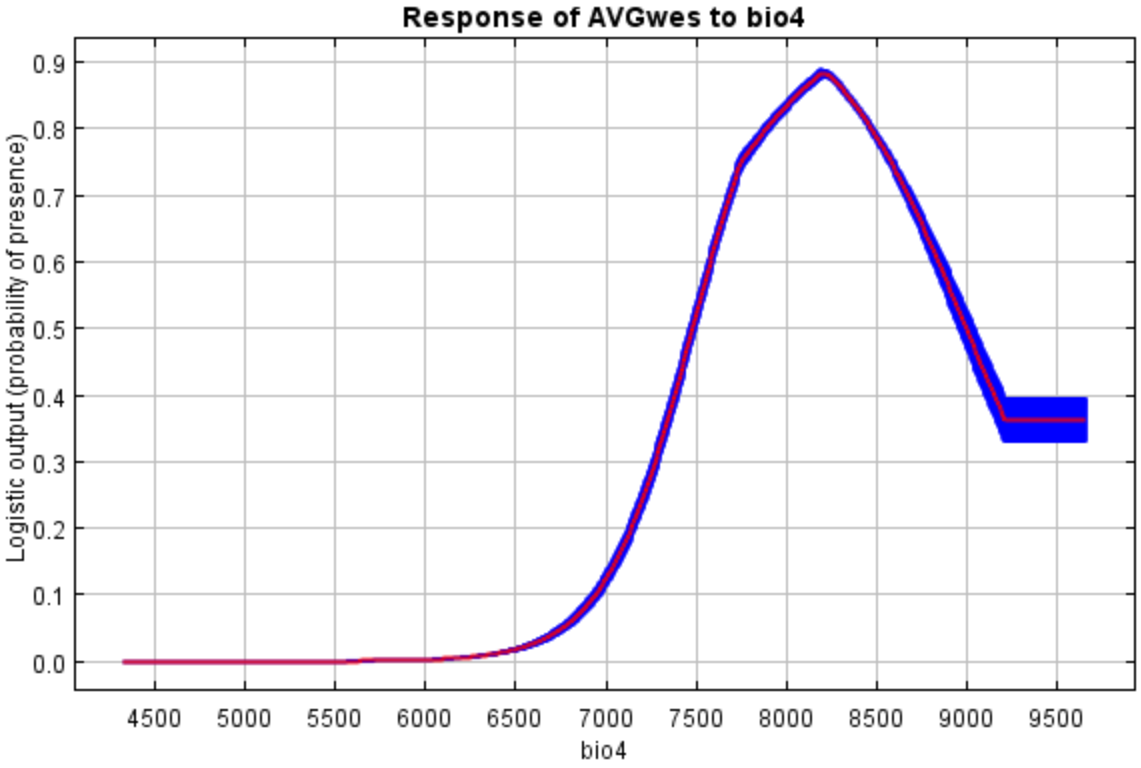

Figure 19. Mean response curve with 58 replicate Maxent runs (red) of environmental variable: temperature seasonality for the western massasauga, *Sistrurus catenatus tergeminus*. Blue area represent +/- one standard deviation

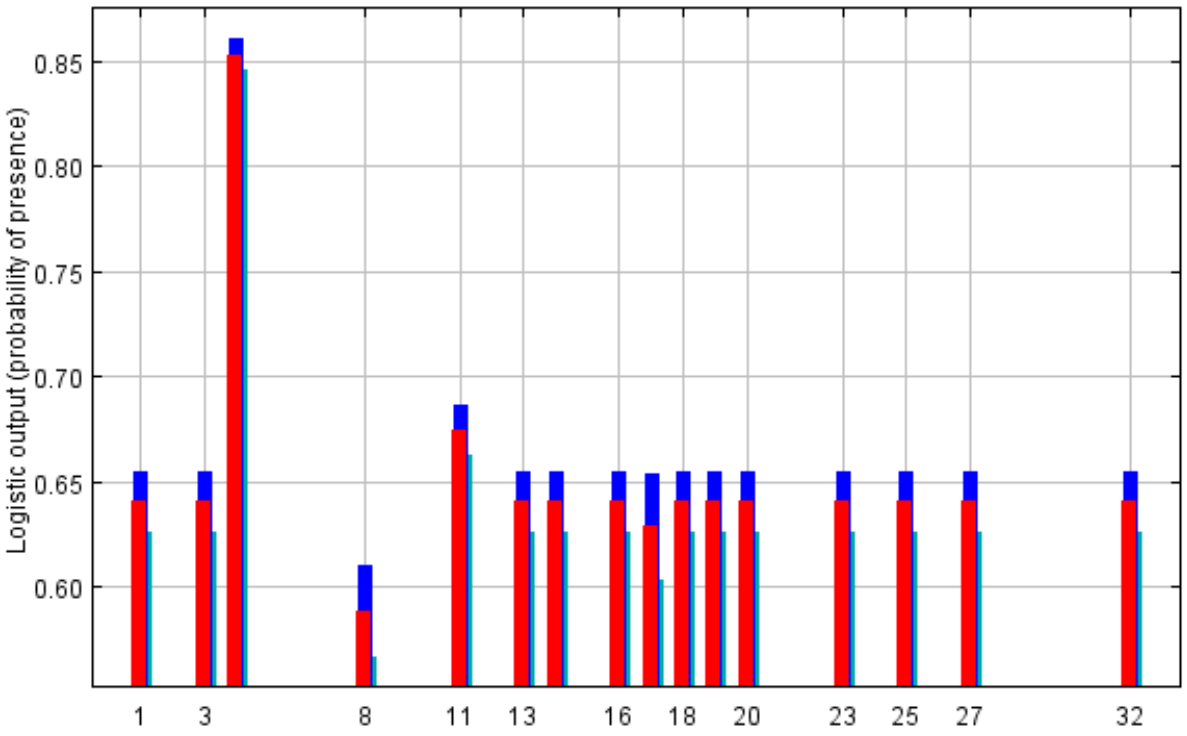

Figure 20. Mean response curve with 58 replicate Maxent runs (red) of environmental variable: landform for the western massasauga, *Sistrurus catenatus tergeminus*. Blue area represent + one standard deviation. X-axis corresponds to unique values found in Table 12

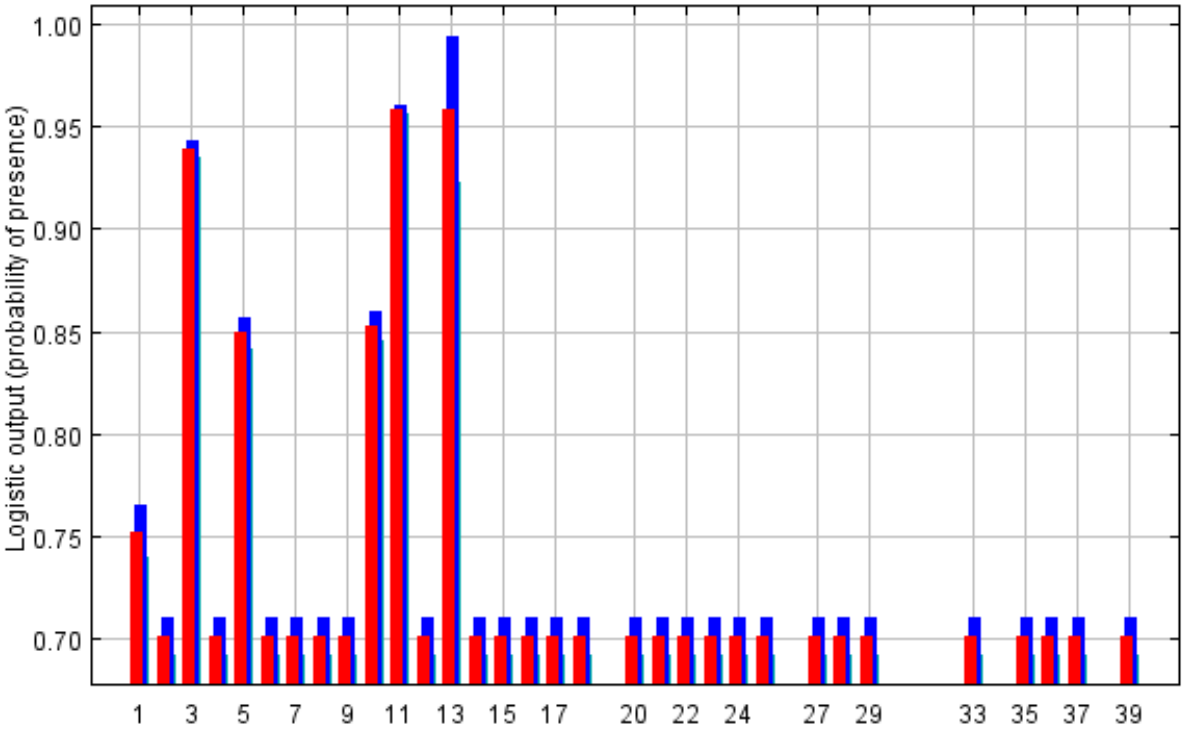

Figure 21. Mean response curve with 58 replicate Maxent runs (red) of environmental variable geology for the western massasauga, *Sistrurus catenatus tergeminus*. Blue area represent + one standard deviation. X-axis corresponds to unique values found in Table 13

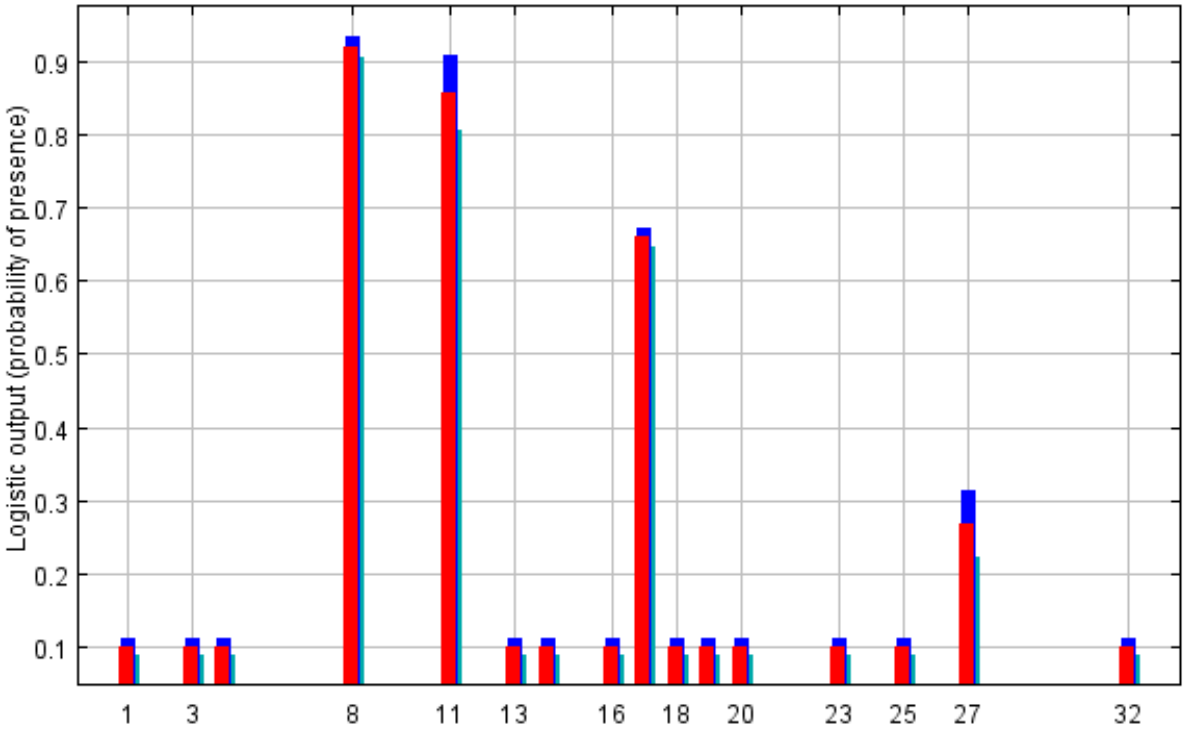

Figure 22. Mean response curve with 58 replicate Maxent runs (red) of environmental variable landform for the desert massasauga, *Sistrurus catenatus edwardsii*. Blue area represent + one standard deviation. X-axis corresponds to unique values found in Table 14

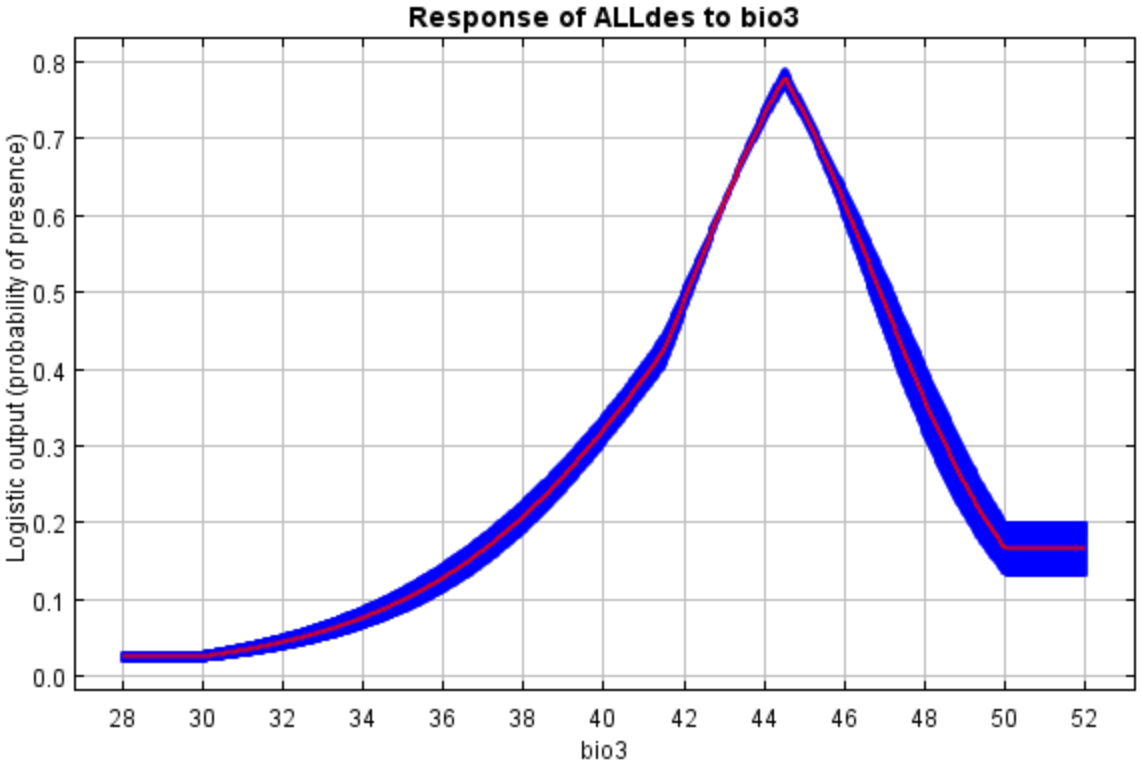

Figure 23. Mean response curve with 58 replicate Maxent runs (red) of environmental variable: isothermality (Mean diurnal temperature range/temperature annual range) for the desert massasauga, *Sistrurus catenatus edwardsii*. Blue area represent +/- one standard deviation

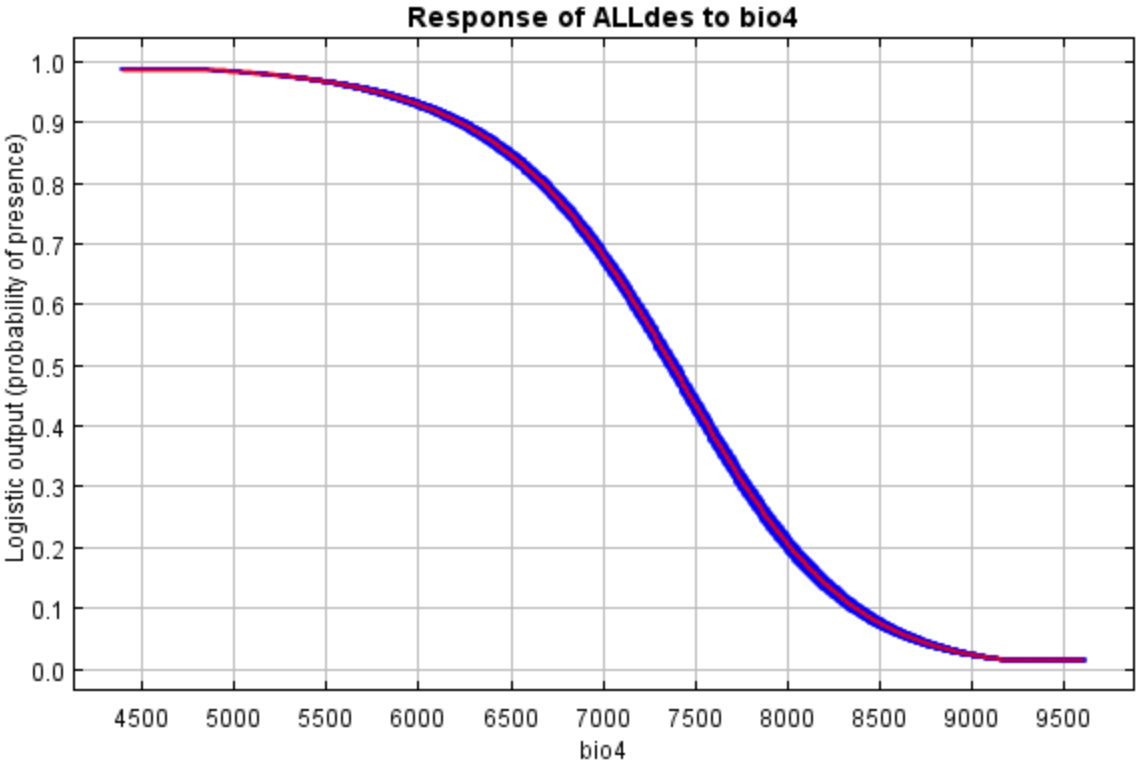

Figure 24. Mean response curve with 58 replicate Maxent runs (red) of environmental variable: temperature seasonality for the desert massasauga, *Sistrurus catenatus edwardsii*. Blue area represent +/- one standard deviation

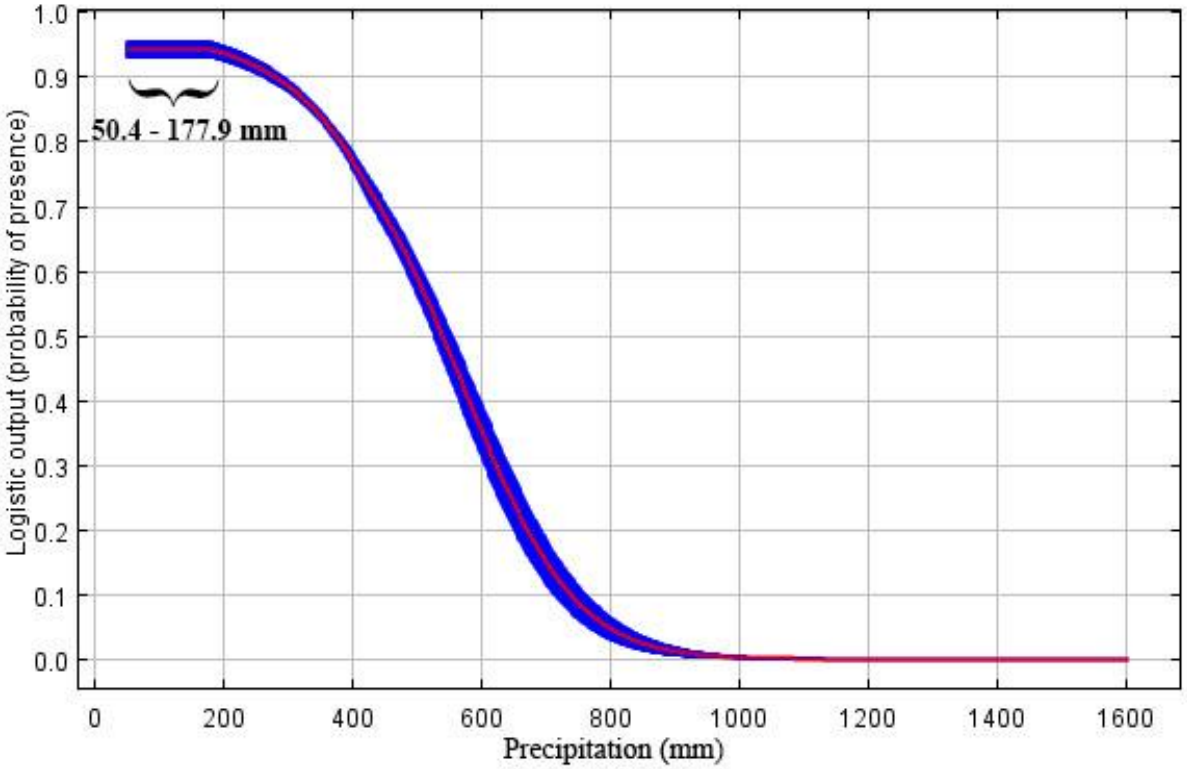

Figure 25. Mean response curve with 58 replicate Maxent runs (red) of environmental variable: annual precipitation for the desert massasauga, *Sistrurus catenatus edwardsii*. Blue area represent +/- one standard deviation

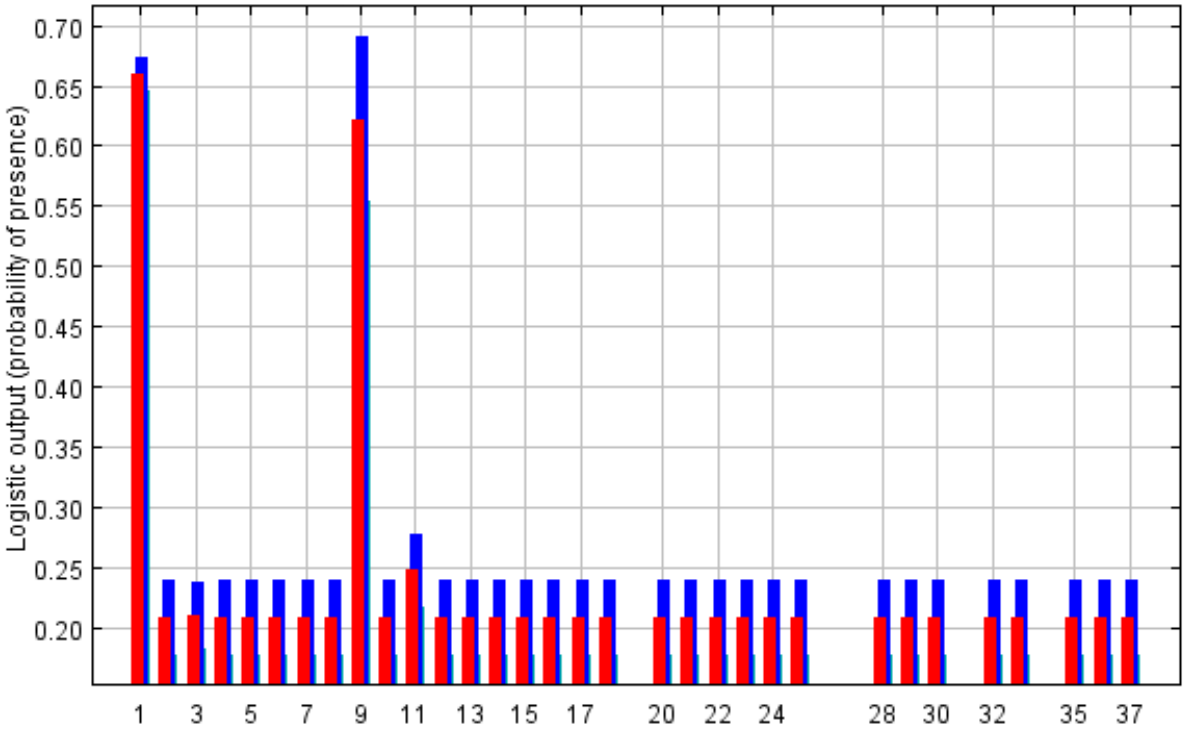

Figure 26. Mean response curve with 58 replicate Maxent runs (red) of environmental variable geology for the desert massasauga, *Sistrurus catenatus edwardsii*. Blue area represent + one standard deviation. X-axis corresponds to unique values found in Table 15

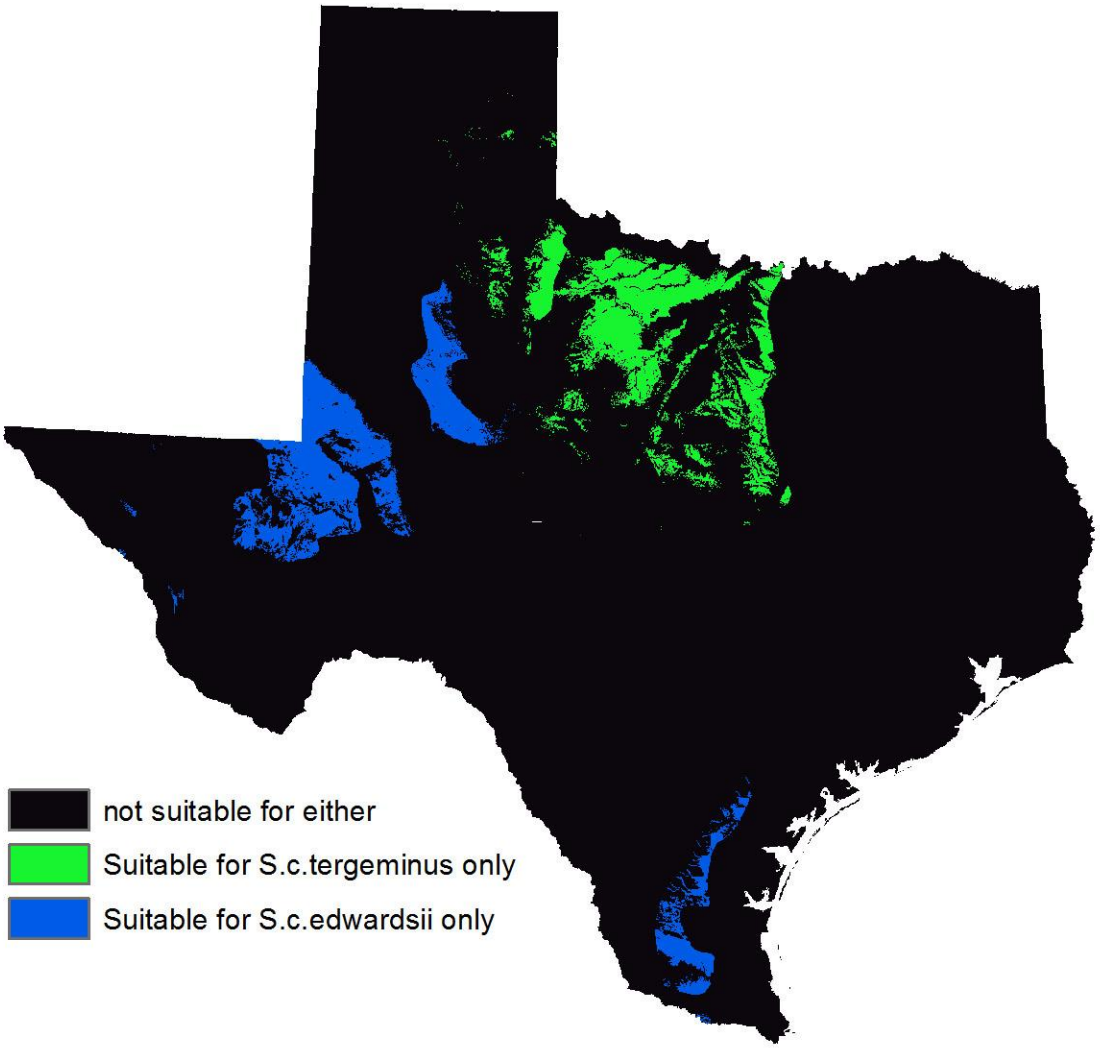

Figure 27. Comparative binary ecological niche model displaying areas of suitable habitat for both the western massasauga, *Sistrurus catenatus tergeminus* and the desert massasauga, *S. c. edwardsii*

Chapter 4

Concluding Remarks

This study has provided very useful insight into the evolutionary history of the massasauga rattlesnake, *Sistrurus catenatus* as well as the usefulness and power of taking an integrative approach to taxonomy and conservation. My study found strong genetic evidence within the mitochondrial DNA sequences to support the previously suggested elevation of the eastern massasauga, *S. c. catenatus*, to its own species separated from the two western subspecies. This is a particularly important change in taxonomy for this species because the elevation to species from subspecies will increase its priority level as per the Endangered Species Act.

Additionally, I found evidence of ecological lineage distinction within the western subspecies complex containing the western, *S. c. tergeminus*, and the desert massasauga, *S. c. edwardsii*. The ecological data shows that these two subspecies are likely undergoing ecological speciation, which is supported by the previous findings of Wooten and Gibbs (2012). Taking into account the recent divergence of these two subspecies, ecological differentiation provides the strongest evidence that *S. c. tergeminus* and *S. c. edwardsii* are representative of two distinct evolutionary lineages within the western *S. catenatus* complex. However, this differentiation is only weakly supported by the mitochondrial DNA and not supported by the nuclear DNA. Mitochondrial intersubspecific divergence estimates show there is some genetic distinction between *S. c. tergeminus* and *S. c. edwardsii*, although this distinction is less clear in the ML gene trees. Therefore, I recommend in order to further elicit the genetic relationship between *S.*

c. tergeminus and *S. c. edwardsii* more sensitive genetic markers such as microsatellites be employed in future research. While I agree with Ryberg et al. (2014) that *S. c. tergeminus* and *S. c. edwardsii* are genetically not divergent enough to consider separate species, I disagree that the subspecies should be collapsed into one. There is some evidence that these two subspecies are genetically distinctive and very strong evidence that they are ecologically divergent.

In conclusion, I believe the eastern massasauga, *S. c. catenatus,* should be elevated to be the sole member of the species *S. catenatus*. This elevation will resurrect the species *S. tergeminus* to represent the two westerns subspecies, reclassifying these subspecies as *S. tergeminus tergeminus* and *S. t. edwardsii*. These subspecific designations, based off the strong ecological evidence, accurately represent a divergence in evolutionary history between *S. t. tergeminus* and *S. t. edwardsii*. Therefore, *S. t. tergeminus* and *S. t. edwardsii,* should remain as viable subspecies and not be collapsed into one species. The decision to keep the subspecific distinction between *S. c. tergeminus* and *S. c. edwardsii* bring with it important biological and conservation decisions. The current petition to afford Federal protection to *S. c. edwardsii* remains valid, whereas, collapsing *S. c. edwardsii* into a single species with *S. c. tergeminus* would likely invalidate the petition. At the very least, collapsing *S. c. edwardsii* into a single species with *S. c. tergeminus* would require the petition to be rewritten and resubmitted, restarting a long evaluation process by the United States Fish and Wildlife Agency. Another important implication is, if or when, any conservation decisions are made in regards to *S. c. tergeminus* or *S. c. edwardsii* they must be treat as biologically distinct entities. These two subspecies respond differently to their environment and are under different selective pressures; therefore, management practices must be matched to the distinct subspecies in question.

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