Genetic determination of the Desert Massasauga distribution in Texas

A report to Texas Parks and Wildlife Department for the Wildlife Conservation Grants program

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Desert Massasauga (*Sistrurus catenatus edwardsii*)
from New Mexico, Lea County

ABSTRACT

The Massasauga (*Sistrurus catenatus*) maintains a fragmented distribution comprised of three subspecies distinguished on the basis of morphological variation and geographic isolation. Recent genetic work supported the distinction of the geographically isolated Eastern Massasauga (*S. c. catenatus*) from both Western (*S. c. tergeminus*) and Desert Massasaugas (*S. c. edwardsii*), but the exact relationships among geographically isolated *S. c. tergeminus* and *S. c. edwardsii* populations remained unresolved due to poor sampling throughout the species range (Kubatko et al. 2011). The unresolved geographic relationship between these subspecies poses a difficult challenge for the conservation and management of this species in Texas where both subspecies exist, because *S. c. tergeminus* has no special state or federal status and *S. c. edwardsii* has been petitioned for listing and is currently under 12-month review for candidacy under the US Endangered Species Act. To address this challenge, we used nuclear and mitochondrial DNA variation to 1) define the geographic relationships between *S. c. tergeminus* and *S. c. edwardsii* in Texas and adjacent states, 2) determine baseline population structure throughout the state and 3) discuss the establishment of potential management units for *S. c. edwardsii* should listing occur. We found strong evidence that *S. c. tergeminus* and *S. c. edwardsii* are genetically indistinguishable for the nuclear and mitochondrial genes investigated. We also found strong evidence supporting earlier conclusions that *S. c. catenatus* is highly divergent from the *S. c. tergeminus-edwardsii* group. Within the *S. c. tergeminus-edwardsii* group, we found some evidence of population structure, which included populations of Massasaugas from 1) Arizona and New Mexico, 2) Colorado and Kansas, 3) Missouri, 4) Oklahoma, and 5) south Texas. These 5 distinct population segments could be considered for listing, but with no clear evidence suggesting relationships among these disjunct populations, we recommend that more research using other molecular markers (e.g., SNP's, microsatellites) be conducted to provide a measure of genetic connectivity capable of revealing more detailed taxonomic and population level structure for identifying potential conservation units. Regardless of federal ruling, we feel that the overall rarity of Massasaugas in south Texas and their geographic isolation from other populations in the *S. c. tergeminus-edwardsii* group means that they deserve continued attention. We recommend continued survey efforts in this region to provide information on the distribution and abundance of this Massasauga population and to monitor changes to its habitat over time.

INTRODUCTION

The Massasauga (*Sistrurus catenatus*) is one of two species in the genus *Sistrurus*. Historically, the Massasauga is known from fragmented populations in southern Ontario, Canada across parts of the Midwest and Great Plains south to several isolated (disjunct) populations in Texas and west to several more isolated populations in New Mexico and Arizona (Fig. 1). Within this fragmented distribution, three subspecies have been distinguished on the basis of morphological variation in scale characters, body size and coloration, and geographic distribution: the Eastern Massasauga (*S. c. catenatus*), the Western Massasauga (*S. c. tergeminus*), and the Desert Massasauga (*S. c. edwardsii*) (Gloyd 1940, 1955, reviewed in Mackessy 2005). *Sistrurus catenatus catenatus* ranges from Illinois east to central New York (Fig. 1) and is distinguished from the other two subspecies by its dark ventral coloration, lower number of ventral scales and lower number of dorsal blotches. *Sistrurus catenatus tergeminus* ranges from Missouri west into Nebraska and south through Kansas and Oklahoma into Texas

(Fig. 1). *Sistrurus catenatus tergeminus* is larger, darker in color, and has higher numbers of ventral scales and dorsal blotches than *S. c. edwardsii*, which ranges northwest from southern Texas up through New Mexico to Colorado and west to Arizona (Fig. 1).

Recently, a large number of phylogenetic studies were conducted to further unravel the geographic relationships among populations of these three currently accepted subspecies (Anderson et al. 2009, Chiucchi and Gibbs 2010, Gibbs et al. 2011, Kubatko et al. 2011, Gibbs and Chiucchi 2012, Ray et al. 2013, DiLeo et al. 2013). To date, only Kubatko et al. (2011) investigated relationships between all three subspecies. Testing for lineage and taxonomic distinctiveness, Kubatko et al. (2011) presented evidence supporting the genetic distinctiveness of all subspecies (Fig. 1). The strongest support, however, was observed for the separation of *S. c. catenatus* from both *S. c. tergeminus* and *S. c. edwardsii*. The geographic point of genetic distinctiveness between these subspecies coincided with the Mississippi River, a result which was later corroborated by Gibbs et al. (2011). The Mississippi River has been associated with splits in many other vertebrate species (see Soltis et al. 2006 for review) including some snakes (e.g., Burbrink et al. 2000; Burbrink 2002). Based on these genetic results, morphological differences, and the allopatric distributions of each subspecies, Kubatko et al. (2011) concluded that elevation of *S. c. catenatus* to full species status was warranted.

Support for the distinction between *S. c. tergeminus* and *S. c. edwardsii* was relatively weak compared to *S. c. catenatus*. This weaker distinction was thought to result from the lack of a large-scale geographical feature that could act as an isolating barrier in north central Texas, where the distributions of *S. c. tergeminus* and *S. c. edwardsii* nearly overlap (Fig. 1; Campbell and Lamar 2004). Indeed, several other reptile species maintain continuous distributions through this area and show weak to no genetic discontinuities (Burbrink 2002; Leach´e and Reeder 2002; Leach´e and McGuire 2006; but see Fontanella et al. 2008). Alternatively, the weaker distinction between these subspecies might also be an artifact of incomplete sampling. Kubatko et al. (2011) acknowledged the geographic scope of their sampling was limited for those subspecies, possibly weakening the power to detect phylogeographic structure. On the other hand, this incomplete sampling might also cause an overestimation of genetic distinctiveness between these subspecies, because samples are completely lacking from the geographic area that represents the subspecies boundary in Texas (Leach´e 2009). If undetected gene flow has lead to the sharing of genotypes across the north central Texas region, then the observed weak genetic distinction between *S. c. tergeminus* and *S. c. edwardsii* could be overestimated. If substantial gene flow has gone undetected, then these presumed subspecies could actually be part of a large collection of patchy populations.

The lack of samples from Texas in this study also leaves the taxonomic affinity of the south Texas population of Massasaugas unresolved. There are several competing hypotheses concerning the geographic relationships of these subspecies in south Texas as inferred from morphological characters. Along with Werler and Dixon (2000), Kubatko et al. (2011) identify that population as part of the *S. c. edwardsii* distribution (Fig. 1). Alternatively, Stebbins (1980) predicts the disjunct south Texas population is part of the *S. c. tergeminus* distribution. Finally, Klauber (1982) and Conant and Collins (1998) suggest that both subspecies maintain populations within that disjunct portion of the Massasauga's distribution.

These unresolved geographic relationships among subspecies pose difficult challenges for the conservation and management of this species in Texas and elsewhere, because each subspecies maintains a different threatened or endangered designation (Mackessy 2005). For example, *S. c. tergeminus* has no federal status, but is considered state threatened in Missouri.

Sistrurus catenatus edwardsii has been petitioned for listing (currently under 12-month review for candidacy) under the US Endangered Species Act (ESA) (US Federal Register August 9, 2012), and is protected in Arizona and listed as a species of concern in Colorado (designation confers no protection). *Sistrurus catenatus catenatus* has already been named by the US Fish and Wildlife Service as a candidate species for listing under the US Endangered Species Act (ESA) (listing priority number = 9; US Federal Register 1999) due to significant population declines, habitat destruction and degradation (Szymanski 1998), and because the observed reproductive isolation makes it a "distinct population segment" (Kubatko et al. 2011). Finally, Texas, New Mexico, Oklahoma, and Kansas lack any protections for Massasaugas, but they do require hunting permits or licenses for collecting.

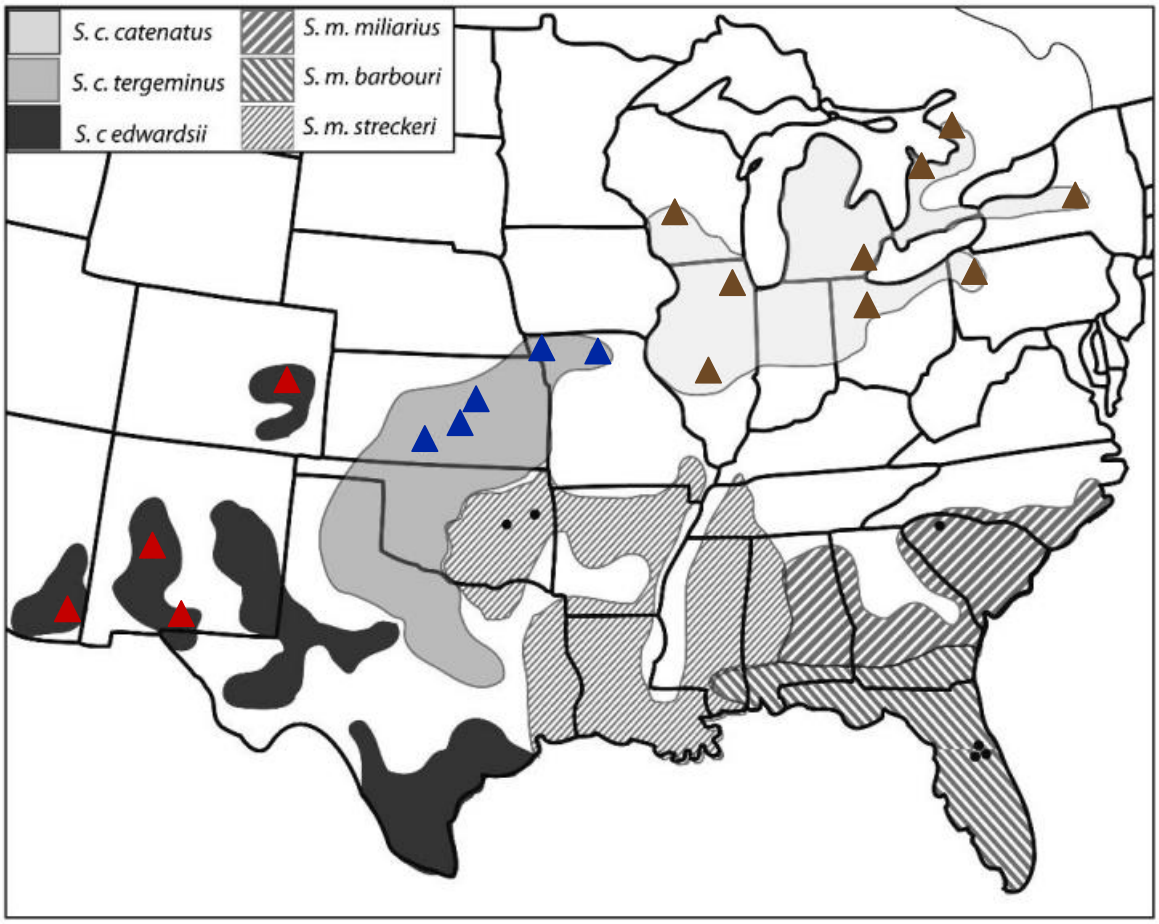


Figure 1. Map showing the geographic distribution of each subspecies of *Sistrurus* and the approximate locations of samples used in analyses from Kubatko et al. (2011). (A) Triangles mark locations of sampled Desert (Red, *S. c. edwardsii*), Western (Blue, *S. c. tergeminus*), and Eastern (Brown, *S. c. catenatus*) subspecies of Massasauga.

If *S. c. edwardsii* is declared a candidate for listing, then designation of critical habitat for the subspecies will be proposed under section 4 of the ESA. Determining the subspecific identity of Massasauga populations in Texas and adjacent states will be the first step toward initiating a conservation and management plan for the species and critical habitat throughout its range. In this study, we take this first step by using nuclear and mitochondrial DNA variation to

1) define the geographic relationships between *S. c. tergeminus* and *S. c. edwardsii* in Texas and adjacent states, 2) determine baseline population structure throughout the state and 3) discuss the establishment of potential management units for *S. c. edwardsii* should listing occur.

METHODS

Data Collection

We conducted a state-wide survey for Massasaugas in Texas, where the distributions of *S. c. tergeminus* and *S. c. edwardsii* are predicted to nearly overlap. We used day and night road searches to find dead Massasaugas along roads proven to have a strong and continuous history of museum collecting records (Werler and Dixon 2000). Road searching is regarded as the most efficient way to collect specimens and tissues in this species, because Massasaugas have a tendency to bask on roads, which increases the likelihood of fatal encounters with cars (Werler and Dixon 2000, Mackessy 2005). Thirty-six road searches were conducted by the principal investigators in this study between March and August 2013, although many more road searches were conducted by our contacts from collections and museums, herpetological societies and events (e.g., SnakeDays), and social media (e.g., iNaturalist – Herps of Texas, Facebook). These contacts formed a 'Sauga' network that dramatically extended our survey reach and sampling extent throughout the state. To supplement tissues collected from road searches, we also queried natural history collections and museums for existing Massasauga tissues from Texas, New Mexico, Oklahoma, and Kansas.

DNA extraction, sequencing, and analysis

We selected for amplification two genes, one mitochondrial and one nuclear, that previously indicated usefulness in intraspecies delimitation of Massasauga subspecies (Kubatko et al. 2011). The mitochondrial genes, *ATP synthase 6 and 8*, amplified as a single fragment due to overlapping gene fragments (*ATP*~665 bps) (8.2 L8331, CO3.2 H9236) (Bermingham and Martin, 2009). The nuclear intron, *TATA box binding protein gene* (*TBP*~796 bps), was amplified via intron crossing EPIC primers TRIMTBP13F, TRIMPTBP13R (Creer et al. 2005) and has previously been identified as a rapidly evolving marker (Douglas et al. 2002).

Samples for tissues and shed skin were collected from New Mexico (n=7 individuals across 6 counties), Oklahoma (n=3 across 3 counties), and Texas (n=41 across 16 counties). This sampling was used along with previous sampling for *S. c. catenatus*, *S. c. tergeminus*, *S. c. edwardsii*, and *Agkistrodon* outgroups (Kubatko et al. 2011). DNA was extracted using E.Z.N.A Tissue Extraction Kit and standard protocols (Omega Bio-Tek, Norcross, GA). Polymerase chain reaction (PCR) was used for each primer set with a high-fidelity taq polymerase, Platinum Taq (Invitrogen). Automated sequencing was performed using BigDye (Applied Biosystems) and products were run out on an ABI 377 sequencer.

Sequences were verified and aligned by eye using Sequencher 4.10.1 (Gene Codes). We used the PHASE 2.1 implementation for haplotype reconstruction of diploid gametic alleles (Stephens et al. 2001) using DnaSP 5.10.1 (Librado and Rozas, 2009) for the *TBP* intron with a 1000 burn-in, 10 thinning intervals, and 1000 iterations. The most appropriate models of evolution were determined using both MrModletest 2.3 (Nylander, 2004) and jModelTest2 (Darriba et al. 2012; Guindon and Gascuel, 2003). For the *TBP* gene, the most appropriate model as selected by both Akaike information criterion (AIC) and Bayesian information criterion (BIC) criterion was HKY I+G. For the *ATP* gene, the model selected as most appropriate by BIC

criterion was HKY+G. Single-gene phylogenies were assessed using a maximum likelihood (ML) and Bayesian inference framework. ML was assessed using PAUP* 4.0b.10s (Swofford, 2002) with bootstrap support calculated with 1000 replicates. Maximum likelihood analysis was also implemented using Garli v2.01 (Zwickl, 2006). Bayesian inference was conducted using MrBayes for each gene (Ronquist and Huelsenbeck, 2003) for two Monte Carlo Markov Chain (MCMC) analyses (Geyer, 1991), which were run for 10 million generations and sampling every 1000. Convergence was assessed using Tracer for appropriate burn-in at 2000 runs (20%) for analyses of both genes independently (Rambaut, 2007). Median-joining haplotype networks were constructed using Network 4.611 (Fluxus-engineering.com).

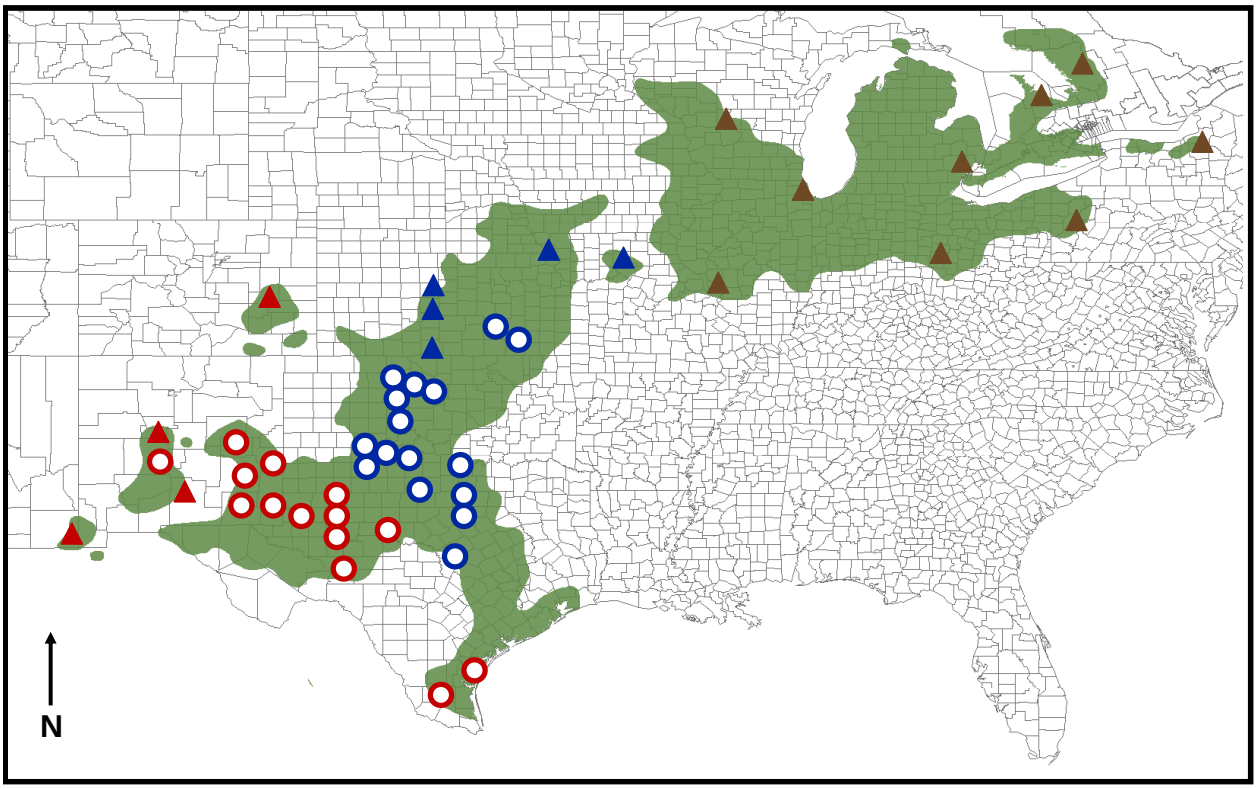


Figure 2. Map showing historic Massasauga distribution (green, IUCN database) and sample localities from Kubatko et al. 2011 (triangles) and this study (circles). Brown, red, and blue symbols are predicted to be samples from populations of *Sistrurus catenatus catenatus*, *S. c. edwardsii*, and *S. c. tergeminus*, respectively (Kubatko et al. 2011). Note that Massasauga populations are not continuous throughout the entire area shaded.

RESULTS

We obtained 55 samples from road surveys, the 'Sauga' network, and museums. Twenty percent (n = 11) of our samples were provided by the network, 78% (n = 43) came from existing museum collections and the collectors associated with them, and one sample was provided by the National Natural Toxins Research Center (NNTRC) at Texas A&M University-Kingsville (Appendix 1). These samples were distributed across 17 counties in Texas (n = 41), 6 New Mexico counties (n = 7), 5 Oklahoma counties (n = 5), and 2 Kansas counties (n = 2) (Fig. 2). No samples were obtained from populations connecting north-central and south Texas. The last

known specimen from this region was collected in 1922, near Rock Island in Colorado County, Texas, which suggests a recent break in this portion of the Massasauga's historical distribution.

Phylogenetic analysis of *DNA variation*

Phylogenetic analyses indicated that *S. c. edwardsii* and *S. c. tergeminus* are indistinguishable at the *ATP* mtDNA region. Median-joining haplotype network analysis revealed 3 discrete haplogroups including 1) *S. c. catenatus*, 2) *S. c. edwardsii* and *S. c. tergeminus*, and 3) *Agkistrodon* (outgroup) (Fig. 3). Consistent with Kubatko et al. (2011), the *S. c. catenatus* haplogroup exhibited high sequence divergence (10.9%) from the *S. c. edwardsii* and *S. c. tergeminus* haplogroup. The *ATP* network resulted in a conserved number of haplotypes with 34 observed. All 64 *S. c. edwardsii* and *S. c. tergeminus* samples from this study and Kubatko et al. (2011) comprised one haplogroup with a high degree of shared haplotypes (ancestral haplotypes central). Specifically, one broadly shared ancestral haplotype was found among 20 individuals from across Texas and one individual from Oklahoma. Many unique (recent) haplotypes (1-4 mutational changes) were also observed for *S. c. tergeminus* and *S. c. edwardsii* suggesting the presence of population-level structuring.

Maximum likelihood and Bayesian tree-based analyses recovered similar overall topologies for both genes, *ATP* and *TBP*. Analyses for *ATP* generated two clear clades composed of a highly supported *S. c. catenatus* clade (1.0 posterior probability and 100% ML bootstrap support) and a well-supported clade consisting of *S. c. tergeminus* and *S. c. edwardsii* (100% ML bootstrap support) with the latter group resulting in a polytomy (Fig. 4). Sample sequences of *S. c. catenatus* from Kubatko et al. (2011) were used as the outgroup in this analysis due to the deep divergence observed in preliminary analyses from the *S. c. edwardsii* and *S. c. tergeminus* clade. There was no clear evidence supporting genetic differentiation of the *S. c. edwardsii* and *S. c. tergeminus* subspecies. However, there was significant support for several discrete inner clades which grouped populations from Arizona/New Mexico, Colorado/Kansas, Missouri, Oklahoma, and South Texas (all with 100% ML bootstrap support).

The *TBP* network revealed high haplotype diversity with 66 observed haplotypes across 4 distinct haplogroups: 1) *S. c. catenatus*, 2) and 3) *S. c. edwardsii* and *S. c. tergeminus*, and 4) *Agkistrodon* (outgroup) (Fig. 5). The *S. c. catenatus* haplogroup was again distinct from the *S. c. edwardsii* and *S. c. tergeminus* haplogroups, although with less sequence divergence compared to the mtDNA network. The 64 *S. c. edwardsii* and *S. c. tergeminus* samples were split between two haplogroups indicating that these two subspecies share many haplotypes (n=39 individuals across 9 haplotypes). Many uniquely divergent haplotypes (mutational steps 2-17) from both *S. c. edwardsii* and *S. c. tergeminus* samples were also split between the two haplogroups. Some of these unique haplotypes shared across the two haplogroups were individual gametic haplotypes (n=9 individuals).

Tree-based analyses for *TBP* using ML and Bayesian inference also revealed two distinct clades with strong support. Both *S. c. tergeminus* and *S. c. edwardsii* were found in each clade while *S. c. catenatus* was found in one (Fig. 6). Both major clades exhibited high posterior probability support (respectively 0.94 and 1.0). Individual gametic haplotypes, however, were shared across this major clade division (n=9 individuals). All together, these data indicate that *S. c. edwardsii* and *S. c. tergeminus* are indistinguishable at both the *TBP* nuclear and *ATP* mtDNA genes.

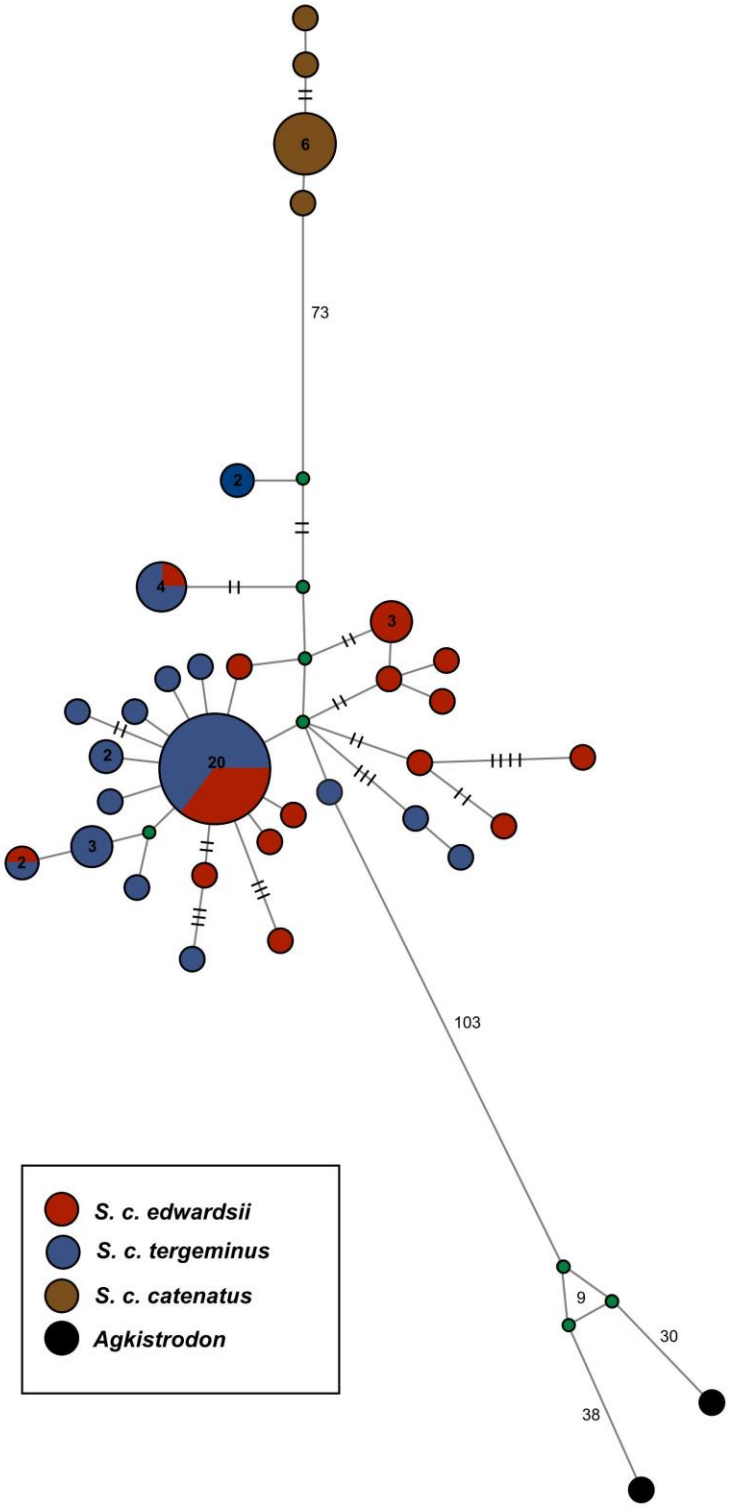


Figure 3. Median-joining network of the observed 34 haplotypes for *ATP* mitochondrial gene sequences. Circle sizes are proportional to frequencies of haplotypes. Green circles indicate missing intermediates (unsampled). Cross hatches represent mutational steps with all greater then 4 denoted by the number.

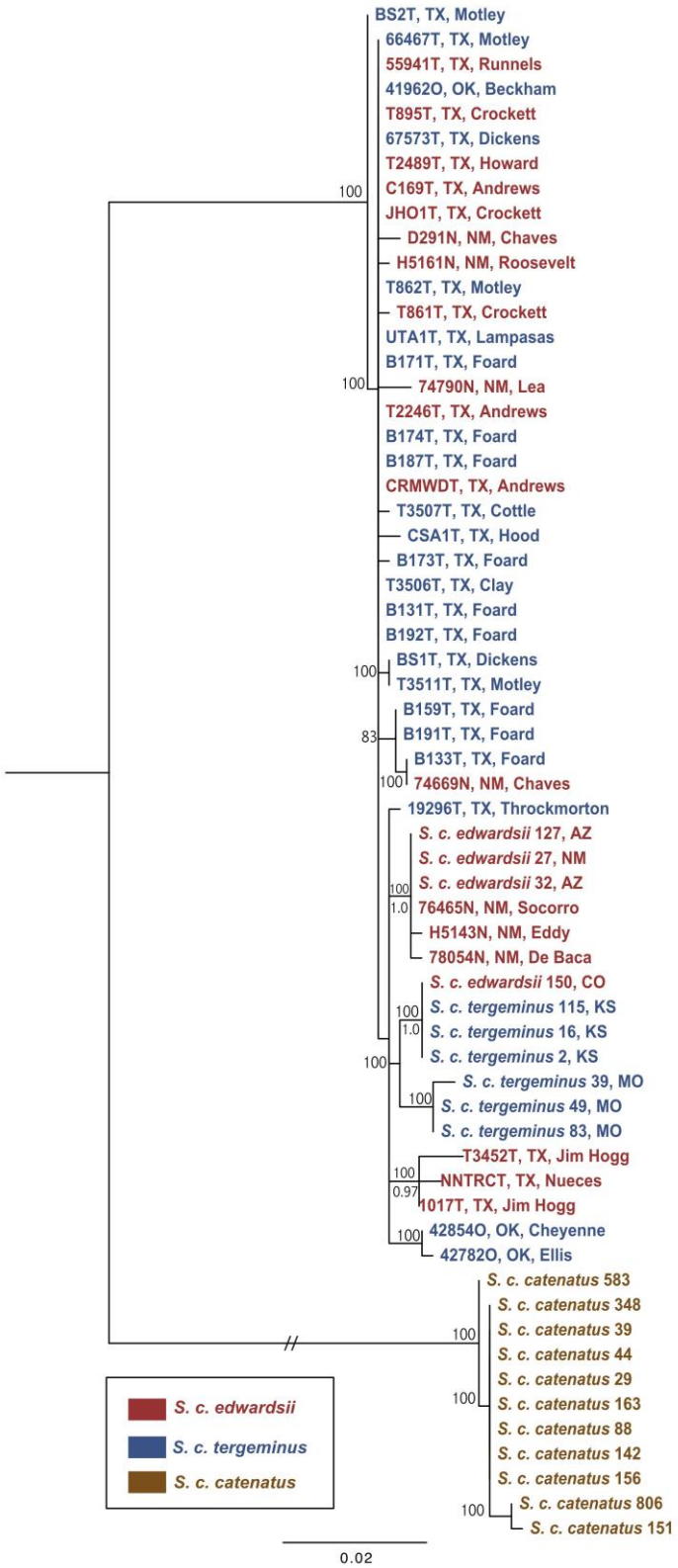


Figure 4. Bayesian analysis of the *Sistrurus catenatus* mtDNA *ATP* gene. Number above the node are ML bootstrap support values. Numbers below the node are posterior probabilities values.

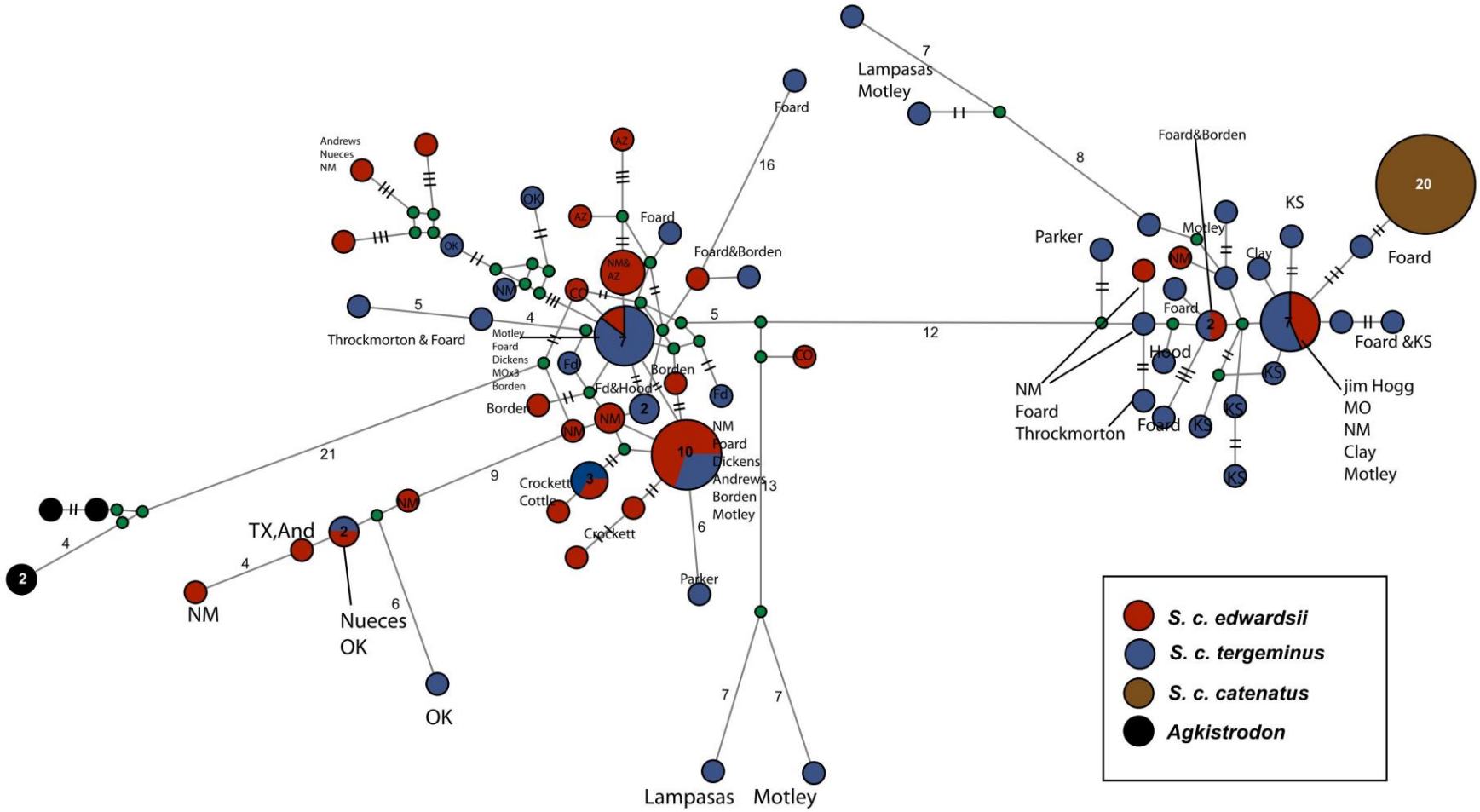
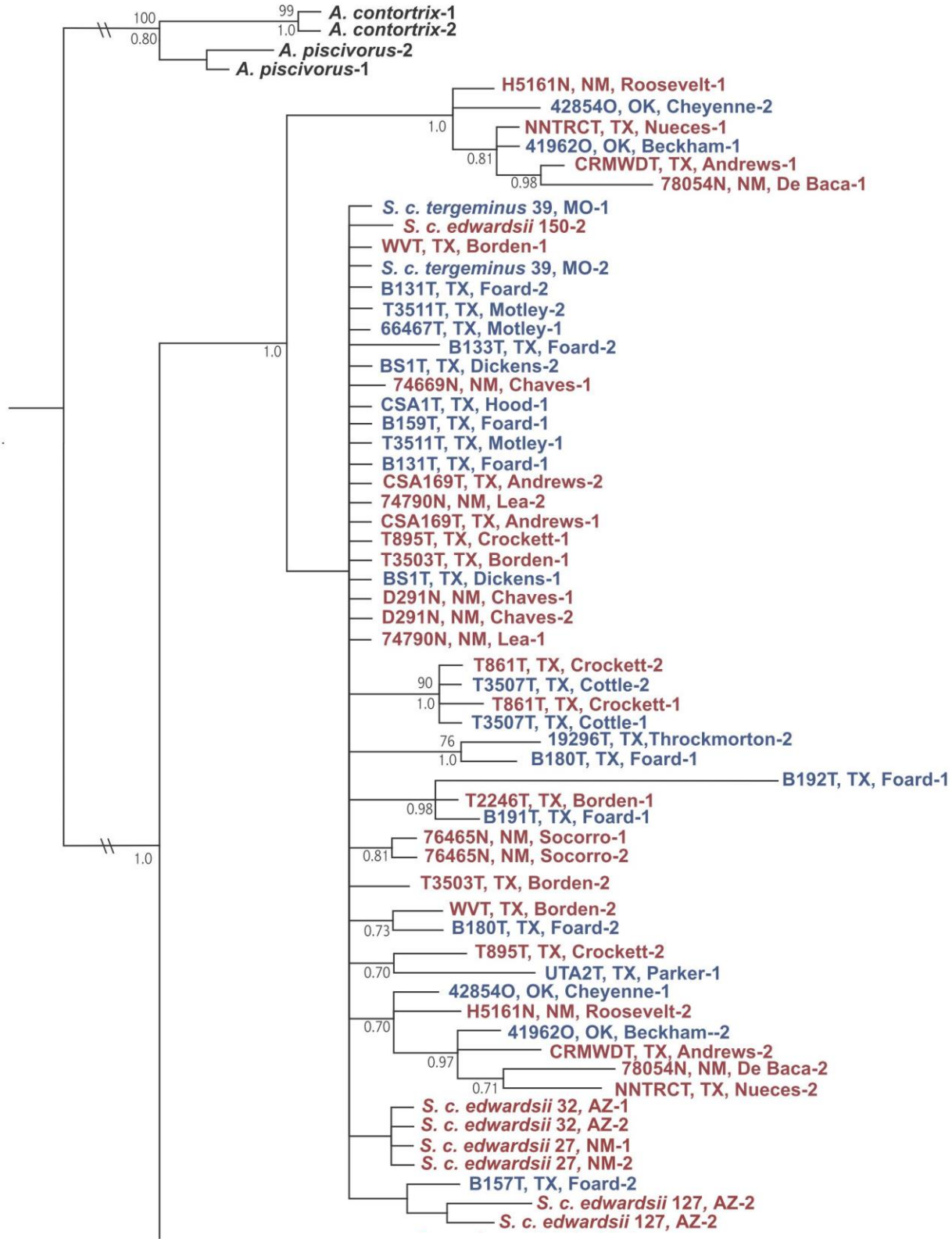


Figure 5. Median-joining network of the observed 72 haplotypes for TBP nuclear gene sequences. Circle sizes are proportional to frequencies of haplotypes. Green circles indicate missing intermediates (unsampled). Cross hatches represent mutational steps with all greater then 3 denoted by the number.



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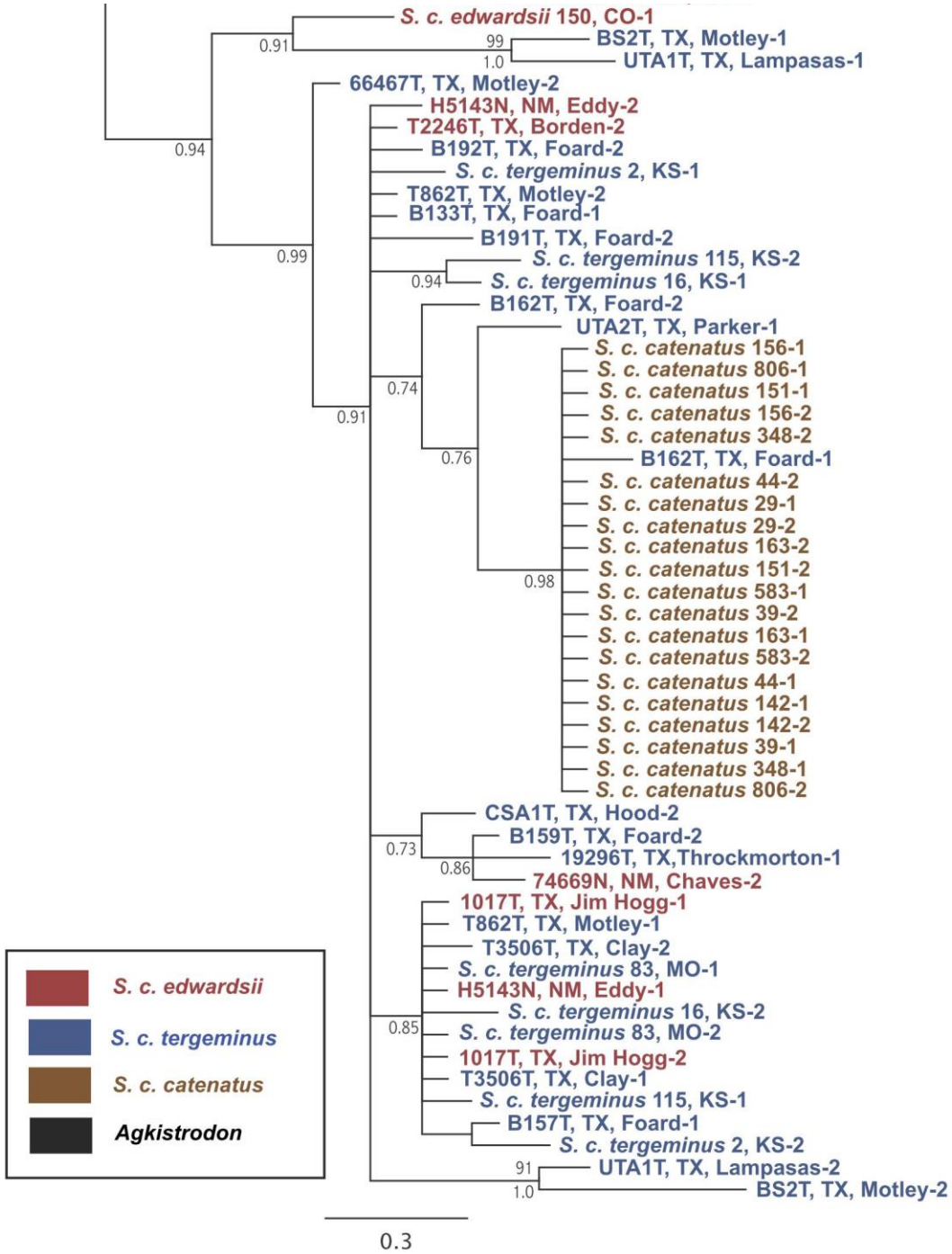

Figure 6. Bayesian analysis of the nuclear intron *TBP* gene data. Number above the node are ML bootstrap support values. Numbers below the node are posterior probabilities values.

DISCUSSION

In this study, we provide strong evidence that Massasaugas west of the Mississippi River that are currently classified as *S. c. edwardsii* and *S. c. tergeminus* are genetically indistinguishable at both the *TBP* nuclear and *ATP* mtDNA genes. We also provide strong evidence that Massasaugas east of the Mississippi River identified as *S. c. catenatus* are highly divergent from both western forms. Our results corroborate the major conclusion of Kubatko et al. (2011), who found clear genetic evidence across 18 nuclear and one mitochondrial gene that *S. c. catenatus* was a phylogenetically distinct lineage relative to *S. c. tergeminus* and *S. c. edwardsii* and suggested it should be elevated to full species status under a genealogical species concept. Using the same mtDNA *ATP* gene from that study, but more thorough sampling throughout the Massasauga's range, our study revealed > 10% sequence divergence between *S. c. catenatus* and both western forms. As such, we support suggestions from Kubatko et al. (2011) to elevate the status of *S. c. catenatus* to full species.

Kubatko et al. (2011) also found weak evidence for the taxonomic distinctiveness of *S. c. tergeminus* and *S. c. edwardsii* from each other. Using the same two genes from that study, but more thorough geographic sampling around the predicted point of contact between both subspecific distributions, our study uncovered few genetic differences among these subspecies and therefore refuted this conclusion. The taxonomic distinctiveness of *S. c. tergeminus* and *S. c. edwardsii* reported in Kubatko et al. (2011) was largely supported by the monophyly of *S. c. edwardsii* for the *TBP* nuclear gene, although the remaining 17 nuclear genes and the single mitochondrial gene all indicated the *S. c. edwardsii* group was not monophyletic. Our results illustrated that *S. c. edwardsii* was indeed not monophyletic for *TBP* as well, which suggests that the monophyly observed in Kubatko et al. (2011) for the *TBP* gene was possibly a result of sample bias. Given the results of both Kubatko et al. (2011) and this study, evidence was strongest for *S. c. catenatus* being distinct from the other two subspecies and for *S. c. tergeminus* and *S. c. edwardsii* being indistinguishable from each other. Gibbs et al. (2011) suggested this pattern of divergence is likely due to the influence of differences in the ages of each clade (*S. c. catenatus*:*3.0 million ybp; *S. c tergeminus* and *edwardsii*:*0.5 million ybp) and its effect on the genetic distinctiveness of each taxa through the influence of retained ancestral polymorphism, as seen in the degree of shared haplotypes across broadly distributed individuals in this study.

Within the *S. c. tergeminus-edwardsii* group, we found some evidence of limited population structure. In the *ATP* mtDNA haplotype network, we observed 27 unique haplotypes that were 1-4 mutational changes from ancestral shared haplotypes. We also found strong support for several inner clades in the *ATP* mtDNA maximum likelihood tree. These clades included populations of massasaugas from 1) Arizona and New Mexico, 2) Colorado and Kansas, 3) Missouri, 4) Oklahoma, and 5) south Texas. The remaining populations of Massasaugas from north-central and west Texas, as well as some populations from New Mexico and Oklahoma, comprised a large polytomy which lacked well-supported inner clades. This evidence of population structure is consistent with results from past research using microsatellite markers to assess population-level variation in Massasaugas. In populations of *S. c. edwardsii* from Arizona and New Mexico, Anderson et al. (2009) observed high genetic differentiation and concluded that individual populations can have high conservation value for the species. Chiucchi and Gibbs (2010) also observed high genetic differentiation among 19 populations (even populations less than 7 km apart) of *S. c. catenatus* throughout its range. From these patterns of genetic variation described in both microsatellite studies and our study, we conclude

that this species existed historically as a large collection of small isolated populations, which has recently become fragmented by long-term, climate-driven changes in habitat and anthropogenic habitat loss (Mackessy 2005).

The most significant conservation implication of these results is that *S. c. tergeminus* and *S. c. edwardsii* were genetically indistinguishable. This finding complicates the petition for listing *S. c. edwardsii* as endangered under the ESA (US Federal Register August 9, 2012), because no reliable sub-specific distribution can be produced to guide conservation and the designation of critical habitat under section 4 of the Act should listing be warranted. The USFWS could decide to name the entire *S. c. tergeminus-edwardsii* group as a candidate for listing, because distribution data for the collective group is readily available. While this action would offer protection for the entire species given the candidate status of *S. c. catenatus*, we believe it is extremely unlikely and offer two alternative outcomes for the proposed listing of *S. c. edwardsii* given the results of this study. Under the first scenario, the *S. c. edwardsii* listing is not warranted, but the species (*S. c. tergeminus-edwardsii*) likely retains existing state protection and possibly gains protected status in those states currently lacking protection (e.g., Texas, New Mexico, Oklahoma, and Kansas). Under the second scenario, the *S. c. edwardsii* listing is not warranted, but several distinct population segments of the *S. c. tergeminus-edwardsii* group in Colorado, Kansas, and Oklahoma are listed. This scenario comes directly out of the petition to list *S. c. edwardsii* that was submitted to USFWS by WildEarth Guardians on October 28, 2010. In this petition, WildEarth Guardians first sought listing of the entire subspecies, but also requested listing of a distinct population segment of *S. c. edwardsii* in Colorado, Kansas, and Oklahoma if listing the entire subspecies was not warranted. Given the population genetic structure observed in this study, we recognize at least 5 distinct population segments of the *S. c. tergeminus-edwardsii* group that could be listed, if listing the entire subspecies is not warranted. Those distinct segments include currently disjunct populations from 1) Arizona and New Mexico, 2) Colorado and Kansas, 3) Missouri, 4) Oklahoma, and 5) south Texas. With no clear evidence suggesting relationships among these disjunct populations, however, we recommend that future research use other molecular markers (e.g., SNP's, microsatellites) to provide a measure of genetic connectivity capable of revealing more detailed taxonomic and population level structure for identifying potential conservation units.

Across the range, population sizes and trends for *S. c. edwardsii* are largely unknown (but see Mackessy 2005 for CO populations). However, through our survey efforts we have anecdotal data that indicate this species is common in some localities and extremely rare others. For Texas in particular, several collectors searching in north-central and west Texas found multiple individuals in a single night or over several consecutive nights of searching in 2013. These collectors describe this searching success as consistent with past years in those areas. Alternatively, a single collector from south Texas found 2 individuals this year after regularly searching for the last 17 years with only one observation. We lack such anecdotal data from the other distinct population segments listed above, although the petition provides evidence that *S. c. edwardsii* has undergone some range reduction over time as a result of population declines in those portions of its range. In addition, information is presented that indicates these population declines are associated with habitat degradation from land conversion to cultivated croplands and heavy livestock grazing as well as heavy road mortality (US Federal Register August 9, 2012). The disjunct south Texas population segment occupies a region of the state where such land use practices are common, often on large, privately owned ranches 10,000 to 100,000 acres or more. If USFWS determines that listing (entire subspecies group or population segments) is warranted,

we anticipate that conservation easement agreements with private property owners in this region are likely to be a productive means of providing broad protection for this disjunct population in the state. Regardless of federal ruling, we feel that the overall rarity of these snakes in south Texas and the fact that they appear to have recently (~100 ybp) undergone geographic isolation from other populations in the *S. c. tergeminus-edwardsii* group throughout the state means that they deserve continued attention. We strongly recommend continued survey efforts in this region to provide baseline information on the distribution and abundance of this Massasauga population and to monitor potential changes to its habitat over time.

Acknowledgments

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Appendix 1. List of Massasauga samples collected from road surveys, the 'Sauga' network, and museums.									
Tree Label	Museum-Collector Number	State	County	Latitude	Longitude	Locality	Date	Museum-Collector	Contact Method
67573	67573	TX	Dickens	33.74831	-100.65032		5/29/2007	TNHC	HerpNET
1017T	1017	TX	Jim Hogg	-	-	South of Hebbronville on 1017	-	T. Petty	Facebook
19296T	19296	TX	Throckmorton	-		unknown	-	UTEP	HerpNET
2612O	OCGR2612	OK	Blaine	-	-	~2.5 mi S of Hitchcock on Hwy 8, E side of Roman Nose SP	-	OMNH	HerpNET
2613O	OCGR2613	KS	Butler	-	-	unknown	-	OMNH	HerpNET
2682O	OCGR2682	OK	Dewey	-	-	1.8 mi S of Leedey, jct. Hwy 47 and Hwy 34	6/17/2006	OMNH	HerpNET
2683O	OCGR2683	KS	Elk	-	-	unknown	-	OMNH	HerpNET
41962O	41962	OK	Beckham	-	-	Sandy Sanders Wildlife Management Area	-	OMNH	HerpNET
42782O	42782	OK	Ellis	-	-	Packsaddle WMA	-	OMNH	HerpNET
42854O	42854	OK	Cheyenne	-	-	2.9 mi E Cheyenne	-	OMNH	HerpNET
55941T	55941	TX	Runnels	-	-	Ballinger, SE of near the Colorado River	5/1/1993	TNHC	HerpNET
66467T	66467	TX	Motley	-	-	TX FM 97, 10.3 rd mi E TX FM 1065	5/28/2004	TNHC	HerpNET
67573T	TJH2489	TX	Howard	-	-	unknown	-	TCWC	HerpNET
74669N	MSB74669	NM	Chaves	33.88370	-103.95517		-	MSB	HerpNET
74790N	MSB74790	NM	Lea	32.52560	-103.09759		-	MSB	HerpNET
76465N	MSB76465	NM	Socorro	-	-	Highway 60, east of Bernardo	-	MSB	HerpNET
78054N	MSB78054	NM	De Baca	34.16701	-103.99699		-	MSB	HerpNET

B131T	BCB131	TX	Foard	34.01068	-99.91764		3/27/2008	B. Bowers	TCWC collector
B133T	BCB133	TX	Foard	34.01272	-99.91134		4/5/2008	B. Bowers	TCWC collector
B157T	BCB157	TX	Foard	34.00158	-99.86946		4/6/2008	B. Bowers	TCWC collector
B158T	BCB158	TX	Foard	34.07398	-100.00742		4/8/2008	B. Bowers	TCWC collector
B159T	BCB159	TX	Foard	34.00262	-99.87195		4/8/2008	B. Bowers	TCWC collector
B162T	BCB162	TX	Foard	34.04769	-99.92277		4/5/2008	B. Bowers	TCWC collector
B171T	BCB171	TX	Foard	34.03389	-99.47785		4/11/2008	B. Bowers	TCWC collector
B173T	BCB173	TX	Foard	33.98406	-99.71281		4/11/2008	B. Bowers	TCWC collector
B174T	BCB174	TX	Foard	33.99860	-99.51157		4/11/2008	B. Bowers	TCWC collector
B180T	BCB180	TX	Foard	34.00781	-99.92745		4/11/2008	B. Bowers	TCWC collector
B182T	BCB182	TX	Foard	34.01272	-99.90037		4/11/2008	B. Bowers	TCWC collector
B187T	BCB187	TX	Foard	33.98305	-99.67075		4/12/2008	B. Bowers	TCWC collector
B191T	BCB191	TX	Foard	34.00778	-99.92467		4/13/2008	B. Bowers	TCWC collector
B192T	BCB192	TX	Foard	34.04991	-99.92468		4/13/2008	B. Bowers	TCWC collector
BS1T	BS1	TX	Dickens	-	-	unknown	-	B. Stupavsky	Facebook
BS2T	BS2	TX	Motley	-	-	unknown	-	B. Stupavsky	Facebook
CRMWDT	CRMWD	TX	Andrews	32.12301	-102.72954		6/23/2013	S. Wahlberg	TCWC collector
CSA169T	CSA169	TX	Andrews	32.38266	-102.42353		5/16/2013	C. Adams	iNaturalist
CSA1T	CSA1	TX	Hood	32.54450	-97.64288		5/20/2013	C. Adams	iNaturalist

D291N	DJL291	NM	Chaves	33.88680	-103.95780	NW Kenna	-	TCWC	HerpNET
H5143N	H5143	NM	Eddy	-	-	5 mi S, 3.5 mi W Maljamar	5/18/1999	TCWC	HerpNET
H5161N	H5161	NM	Roosevelt	-	-	2 mi S, 5 mi W Lingo	5/25/1999	TCWC	HerpNET
JHO1T	JH01	TX	Crockett	-	-	County Rd 209	-	J. Holmes	TCWC collector
NNTRCT	NNTRC	TX	Nueces	-	-	North Padre Island	-	NNTRC	Web
T2246T	TJL2246	TX	Borden	32.55636	-101.26105		5/9/2010	T. Dimler	iNaturalist
T3452T	TJH3452	TX	Jim Hogg	27.09735	-98.58955		5/30/2013	T. Petty	Facebook
T3503T	TJH3503	TX	Borden	32.60025	-101.38160		5/26/2013	T. Dimler	iNaturalist
T3506T	TJH3506	TX	Clay	33.79472	-98.14044		5/17/2013	B. Box	TCWC collector
T3507T	TJH3507	TX	Cottle	33.90504	-100.32743		5/1/2013	M. Box	TCWC collector
T3511T	TJH3511	TX	Motley	33.89204	-100.75378		7/5/2013	B. Stupavsky	Facebook
T853T	TJL853	TX	Andrews	-	-	TX Hwy 128, 8.4 rd mi E NM State line	5/29/2001	TNHC	HerpNET
T861T	TJL861	TX	Crockett	-	-	TX Hwy 163, 4.5 mi S Irion Co line	5/6/2001	TNHC	HerpNET
T862T	TJL862	TX	Motley	-	-	Double Helix Ranch, 5.5 km N, 4 km W Dumont	5/26/2001	TNHC	HerpNET
T895T	TJL895	TX	Crockett	-	-	Crockett Co Rd 209, 0.2 rd mi W TX Hwy 163	6/26/2001	TNHC	HerpNET
UTA1T	UTA1	TX	Lampasas	-	-	Hwy 281 ca 15 miles N of Lampasas	-	C. Franklin	HerpNET
UTA2T	UTA2	TX	Parker	-	-	Benbrook Aledo Road	-	C. Franklin	HerpNET
WAR8T	WAR8	TX	Shackelford	32.75800	-99.60600		9/5/2013	M. Haynie	Facebook
WVT	Willow Valley Rd	TX	Borden	-	-	East of Willow Valley Road on RM 1785, 2317	-	T. Dimler	iNaturalist