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Diversity-dependent cladogenesis throughout western Mexico: evolutionary biogeography of rattlesnakes (Viperidae: Crotalinae: *Crotalus* and *Sistrurus*)

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Abstract

Rattlesnakes (*Crotalus* and *Sistrurus*) represent a radiation of approximately 42 species distributed throughout the New World from southern Canada to Argentina. Interest in this enigmatic group of snakes continues to accrue due, in part, to their ecomorphological diversity, contributions to global envenomations, and potential medicinal importance. Although the group has garnered substantial attention from systematists and evolutionary biologists for decades, little is still known regarding patterns of lineage diversification. In addition, few studies have statistically quantified broad-scale biogeographic patterns in rattlesnakes to ascertain how dispersal occurred throughout the New World, particularly among the different major biomes of the Americas. To examine diversification and biogeographic patterns in this group of snakes we assemble a multilocus data set consisting of over 6700 bp encompassing three nuclear loci (NT-3, RAG-1, C-mos) and seven mitochondrial genes (12S, 16S, ATPase6, ATPase8, ND4, ND5, cytb). Fossil-calibrated phylogenetic and subsequent diversification rate analyses are implemented using maximum likelihood and Bayesian inference, to examine their evolutionary history and temporal dynamics of diversity. Based on ancestral area reconstructions we explore dispersal patterns throughout the New World. Cladogenesis occurred predominantly during the Miocene and Pliocene with only two divergences during the Pleistocene. Two different diversification rate models, advocating diversity-dependence, are strongly supported. These models indicate an early rapid radiation followed by a recent speciation rate decline. Biogeographic analyses suggest that the high elevation pine-oak forests of western Mexico served as a major speciation pump for the majority of lineages, with the desert biome of western North America colonized independently at least twice. All

49 together, these results provide evidence for rapid diversification of rattlesnakes
50 throughout the Mexican highlands during the Neogene, likely in response to continual
51 orogenesis of Mexico's major mountain systems, followed by more recent dispersal into
52 desert and tropical biomes.
53
54 Key Words: ancestral area reconstruction, diversification, Mexico, rattlesnakes, tropical
55 pine-oak forests

1. Introduction

Rattlesnakes (Viperidae: Crotalinae: *Crotalus* and *Sistrurus*) represent a radiation of approximately 42 pitviper species distributed throughout the New World from Canada to Argentina (Campbell and Lamar, 2004; Gloyd, 1940; Klauber, 1956,1972). These snakes have attracted substantial attention from biologists due, in part, to their broad distribution and potential medicinal importance. However, despite extensive effort over the last 60+ years, we still know relatively little regarding diversification patterns and biogeographic history of this enigmatic group. This lack of knowledge is due, in part, to a largely unresolved phylogenetic hypothesis for rattlesnakes. Indeed, the majority of recent molecular studies that focus solely or largely on rattlesnakes (e.g. Anderson and Greenbaum, 2012; Bryson et al., 2011c; Castoe and Parkinson, 2006; Murphy et al., 2002; Reyes-Velasco et al., 2013) conflict with both each other and with earlier studies that used morphology or venom proteins to elucidate relationships (e.g. Brattstrom, 1964; Gloyd, 1940; Foote and MacMahon, 1977; Klauber 1956,1972). More recent large-scale phylogenies that include all major groups of advanced snakes also show low support for many nodes within rattlesnakes (Pyron et al., 2011; Pyron et al., 2013). Thus, this lack of a well-supported phylogenetic hypothesis is problematic if patterns of diversification and biogeographic history are to be determined.

Although several studies have utilized molecular data to infer rattlesnake and other pitviper relationships, the overwhelming majority relies solely on mtDNA sequence data (e.g. Bryson et al., 2011b; Bryson et al., 2011c; Castoe and Parkinson, 2006; Murphy et al., 2002; Parkinson, 1999; Parkinson et al., 2002). The limitations of relying on a single molecular marker are now well established, and this reliance may in part

explain the lack of resolution for many nodes in the rattlesnake tree. Support for this assumption comes from a more recent investigation of rattlesnake phylogeny, which utilizes multiple mitochondrial and nuclear markers to determine the phylogenetic placement of the long-tailed rattlesnakes with respect to the majority of remaining species (Reyes-Velasco et al., 2013). Unlike previous studies, these authors find strong support for several nodes in the rattlesnake tree highlighting the power of a multilocus approach to understanding rattlesnake evolution. However, the phylogenetic position of several species remains ambiguous, including the placement of *C. horridus* and *C. willardi*. In addition, many deeper nodes in the phylogeny were poorly supported and thus the relationships of currently defined species groups remain unresolved.

Few studies to date have formulated a comprehensive data-driven hypothesis for patterns of rattlesnake diversification and historical biogeography. Although recent empirical evidence suggests that rattlesnakes likely originated in the Mexican highlands due to both the relatively basal position of several small montane species (Reyes-Velasco et al., 2013; Pyron et al., 2013) and previous biogeographic analyses (Place and Abramson, 2004), we still known little about spatial patterns of diffusion through time throughout the Americas. Because of the numerous habitat-types occupied by rattlesnakes ranging from desert to pine-oak forest, it is also possible that the group experienced an early burst of diversification followed by gradual declines as ecological niche space was filled.

We utilize a comprehensive multilocus data set to elucidate temporal and spatial patterns of rattlesnake diversification and colonization throughout the New World. We are specifically interested in addressing the following key questions: 1) Where in the

New World did rattlesnakes likely originate? 2) How did the major biogeographic regions and biomes of the Americas shape cladogenesis and dispersal? 3) Do rattlesnakes show evidence for an early burst of diversification followed by recent declines as ecological niche space was filled? To address these questions, we assemble the largest molecular data set to date for the majority of rattlesnake species consisting of both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) sequences.

2. Materials and Methods

2.1 Sampling

As the majority of previous pitviper and rattlesnake phylogenetic and phylogeographic studies have utilized mtDNA genes only (e.g. Ashton and Queiroz, 2001; Bryson et al., 2011b; Bryson et al., 2011c; Castoe and Parkinson, 2006; Castoe et al., 2007; Douglas et al., 2006; Murphy et al., 2002; Pook et al., 2000; Wüster et al., 2005), we first mined GenBank for all available mtDNA orthologs across that taxonomic spectrum of *Crotalus* and *Sistrurus*. More recently, nuclear genes have been utilized to help resolve pitviper relationships (Anderson and Greenbaum, 2012; Bryson et al., 2014; Pyron et al., 2011; Pyron et al., 2013; Reyes-Velasco et al., 2013). Therefore, we also mined GenBank for available nuclear orthologs for rattlesnakes. We were careful not to utilize GenBank entries with putative errors (Bryson et al., 2011c; Reyes-Velasco et al., 2013). We note that the taxonomy of rattlesnakes continues to be modified as new data become available, and follow the recommendations of recent studies (e.g. Douglas et al., 2007; Meik et al., 2015; Wüster et al., 2005) that advocate the elevation of several subspecies to species status (e.g. *C. culminatus, C. tzabcan, C. pyrrhus, C. stephensi*). However, due to lack of

125 data we continue to treat *C. oreganus cerberus* as a subspecies of *C. oreganus* (Ashton
126 and Queiroz, 2001; Pook et al., 2000). The final mtDNA data set consisted of sequences
127 from the following genes: 12S rRNA, 16S rRNA, ATPase 6, ATPase 8, ND4, ND5, and
128 cytb totaling 4,717 bp. Nuclear loci consisted of RAG-1 (926 bp), NT-3 (512 bp), and C-
129 mos (572 bp). The total concatenated matrix consisted of 6,727 bp. Orthologs were also
130 obtained for *Agkistrodon contortrix* and *A. piscivorous* to serve as outgroup taxa. All
131 GenBank sequences used in this study can be found in Supplementary Online Table S1.
132 Sequences were obtained from 39 out of 42 currently recognized rattlesnake species
133 (Reptile Database, 2015).
134
135 *2.1. Multiple sequence alignment and phylogenetic analysis*
136 Multiple sequence alignments were performed for each gene using the MAFFT L-INS-i
137 algorithm (Katoh et al., 2002; Katoh and Standley, 2013) implemented through AliView
138 v. 1.17.1 (Larsson, 2014). Alignment quality was checked by eye to look for premature
139 stop codons within protein coding genes (none were found). Concatenated matrices were
140 formed using custom perl scripts (S. Sanchez-Ramirez). We then used BEAST v. 2.3.1
141 (Bouckaert et al., 2014) to estimate the phylogenetic relationships and divergence times
142 of rattlesnakes using the concatenated mtDNA and nDNA data. We did not utilize species
143 tree methods (e.g. *BEAST, BEST) for inference due to the relatively large quantity of
144 missing data for each nuclear gene and due to the fact that most rattlesnake species only
145 contain sequences for a single individual. Further, many rattlesnake species readily
146 hybridize (Campbell et al., 1989; Campbell and Lamar, 2004; Murphy and Crabtree,
147 1988), which violates assumptions of many species tree methods. However, we did test

148 the potential efficacy of *BEAST by assembling a second data set consisting of multiple
149 individuals per select species. In all *BEAST runs parameter estimation was problematic,
150 and thus these analyses were not pursued further.
151 We specified the reversible-jump (RB) algorithm in BEAUti for model selection,
152 which selected the best substitution model across multiple parameter dimensions
153 (Bouckaert et al., 2013) for each independent unlinked data set (i.e. mtDNA, RAG-1,
154 NT-3, C-mos). Due to the large number of nucleotide differences in the mtDNA data we
155 also included parameters for the proportion of invariant sites and gamma shape
156 heterogeneity for this partition. An uncorrelated lognormal relaxed clock with a Yule tree
157 prior was used for each locus. To calibrate the analysis and estimate divergence times, we
158 took advantage of available information from two fossils: (1) a fossil record from
159 *Sistrurus* (Late Miocene-Clarendonian [~9 mya]; Parmley and Holman, 2007) and (2)
160 from *Agkistrodon contortrix* (Miocene-Late Hemphillian [~10 mya]; Holman, 2000). The
161 former was used as calibration prior for the divergence of *S. catenatus* and *S. miliarius*
162 (Reyes-Velasco et al., 2013), where we specified a lognormal prior distribution with an
163 offset of 8, mean of 1.5, and standard deviation of 0.75. This resulted in a 95% interval of
164 8.26–12.9 mya. The latter used as calibration prior for the divergence of *A. contortrix* and
165 *A. piscivorus*, where we specified a lognormal prior distribution with an offset of 6, mean
166 of 1.5, and standard deviation of 0.75. This resulted in a 95% interval of 6.26–10.9 mya.
167 The analysis ran for 50 million generations, sampling every 5,000 for a total of 10,000
168 posterior samples. Tracer v.1.5. (Rambaut and Drummond, 2007) was used to assess
169 convergence and mixing and to monitor ESS values (target >200). Due to some
170 suboptimal ESS values, an additional 50 million generations were run to improve

parameter estimation. TreeAnnotator was used to generate a maximum-clade-credibility (MCC) tree following a burn of 10%. LogCombiner was used to generate a post-burnin trees file for subsequent analysis. We then ran a second analysis sampling from the prior only to ascertain the information content of the data.

We also used RAxML v. 8.0.0 (Stamatakis, 2006,2014; Stamatakis et al., 2008) to estimate phylogenetic relationships under a maximum likelihood (ML) framework using the concatenated data. All nDNA loci were partitioned by gene and codon position within genes, whereas the mtDNA data were treated a single linked unit to minimize overparameterization of models. Separate GTRGAMMA models were assigned to each partition and a full ML search was implemented using the autoMRE bootstopping criterion (-f a -# autoMRE option) to assess nodal support.

2.3. Diversification analysis

We utilized three different approaches in order to assess diversification rate dynamics within the rattlesnake phylogeny. All diversification analyses assumed 93% taxon sampling (Reptile Database, 2015). Prior to performing diversification analyses, we used the function *drop.tip* from the R package APE (Paradis et al., 2004) to remove *Agkistrodon contortrix* and *A. piscivorus* from both the MCC tree and 100 post-burnin trees. APE was then used to create lineage-through-time (LTT) plots for the 101 trees. We then used LASER v.2.4 (Rabosky, 2006) to calculate the γ-statistic (Pybus and Harvey, 2000) based on the MCC tree to test for deviations of diversification rate constancy. Significance was assessed using the mccrTest in LASER using 5,000 replicates and assuming an incompletely sampled phylogeny (3 missing species). In

addition, to account for phylogenetic uncertainty we calculated the γ-statistic for 100 trees and used the estimated mean in a second mccrTest to estimate a new γ-critical and test for significant departures from diversification rate constancy.

Next, we fitted six diversification rate models to the 100 subsampled post-burnin ultrametric trees. The models consisted of two rate-constant; namely the (1) *pure-birth*, with a single speciation rate (λ) parameter, and the (2) *birth-death* model, with two parameters, λ and the extinction rate (μ); and four rate-variable, namely three *birth-death diversity-dependent* models (Etienne et al., 2012): (3) linear dependence on speciation rate where *K* (clade-level carrying capacity) is the diversity when λ = μ, (4) linear dependence on speciation rate where *K'* (clade-level carrying capacity) is the diversity when λ = 0, and (5) 1/*n* dependence on speciation rate, where *n* is the number of lineages. In addition, we explored the (6) *birth-death protracted speciation* model, as it has been shown that this phenomenon can also cause diversification slowdowns towards the present (Etienne and Rosindell, 2012; Moen and Morlon, 2014). In this last model, we estimated the speciation initiation rate (λ_b), the rate of speciation completion (λ_1), and a single μ. All models were optimized in a maximum likelihood framework using the R packages DDD v2.7 (Etienne et al., 2014a; Etienne et al., 2012) and PBD (Etienne and Rosindell, 2012) available at the Comprehensive R Archive Network (http://cran.r-project.org/). Models were compared using AIC and Akaike weights (Wagenmakers and Farrell, 2004) for each posterior tree (100).

Finally, we used the program BAMM (Rabosky, 2014), which uses MCMC simulations and reversible-jump sampling to estimate time-varying rates of speciation and extinction, and to find the optimal set(s) of rate-shift configurations. Here we only

used the MCC tree on which we ran four metropolis-coupled chains and 100,000 generations sampling every 10^{th} state. Convergence was assessed by plotting likelihood values for each generation for all four chains. We discarded 10% as burnin. The data were then processed using the R package BAMMtools (Rabosky et al., 2014; http://bamm-project.org/documentation.html).

2.4 Biogeographic analysis

RASP (Reconstruct Ancestral State in Phylogenies) v. 3.2 (Yu et al., 2010; Yu et al., 2015) was used to examine the biogeographic history of rattlesnakes and elucidate how snakes dispersed throughout the New World. RASP is a recently published package that implements multiple methods of biogeographic inference including S-DIVA (Nylander et al., 2008; Yu et al., 2010), dispersal-extinction-cladogenesis (DEC) (Ree and Smith, 2008), Statistical DEC (S-DEC; Yu et al., 2015) and BayArea (Landis et al., 2013). The S-DEC addition to RASP is similar to the S-DIVA algorithm in the sense that ancestral states are estimated from multiple trees, thus explicitly incorporating phylogenetic uncertainty into the analysis. After the DEC model is applied to each tree in the analysis, the following formula is used to calculate the probability *p* of ancestral range *x* at node *n* of the summary (e.g. MCC) tree:

$$p(x_n) = p_n * \sum_{t=1}^{m} w(x_n)t$$

where t represents the present tree out of m total trees, the expression $w(x_n)t$ represents the AIC weight for ancestral range x at node n for tree t and p_n represents the support for node n.

Contemporary geographic ranges for all rattlesnake species were obtained from the literature (Campbell and Lamar, 2004) and cross-referenced with information provided in the IUCN Red List of Threatened Species (www.iucnredlist.org). We then classified species into different areas based on a comprehensive map of ecoregions from The Nature Conservancy (http://maps.tnc.org/gis_data.html). To reduce the complexity of the data, ecoregions were merged into eight continuous units for ancestral area estimation based on both geography and similar bioclimatic characteristics (see Online Supplementary Table S2). All map manipulations were performed in R.

Ancestral states were estimated using both the S-DIVA and S-DEC methods in RASP. To explicitly account for phylogenetic uncertainty, all analyses used a set of 100 post-burnin trees from the BEAST analysis. Both species of *Agkistrodon* were removed from trees prior to analysis using the *drop.tip* function in APE. The total number of geographic units for analysis was eight. Initial S-DEC analyses were unconstrained and we compared results when specifying a maximum number of areas of 3 versus 4. Larger values were not tested due to substantial computational burden with S-DEC (Yu et al., 2015). We also ran additional analyses constraining ranges to adjacent areas. For S-DIVA analyses we utilized the 'Allow Reconstruction' option with default parameters and 1000 max reconstructions for the final tree. Like the S-DEC analyses, we also compared reconstructions when selecting a max areas value of 3 or 4. Optimal S-DEC and S-DIVA reconstructions were summarized on the pruned BEAST MCC tree.

3. Results

3.1 Phylogenetic analysis and divergence times

The vast majority of ESS values from the BEAST analysis were >200 (most >1000) indicating adequate sampling of the posterior. The only parameter with ESS <200 was the proportion of invariable sites for the mtDNA partition (ESS = 191). However, mean and 95% HPD values for this parameter appeared to have stabilized. RBCount 95% HPD's for the reversible-jump model selection included the following: mtDNA (5–5), RAG-1 (1–3), NT-3 (1–2), C-mos (1–3). These numbers corresponded to the following models: 0 = F81, 1 = HKY85, 2 = TAN93, 3 = TIM, 4 = SYM, 5 = GTR. Note that higher numbers were inclusive of all lower rates. For example, the mtDNA partition included rates from all other models. Sampling from the prior only (no data) resulted in substantially different parameter estimates (results not shown) indication the information content of the data.

The Bayesian results indicated strong support for many nodes, especially towards the present (Fig. 1). Results from the ML analysis were similar to the BEAST tree and mostly differed in the placement of poorly supported taxa (Supplementary Online Fig. S1; ML bootstrap support shown below Bayesian tree in Fig. 1). Only six nodes in the tree had Bayesian posterior probability (BPP) values <0.9 or bootstrap (BS) values <0.6, the majority of which were deeper in the phylogeny. Monophyly of *Crotalus* was strongly supported (BPP = 1; BS = 83). In the ML tree a clade containing *C. willardi*, *C. pricei*, *C. intermedius*, *C. tancitarensis* and *C. transversus* branched off first and was sister to all remaining *Crotalus*. Membership in this clade was identical in the Bayesian analysis, but the phylogenetic position of the clade as a whole differed. In both analyses,

Crotalus willardi rooted the base of the clade with moderate support values (BPP = 1; BS = 63) and all remaining relationships received maximum support (BPP = 1; BS = 100).

The phylogenetic position of *C. polystictus* was ambiguous in both Bayesian and ML analyses, whereas the sister relationship between *C. enyo* and *C. cerastes* was strongly supported (BPP = 1; BS = 80). The Bayesian analysis placed *C. cerastes* and *C. enyo* near the base of all *Crotalus*, albeit with a fair degree of uncertainty (Fig. 1). Although support was low (BS = 37) the ML tree placed a clade containing *C. cerastes*, *C. enyo*, and *C. polystictus* as sister to a strongly supported clade of primarily montane rattlesnakes including *C. ravus*, *C. armstrongi*, *C. pusillus*, *C. lepidus*, *C. aquilus*, and *C. triseriatus*. Support for all nodes within this clade of montane rattlesnakes was high in both the Bayesian and ML analyses.

The Bayesian analysis placed *Crotalus horridus* at the base of all *Crotalus* with relatively strong support. Conversely, ML placed this species as sister to a strongly supported clade of long-tailed rattlesnakes, but with weak support (BS = 24). The sister relationship between *C. lannomi* and *C. ericsmithi* was moderately to strongly supported (BPP = 1; BS = 66). ML placed these species sister to a clade consisting of the *C. atrox*, *C. durissus* and *C. viridis* species groups, whereas in the Bayesian analysis the *C. durissus* group branched off first. Relationships within the *C. durissus* group were strongly supported in both analyses (Fig. 1).

The final two strongly supported clades contained species from the *C. atrox* and *C. viridis* groups. *Crotalus ruber* was sister to a clade containing *C. catalinensis* and *C. atrox* in both analyses. Relationships within the *C. viridis* group were identical using both Bayesian and ML, with the majority of nodes strongly supported. Uncertainty in

placement was restricted to *C. adamanteus* and relationships between *C. mitchellii*, *C. pyrrhus*, and *C. stephensi*.

Rattlesnakes diverged from the genus *Agkistrodon* in the Miocene *ca*. 15 mya. The majority of speciation within *Crotalus* and *Sistrurus* occurred over a relatively short temporal period in the Neogene (Fig. 1). Only two species pairs showed Pleistocene divergence and included the split between *C. durissus* and *C. simus* and the split between *C. transversus* and *C. tancitarensis*. The split between *C. oreganus* and *C. viridis* appeared to occur at the Neogene-Quaternary boundary. Although the present study showed high support for the majority of nodes, some deeper nodes still lacked resolution and will no doubt require additional nDNA data to be fully resolved.

3.2. Diversification analysis

The LTT plots indicated a recent decline in speciation rate towards to present (Fig. 1). Using the MCC tree, we calculated a γ-value of -4.222212. Using this value we simulated a null distribution of γ (assuming a constant rate *pure-birth* process) using 5,000 replicates and assuming an incompletely sampled phylogeny. The mccrTest in LASER calculated a γ-critical value of -1.761971 and thus, the empirical γ was significantly different from a constant rate *pure-birth* model ($p = 0.0004$). We also calculated γ for 100 post-burnin trees from the posterior distribution and obtained a mean value of -4.166968 (Supplementary Online Fig. S2), which was also significantly different from null expectations (γ-critical = -1.741022; $p = 0.0002$).

Results from DDD and BAMM analyses also suggested that diversification rates have slowed towards the present in the rattlesnake phylogeny. In DDD, models with

linear dependence on the speciation rate (models 3 and 4) had by far a better fit that any other model, even across posterior trees (Fig. 2), indicating that phylogenetic uncertainty was not an issue. Although models 3 and 4 had a different mathematical basis, parameter estimates from both models were essentially indistinguishable (Fig. 2; Supplementary Online Fig. S3). This was mainly because μ estimates were approximating 0. In addition, the protracted speciation model performed better than the two rate-constant models and the model with 1/*n* dependence on the speciation rate. According to Etienne et al. (2014b), the expected time for speciation completion (speciation duration) can be accurately estimated from phylogenies. Based on our results, we report that in rattlesnakes it takes on average 2.54 [± 1.43] myr for speciation to complete. BAMM did not detect any significant rate-shift configuration, however diversification rate estimates were lower towards the tips compared early lineages (Supplementary Online Fig. S4), suggesting a diversification slowdown. Likewise, λ and μ estimates were similar in both DDD and BAMM.

3.3. Biogeography

There was no discernable difference between an unconstrained or constrained S-DEC model, or models using a max areas value of 3 versus 4 for either S-DEC or S-DIVA analyses. Maximum likelihood ancestral state reconstruction based on 100 phylogenies suggested that Western Mexico (Area E) was an important region of diversification throughout the evolutionary history of rattlesnakes, as this area was the most likely ancestral state for the vast majority of nodes with relatively little uncertainty (Fig. 3A). Ancestral state estimation for the MRCA of *Crotalus* and all rattlesnakes was more

ambiguous, although results suggested that speciation occurred relatively rapidly during the Neogene throughout tropical dry forest and/or pine-oak forests. S-DEC results suggested that the North American deserts were initially colonized independently at least twice *ca*. 7 mya—once in the lineage leading to *C. cerastes* and *C. enyo* and once in the MRCA of the *C. atrox* and *C. viridis* groups (note that Area D was inferred with low probability prior to 7 mya). Initial colonization of tropical moist forest habitat of Eastern Mexico (Area F) occurred *ca*. 4 mya within the *C. durissus* group. Central and South America were colonized relatively recently *ca*. 1 mya. Dispersal into all remaining areas occurred following cladogenesis. S-DIVA results also suggested that Western Mexico was an important center of diversification in rattlesnakes (Fig. 3B). Like the S-DEC analysis, ancestral states near the base of the tree were more ambiguous. Overall biogeographic patterns inferred with S-DIVA were congruent with S-DEC. Again, most diversification occurred rapidly in high elevation pine-oak forest and/or intervening tropical dry forest, with desert habitats being colonized independently twice *ca*. 7-8 mya. One minor difference between S-DEC and S-DIVA was the most likely ancestral state inferred for the MRCA of the two *Sistrurus* species. S-DEC suggested Areas CFG, whereas S-DIVA inferred Area C. However, there were relatively high levels of uncertainty at this node with both methods.

4. Discussion

4.1. Phylogenetic relationships and divergence times

Our inferred phylogenetic relationships and divergence times for rattlesnakes are highly congruent with many recent studies utilizing molecular data to infer rattlesnake

relationships (e.g. Bryson et al., 2011b; Bryson et al., 2011c; Castoe and Parkinson, 2006; Meik et al., 2015; Murphy et al., 2002; Pyron et al., 2011; Pyron et al., 2013; Reyes-Velasco et al., 2013). However, our results do provide some additional findings. For example, we find moderate support for the placement of *C. willardi* as sister to the *C. intermedius* group of snakes, similar to the preferred phylogeny of Murphy et al. (2002). *Crotalus willardi* has also been recently associated with *C. horridus* (Pyron et al., 2013), the *C. durissus* group (Reyes-Velasco et al., 2013), the *C. viridis* group (Castoe and Parkinson, 2006) and branching off in the center of *Crotalus* (Reyes-Velasco et al., 2013). As *C. willardi* is a montane species closely associated with pine-oak forests of the Sierra Madre Occidental (Campbell and Lamar, 2004), a close affinity with the *C. intermedius* group would not be surprising.

The phylogenetic affinities of *C. polystictus* are uncertain. Our ML tree places *C. polystictus* sister to *C. enyo* and *C. cerastes* similar to the concatenated Bayesian tree and species tree of Reyes-Velasco et al. (2013), but with relatively low support in our analyses. Previous studies have also shown close affinities of *C. polystictus* with members of the *C. cerastes* group, albeit with low support (Murphy et al., 2002; Pyron et al., 2013). In contrast, our Bayesian analysis places *C. cerastes* and *C. enyo* near the base of all *Crotalus*, but with low support. This placement has not been supported by other recent studies and we suggest that these relationships are likely erroneous due to relatively high levels of uncertainty (Fig. 1).

ML analysis weakly supports (BS = 37) a *C. cerastes* + *C. enyo* + *C. polystictus* clade as sister to the *C. triseriatus* group of primarily montane rattlesnakes including *C. ravus*, *C. armstrongi*, *C. pusillus*, *C. lepidus*, *C. aquilus*, and *C. triseriatus*. These results

399 are similar to the ML tree of Reyes-Velasco et al. (2013) and Pyron et al. (2013), but
400 differ from other analyses (e.g. Murphy et al., 2002; Reyes-Velasco et al., 2013). Pyron et
401 al. (2013) associate *C. ravus* with *C. cerastes*, whereas both our Bayesian and ML
402 analyses strongly places *C. ravus* at the base of a clade with *C. triseriatus* and allies,
403 similar to other molecular studies (e.g. Bryson et al., 2011c; Bryson et al., 2014; Castoe
404 and Parkinson, 2006; Reyes-Velasco et al., 2013). Pyron et al. (2013) also place *C.*
405 *triseriatus* and *C. pusillus* as sister taxa, in contrast to the strong sister relationship
406 between *C. triseriatus* and *C. aquilus* inferred in this study. Similar conflict within this
407 clade can be found in other single- and multilocus studies (e.g. Bryson et al., 2011c;
408 Bryson et al., 2014; Castoe and Parkinson, 2006; Reyes-Velasco et al., 2013), but to our
409 knowledge the present study exhibits the highest support values for the *C. triseriatus*
410 group to date even though we are missing two newly described species (Bryson et al.,
411 2014).

412 The phylogenetic placement of *Crotalus horridus* has been contentious and
413 unfortunately remains so. Some analyses have associated this species with small montane
414 rattlesnakes (Reyes-Velasco et al., 2013), long-tailed rattlesnakes (Reyes-Velasco et al.,
415 2013), a Neotropical clade (Reyes-Velasco et al., 2013), members of the *C. viridis* group
416 (Murphy et al., 2002), and *C. willardi* (Pyron et al., 2013). Our ML results place *C.*
417 *horridus* sister to a strongly supported clade of long-tailed rattlesnakes, but with weak
418 support. Conversely, our Bayesian analysis places this species at the base of all *Crotalus*
419 with relatively strong support—a placement not supported by other recent molecular
420 studies. Clearly, additional data are required to resolve the enigmatic placement of this
421 species.

A close relationship between *C. basiliscus* and *C. molossus* has been inferred previously (Anderson and Greenbaum, 2012; Murphy et al., 2002; Pyron et al., 2013; Reyes-Velasco et al., 2013; Wüster et al., 2005) and indeed these two species likely hybridize readily in the field (Campbell and Lamar, 2004). Our results are also concordant with previous studies suggesting that *C. ornatus* and *C. totonacus* are sister taxa (Anderson and Greenbaum, 2012). *Crotalus simus* was previously considered a subspecies of *C. durissus* (Campbell and Lamar, 2004). Our results suggest a divergence time of *ca*. 1 mya for these species indicating that gene flow has been severed for a substantial amount of time and that *C. simus* warrants species status. Due to non-monophyly within *C. simus* and distinct morphological differences, Wüster et al. (2005) erected two new species, *C. culminatus* and *C. tzabcan* to obtain taxonomic stability. Indeed, our results suggest that *C. culminatus* and *C. tzabcan* are fairly divergent from *C. simus* and C. *durissus sensu stricto* with initial cladogenesis *ca*. 4 mya. Additional taxonomic work is needed on the *C. durissus* group.

In contrast to previous studies (e.g. Castoe and Parkinson, 2006; Murphy et al., 2002; Pyron et al., 2013), our results place *Crotalus ruber* as sister to a clade containing *C. catalinensis* and *C. atrox*. In a previous study, Castoe et al. (2007) synonymize *C. tortugensis* with *C. atrox* based on mtDNA data, and thus we treat *C. tortugensis* as a junior synonym of *C. atrox*. Castoe et al. (2007) hypothesize that early divergence within the *C. atrox* group was a result of the formation of the Gulf of California in the Pliocene. Our divergence time results lend additional support for this hypothesis as *C. ruber* diverged from the remaining species *ca*. 3.5 mya. However, a sister relationship between C. *ruber* and C. *catalinensis* is highly probable because of 1) the low support for a *C.*

445 *atrox* + *C. catalinensis* grouping, and 2) the relative proximity of Isla Santa Catalina to
446 Baja California Sur where *C. ruber* is found. Results for relationships within the *C.*
447 *viridis* group are similar to Reyes-Velasco et al. (2013), but differ from several previous
448 studies (e.g. Anderson and Greenbaum, 2012; Murphy et al., 2002; Pyron et al., 2013).
449 Differences mostly involve the phylogenetic placement of *C. tigris*, which is strongly
450 supported as sister to a clade containing *C. scutulatus* + *C. oreganus* + *C. viridis* in our
451 study, and *C. adamanteus*, which is placed near the base of the clade. Several subspecies
452 of *C. mitchellii* were recently elevated to species status based on comprehensive data sets
453 (Douglas et al., 2007; Meik et al., 2015). Previous coalescent-based species tree analysis
454 (2409 SNPs) of these taxa suggests a sister relationship between *C. tigris* and *C.*
455 *stephensi*, forming a clade sister to *C. pyrrhus*, *C. mitchellii* and *C. angelensis* (Meik et
456 al., 2015). Conversely, our results suggest that *C. stephensi* is much more closely related
457 to *C. pyrrhus* and *C. mitchellii* than to *C. tigris*, consistent with concatenated ML analysis
458 of the SNP data (Meik et al., 2015).
459
460 *4.2. Diversification and biogeography*
461 Combining mutilocus phylogenetic analyses with ancestral state reconstruction and
462 diversification analyses provides a powerful approach to elucidate evolutionary patterns
463 and processes through space and time. We focus our efforts on understanding how
464 rattlesnakes diversified throughout major geographic regions and biomes of the Americas
465 following the colonization of the New World. Our results suggest that speciation
466 occurred rapidly throughout Western Mexico during the Neogene, with at least two
467 subsequent invasions into desert habitats of Western North America. The current

distribution of species throughout multiple habitat-types is likely due to recent dispersal following rapid cladogenesis.

Although inferred ancestral states throughout most of the phylogeny show little uncertainty, ancestral states near the base of the phylogeny are more ambiguous. However, based on our phylogenetic and biogeographic results and in concert with current distribution data, we conclude that rattlesnakes likely evolved in the montane pine-oak forests of Mexico. Within Mexico, these forests are primarily restricted to major mountain systems including the Sierra Madre Occidental, Sierra Madre Oriental, Sierra Madre del Sur, and the Trans-Mexican Volcanic Belt (TMVB). These Mexican highlands are considered a biodiversity hotspot and encompass a high degree of biotic endemism (Peterson et al., 1993; Peterson and Navarro‐Sigüenza, 1999; Ramamoorthy et al., 1993). The Sierra Madre Occidental, Sierra Madre Oriental, and Sierra Madre del Sur are relatively ancient, with initial orogenesis occurring during the Eocene and Oligocene (Becerra, 2005; Ferrusquía-Villafranca and González-Guzmán, 2005). A previous biogeographic study of rattlesnakes suggests that the pine-oak forests of the Sierra Madre Occidental are the likely ancestral area of the clade (Place and Abramson, 2004). However, this study is based on a single mtDNA-only tree (Murphy et al., 2002), examines only Mexican biogeography, does not examine all nodes in the phylogeny, and utilizes a single method of ancestral state reconstruction with no temporal component that may not be appropriate. Our results suggest that the ancient formation of the Sierra Madre Occidental pre-dates diversification within rattlesnakes by *ca*. 15 my, indicating that the initial uplift may have had little effect on rattlesnake diversification. With that said, a tenable hypothesis is that subsequent orogenesis throughout the Neogene

contributed to rapid diversification in rattlesnakes (Hafner and Riddle, 2005; Riddle and Hafner, 2006).

An alternative hypothesis is that the majority of speciation occurred in the high elevation forests of the TMVB. This system is younger in age (Miocene; Ferrusquía-Villafranca and González-Guzmán, 2005), with multiple volcanic periods occurring throughout the Neogene (Ferrusquia-Villafranca, 1993; Gómez-Tuena et al., 2007). Two specific temporal periods of high volcanic activity relevant to our study are 19–10 mya and 7.5–3 mya, as nearly all speciation events in rattlesnakes occurred during this time. It was also during this time that the Mexican dry forest was rapidly expanding throughout much of western Mexico (Becerra, 2005), which may have caused rapid cladogenesis in some groups (Blair et al., 2015). Our divergence times for rattlesnakes are highly congruent with multiple other studies on diverse taxonomic groups that find evidence for deep lineage divergences associated with the TMVB (e.g. Bryson et al., 2011a; Bryson et al., 2011b; Bryson et al., 2011c; Ferval et al., 2013; Hulsey et al., 2004; McCormack et al., 2008; Mulcahy and Mendelson III, 2000; Ruiz-Sanchez and Specht, 2013). Interestingly, our divergence time estimates based on a multilocus data set are congruent with many of these studies that rely on mtDNA data only, indicating that the controversial mtDNA-only approach may have utility in many instances. Although many studies suggest a dual effect of Neogene vicariance and Pleistocene climate change on lineage diversification for the Mexican pine-oak biota (e.g. Bryson et al., 2012a; Bryson et al., 2011b; Bryson et al., 2011c and references therein), our results show limited evidence for Quaternary climate change as a catalyst for rattlesnake diversification at the species level in these forests.

514 Combining our biogeographic results with our diversification results suggests that
515 rapid speciation occurred throughout the Mexican highlands for *ca*. 6 myr until the clade-
516 level carrying capacity was reached and diversification declined. Rattlesnakes then
517 dispersed out of high elevation pine-oak forest into a multitude of habitat-types. Recent
518 declines in diversification rates have also been inferred for other taxa inhabiting the
519 Mexican highlands (e.g. Bryson et al., 2012b). As our data strongly support a diversity-
520 dependent diversification model, two primary hypotheses can be invoked to explain the
521 observed patterns (Moen and Morlon, 2014). The first hypothesis (hypothesis 1) is the
522 generally accepted null hypothesis that diversification slowed due to competition for
523 resources as ecological niche space was filled (i.e. niche saturation). An alternative
524 diversity-dependent hypothesis (hypothesis 2) is that speciation rates declined as
525 geographic ranges of ancestral species became subsequently smaller due to vicariance.
526 Both hypotheses are tenable in this system—hypothesis 1 is supported due to the fact that
527 competition would likely play a part because the majority of ancestral species were
528 limited to a single area (Western Mexico—Area E); hypothesis 2 is supported because of
529 the potential for continued vicariance throughout the Neogene due to orogenesis of the
530 Sierra Madre Occidental and TMVB. In reality, both hypotheses are likely involved in
531 this system. Although it can often be difficult to disentangle alternative explanations for
532 diversification rate slowdowns (Moen and Morlon, 2014), we are able to conclude that
533 the protracted speciation model may be an unlikely explanation for these dynamics.
534 Likewise, a peripatric speciation model may not be supported as a single area is inferred
535 throughout much of the tree. Finally, the Late Miocene–Pliocene formation and
536 expansion of North America's desert regions (Axelrod, 1979; Douglas et al., 2006; Jaeger

et al., 2005; Riddle and Hafner, 2006) are also highly concordant with our biogeographic results of potentially two independent invasions into deserts during this time frame.

4.3. Conclusions

We assemble a comprehensive data set to elucidate the diversification and biogeographic history of rattlesnakes. Results suggest that the vast majority of diversification occurred in the Neogene throughout the montane pine-oak forests of western Mexico. An important caveat to our analysis is that we assume that major biomes have been static throughout the evolution of rattlesnakes. Although this is unlikely to be true due to various geologic and climatic events, palynological evidence suggests that the Mexican pine-oak forest was well established during the Mid-Late Tertiary (Graham, 1987, 1989). As tropical pine-oak forests are primarily restricted to the Mexican highlands, our results also corroborate previous hypotheses that Mexico was the likely ancestral area for rattlesnakes (Armstrong and Murphy, 1979; Klauber, 1972). As speciation continued due to orogenesis of the TMVB and Sierra Madre Occidental and carrying capacity was reached, rattlesnakes began to disperse out of tropical montane biomes and into more lowland habitats including desert and tropical forests. As Mexican montane forests constitute a major biodiversity hotspot (Peterson et al., 1993; Ramamoorthy et al., 1993), additional biogeographic studies are needed to ascertain the relative importance of this biome as a species pump.

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Figure Legends

Fig. 1. Fossil-calibrated Bayesian time-tree of rattlesnakes (A) inferred from 6,727 bp of combined mitochondrial and nuclear DNA. Branch values represent Bayesian posterior probabilities (>0.9) inferred in BEAST (top) and maximum likelihood bootstrap proportions (>60; bottom) based on the autoMRE bootstopping criterion in RAxML. DensiTree cloudogram under the BEAST maximum clade credibility tree illustrates the degree of phylogenetic uncertainty towards the root. Lineage-through-time (LTT) plot (B) with the 95% highest posterior density intervals (grey) from 100 post-burnin trees and the maximum clade credibility tree (dotted line). Both species of *Agkistrodon* were excluded in the LTT plots. *Crotalus polystictus* photo courtesy of Eric Centenero Alcala.

Fig. 2. Akaike weights for six diversification rate models across a sample of 100 post-burnin evolutionary trees. On the x-axis, each bar represents one posterior tree, and on the y-axis, Akaike weights (AIC scores scaled from 0 to 1) show the relative fit of each model; linear diversity-dependence with *K* equals the diversity where $\lambda = \mu$ (orange, DDL1), linear diversity-dependence with *K*' equals the diversity where $\lambda = 0$ (red, DDL1.3), birth-death protracted speciation model (royal blue, PBD), 1/n diversity-dependence (green, DDX2.2), standard pure-birth $\mu = 0$ (light blue) and birth-death (pink) models.

Fig. 3. Biogeographic history of rattlesnakes inferred through the Statistical Dispersal-Extinction-Cladogenesis (S-DEC) model (A) and Statistical Dispersal Vicariance Analysis (S-DIVA; B) in RASP. Ancestral states were estimated using 100 post-burnin trees from time-calibrated BEAST analyses and summarized on the BEAST maximum clade credibility tree (shown). Ranges in S-DEC were constrained to adjacent geographic areas (C). Colors on map (C) and trees represent different areas that were constructed by merging ecoregions defined by The Nature Conservancy (http://maps.tnc.org/gis_data.html). For clarity, only the most likely ancestral states are presented. Nodes with more than one colored bar represent ancestral areas encompassing multiple regions. Black and white boxes at tips represent the contemporary distribution of each species in the different areas. Dotted lines indicate boundary between the Neogene and Quaternary. All map manipulations were performed in R. Boxes below scale axis represent the proposed origin of North American deserts (orange) and high volcanic activity throughout the Trans-Mexican Volcanic Belt (light blue). *Crotalus willardi* photo courtesy of Eric Centenero Alcala.