



## Phylogenetic relationships of the enigmatic longtailed rattlesnakes (*Crotalus ericsmithi*, *C. lannomi*, and *C. stejnegeri*)

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### ABSTRACT

The longtailed rattlesnakes of western Mexico represent an enigmatic group of poorly known venomous snake species: *Crotalus ericsmithi*, *C. lannomi*, and *C. stejnegeri*. In the 120 years since their discovery, fewer than twenty individuals have been deposited in natural history collections worldwide. These three species share similar morphological traits, including a particularly long tail that has been interpreted as either an ancestral condition among rattlesnakes or as derived within the longtailed group. An understanding of the phylogenetic distinctiveness and relationships among the longtailed rattlesnakes, and their relationships to other rattlesnake groups, has previously been hampered by a dearth of comparative material and tissues for collection of DNA sequence data. Facilitated by the recent availability of tissue samples from multiple individuals of each species, we estimate the phylogenetic relationships among the longtailed rattlesnakes and their placement among other rattlesnake groups, using DNA sequence data from three mitochondrial and three nuclear gene fragments. We explore phylogenetic signal in our data using Bayesian and maximum likelihood methods, species tree analyses and hypothesis testing. Our results strongly support the monophyly of longtailed rattlesnakes and suggest the three species diverged from each other during the mid to late Pliocene or early Pleistocene (~1.5–5.6 mya). Contrary to prevailing hypotheses, we find no evidence for an early or basal divergence of the longtailed clade within the rattlesnake tree, and instead estimate that it diverged relatively recently (~6.8 mya) from its sister lineage, composed of the diamondback rattlesnakes (*C. atrox* group) and the prairie rattlesnakes (*C. viridis* group). With our added sampling of lineages and identification of previously used problematic sequences, we provide a revised hypothesis for relationships among *Crotalus* species, yet underscore the need for future studies and new data to deliver a well-supported robust estimate of rattlesnake relationships.

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### 1. Introduction

Rattlesnakes are a unique and distinctive group of venomous snakes exclusive to the Western Hemisphere that have intrigued biologists and laymen alike for centuries. Their distinctive morphological features, potent venom, and wide geographic range have contributed to both their medical and cultural importance (Greene, 2000). Rattlesnakes range from Canada to Argentina, and include 36 species placed within two genera, *Crotalus* and *Sistrurus* (The Reptile Database, accessed March 2013; (Uetz and Etzold, 1996). Since Linnaeus described the first rattlesnake species (Linnaeus, 1758) they have become among the most studied group of reptiles (e.g. >2000 citations on PubMed). Multiple hypotheses concerning the systematic relationships of the rattlesnakes have been proposed

based on internal and external morphology (Brattstrom, 1964; Gloyd, 1940; Klauber, 1956, 1972), venom properties (Foote and MacMahon, 1977; Githens and George, 1931; Minton, 1956), immunological and electrophoretic data (Cadle, 1992; Minton, 1992) and molecular data (Castoe and Parkinson, 2006; Murphy et al., 2002; Parkinson, 1999, 2002). Despite substantial attention, a cohesive and well-supported phylogenetic hypothesis for the relationships among rattlesnake species remains absent, particularly at deeper nodes in the rattlesnake tree. Among published phylogenies there is much conflict between morphological and molecular-based analyses, and even among molecular-based estimates {Castoe, 2006 #91; Murphy, 2002 #72}.

It is notable that the majority of molecular phylogenies that include rattlesnakes (e.g. Castoe and Parkinson, 2006; Lawing and Polly, 2011; Pyron et al., 2011) have recycled the same GenBank sequences of earlier studies, many published more than a decade ago (Murphy et al., 2002; Parkinson, 1999). Thus, despite many studies

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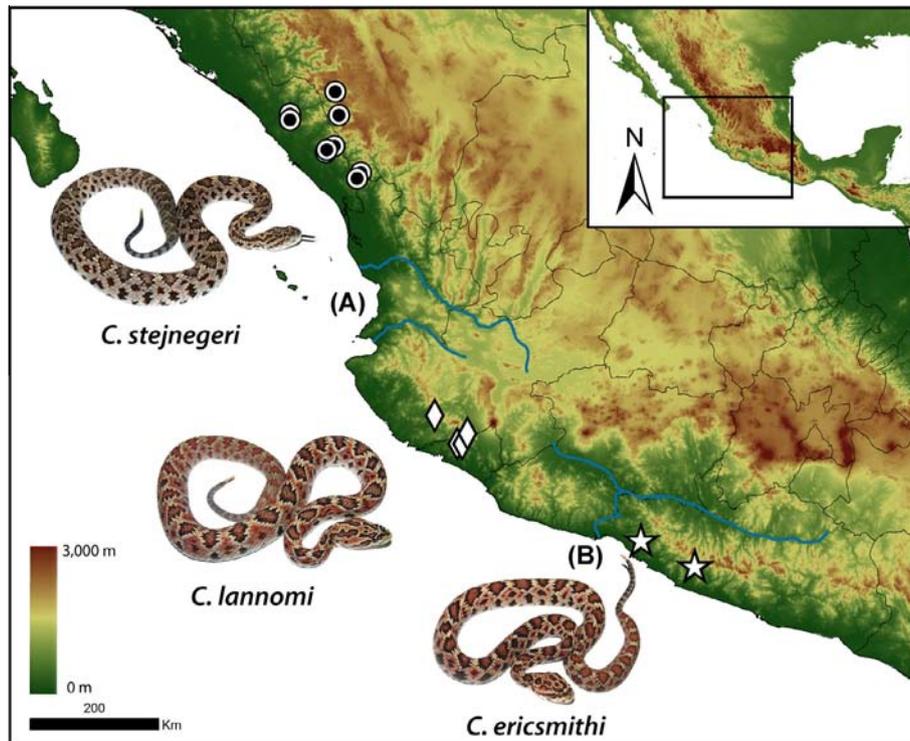
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including rattlesnake DNA sequences, there have been little new data added to refine estimates of rattlesnake relationships. In addition to the issue of minimal additions to gene sequences being used to resolve rattlesnake phylogeny, there are several rare rattlesnake species that have never been included in any molecular study, and this systematic exclusion of lineages may lead to decreased accuracy of inferred phylogenies (Rannala et al., 1998; Zwickl and Hillis, 2002).

One group of species collectively referred to as the “longtailed” rattlesnakes has never been included in a molecular phylogenetic analysis and contains the rarest rattlesnake species in museums worldwide. The group is composed of three species that inhabit the coastal foothills of western Mexico (Campbell and Flores-Villela, 2008; Campbell and Lamar, 2004; Reyes-Velasco et al., 2010), from Sinaloa to Guerrero (see Fig. 1). Although at least one species of the longtailed rattlesnakes has been known to science for more than 115 years (Boulenger, 1896), they have remained particularly rare in biological collections. The first species to be described was *C. stejnegeri* Dunn (1919); this species inhabits the lower foothills of the Sierra Madre Occidental, in the Mexican states of Durango and Sinaloa (and possibly Nayarit, Sonora and Chihuahua). It is known from fewer than 15 specimens, and had not been collected since 1976 (Armstrong and Murphy, 1979). The second species, *C. lannomi*, was described from a single specimen collected in the state of Jalisco in 1966 (Tanner, 1966). For many years no additional specimens were reported until the species was recently rediscovered in the mountains of Colima (Reyes-Velasco et al., 2010). The third species, *C. ericsmithi*, was recently described from a single specimen collected in the Sierra Madre del Sur of Guerrero (Campbell and Flores-Villela, 2008). Newly acquired material for all three species has also shown that some characters used to distinguish these species from one another are not consistent; for example, head scalation and coloration characters show overlap among species (Reyes-Velasco et al., 2010).

The phylogenetic relationships among the longtailed rattlesnakes, and their position in the rattlesnake phylogeny, have been historically difficult to establish based solely on morphological analysis of the small number of available specimens (Gloyd, 1940). Several authors have proposed close affinities between *C. stejnegeri* and the Mexican lance-headed rattlesnake, *C. polystictus*, as well as with members of the *C. triseriatus* group (Amaral, 1929; Brattstrom, 1964; Dunn, 1919; Klauber, 1972). In the description of *C. lannomi* and *C. stejnegeri*. Later, other authors suggested that these two species were among the most basally-diverged rattlesnake lineage, but were not each other’s closest relatives (Klauber, 1972; Stille, 1987). Most recently, Campbell and Flores-Villela (2008) proposed that *C. stejnegeri*, *C. lannomi* and the newly described *C. ericsmithi* were closely related, although no explicit inferences were made regarding their relationships to other rattlesnake species. Recent fieldwork in Mexico has substantially increased the number of specimens of longtailed rattlesnakes, thereby facilitating the inclusion of these enigmatic species in molecular phylogenetic analyses and providing the first opportunity to examine previous hypotheses about relationships of longtailed rattlesnake species.

In this study we bring new mitochondrial and nuclear gene sequence data from all three longtailed rattlesnake species to bear on questions relating to the relationships among these species and their placement in the phylogeny of rattlesnakes. We also add new data to supplement existing GenBank sequences for several other rattlesnake species, to fill in sampling for major lineages and to replace GenBank data we identify as questionable. With this data set we evaluate the following questions: (1) Are the longtailed rattlesnake species valid and moderately divergent from one another? (2) Do the three longtailed rattlesnake species form a monophyletic group, and if so, how are they related to one another? (3) Where do longtailed rattlesnakes fall within the phylogeny of all rattlesnakes and what lineages are most closely related to them?



**Fig. 1.** Map of central Mexico showing topographic relief and indicating the known ranges of each of the three species of longtailed rattlesnakes, as well as possible biogeographic barriers. Rivers are indicated in blue, as either (A) Rio Grande de Santiago/Rio Ameca, or (B) Rio Balsas. Icons represent the only known localities of the longtailed rattlesnakes: circles – *Crotalus stejnegeri*; diamonds – *C. lannomi*; stars – *C. ericsmithi*.

(4) When did longtailed rattlesnakes diverge from one another and from other rattlesnake lineages? (5) Can estimated divergence times be plausibly linked to spatio-temporal biogeographic events?

## 2. Materials and methods

### 2.1. Taxon sampling

We collected all three species of longtailed rattlesnakes, including two specimens of *C. ericsmithi*, three specimens of *C. lannomi* and three specimens of *C. stejnegeri*, between 2007 and 2011. These specimens represent the only individuals of two of the species (*C. ericsmithi* and *C. lannomi*) and three of only four specimens of *C. stejnegeri* known to have been collected in over 30 years (Campbell and Flores-Villela, 2008; Villa and Uriarte-Garzon, 2011). Tissue samples (muscle or liver) were preserved in either 95% ethanol or tissue lysis buffer (Burbrink and Castoe, 2009). Whole preserved specimens were fixed in formalin and deposited at the Museo de Zoología, Facultad de Ciencias, Universidad Nacional Autónoma de México (MZFC-UNAM) and the University of Texas at Arlington Amphibian and Reptile Diversity Research Center (UTA-ARDRC). We obtained tissues of additional species of rattlesnakes from the frozen tissue collection at the UTA-ARDRC. In addition to new data generated, we retrieved DNA sequences from GenBank from other *Crotalus* species and outgroup taxa. Except in the case of the longtailed rattlesnakes, all sequences (from multiple voucher individuals in some cases) from a particular taxon were combined to represent that taxon in phylogenetic analyses. Data for all specimens and sequences used in this study are provided in the [Supplementary Online Table 1](#).

### 2.2. Laboratory techniques

Genomic DNA was extracted using the Qiagen DNeasy kit (Valencia, CA, USA). We PCR amplified and sequenced three mitochondrial DNA fragments, including ATPase subunits 6 and 8 (ATP6\_8), cytochrome B (cyt-b), and NADH dehydrogenase subunit 4 (ND4). We also amplified and sequenced three nuclear gene fragments: oocyte maturation factor mos (C-mos), neurotrophin-3 (NT3) and recombination activating gene-1 (RAG-1). Gene fragments were amplified using previously published primer sets and PCR protocols ([Supplementary Online Table 2](#)). Bi-directional sequencing of DNA fragments was performed by the University of Texas Arlington Genomics Core Facility on an ABI 3130 capillary sequencer (Applied Biosystems). Raw sequence chromatographs were trimmed for quality, assembled, and consensus sequences for gene fragments were estimated using Sequencer 4.8 (Gen Codes Corp., Ann Arbor, MI, USA).

### 2.3. Screening problematic GenBank sequences

In preliminary analyses of sequences, we discovered multiple instances in which GenBank sequences appeared to have either been labeled incorrectly upon original deposition, or to represent anomalous or chimeric sequences. In the supplementary material we summarize the evidence for these assumptions ([Supplementary Online Table 3](#)). Many discrepancies were diagnosed by a first-pass phylogenetic screening of all *Crotalus* GenBank sequences using neighbor joining; problematic sequences were identified when the same species or lineage clustered with taxa known to be distantly related (rather than grouping with conspecific or congeneric species) or where species known to be distantly related had identical sequences for rapidly-evolving mitochondrial loci. Other problematic sequences were identified by blastn searches against the NCBI nr database in which only portions of their length aligned

to other rattlesnakes, or where they aligned to non-rattlesnake species (details in [Supplementary Online Table 3](#)). Many discrepancies involved apparent mismatching of information between that listed in GenBank and that provided in the referenced publications (i.e., in many cases the original publication results seemed correct but the GenBank details were incorrect), but in other instances, mislabeling of sequences or presumed contamination appear have been responsible for the errors. Several of these problematic sequences could be the cause of erroneous phylogenies in previous works, for example the nesting of *Crotalus enyo* in the *C. durissus* group (Castoe and Parkinson, 2006; Murphy et al., 2002), or the apparent paraphyly of *Crotalus* found by (Parkinson (1999).

Based on concerns with some existing data on GenBank for several rattlesnake species, we took multiple steps to increase our confidence in the quality of the data used in this study. First, we filled in new data from six species that seemed particularly phylogenetically unstable (based on preliminary analyses): *C. adamanteus*, *C. cerastes*, *C. enyo*, *C. horridus*, *C. polystictus* and *C. willardi*. Second, we generated new data for seven species that we identified as having questionable GenBank accessions or missing data: *C. aquilus*, *C. atrox*, *C. basiliscus*, *C. pricei*, *C. scutulatus*, *C. tigris* and *C. triseriatus*. Lastly, we excluded the following sequences from GenBank due to probable errors: AF259175.1 (cyt-b of *C. enyo*), HM631837.1 (ND4 of *C. horridus*) and HQ257775.1 (ND4 of *C. triseriatus armstrongi*).

### 2.4. Phylogenetic analysis

We aligned all sequences using ClustalW (Thompson et al., 1994). All protein-coding genes were translated to their predicted amino acid sequences to check for the presence of stop codons (none were detected). Only two individual sequences of nuclear genes had heterozygote sites, *Crotalus lannomi* (JRV-BM) and *C. scutulatus* (JAC-29076), both in the NT3 loci. We phased these sequences manually (based on re-analysis of the raw chromatogram files) and included each individual allele separately in downstream analyses. We used TOPALI version 2 (Miine et al., 2009) to test for recombination in nuclear loci using the difference of sums of squares (DSS) method with a sliding window of 100-bp and 10-bp step size. No significant recombination was detected in any of the nuclear loci. Best-fit models of nucleotide evolution for each gene (or partition) were estimated using Akaike Information Criterion (AIC) in the program JModelTest (Posada, 2008). Individual gene fragments were concatenated using Sequence Matrix (Vaidya et al., 2011). When all genes were concatenated the total length of aligned positions was 3496 bases. The final data matrix was ca. 71% complete at the level of gene loci per species, and 68% complete at the nucleotide level.

We estimated phylogenetic trees using Bayesian Metropolis-Hastings coupled Markov chain Monte Carlo inference (BI) and maximum likelihood (ML) phylogenetic approaches using all concatenated genes. BI was used to estimate the posterior probabilities of phylogenetic trees based on a total of  $10^8$  generations Metropolis-coupled Markov chain Monte Carlo (MCMC) with MrBayes version 3.2.1 (Huelsenbeck and Ronquist, 2001). BI analyses consisted of four simultaneous runs, each with four chains (three heated and one cold), sampled every 1000 generations. We visualized the output from BI in the program TRACER v. 1.5 (Drummond and Rambaut, 2007) to verify that independent runs had converged. Potential scale reduction factor (PSRF) estimates comparing chain likelihood values indicated convergence by  $10^7$  generations. We therefore conservatively discarded the first 25% of BI samples as burn-in. A majority-rule consensus phylogram was estimated from the combination of the post-burnin samples from the four BI runs. ML analysis was performed with raxmlGUI 1.3 (Silvestro and Michalak, 2012). Nodal support for ML analyses

was assessed using the rapid bootstrap algorithm with  $10^4$  replicates (Stamatakis et al., 2008).

We estimated BI phylogenetic trees in MrBayes for each individual locus separately, and also ran independent analyses for both the mitochondrial (ATP6\_8, cyt-b, ND4) and nuclear (C-mos, NT3 and RAG-1) data sets. We conducted further BI analyses on the concatenated set of all loci combined. For the sake of discussion, nodes with  $\geq 95\%$  Bayesian posterior probabilities were considered to be strongly supported (Felsenstein, 2004); in the ML analysis, nodes with  $\geq 70\%$  bootstrap support were considered strongly supported (Hillis and Bull, 1993).

We used comparisons of tree likelihoods for different tree topologies to evaluate relative support for alternative trees. For this, we implemented the stepping-stone sampling method (Xie et al., 2011) in MrBayes v3.2 to estimate the marginal likelihood for each topological constraint. For each hypothesis, we evaluated the complete concatenated dataset using the best-fit partitioned model based on  $5 \times 10^5$  generations of each of the 49 steps, sampling every 1000 generations, for a total of  $2 \times 10^7$  generations.

### 2.5. Species tree analysis

In addition to concatenated phylogenetic inferences made using MrBayes, we also implemented a multispecies coalescent model to estimate the 'species tree' based on multi-locus data. Given the lack of substantial intraspecific sampling and the moderate amount of missing data, our dataset is not particularly well suited for species tree analysis. We therefore use species tree analyses as a means to further explore phylogenetic signal in the data, but with the above caveats. We used the program \*BEAST (Heled and Drummond, 2010), within the BEAST software package (Drummond and Rambaut, 2007), to estimate a species tree from the three separate nuclear loci (C-mos, NT3 and RAG-1) plus a concatenated mitochondrial dataset (ATP6\_8, cyt-b and ND4) that was treated as a fourth locus. We used a relaxed molecular clock model for all loci and an HKY +  $\Gamma$  model of nucleotide substitution for each data partition, with the exception of RAG-1, for which we used a JC model. We chose the models of nucleotide substitution based on the Akaike Information Criterion (AIC) estimated using JModelTest (Posada, 2008). The tree prior was set to the Yule process, and other priors in \*BEAST were set to default values. Analyses were run in duplicate, each for  $1 \times 10^9$  generations, sampling every 20,000 generations, for a total of  $5 \times 10^4$  sampled trees. We used TreeAnnotator v1.7.4 (Drummond and Rambaut, 2007) to discard the first 10% of the samples as burn-in, and to map nodal support for the remaining samples on the tree.

### 2.6. Allele networks

Parsimony haplotype networks for the nuclear C-mos and NT3 data sets of the longtailed rattlesnakes were calculated using TCS 1.21 (Clement et al., 2000). All three species of longtailed rattlesnakes shared an identical haplotype of the Rag-1 gene, so it was excluded from this analysis. Haplotype networks were inferred using a statistical parsimony framework (Templeton, 1998), with gaps treated as missing data and a connection limit of 95%. Identical sequences were collapsed into a unique haplotype set.

### 2.7. Phylogenetic hypothesis tests

To evaluate relative evidence for different hypotheses regarding the phylogenetic placement of the longtailed rattlesnakes among *Crotalus* species, and the relationships among the three longtailed species, we used Bayes Factors in MrBayes to compare the likelihood of alternative trees based on the concatenated dataset. We used the criterion of  $2\ln[bf]$  ranging from two to six

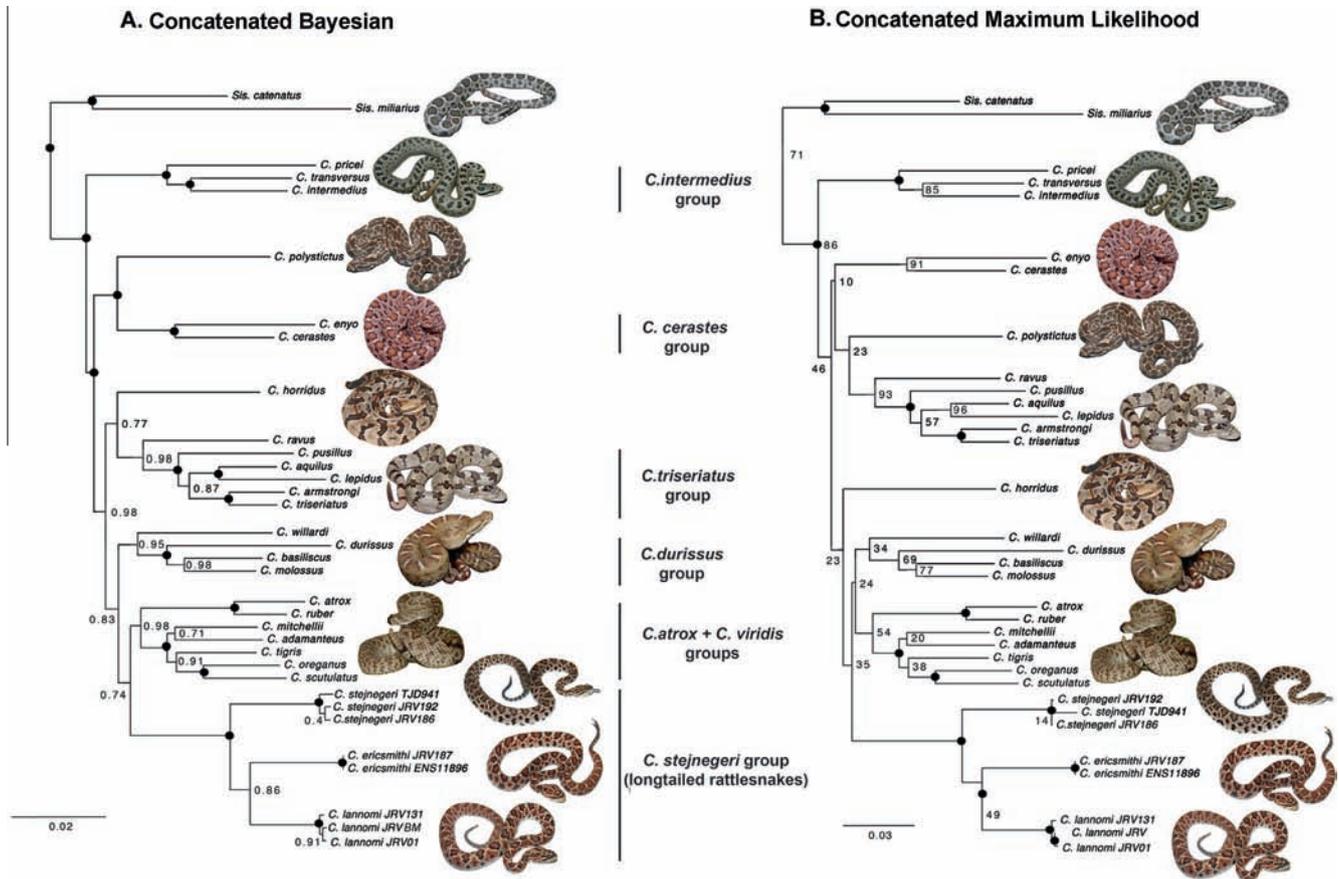
as positive evidence, six to ten as strong evidence and  $>10$  as very strong evidence against the alternative hypotheses (Kass and Raftery, 1995; Miller and Bergsten, 2012).

### 2.8. Divergence dating

We performed a likelihood ratio test to test the null hypothesis that substitutions in the genes used follow a strict molecular clock of evolution. At a significance threshold of  $p < 0.05$ , the set of all mitochondrial genes rejected the strict molecular clock, while the set of all nuclear genes failed to reject it. The concatenated analysis of all genes also rejected the strict molecular clock. We estimated divergence times across the rattlesnake phylogeny using BEAST 2 (Drummond and Rambaut, 2007) instead of incorporating divergence estimation in our \*BEAST analysis of the species tree. We took this approach because our species tree analysis had considerable missing data ( $>30\%$ ), which presumably contributed to the failure of these \*BEAST runs to reach convergence in  $>1$  billion generations. Additionally, most nodes in our species tree analysis had extremely low support values. Because preliminary analyses in BEAST 2 implementing nucleotide models partitioned across genes and codon position showed poor convergence, our final analysis used an unpartitioned model to estimate divergences using the entire dataset. The concatenated data set rejected the strict clock hypothesis, so we implemented an uncorrelated log-normal relaxed clock model with a Yule tree prior using the HKY +  $\Gamma$  model of sequence evolution applied to the combined data set. Two independent analyses were run for  $1 \times 10^8$  generations, sampling every 10,000 generations. Dates used to constrain nodes were obtained from estimates based on the fossil record or biogeographic divergence events published in previous studies (Holman, 2000; Castoe et al., 2007; Parmley and Holman, 2007), and many of the priors we use here follow a recent study that has dated a similar phylogenetic tree (Bryson et al., 2011b). We used the program Tracer v. 1.5 (Drummond and Rambaut, 2007) to confirm stationarity of the Markov chain Monte Carlo (MCMC) analysis, adequate effective sample sizes of the posterior ( $>200$  for each estimated parameter), and the appropriate percent to discard as burn-in (which we estimated conservatively to be 10%, or 1000 trees). We used two fossil and one geologic calibration for our divergence estimates. First, we used the oldest *Sistrurus* fossil (Late Miocene, Clarendonian; Parmley and Holman, 2007). We constrained the ancestral node of *Sistrurus* with a zero offset of 8 million years ago (mya), with a log-normal mean of 0.01, and a log-normal standard deviation of 0.76, resulting on a median age of 7 my and a 95% prior credible interval (PCI) that extended to the Late Clarendonian,  $\sim 11.5$  mya (Holman, 2000). Second, we used the oldest fossil of *Agkistrodon contortrix* (Late Hemphillian; Holman, 2000). This node was constrained with a zero offset of 6 mya, a log-normal mean of 0.01, and a log-normal standard deviation of 0.42, resulting on a median age of 7 my and a 95% PCI that extended to the Late Hemphillian,  $\sim 8$  mya (Holman, 2000). Third, we used the estimated time of divergence between *C. atrox* and *C. ruber* as approximately 3.2 mya (Castoe et al., 2007). This node was given an offset of 3.2, a normal mean of 0 and a normal standard deviation of 1, resulting on a median age of 3.2 my and a 95% PCI that extended to  $\sim 4.8$  mya. After discarding burn-in samples, the trees and parameter estimates from the independent runs were combined using LogCombiner v. 1.7.4 (Drummond and Rambaut, 2007). We summarized parameter values of the samples from the posterior on the maximum clade credibility tree using the program TreeAnnotator v. 1.7.4 (Drummond and Rambaut, 2007).

### 2.9. Revision of skeletal material

The absence of teeth in the palatine bone has been considered a synapomorphy uniting *Crotalus polystictus* and *C. stejnegeri*



**Fig. 2.** Phylogenetic estimates based on concatenated data analyses. (A) Majority-rule consensus tree from Bayesian phylogenetic tree estimates based on all genes concatenated, with bipartition posterior probabilities indicated by numbers or a filled circle if equal to 1.0. (B) Maximum likelihood phylogenetic tree estimate based on all genes concatenated, with bipartition bootstrap values indicated by numbers or a filled circle if equal to 100%. Outgroup species are omitted from figures.

(Klauber, 1952; Brattstrom, 1964). To re-evaluate this supposition, we looked for the presence or absence of teeth in the palatine bone in the skulls of specimens of ten species of the genus *Crotalus*, as well as one species of each of the genera *Sistrurus* and *Agkistrodon*. All specimens are deposited at the UTA-ARDRC. A list of the specimens examined and their locality data is given in [Supplementary Online Table 4](#).

### 3. Results

#### 3.1. DNA sequence characteristics

The combined set of mitochondrial loci contained 1610 bp, 801 of which were variable. The total length of ATP6\_8 was 444 bp, with 245 (45%) variable sites. For cyt-b, the total length was 564 bp, with 260 (46%) variable sites. The total length of ND4 was 602 bp, with 296 (49%) variable sites. The combined set of nuclear loci contained 1887 bp, 91 of which were variable. The C-mos fragment contained 553 bp, with 29 (5%) variable sites. NT3 had a total length of 512 bp, 41 sites (8%) were variable. RAG-1 had a length of 822 bp, and only 21 sites (2%) were variable.

#### 3.2. Individual gene tree estimates

There was broad support that the three longtailed rattlesnake species formed an exclusive clade across BI trees estimated from individual loci, although there were several differences in topology between individual gene trees (figures not shown). Nuclear genes had a low number of polymorphic sites and tended to provide lower phylogenetic resolution and support (see above).

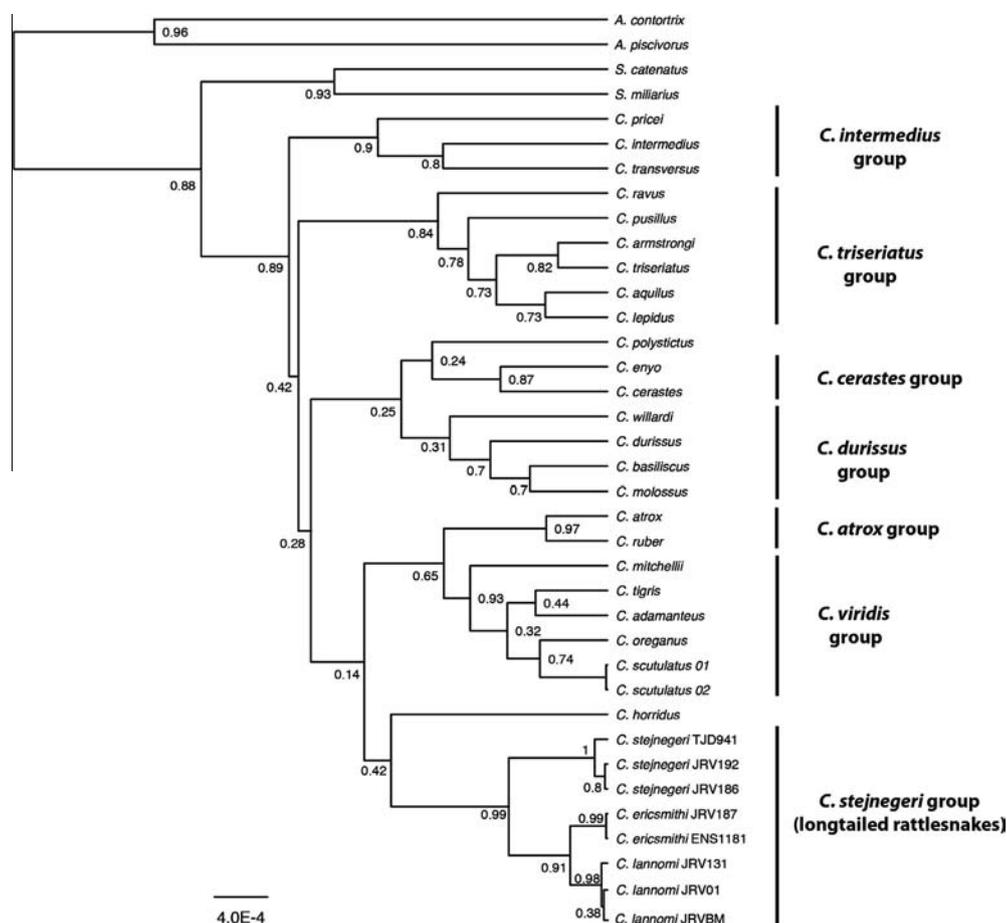
Longtailed rattlesnakes were recovered as monophyletic in all BI trees based on analyses of individual genes except for that based on the nuclear gene C-mos, which resulted in a polytomy that included the longtailed rattlesnakes, *C. horridus* and *C. molossus*. In the case of NT3, *C. ericsmithi* was nested within a cluster of *C. lannomi* samples, and *C. stejnegeri* was sister to this clade.

The relationships among the three species of longtailed rattlesnakes differed somewhat among BI phylogenetic estimates based on individual loci. A clade containing *C. lannomi* and *C. stejnegeri*, sister to *C. ericsmithi*, was inferred based on BI analysis of the mitochondrial loci ATP6\_8 and ND4 (posterior probability [pp] = 1). In contrast, a clade containing *C. lannomi* and *C. ericsmithi* as the sister lineage to *C. stejnegeri* was inferred based on the mitochondrial cyt-b fragment and the nuclear fragments NT3 and RAG-1 (pp = 0.99, 1 and 0.73, respectively).

The phylogenetic placement of the longtailed rattlesnake clade within the phylogeny of the rattlesnakes was weakly and differentially resolved by individual gene BI estimates. The longtailed rattlesnake clade formed a polytomy with several other rattlesnake lineages based on ATP6\_8. The ND4 BI tree recovered the longtailed clade as the sister-lineage to *C. horridus* plus *C. cerastes* (pp = 0.68), and cyt-b showed a different topology in which the longtailed clade formed the sister lineage to all other *Crotalus* species (pp = 0.98).

#### 3.3. Concatenated phylogenetic analyses

The concatenated nuclear dataset analyzed using BI (not shown) recovered *C. stejnegeri* as sister to a clade containing *C. lan-*



**Fig. 3.** Species tree estimate for the rattlesnakes based on analysis using <sup>†</sup>BEAST incorporating all six gene fragments (ATP6\_8, C-mos, cyt-b, ND4, NT3 and RAG-1). Posterior probability values are given adjacent to respective nodes.

*nomi* and *C. ericsmithi*, with a single representative of *C. ericsmithi* nested within a clade of three *C. lannomi* samples. The longtailed species were one of the only *Crotalus* clades with posterior support >0.95 (the other groups being *C. tigris* + *C. oreganus* and *C. basiliscus* + *C. polystictus*), although their placement among other lineages of *Crotalus* was unresolved. The BI analysis of concatenated mitochondrial genes (not shown) also recovered the longtailed rattlesnakes as monophyletic, but with *C. ericsmithi* as sister to a clade comprising *C. lannomi* plus *C. stejnegeri* (pp = 1); this longtailed rattlesnake clade was inferred to be the sister group to the *C. durissus* + *C. atrox* + *C. viridis* groups (pp = 0.96).

When all genes were combined for BI and ML analyses, a slightly different topology was recovered (Fig. 2). The monophyly of the longtailed group was strongly supported in both BI and ML analyses (pp = 1; bootstrap support [bs] = 100%), with *C. stejnegeri* as the sister lineage to a clade containing *C. lannomi* and *C. ericsmithi*. In the BI estimate, the longtailed group was supported by 0.74 posterior probability as sister to the *C. atrox* + *C. viridis* groups, while the ML tree placed the longtailed rattlesnakes sister to a clade consisting of the *C. atrox*, *C. viridis* and *C. durissus* groups (like the BI tree of mitochondrial genes). Another difference between the BI and ML inferences was the position of *C. horridus*, which was the sister to the *C. triseriatus* group in the BI tree, but recovered as sister to a clade containing the *C. durissus*, *C. atrox*, *C. viridis*, and longtailed rattlesnakes in ML. Both BI and ML inferred that *C. enyo* and *C. cerastes* formed a clade (the *C. cerastes* group), but they differed in their placement of *C. polystictus*, which was sister to the *C. triseriatus* group in ML, and sister to the *C. cerastes* group in BI. In

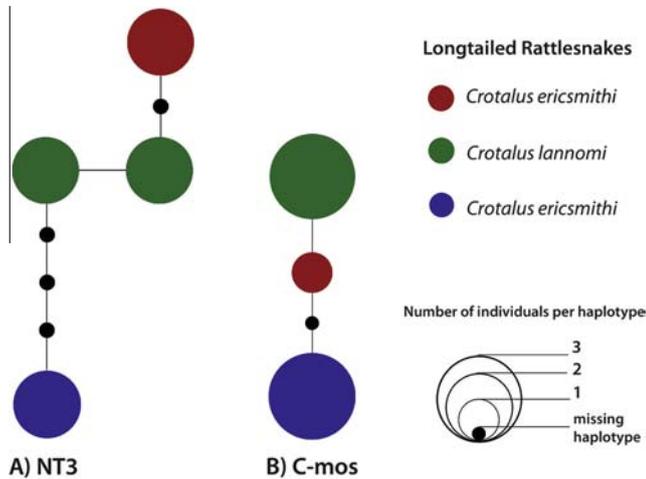
both analyses, the *C. intermedius* group was recovered as the sister group to all other species of *Crotalus*.

### 3.4. Species tree analysis

The species tree analysis of all loci using <sup>†</sup>BEAST recovered an exclusive longtailed rattlesnake species clade, with *C. stejnegeri* sister to *C. ericsmithi* + *C. lannomi*; this clade was recovered with strong support (pp = 0.99; Fig. 3). Contrary to the BI and ML analyses, the longtailed rattlesnakes were placed as sister to *C. horridus*, and this clade was the sister group to the *C. atrox* plus *C. viridis* groups. Unlike results from concatenated BI and ML analyses, species tree analyses implied that the *C. cerastes* group and *C. polystictus* formed a clade sister to the *C. durissus* group.

### 3.5. Allele networks

We found no evidence for recombination within any of the three nuclear genes within the longtailed rattlesnake samples. For these longtailed rattlesnake samples, the six sequences of C-mos grouped into three distinct haplotypes, each haplotype was unique to each of the three species. In the case of NT3, the eight individuals grouped into 4 different haplotypes. *Crotalus lannomi* had two distinct haplotypes, each of which were homozygous in one individual, and heterozygous in a third individual. Samples of *C. ericsmithi* and *C. stejnegeri* were homozygous for a single variant unique to each species (Fig. 4). In sum, within the longtailed



**Fig. 4.** Allele network for the variable nuclear genes (NT3 and C-mos) constructed for the longtailed rattlesnakes. All specimens of longtailed rattlesnakes shared the same RAG-1 haplotype, so it was excluded from this analysis.

rattlesnake species, all nuclear variants observed are unique to a recognized species (as are all mitochondrial variants).

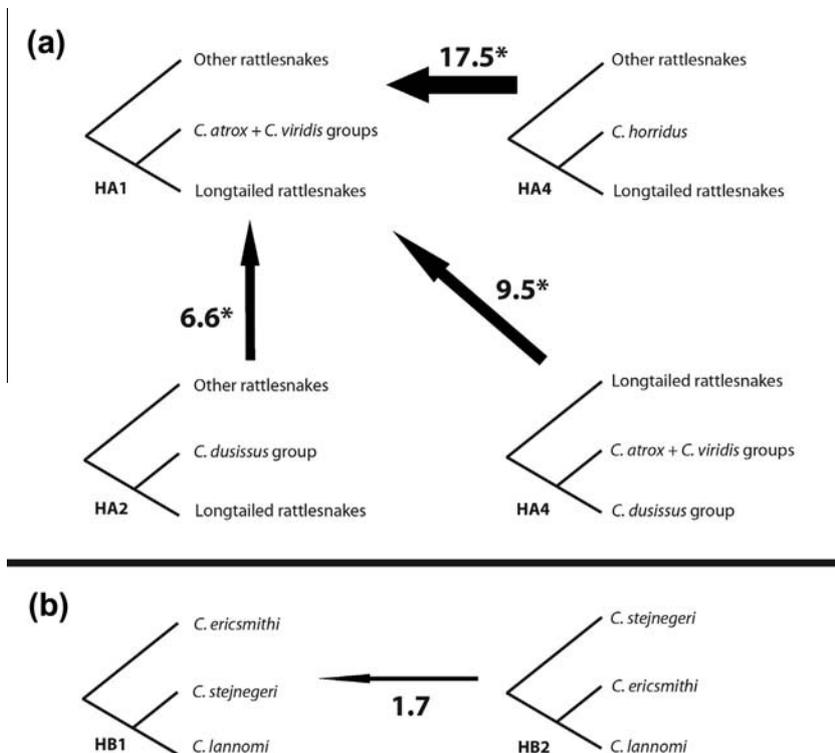
### 3.6. Phylogenetic hypothesis test

Because different analyses resulted in different phylogenetic estimates, we tested two sets of hypotheses regarding the relationships of the longtailed rattlesnakes. Set A, which represents hypotheses regarding the placement of the longtailed rattlesnakes among rattlesnakes: HA1) longtailed rattlesnakes sister to *C. atrox* + *C. viridis* groups – this topology was obtained from BI

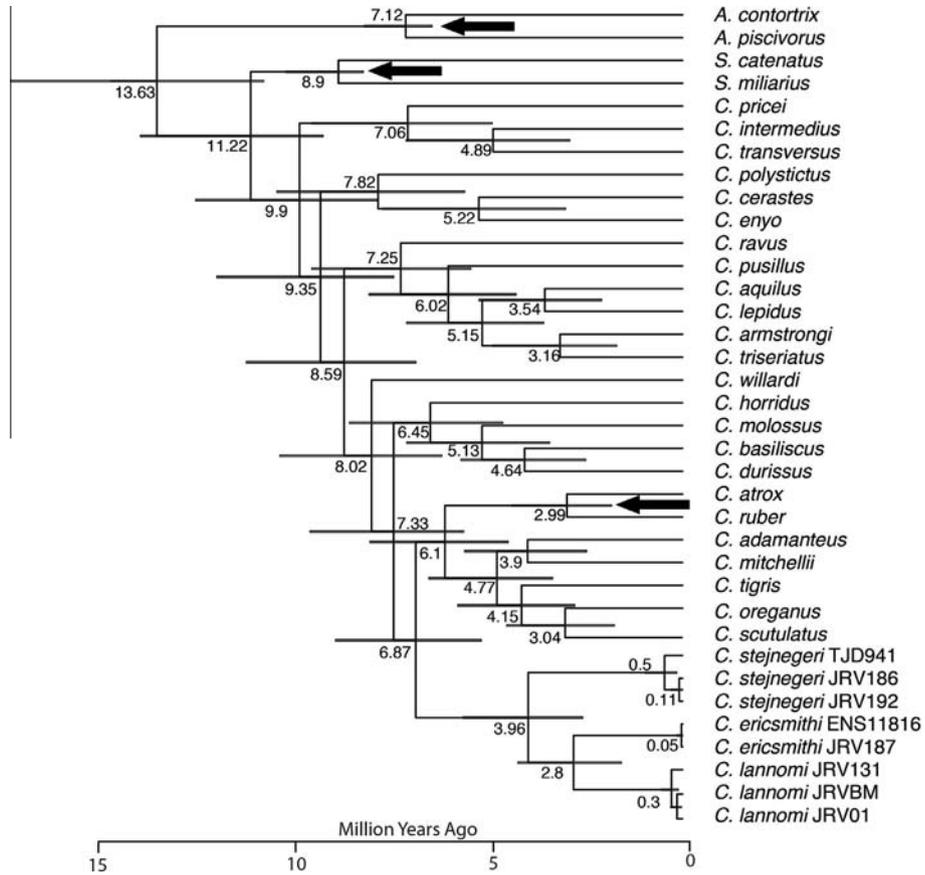
analysis of all genes; HA2) longtailed rattlesnakes sister to the *C. durissus* group – this topology was obtained in some of the BEAST runs; HA3) longtailed rattlesnakes sister to a clade containing the *C. durissus*, *C. atrox*, and *C. viridis* groups – this topology was recovered by the BI analysis of mitochondrial genes and RaxML analysis of all genes; HA4) longtailed rattlesnakes sister to *C. horridus* – recovered in the species trees analysis in BEAST, although with very low support. Our second set (set B) focused on the branching order of the three longtailed rattlesnakes: HB1) *C. lannomi* sister to *C. stejnegeri* – recovered from BI analysis of individual ATP6.8 and ND4 genes. HB2) *C. lannomi* sister to *C. ericsmithi* – obtained from all other analyses. In tests of these hypotheses using Bayes factors [*bf*] based on the concatenated dataset, we found strong support ( $bf = 6.6 - 17.5$ ) for the longtailed rattlesnakes as sister to the *C. atrox* plus *C. viridis* groups (HA1), but we found no notable support ( $bf = 1.7$ ) for a particular branching order among the three longtailed rattlesnakes (Fig. 5).

### 3.7. Divergence time estimates

Our divergence estimates are similar to previous studies of pitviper evolution (e.g. Bryson et al., 2011b; Daza et al., 2010; Douglas et al., 2002), which is expected because many calibration points, and much sequence data are shared with these studies. Due to the lack of substantial intra-specific sampling, missing data, and strong support for the topology recovered in concatenated BI analyses (Fig. 5), we base our divergence time estimates on concatenated (non species tree) BI analysis. Based on the divergence time analysis implemented in BEAST 2, we estimate that the split between the *C. intermedius* group and the rest of *Crotalus* occurred ~9.9 mya (7.8–12.3 mya, 95% highest posterior densities [HPD]). Following this event, most other major lineages of *Crotalus* (i.e., species groups) diverged in



**Fig. 5.** Topological hypotheses tested for the placement and branching order of the longtailed rattlesnakes. HA 1–4: Hypothesis for which lineages are the sister group to the longtailed rattlesnake clade. HB 1–2: Hypotheses for the branching order among the three species of longtailed rattlesnakes. Arrows point towards the hypothesis that is favored by Bayes Factors. Numbers represent relative support based on Bayes factors ( $2\ln[bf]$ ) between topologies trees, which are considered as positive evidence for a particular topology if they range from two to six, strong evidence from six to ten, and as very strong evidence if  $>10$ .



**Fig. 6.** Bayesian relaxed clock estimate of divergence times among rattlesnake lineages with 95% credibility intervals shown over nodes by shaded bars. Dark arrows represent calibration points used in the analysis.

relatively rapid succession during the late Miocene, from ~9 to 6 mya (Fig. 6). Our estimates of the divergence dates among most *Crotalus* lineages are mostly similar to previous studies (e.g. Douglas et al., 2006; Bryson et al., 2011a,b; Anderson and Greenbaum, 2012), with the exception of the divergence between *C. durissus* and *C. molossus*, as our estimate is substantially more recent than previous estimates (Wuster et al., 2005). We estimate that the ancestor of the longtailed rattlesnake group diverged from a common ancestor with the *C. atrox* + *C. viridis* group clade during the late Miocene, ~6.8 mya (5.1–8.6 mya, 95% HPD). The extant longtailed rattlesnake lineages are estimated to have split from one another during the Pliocene (Fig. 6), with the first division occurring when *C. stejnegeri* diverged from the other two longtailed species ~3.96 mya (2.5–5.46 mya, 95% HPD), followed by the divergence of *C. lannomi* from *C. ericsmithi* ~2.7 mya (1.6–4.1 mya, 95% HPD).

3.8. Revision of skeletal material

Among the pitviper species examined for palatine teeth, the only species without teeth in the palatine bone is *C. polystictus*, and this trait was consistent across three specimens. *Crotalus stejnegeri* was reported by Klauber (1956) and Brattstrom (1964) to lack teeth on the palatine bone, but the specimen we examined (UTAR-10499) had three palatine teeth (Fig. 7).

4. Discussion

4.1. Monophyly and distinctiveness of the longtailed rattlesnakes

The importance of rattlesnakes transcends academic interests in many ways, including their medical importance and their central role in the imagery and folklore of North America (Greene and Cundall, 2000). Furthermore, this group of 36 species is one of the most heavily studied lineages of reptiles, particularly when



**Fig. 7.** Photographs of skulls of *Crotalus stejnegeri* (left) and *C. polystictus* (right). Red arrows point to the palatine bone. Notice the presence of palatine teeth in *C. stejnegeri* and their absence in *C. polystictus*.

their relatively low diversity (equivalent to ~1% of all snake species) is considered. Among rattlesnake species, the longtailed rattlesnakes have remained the most enigmatic, largely because of the dearth of scientific material available for these species (e.g., a single specimen for *C. lannomi* for almost 50 years) and the recent discovery of *C. ericsmithi* (Campbell and Flores-Villela, 2008). Thus, in the absence of sufficient comparative material, the origins, distinctiveness, and relationships among longtailed rattlesnakes have been much debated but insufficiently tested.

Our phylogenetic estimates provide unilateral evidence that the longtailed rattlesnakes form a well-supported monophyletic clade (Figs. 2–6). Most authors have considered the long tail of these species to be an ancestral character state (Gloyd, 1940; Klauber, 1952, 1972; Tanner, 1966), and therefore not a synapomorphy supporting the monophyly of the group (Campbell and Flores-Villela, 2008). Our results instead indicate that the long tail condition is a shared derived character uniting these three species, as is the mediolateral compression of the hemipenial lobes (Jadin et al., 2010).

Although each of the three longtailed rattlesnake species share characteristics of their internal and external morphology (Jadin et al., 2010; Reyes-Velasco et al., 2010; Reyes-Velasco, unpublished), we find each to constitute reciprocally monophyletic groups based on all mitochondrial gene analyses, analysis of the nuclear gene *C-mos*, and the species tree analysis of the combined data (Figs. 2 and 3). Furthermore, for nuclear genes that show variation in these three species (*NT3* and *C-mos*), each species contains species-specific alleles and no alleles are shared among species (Fig. 4). We estimate that the three species have most likely diverged from one another during the Mid-Late Pliocene (Fig. 6). Based on our analyses, together with previous evidence for their morphological distinctiveness, there is broad agreement that these three species are indeed distinct.

#### 4.2. Phylogenetic placement of the longtailed rattlesnakes

The long, attenuated tails and minute rattles characteristic of species of the longtailed rattlesnakes have led most researchers to conclude that these species where the sister group to all other *Crotalus* (Gloyd, 1940; Klauber, 1952, 1972; Tanner, 1966). Based on morphological similarities, including high ventral counts, high number of dorsal scale rows, and a tendency toward subdivided head scales, Klauber (1952) noted that *C. stejnegeri* more closely resembled *C. viridis* and *C. atrox* than other rattlesnakes. Longtailed rattlesnake species also, however, possess high numbers of spines on each hemipenial lobe, a trait that they shared with *C. polystictus* (Jadin et al., 2010). Further linking *C. polystictus* and *C. stejnegeri*, the absence of teeth on the palatine bone was considered a synapomorphy uniting these two species (Brattstrom, 1964; Klauber, 1956, 1972), although LaDuc (2003) reported palatine teeth from a specimen of *C. stejnegeri* (UTA R-10499). We reexamined this specimen as well as various other rattlesnake species (including *Sistrurus catenatus*, *Crotalus aquilus*, *C. atrox*, *C. lepidus*, *C. molossus*, *C. pricei*, *C. stejnegeri*, *C. willardi*, and several *C. polystictus*; see Supplementary Online Table 4), and the lack of palatine teeth was found to be unique to *C. polystictus*, and the presence of palatine teeth in *C. stejnegeri* was confirmed (Fig. 7). Due to the lack of comparative skeletal material, we were not able to assess the presence of palatine teeth in *C. lannomi* and *C. ericsmithi*. The absence of teeth in the palatine bone is therefore an autapomorphy of *C. polystictus* and not a synapomorphy linking *C. polystictus* and *C. stejnegeri*.

The ML analysis of all genes placed the longtailed rattlesnakes as sister to a clade consisting of the *C. durissus* + (*C. atrox* and *C. viridis*) groups, but with little support (bs = 35%). Concatenated BI analysis estimated a sister relationship between the longtailed

rattlesnakes and the *C. atrox* + *C. viridis* groups, but with relatively weak support (pp = 0.74). Our species tree inference from BEAST resulted in the longtailed rattlesnakes placed as the sister to *C. horridus*, but with extremely low support (pp = 0.42), as was recovered at most other nodes of this tree (Fig. 3). Because we inferred multiple competing hypotheses for relationships of longtailed rattlesnakes across different phylogenetic methods, we tested these hypotheses using Bayes Factors implemented in MrBayes based on the concatenated data set. Our results strongly favored the sister relationship between the longtailed rattlesnakes and the *C. atrox* + *C. viridis* groups (Fig. 5), as was inferred by the BI concatenated analysis. The close relationship between the longtailed rattlesnakes and the *C. atrox* and *viridis* groups has never been explicitly inferred by phylogenetic analyses, although there are several similarities between these groups of rattlesnakes that others have noted (Klauber, 1952). We find strong evidence countering previous hypotheses that the longtailed rattlesnakes are sister to all other *Crotalus*, and also against the hypothesis that they are close relatives of *C. polystictus*, as previously suggested based on hemipenial characters (Jadin et al., 2010) and the presumed synapomorphy of the absence of palatine teeth that we confirm to have been incorrect (Fig. 7).

#### 4.3. Insights into rattlesnake phylogeny

Estimating the phylogenetic placement of the longtailed rattlesnake clade within the context of rattlesnake phylogeny requires at least partial resolution of the phylogeny of rattlesnakes, which has historically been difficult. Although our sampling of *Crotalus* species was not exhaustive, we included multiple taxa from all major rattlesnake species groups, together with new data for other lineages, and recovered several well-supported clades within *Crotalus* (Fig. 2). Our phylogenetic results are largely congruent with many previous hypotheses (Bryson et al., 2011a,b; Castoe and Parkinson, 2006), although there are some notable differences. Because our data and species coverage allow us to make inferences that were previously untenable, we briefly discuss salient findings below.

In contrast to other molecular studies (Castoe and Parkinson, 2006; Murphy et al., 2002), our data provided support for the *C. intermedius* group as sister to all other species of *Crotalus* (combined data: pp  $\geq$  0.95, bs  $\geq$  70%; Fig. 2). We inferred that the next lineage to diverge from the remaining species of *Crotalus* is a clade containing *C. polystictus*, *C. enyo*, and *C. cerastes*, with these last two forming a clade. A close relationship between *C. enyo* and *C. cerastes* is not novel, and has been previously suggested by analyses of venom electrophoresis, skull morphology and molecular data (Brattstrom, 1964; Douglas et al., 2006; Minton, 1956). While support for the sister relationship between *C. enyo* and *C. cerastes* was consistently high in BI and ML concatenated analyses (pp  $\geq$  0.95, bs  $\geq$  0.90), the sister relationship between *C. polystictus* and the *C. cerastes* group was not supported by the ML analysis, which instead placed *C. polystictus* as sister to the *C. triseriatus* group with extremely low support (bs = 23%). The instability of support values and topology suggests that the inclusion of *C. polystictus* within this clade is tentative, and may be an artifact of long-branch attraction (Bergsten, 2005 and references therein). *Crotalus enyo* had previously been assumed to be the northernmost member of the neotropical rattlesnake (*C. durissus*) group (Castoe and Parkinson, 2006; Murphy et al., 2002), but our results strongly support the exclusion of *C. enyo* from this group. The inclusion of *C. enyo* in the *C. durissus* group seems to be based on previous use of a single sequence of *cyt-b*, which our analysis suggests represents a chimeric sequence (see Supplementary Online Table 3). Instead of a close relationship between *C. enyo* and *C. durissus*, our results find weak to moderate support for *C. willardi* as a basally-diverging member of the *C. durissus* group (Figs. 2 and 3).

Our results support an expanded definition of the *C. viridis* group that includes species of the former *C. mitchellii* group as well as *C. adamanteus*; this conclusion parallels that of previous studies (Castoe and Parkinson, 2006). Although this clade is strongly supported in all of our analyses, the precise order of basal divergences within this clade remains poorly resolved (Figs. 2 and 3). The close phylogenetic affinity of *C. mitchellii* and *C. tigris* with the *C. viridis* group has been previously suggested on the basis of morphological data (Gloyd, 1940; Klauber, 1956). Although it has been assumed that the two “diamondback rattlesnakes” *C. adamanteus* and *C. atrox*, might be sister taxa, the accumulation of molecular data from this and other studies (Castoe and Parkinson, 2006; Pyron et al., 2011) provide evidence against this.

Early morphological studies considered *C. horridus* to be closely related to *C. molossus* (Brattstrom, 1964; Gloyd, 1940; Klauber, 1956). More recently, Murphy et al. (2002) recovered *C. horridus* as sister to *C. viridis* plus *C. scutulatus*, and Castoe and Parkinson (2006) placed *C. horridus* as a lineage roughly in the center of the *Crotalus* radiation. Our BI analysis of mtDNA sequences and ML analysis of all genes supported *C. horridus* as sister to a clade of “derived” rattlesnake species groups (*C. atrox*, *C. durissus*, *C. stejnegeri*, and *C. viridis* groups). This node, however, was not strongly supported in our ML results (bs = 23%), similar to other previous studies (Castoe and Parkinson, 2006). In contrast, the BI analysis of combined data placed *C. horridus* as the earliest diverging lineage within the *C. triseriatus* group with moderate support (pp = 0.77; Fig. 2), while the species tree analysis in BEAST placed this species as sister to the longtailed rattlesnakes, but with almost no support (pp = 0.42; Fig. 3). Despite substantial progress, including contributions of this study, the phylogeny of the rattlesnakes is far from resolved, and the phylogenetic relationships of several rattlesnake taxa should be re-evaluated with additional loci and perhaps even additional sampling. Lineages that are particularly in question with regard to their placement on the rattlesnake tree include *C. horridus*, *C. polystictus* and *C. willardi*, as well as the *C. cerastes* group (*C. cerastes* and *C. enyo*).

#### 4.4. Divergence and biogeography of the longtailed rattlesnakes

During the Pliocene, major volcanism occurred in what is now the boundary between the Mexican states of Jalisco and Nayarit, between the Río Grande de Santiago and Ameca rivers (Frey et al., 2007). This period of volcanic activity extended from 5 to 3 mya, which coincides with our estimates of the time that *C. stejnegeri* diverged from the ancestor of the two southern species of longtailed rattlesnakes. Regional changes in habitat distributions associated with these periods of volcanism may have split the putative ancestor of *C. stejnegeri* from the ancestor of *C. lannomi* + *C. ericsmithi* (Figs. 1 and 6). On the other end, the Balsas Basin has been implicated as an important biogeographic barrier for other vertebrate groups, including snakes (Bryson et al., 2008; Devitt, 2006), mammals (Amman and Bradley, 2004) and birds (Navarro-Siguenza et al., 2008). At the heart of the Balsas Basin, the Río Balsas is currently located at the border between the states of Michoacán and Guerrero and is a likely candidate for causing the divergence between ancestral lineages of *C. ericsmithi* and *C. lannomi* (Figs. 1 and 6).

Longtailed rattlesnake species tend to occur at middle elevations in tropical deciduous and tropical oak forests (Campbell and Flores-Villela, 2008; Campbell and Lamar, 2004; Reyes-Velasco et al., 2010). One of the most intriguing regions not yet thoroughly examined for the presence of these enigmatic snakes is the Sierra de Coalcomán, which is a small coastal mountain range in the state of Michoacán, West of the Río Balsas. Although no longtailed rattlesnake species have been recorded from this locality, convincing reports from local residents indicate that a population of longtailed

rattlesnake is likely to exist there. As additional collections are made in the region, it is therefore possible that yet another population of longtailed rattlesnakes will be discovered that may represent a new species, or possibly a population allocable to either *C. lannomi* (which is known from ca. 150 km away), or to *C. ericsmithi*, found farther to the southeast.

#### 4.5. Conclusions

Our results provide new conclusive evidence for the distinctiveness, monophyly and phylogenetic placement of the longtailed rattlesnakes. A well-resolved phylogeny for the rattlesnakes has been elusive despite a substantial number of studies that have addressed this conspicuous group (e.g. Castoe and Parkinson, 2006; Murphy et al., 2002; Parkinson, 1999; Pyron et al., 2011). By adding new data from the three most rare and enigmatic species of *Crotalus*, this study contributes important sampling for resolving *Crotalus* phylogeny. We also identified multiple instances where errors in GenBank submissions might have contributed to poor and conflicting resolution in previous studies. The fact remains, however, that although many studies have inferred rattlesnake phylogenies, most have essentially used a common set of data from a few mitochondrial and nuclear gene loci that (in some cases) have existed for more than a decade. We expect that definitive resolution of the phylogeny of rattlesnakes will ultimately require a new influx of molecular data to resolve remaining questions about the relationships among major *Crotalus* lineages and species groups.

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#### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2013.07.025>.

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