



<http://dx.doi.org/10.11646/zootaxa.3826.3.3>

<http://zoobank.org/urn:lsid:zoobank.org:pub:8D8FCB6B-E1DC-4A00-9257-BCAB7D06AE22>

Multilocus species delimitation in the *Crotalus triseriatus* species group (Serpentes: Viperidae: Crotalinae), with the description of two new species

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Abstract

Members of the *Crotalus triseriatus* species group of montane rattlesnakes are widely distributed across the highlands of Mexico and southwestern USA. Although five species are currently recognized within the group, species limits remain to be tested. Genetic studies suggest that species may be paraphyletic and that at least one cryptic species may be present. We generate 3,346 base pairs of DNA sequence data from seven nuclear loci to test competing models of species delimitation in the *C. triseriatus* group using Bayes factor delimitation. We also examine museum specimens from the Trans-Mexican Volcanic Belt for evidence of cryptic species. We find strong support for a nine-species model and genetic and morphological evidence for recognizing two new species within the group, which we formally describe here. Our results suggest that the current taxonomy of the *C. triseriatus* species group does not reflect evolutionary history. We suggest several conservative taxonomic changes to the group, but future studies are needed to better clarify relationships among species and examine genetic patterns and structure within wide-ranging lineages.

Key words: Bayes factor delimitation, cloud forest, Mexico, Trans-Mexican Volcanic Belt

Resumen

Miembros del grupo *Crotalus triseriatus* se encuentran ampliamente distribuidos en las tierras altas de México y el suroeste de Estados Unidos. Aunque actualmente se reconocen cinco especies dentro del grupo, los límites entre especies no han sido formalmente evaluados. Estudios genéticos sugieren que las especies pueden ser parafiléticas y que al menos una especie críptica puede estar presente. Generamos una secuencia de datos de 3,346 pares de bases de ADN provenientes de siete loci nucleares para evaluar modelos contrastantes de delimitación de especies en el grupo *C. triseriatus* usando el factor de delimitación de Bayes. En la búsqueda de especies crípticas, también examinamos ejemplares de museo provenientes del Eje Neovolcánico. Encontramos fuerte soporte para un modelo de nueve especies y evidencia genética y morfológica para reconocer dos nuevas especies dentro del grupo, las que formalmente describimos aquí. Nuestros resultados sugieren que la taxonomía actual de las especies del grupo *C. triseriatus* no refleja la historia evolutiva. Sugerimos varios cambios taxonómicos conservadores al grupo, requiriéndose de estudios futuros para delinear de manera más fina las relaciones entre especies y para examinar la estructura filogeográfica dentro de linajes de amplia distribución.

Palabras clave: factor de delimitación de Bayes, bosque de niebla, México, Eje Neovolcánico

Introduction

Members of the *Crotalus triseriatus* species group are widely distributed across the highlands of Mexico and the southwestern USA. Currently the group contains five species (Murphy *et al.* 2002; Bryson *et al.* 2011). The nominate species, *C. triseriatus* (Wagler 1830), contains the subspecies *C. t. triseriatus* Klauber 1952 and *C. t. armstrongi* Campbell 1979, which occur in mixed pine-oak forests across the Trans-Mexican Volcanic Belt. *Crotalus pusillus* Klauber 1952 ranges across the highlands of the Sierra de Coalcomán and the western portion of the Trans-Mexican Volcanic Belt. *Crotalus aquilus* Klauber 1952, previously considered a subspecies of *C. triseriatus* (Dorcas 1992), occurs north of the Trans-Mexican Volcanic Belt along the Central Mexican Plateau in mixed pine-oak and rocky mesquite grasslands. *Crotalus lepidus* (Kennicott 1861) is the widest ranging species in the group. It contains four subspecies distributed across a variety of habitats in northern Mexico and southwestern USA. *Crotalus l. lepidus* occurs in rocky regions of the Chihuahuan Desert and adjacent uplands, *C. l. klauberi* Gloyd 1936 inhabits the Sierra Madre Occidental and sky islands of the southwestern USA and northern Mexico, *C. l. morulus* Klauber 1952 occurs in the northern Sierra Madre Oriental, and *C. l. maculosus* Tanner, Dixon & Harris 1972 occupies the Pacific slopes of the southern Sierra Madre Occidental. *Crotalus ravus* Cope 1865 was recently added to the *C. triseriatus* group (Bryson *et al.* 2011) and it includes three subspecies, *C. r. ravus*, *C. r. brunneus* (Harris & Simmons 1978), and *C. r. exiguus* (Campbell & Armstrong 1979), found along the eastern slopes of the Trans-Mexican Volcanic Belt and Sierra Madre del Sur.

Species composition of the *C. triseriatus* group has changed several times over the past 70 years (Gloyd 1940; Smith 1946; Klauber 1952; Brattstrom 1964; Klauber 1972; Dorcas 1992; Murphy *et al.* 2002; Bryson *et al.* 2011). The most recent molecular studies of the group (Castoe & Parkinson 2006; Bryson *et al.* 2011; Reyes-Velasco *et al.* 2013) found strong support for a monophyletic assemblage that includes *C. triseriatus*, *C. pusillus*, *C. aquilus*, *C. lepidus*, and *C. ravus*. One of these studies (Bryson *et al.* 2011) also found evidence that *C. triseriatus* and *C. lepidus* are paraphyletic and that at least one cryptic species was present within the *C. triseriatus* group (Fig. 1). Although this study extensively sampled the geographic range of the *C. triseriatus* group, analyses reconstructed matrilineal relationships only because of a reliance on mitochondrial DNA (mtDNA). Reliance on single genes and mtDNA alone can mislead phylogenetic inferences (e.g., Bossu & Near 2009; Bryson *et al.* 2010; Leaché 2010; Myers *et al.* 2013; Ruane *et al.* 2014).

Despite seven decades of systematic study, no study has tested species limits in the *C. triseriatus* group. Species within the group were recognized and classified long ago based on morphology alone (Gloyd 1940; Smith 1946; Klauber 1952; Brattstrom 1964; Klauber 1972). Recent research has focused on reconstructing phylogenies (Murphy *et al.* 2002; Reyes-Velasco *et al.* 2013) or on using phylogenies to address evolutionary and biogeographic questions (Bryson *et al.* 2011). However, it is important to explicitly delimit taxa prior to constructing phylogenies (Myers *et al.* 2013; Ruane *et al.* 2014). Unrecognized species diversity can decrease the accuracy of phylogenetic, phylogeographic, and biogeographic studies (reviewed in Ruane *et al.* 2014).

Herein, we use data from seven nuclear loci to test competing models of species delimitation in the *C. triseriatus* group. We test different models of species delimitation using the recently developed Bayes factor delimitation (BFD) method, which has a number of advantages over other Bayesian species delimitation approaches (Grummer *et al.* 2014; Leaché *et al.* 2014). We explicitly compare models that reflect historical taxonomy against models that reflect phylogeographic structure and contain cryptic species. We also examine museum specimens for morphological congruence to cryptic species along the Trans-Mexican Volcanic Belt hypothesized in a previous study (Bryson *et al.* 2011).

Material and methods

Taxon sampling and molecular data assembly. We sequenced 39 samples to cover broadly the geographic range of the *C. triseriatus* group (Appendix 1). Each of the eight major mitochondrial lineages in Bryson *et al.* (2011) was represented by 3–6 samples (Fig. 1). We also evaluated the deeply divergent and geographically isolated lineage of *C. t. armstrongi* from western Jalisco and Colima (Bryson *et al.* 2011). We grouped the three subspecies of *C. ravus* into one lineage and three of the four subspecies of *C. lepidus* (*C. l. lepidus*, *C. l. klauberi*, and *C. l. maculosus*) into one lineage for model testing. Genetic structure among these taxa was considerably less pronounced than among the eight major mitochondrial lineages (Bryson *et al.* 2011).

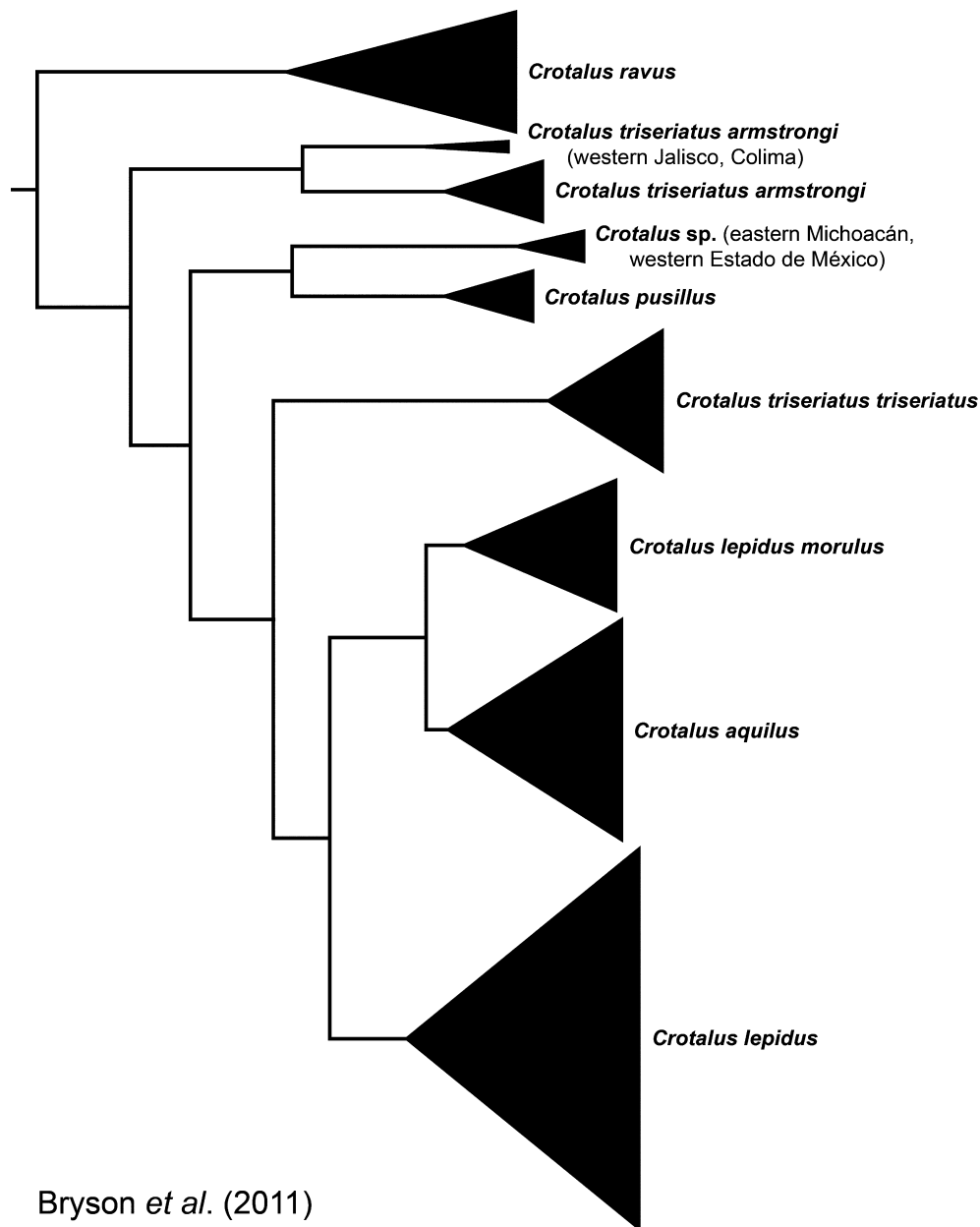


FIGURE 1. Simplified phylogeny of the *Crotalus triseriatus* species group based on Bayesian analysis of 2,408 base pairs of mitochondrial DNA obtained from 130 snakes (from Bryson *et al.* 2011).

We sequenced seven nuclear genes, including TATA box-binding protein (*TBP*), ETS oncogene (*ETS*), prolactin receptor and recombination-activating gene 2 (*Rag2PY*), and three anonymous nuclear loci (Locus25, Locus23, and Locus A). Five of these genes (*TBP*, *ETS*, Locus25, Locus23, and LocusA) were used in previous research on rattlesnake phylogenetics (Kubatko *et al.* 2011). We extracted total genomic DNA from liver, shed skins, or ventral scale clips following methods specified in Bryson *et al.* (2011). All gene regions were amplified via polymerase chain reaction (PCR) using previously published primers (Appendix 2) in a 25 µl reaction volume containing 0.8 µl deoxynucleoside triphosphates (dNTPs) (10 mM), 19.0 µl double-distilled water, 1.0 µl each primer (10 pM), 2.5 µl 1x PCR buffer (1.5 mM MgCl₂; Fisherbrand, Pittsburgh, PA, USA), 0.75 U Taq DNA polymerase (Fisherbrand), and 1.0 µl template DNA. PCR parameters included denaturation at 94 °C for 2 min, followed by 39 cycles of: 94 °C for 30 s, 49–55 °C for 45 s (Appendix 2), 72 °C for 45 s. A final extension phase of 72 °C for 7 min terminated the protocol. We visualized the entire 25 µl reaction on a 1% agarose gel containing

ethidium bromide. Sharp, clear bands were excised from the gel and placed in a filter tip (Sorenson; 75-30550T). DNA was collected in a 1.7 ml Eppendorf tube after centrifuging the DNA through the filter tip for 10 min at 16.1 rcf. We sequenced in both directions using the amplification primers and Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). We used 4 µl of the cleaned PCR product in one-quarter reaction volume of that recommended by ABI (Applied Biosystems). Samples were analyzed with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Forward and reverse sequences for each individual were edited and manually aligned using BIOEDIT v5.0.9 (Hall 1999). We phased nuclear genes using PHASE v2.1.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003) and retained the most probable pair of alleles for each heterozygous individual. To assess the degree of gene tree discordance among genes, we generated maximum likelihood gene trees for each phased gene using RAxML v7.2.8 (Stamatakis 2006). We used jModelTest v2.0.2 (Guindon & Gascuel 2003; Durrin *et al.* 2012) to calculate best-fit models of evolution for each phased gene, and selected the most parameter-rich model in the AIC 95% credible set as the best model.

Species delimitation. We used BFD (Grummer *et al.* 2014) to test competing models of species delimitation. We preferred this method to other species-delimitation approaches for several reasons, including the ability of BFD to compare non-nested models that contain different numbers of species and its ability to integrate over species trees during the delimitation process rather than relying on a specified guide tree (Grummer *et al.* 2014, Leaché *et al.* 2014). Further, obtaining decisive support with BFD for models that incorrectly split weakly diverged species or species connected by moderate to high gene flow is difficult to obtain (Leaché *et al.* 2014).

We grouped individuals into 16 alternative hypotheses based on prior taxonomic work and morphological assessments (Table 1). In general, models with fewer numbers of species reflected historical groupings (e.g., Klauber 1952, 1972; Campbell 1979; Dorcas 1992), whereas models with higher numbers of species reflected matrilineal (mtDNA) structure (Bryson *et al.* 2011). For each hypothesis, we ran species-tree analyses using the multi-species coalescent algorithm in *BEAST (Heled & Drummond 2010), a part of the BEAST v1.7.4 package (Drummond & Rambaut 2007). We assigned genes to their best-fit model of evolution with a strict-molecular clock in all analyses due to the low complexity of the individual genes sampled and to prevent overparameterization. Preliminary analyses of individual genes showed no difference in gene tree topology when using a strict or relaxed clock. Marginal likelihood values based on path-sampling (Lartillot & Philippe 2006) and stepping-stone sampling (Xie *et al.* 2011) were calculated for each hypothesis based on the methods of Baele *et al.* (2012, 2013) and Grummer *et al.* (2014). We ran the marginal likelihood analyses for 1 million generations with 100 path steps (totaling 100 million generations), logging trees and parameter estimates every 20,000 steps. We ranked and compared the resulting marginal likelihood values for path-sampling and stepping stone sampling using Bayes factors (BF; Kass & Raftery 1995) and the following BF scale (values indicate $2\ln\text{BF}$): 0–6 = positive support, 6–10 = strong support, and >10 = decisive support (Kass & Raftery 1995).

We used the preferred species model based on BFD in a separate *BEAST analysis to produce a preferred species tree. We assigned individuals to species according to the preferred BFD model and used best-fit models of DNA evolution for each gene with a strict molecular clock and a Yule species tree prior and constant population size for the species tree. We ran analyses twice, each for 200 million generations sampling every 20,000 generations. We assessed convergence between runs in Tracer v1.5 (Rambaut & Drummond 2007), ensuring ESS values for all parameters were above 200. Topological convergence was assessed with Are We There Yet (AWTY; Wilgenbush *et al.* 2004; Nylander *et al.* 2008) using the compare function. We combined tree files using LogCombiner v1.7.5 (Drummond & Rambaut 2007) and produced a maximum clade credibility tree using TreeAnnotator v1.7.5 (Drummond & Rambaut 2007). Finally, we generated a cloudogram of the posterior distribution of species trees in Densitree v2.1.7 (Bouckaert 2010) to illustrate the uncertainty in the relationships between species.

TABLE 1. Bayes factor comparisons of 16 competing models of species delimitation in the *Crotalus triseriatus* group. Marginal likelihood estimates and Bayes factor testing results ($2\ln\text{BF}$) shown for stepping-stone (SS) and path-sampling (PS) methods. The model that received the best marginal likelihood score for each estimation method is indicated by a $2\ln\text{BF}$ score of NA.

Model	Species	Groupings	SS	PS	SS Rank	PS Rank	$2\ln\text{BF}$	$2\ln\text{BF}$ Rank
M1	4	<i>triseriatus</i> (+ <i>armstrongi</i> , <i>campbelli</i> , <i>tlaloci</i> , <i>aquilus</i>), <i>lepidus</i> (+ <i>morulus</i>), <i>pusillus</i> , <i>ravus</i>	-10284.07093	-10279.16267	16	16	401.68	16
M2	4	<i>triseriatus</i> (+ <i>armstrongi</i> , <i>campbelli</i> , <i>tlaloci</i>), <i>lepidus</i> (+ <i>morulus</i> , <i>aquilus</i>), <i>pusillus</i> , <i>ravus</i>	-10252.63291	-10248.80267	15	15	338.81	15
M3	5	<i>triseriatus</i> (+ <i>armstrongi</i> , <i>campbelli</i> , <i>tlaloci</i>), <i>aquilus</i> , <i>lepidus</i> (+ <i>morulus</i>), <i>pusillus</i> , <i>ravus</i>	-10243.76786	-10240.16204	14	14	321.08	14
M4	5	<i>triseriatus</i> (+ <i>armstrongi</i> , <i>campbelli</i>), <i>aquilus</i> , <i>lepidus</i> (+ <i>morulus</i>), <i>pusillus</i> (+ <i>tlaloci</i>), <i>ravus</i>	-10218.26104	-10214.87800	13	13	270.06	13
M5	6	<i>triseriatus</i> (+ <i>tlaloci</i>), <i>armstrongi</i> (+ <i>campbelli</i>), <i>aquilus</i> , <i>lepidus</i> (+ <i>morulus</i>), <i>pusillus</i> , <i>ravus</i>	-10177.4481	-10174.33187	12	12	188.44	12
M6	7	<i>triseriatus</i> , <i>armstrongi</i> (+ <i>campbelli</i>), <i>tlaloci</i> , <i>aquilus</i> , <i>lepidus</i> (+ <i>morulus</i>), <i>pusillus</i> , <i>ravus</i>	-10124.60139	-10121.76231	6	6	82.74	6
M7	8	<i>triseriatus</i> , <i>armstrongi</i> (+ <i>campbelli</i>), <i>tlaloci</i> , <i>aquilus</i> , <i>lepidus</i> , <i>morulus</i> , <i>pusillus</i> , <i>ravus</i>	-10114.78151	-10110.87248	4	4	63.10	4
M8	6	<i>triseriatus</i> , <i>armstrongi</i> (+ <i>campbelli</i>), <i>aquilus</i> , <i>lepidus</i> (+ <i>morulus</i>), <i>pusillus</i> (+ <i>tlaloci</i>), <i>ravus</i>	-10157.39388	-10154.65596	11	11	148.33	11
M9	8	<i>triseriatus</i> , <i>armstrongi</i> , <i>campbelli</i> , <i>tlaloci</i> , <i>aquilus</i> , <i>lepidus</i> (+ <i>morulus</i>), <i>pusillus</i> , <i>ravus</i>	-10103.72214	-10100.23466	2	2	40.98	2
M10	7	<i>triseriatus</i> , <i>armstrongi</i> , <i>campbelli</i> , <i>tlaloci</i> , <i>lepidus</i> (+ <i>morulus</i> , <i>aquilus</i>), <i>pusillus</i> , <i>ravus</i>	-10112.24332	-10108.59203	3	3	58.03	3
M11	7	<i>triseriatus</i> (+ <i>aquilus</i>), <i>armstrongi</i> , <i>campbelli</i> , <i>tlaloci</i> , <i>lepidus</i> (+ <i>morulus</i>), <i>pusillus</i> , <i>ravus</i>	-10122.10788	-10119.20973	5	5	77.76	5
M12	6	<i>triseriatus</i> (+ <i>aquilus</i>), <i>armstrongi</i> (+ <i>campbelli</i>), <i>tlaloci</i> , <i>lepidus</i> (+ <i>morulus</i>), <i>pusillus</i> , <i>ravus</i>	-10150.59401	-10146.60773	10	10	134.73	10
M13	7	<i>triseriatus</i> , <i>armstrongi</i> , <i>campbelli</i> , <i>aquilus</i> , <i>lepidus</i> (+ <i>morulus</i>), <i>pusillus</i> (+ <i>tlaloci</i>), <i>ravus</i>	-10128.62644	-10125.02949	8	8	90.79	8
M14	6	<i>triseriatus</i> , <i>armstrongi</i> (+ <i>tlaloci</i>), <i>campbelli</i> , <i>lepidus</i> (+ <i>morulus</i> , <i>aquilus</i>), <i>pusillus</i> , <i>ravus</i>	-10138.21296	-10134.60411	9	9	109.97	9
M15	7	<i>triseriatus</i> , <i>armstrongi</i> (+ <i>tlaloci</i>), <i>campbelli</i> , <i>aquilus</i> , <i>lepidus</i> (+ <i>morulus</i>), <i>pusillus</i> , <i>ravus</i>	-10125.26178	-10122.08557	7	7	84.06	7
M16	9	<i>triseriatus</i> , <i>armstrongi</i> , <i>campbelli</i> , <i>tlaloci</i> , <i>aquilus</i> , <i>lepidus</i> , <i>morulus</i> , <i>pusillus</i> , <i>ravus</i>	-10083.23020	-10079.42920	1	1	NA	1

Morphological data. We examined museum specimens of the *C. triseriatus* group from along the Trans-Mexican Volcanic Belt of central Mexico (Appendix 3) for morphological congruence of cryptic species hypothesized by Bryson *et al.* (2011). Acronyms used for museum specimens follow Sabaj Pérez (2013) except for Universidad Michoacana de San Nicolás de Hidalgo, Michoacán, abbreviated HINIRENA. We examined 19 characters historically used for distinguishing species in the *C. triseriatus* group (Dorcas 1992). We first noted the number or arrangement of meristic characters, including supralabials, infralabials, upper preoculars, canthals, intercanthals, prefrontals, ventrals, subcaudals, and rattle-fringe scales. We then measured tail length and proximal rattle width, and standardized these measurements by dividing them by total length and head length, respectively. Finally, we examined aspects of color pattern, including dorsal blotches (and interspace between lateral blotches), tail bands, ventral mottling, tail color (ventrally), proximal rattle color, and postocular stripe. Scale counts and definitions of external morphological features follow Dorcas (1992). Body and tail lengths were measured using a meter stick to the nearest mm; all other measurements were made to the nearest 0.01 mm with a digital caliper. Scale counts were made with the aid of a dissecting microscope. Meristic asymmetry was noted as left/right. Juvenile snakes less than 300 mm long (Dorcas 1992) were not used in comparisons of tail length, proximal rattle width and color, and tail color.

Results

Sequence data. We obtained a total of 3,346 aligned base pairs of DNA sequence data from the seven loci (Table 2). The number of variable sites within each phased gene ranged from 5 (Locus25) to 55 (LocusA). Individual gene trees (not shown) contained few strongly supported nodes.

TABLE 2. Information on the seven nuclear loci used in this study, including length in base pairs (bp) after alignment, number of variable sites, and model of evolution.

Locus	Aligned length (bp)	Variable sites	Model
TATA box-binding protein (<i>TBP</i>)	862	19	HKY + G
ETS oncogene (<i>ETS</i>)	661	7	HKY + G
Prolactin Receptor (<i>PRLR</i>)	478	36	HKY + G
Recombination-activating gene 2 (<i>Rag2PYY</i>)	408	21	K80
Anonymous 25 (Locus25)	242	5	K80 + G
Anonymous 63 (Locus63)	449	23	F81 + G
Anonymous A (LocusA)	246	55	K80 + G

Species delimitation. Bayes factor comparisons of 16 competing models of species delimitation in the *C. triseriatus* group decisively favored a model with nine species (M16) over competing models based on both stepping-stone and path-sampling (Table 1). The $2\ln\text{BF}$ score of the nine-species model over the next ranked model (M9) was > 40 . Models that lumped species, particularly into historical classifications (e.g., grouping all of the former populations of *C. triseriatus* together, M1; grouping all of the former populations of *C. triseriatus* together except for *C. aquilus*, M3 and M4), consistently ranked low.

The preferred species tree revealed weak support among most species relationships in the *C. triseriatus* group (Fig. 2). Three nodes within the group received moderate support. One node supported a large clade that contained a putative undescribed species (see below), *C. pusillus*, *C. armstrongi*, *C. triseriatus*, *C. aquilus*, *C. morulus*, and *C. lepidus* (0.85 posterior probability). Within this clade, *C. triseriatus* was supported as the sister species (1.0 posterior probability) to a subclade containing *C. aquilus*, *C. morulus*, and *C. lepidus* (0.85 posterior probability). The cloudogram demonstrated uncertainty in species relationships across the posterior distribution of species trees, and this uncertainty was especially evident near the base of the trees (Fig. 2).

Morphology. No single morphological character distinguished any one of the five species distributed along the Trans-Mexican Volcanic Belt of central Mexico (Table 3) with one exception: the lack of intercanthals in *C. pusillus* was not shared with any other lineage. Notwithstanding, unique combinations of morphological characters separated most species, particularly the condition of the upper preocular, arrangement of scales in the prefrontal

region, number of ventrals and subcaudals, tail length and proximal rattle width ratios, number of dorsal and tail blotches, pale interspaces between dorsal and lateral blotches, shape of the postocular stripe, and color of the venter, tail, and proximal rattle. The combination of the mean number of ventrals, subcaudals and dorsal blotches, condition of the upper preocular, and tail length and proximal rattle width ratios separated all species.

Recognition of new species. The species delimitation model with the best marginal likelihood values strongly supported the recognition of two new species within the *C. triseriatus* group. These two new species were also phenotypically distinct from most of their close relatives (Table 3), and the mean number and frequency of occurrence of morphological characters in both species were unique. Each new species also appeared to be geographically isolated from similar species (Fig. 3) and to have occupied different habitats (see below). Collectively, these results suggested that each species represented a distinct lineage with a unique evolutionary history and independent evolutionary trajectory.

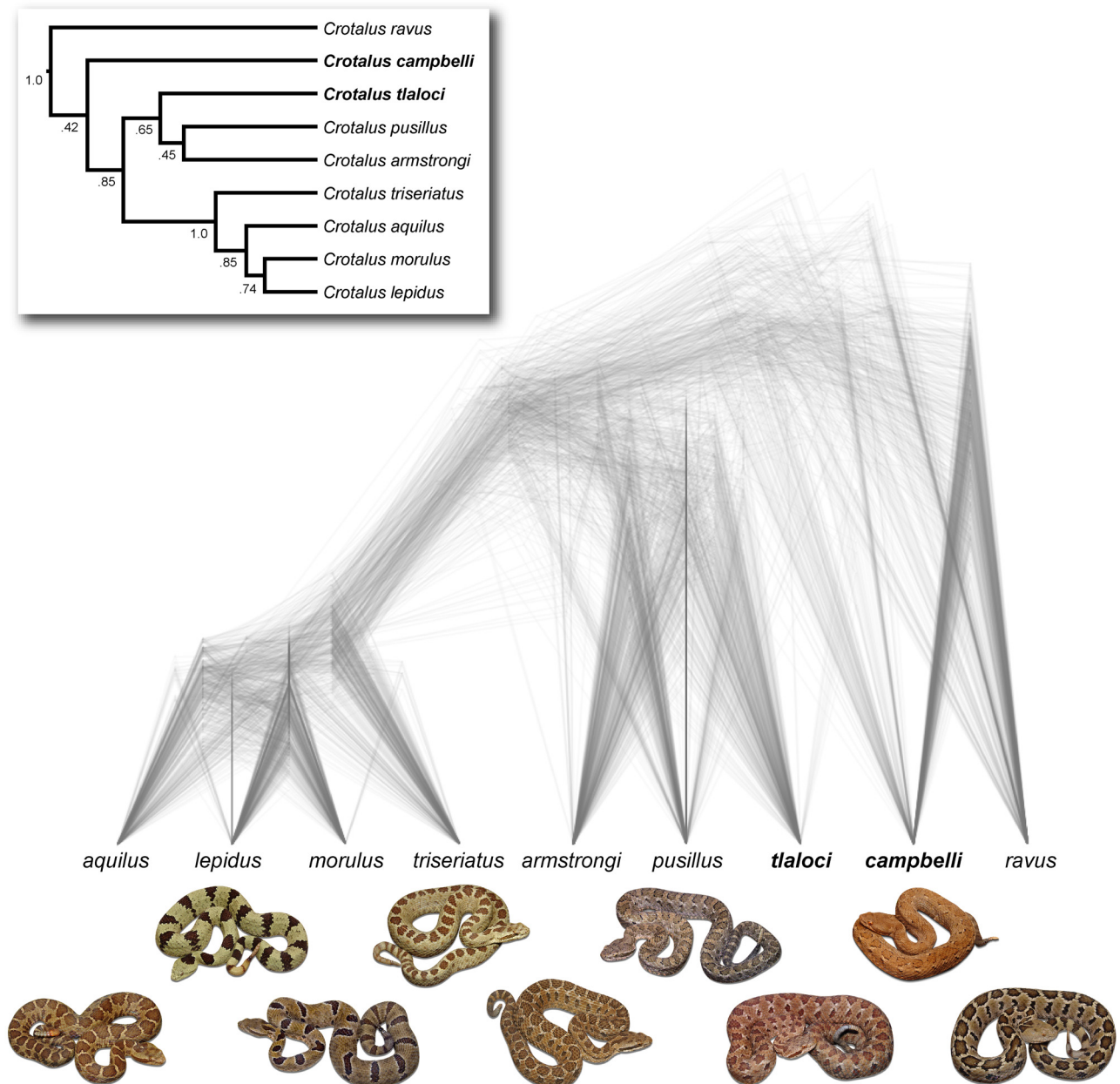


FIGURE 2. Posterior density of species trees (cloudogram) from *BEAST analyses of seven nuclear loci for the *Crotalus triseriatus* species group. Darker areas represent regions of tree space where the majority of trees agree in topology. Upper left inset shows the maximum clade credibility species tree with posterior probability values for each node. *Crotalus tlaloci* **sp. nov.** and *Crotalus campbelli* **sp. nov.** indicated by bold font.

TABLE 3. Summary of meristic, morphometric, and color pattern characters for members of the *Crotalus triseriatus* species group distributed across the Trans-Volcanic Belt. Range followed by mean in parentheses for all characters except for rattle fringe scales, supralabials, infralabials, intercanthals, and tail bands, where range is followed by mode in parentheses. Ventral and subcaudal counts are shown for males (above) and females (below). Tail length ratio for *C. pusillus* from Klauber (1952). Names of species reflect the taxonomy proposed here.

Character	<i>C. pusillus</i> (n = 33)	<i>C. campbelli</i> sp. nov. (n = 6)	<i>C. armstrongi</i> (n = 28)	<i>C. tlaloci</i> sp. nov. (n = 12)	<i>C. triseriatus</i> (n = 21)
Supralabials	11–14 (12)	11–13 (12)	11–13 (12)	11–14 (12)	11–14 (12)
Infralabials	10–13 (11)	10–13 (12)	9–13 (12)	11–13 (12)	10–13 (11)
Upper preocular (% divided)	0	9.1	14.3	0	9.4
Canthals	2	2	2	2	2
Intercanthals	0	1–3 (1)	1–3 (2)	2	1–3 (2)
Posterior intercanthals	0	0–4 (2, 3)	1–4 (3)	1–2 (2)	1–4 (3)
Prefrontals	symmetrical	variable	variable	symmetrical	variable
Ventrals	149–160 (154) 147–160 (154)	150–154 (152) 147–152 (149)	130–151 (141) 138–148 (144)	152–164 (156) 156–165 (159)	134–146 (141) 135–147 (142)
Subcaudals	26–32 (31) 25–27 (26)	31–32 (31) 22–26 (24)	24–31 (28) 19–28 (24)	27–33 (30) 22–32 (28)	21–32 (28) 21–26 (24)
Rattle fringe scales	7–10 (8)	8–9 (8)	7–10 (8)	8–10 (8)	8–10 (8)
Tail length ratio	10.5 8.6	9.1–11.0 (10.3) 7.5–8.9 (8.2)	8.8–10.9 (9.7) 5.7–9.9 (8.3)	8.9–11.3 (10.1) 8.0–10.7 (9.2)	8.0–12.1 (10.7) 7.9–9.4 (8.3)
Proximal rattle width ratio	9.4–13.2 (12.5)	11.0–14.6 (13.0)	11.6–15.9 (14.0)	11.1–14.5 (12.8)	14.3–18.0 (15.8)
Dorsal blotches	36–50 (42)	44–53 (48)	32–52 (42)	35–45 (43)	34–53 (46)
Dorsal and lateral blotches separated by pale interspace	yes	yes	yes	yes	no
Tail bands	6–12 (8)	5–9 (9)	4–9 (6)	8–10 (8)	3–10 (7)
Venter mottling	heavy	heavy	variable	heavy	variable
Tail color	dark	dark	variable	dark	variable
Proximal rattle color	dark	dark	variable	dark	variable
Postocular stripe	uniform width, rarely slight taper near eye	uniform width	uniform width, rarely slight taper near eye	distinct narrowing near eye	uniform width

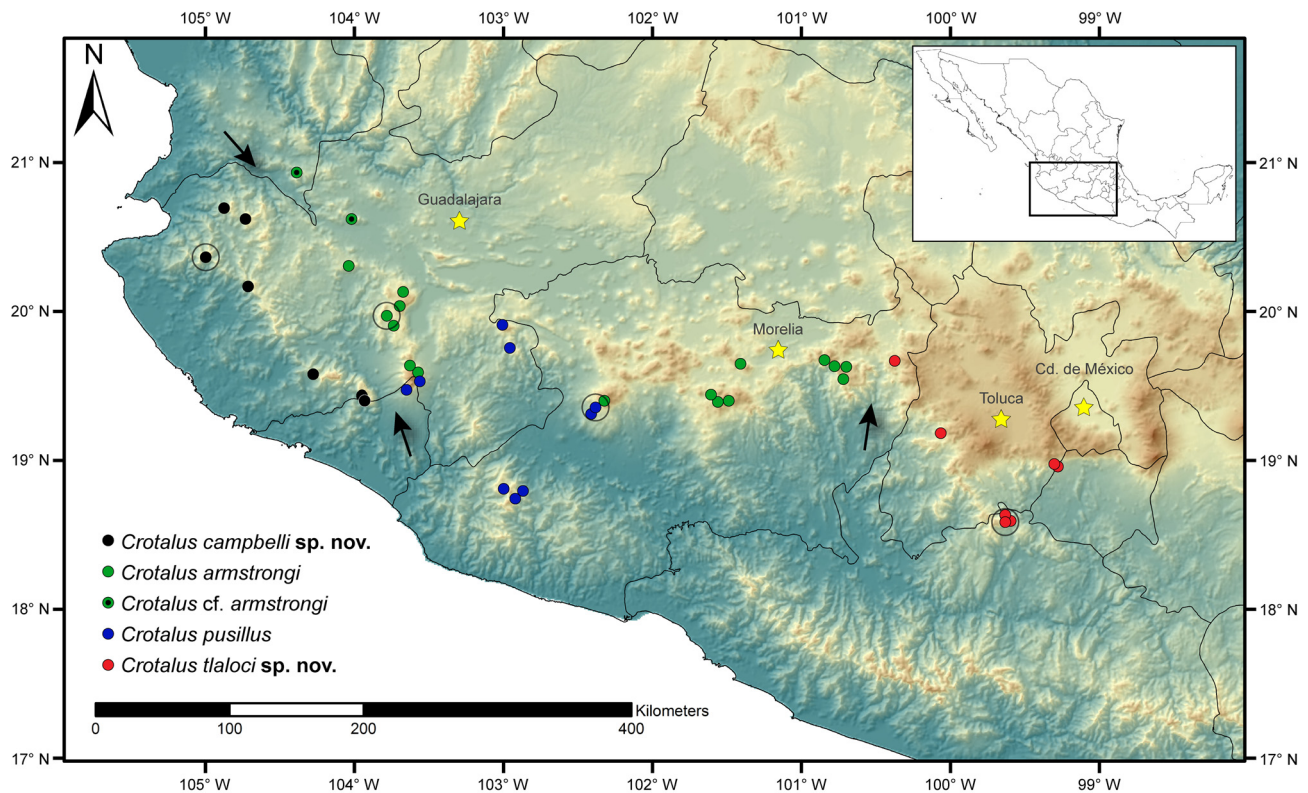


FIGURE 3. Geographic distribution of species in the *Crotalus triseriatus* species group distributed across the Trans-Volcanic Belt. Circled dots indicate type localities. Arrows point to low-elevation depressions that are probable barriers to gene flow. Names of species reflect our proposed taxonomy.

***Crotalus tlaloci* sp. nov.**

Figs. 4–5, Table 3

Crotalus triseriatus triseriatus—Campbell & Lamar (2004): 527 (Map 90), 593 (in part).

Crotalus triseriatus triseriatus—Flores-Villela & Hernández-García (1989): 16.

Crotalus triseriatus triseriatus—Pérez-Ramos *et al.* (2000): 34.

Crotalus triseriatus triseriatus—Flores-Villela & Hernández-García (2006): 270.

Holotype. Adult female (MZFC 3666) collected 20 June 1986 by Efraín Hernández-García in “Los Llanos” (18°36'N, 99°37'W; 2200–2300 m above sea level; asl hereafter), 10 km by road from Taxco to Tetipac, Sierra de Taxco, municipality of Tetipac, state of Guerrero, Mexico.

Paratypes. 11 specimens. MEXICO: GUERRERO: Cerro del Huizteco, Sierra de Taxco, municipality of Taxco (18°36'N, 99°36'W; 2300–2520 m asl); collected 22–23 August 1986 by E. Hernández-García (MZFC 3664–3665). “Arroyo las Damas”, Sierra de Taxco, municipality of Tetipac (18°38'N, 99°37'W; 1600–1850 m asl); collected by E. Hernández-García (MZFC 3666). ESTADO DE MÉXICO: Acatitlán, municipality of Valle de Bravo; collected 7 September 1988 by T. Hentschel-Maida (MZFC 4324). Los Álamos, municipality of Valle de Bravo (19°11'20.2"N, 100°03'57.2"W; 2201 m asl; NAD27 Mexico); collected 23 May 2008 by J. Jones, C. I. Grünwald, and R. W. Bryson Jr. (HINIRENA 725–726). Los Álamos, municipality of Valle de Bravo (19°11'20.2"N, 100°03'57.2"W; 2201 m asl; NAD27 Mexico); collected 22 July 2009 by R. W. Bryson Jr. and M. Torocco (MZFC 25114–25115). MORELOS: Km 12, Carr. Cuernavaca-Ocuilán, municipality of Cuernavaca; collected 17 March 1990 by M. Torres Chávez (MZFC 4657). Carr. Cuernavaca-Ocuilán, near state border, municipality of Cuernavaca (18°58'54.43"N, 99°18'20.43"W; 2268 m asl; WGS84); collected 13 June 2009 by J. Jones, C. I. Grünwald, and R. W. Bryson Jr. (MZFC 25111). MICHOACÁN: N Arroyo Seco, municipality of Aporo (19°40'28.3"N, 100°22'35.8"W; 2463 m asl; NAD27 Mexico); collected 24 May 2008 by J. Jones, C. I. Grünwald, and R. W. Bryson Jr. (HINIRENA 724).

Diagnosis. *Crotalus tlaloci* can be distinguished from all members of the *C. triseriatus* species group by the combination of the following characters: (1) presence of intercanthals, (2) undivided upper preocular, (3) 152–164 ventrals in males, 156–165 in females, (4) 27–33 subcaudals in males, 22–32 in females, (5) small rattle (proximal rattle width 11.1–14.5% of head length), (6) long tail (8.9–11.3% of total body length in males, 8.0–10.7% in females), (7) usually two pairs of symmetrical, similarly sized intercanthals, and (8) dark postocular stripe that noticeably narrows before reaching the posterior of the eye.

Crotalus tlaloci is most similar to species of the *C. triseriatus* group distributed along the Trans-Mexican Volcanic Belt. *Crotalus tlaloci* is distinguished from these species by the presence of intercanthals (lacking in *C. pusillus*), undivided upper preocular (divided 14.3% of the time in *C. armstrongi* and 9.4% of the time in *C. triseriatus*), high number of ventrals (overlapping with *C. pusillus*, but mean number in *C. tlaloci* higher than *C. pusillus*: 156 vs. 154, respectively), high number of subcaudals (mean number in females higher than in *C. pusillus*, *C. armstrongi*, and *C. triseriatus*: 28 vs. 26, 24, and 24; in males, higher than in *C. armstrongi* and *C. triseriatus*: 30 vs. 28 in both), proportionately small proximal rattle (mean width smaller than in *C. armstrongi* and *C. triseriatus*: 12.8% of head length vs. 14.0% and 15.8%), and proportionately longer tail (mean length in females higher than in *C. pusillus*, *C. armstrongi*, and *C. triseriatus*: 9.2% of total length vs. 8.6%, 8.3%, and 8.3%). Most specimens (10/12) possess two pairs of symmetrical, similarly sized intercanthals, creating the appearance of butterfly wings. Of the 100 specimens in the *C. triseriatus* group that we examined, this symmetrical paired arrangement of intercanthal scales in the prefrontal region (“butterfly wings”) was observed in only one other specimen (*C. armstrongi*, CNAR 4498). *Crotalus tlaloci* also possess a dark postocular stripe that noticeably narrows before reaching the posterior of the eye. In *C. pusillus*, *C. armstrongi*, and *C. triseriatus*, the postocular stripe is generally of uniform width, although on rare occasions in *C. pusillus* and *C. armstrongi* it tapers slightly before reaching the eye.

Description of the holotype. Rostral broader than high (4.0 x 2.8 mm); two internasals, in medial contact, wider than long, rectangular, convex through center of scale; two canthals, large, convex, separated by two pairs of square intercanthals; four large intersupraoculars posterior to intercanthals, followed by multiple rows of small intersupraoculars. Naris centered between prenasal and postnasal scales, prenasal larger than postnasal and wrapping around anterior aspect of rostrum; two loreals, lower larger; small upper loreal between canthal, internasal, postnasal, and lower loreal. Loreal pit midway between eye and naris, below line from middle of eye to naris, bordered by single prelacunal, postlacunal, lower preocular, and lower loreal; prelacunal contacting second and third supralabials and a single prefoveal (two prefoveals on left); two prefoveals on right, three on left; single postfoveal contacting postlacunal, lower preocular, first subocular, and fourth supralabial. Two preoculars, upper large and convex, contacting supraocular and canthal, lower preocular thin and long; three suboculars, anterior largest and in contact with fourth supralabial; three interoculabials posterior to anterior subocular; two postoculars, dorsal twice as large as ventral. Supralabials 12/12; infralabials 13/13; first infralabials in medial contact posterior to triangular mental; genials together resemble a heart shape. Midbody dorsal scale rows 22–23; preventral single; ventrals 162; subcaudals 27, last subcaudal row divided into three scales; nine rattle fringe scales; tail bearing four rattle segments.

Coloration significantly faded in preservative with body blotches and bands difficult to discern from ground color. Ground color gray; occasional black speckling along body. Blotches faded to ground coloration in many places, approximately 38 blotches visible. Blotches bordered by lighter coloration than ground color, also heavily faded. Ground color of head gray, heavily stippled with dark brown throughout dorsal and anterior lateral regions of the head; rostral and supralabials 1–4 heavily stippled, supralabials 5–7 with lighter stippling in the scale center; infralabials with brown stippling on scale margins with white scale centers, extending to mouth; gular scales lightly stippled, decreasing in frequency towards the midline of the head. Ventral scutes evenly stippled, increasing in intensity posterior to mid-body with scutes becoming almost completely brown by the tail. Six dark tail bands, the first two bordered by white bands; caudal scales dark brown. Proximal rattle black, distal sections brownish.

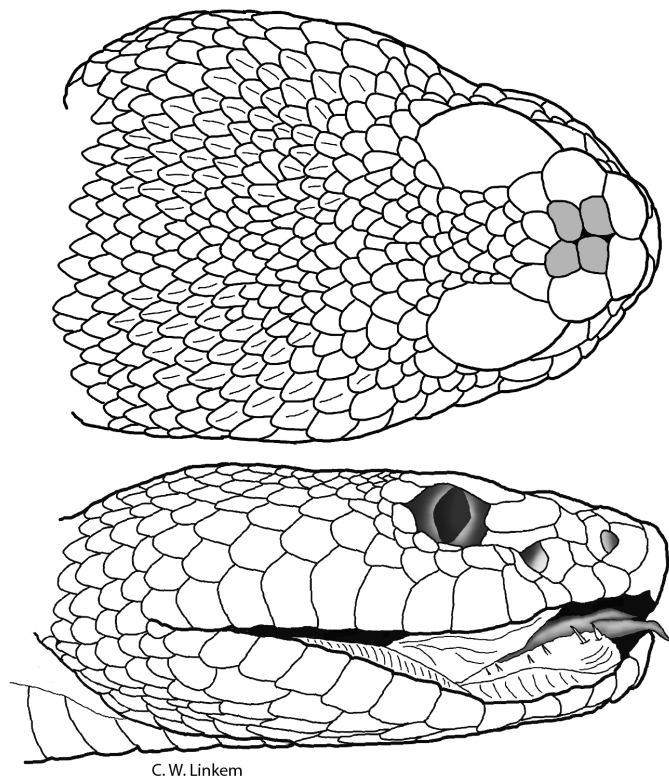


FIGURE 4. Lateral and dorsal view of the holotype of *Crotalus tlaloci* sp. nov. (MZFC 3666). The symmetrical paired arrangement of intercanthal scales, shown here in gray, create the appearance of butterfly wings in the prefrontal region.

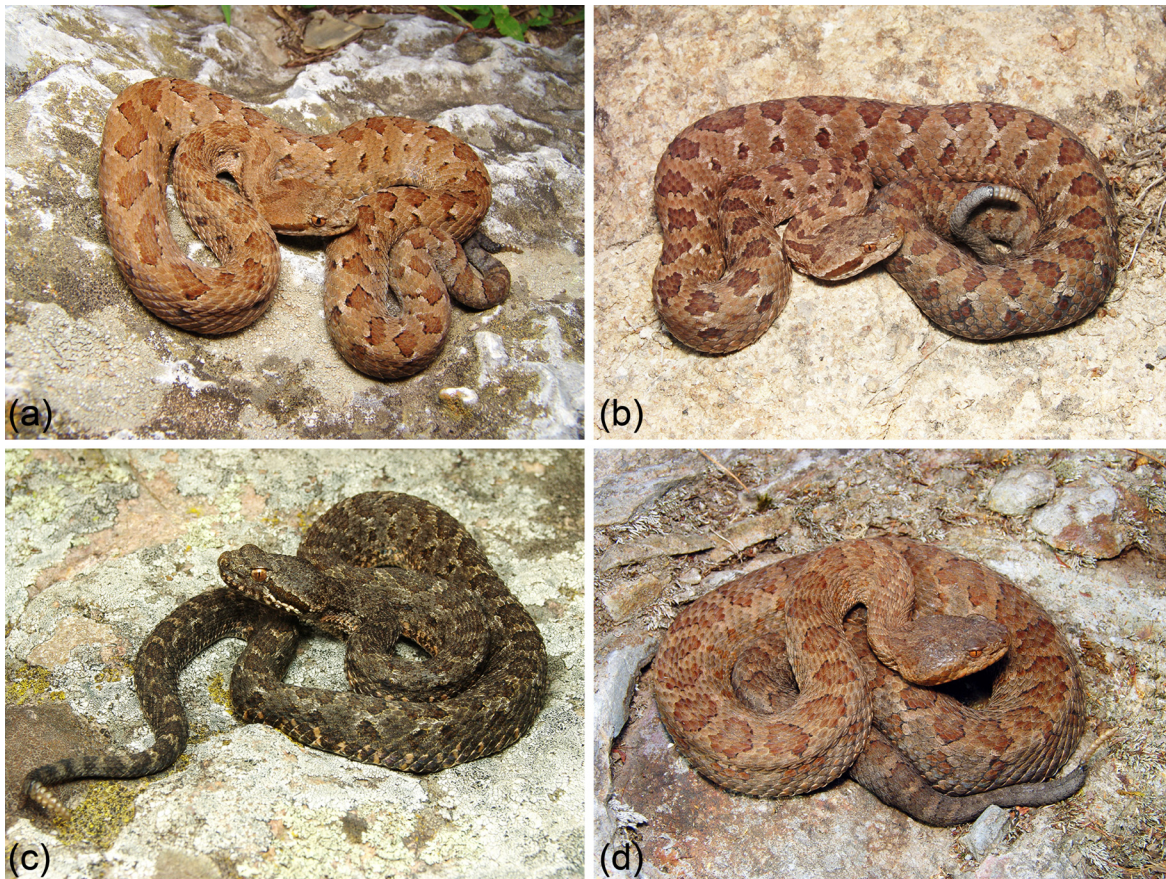


FIGURE 5. *Crotalus tlaloci* sp. nov. in life, (a) MZFC 25114, paratype from Valle de Bravo, Estado de México; (b) HINIRENA 725, paratype from Valle de Bravo, Estado de México; (c) MZFC 25111, paratype from Cuernavaca-Ocuilán highway, Morelos; and (d) HINIRENA 724, paratype from Arroyo Seco, Michoacán.

Color in life. Color in life varies (Fig. 5), with most specimens vibrantly colored, although one is darkly pigmented (MZFC 25111; Fig. 5). The holotype is known only from the preserved specimen.

Variation. Two specimens lack symmetrical paired intercanthals. The first, HINIRENA 725, has one pair of anterior intercanthals but only a single large posterior intercanthal. The second, HINIRENA 724, has one pair of anterior intercanthals, one of which is subdivided, followed by a single large posterior intercanthal. Supralabials in five specimens (MZFC 3664, 3665, 4657, 25111, 25114) are horizontally divided. This split was in the 7th, 8th, or 9th supralabial and when present, occurred in supralabials on both sides of the head. Juveniles (MZFC 3664, 3665, 3667, HINIRENA 726) have cream-colored proximal rattles and pale-colored tails ventrally, typical of juveniles in other species in the *C. triseriatus* group. Variation in meristic, morphometric, and color pattern characters within the type series is listed in Table 3.

Etymology. This species is named for Tláloc, the Aztec god of rain.

Habitat and distribution. *Crotalus tlaloci* inhabits open areas in cloud forest and humid oak-pine forest along the lower slopes of the Trans-Mexican Volcanic Belt. Although one record in the Sierra de Taxco (“Arroyo las Damas”) is at 1850 m (Flores-Villela & Sánchez-H 2003), most records are at around 2000–2400 m asl. This species is known from the states of Guerrero, Estado de México, Michoacán, and Morelos, and may range into western Puebla. The vegetation where *C. tlaloci* is found is characterized by broad-leaf oaks, such as *Quercus candicans* and *Q. laurina*, and dense undergrowth (Fig. 6), and is distinctly different than the drier pine-oak forest inhabited by *C. triseriatus*. The distribution of *C. tlaloci* overlaps the ranges of two alligator lizards, *Barisia herrerae* Zaldívar-Riverón & Nieto-Montes de Oca 2002 and *B. rudicollis* (Wiegmann 1828) (Zaldívar-Riverón & Nieto-Montes de Oca 2002). Interestingly, both of these alligator lizards occur in similar humid forest habitat at elevations of 2000–2500 m asl, and appear ecologically isolated from *B. imbricata* (Wiegmann 1828), which inhabits the surrounding drier pine-oak forest (Zaldívar-Riverón & Nieto-Montes de Oca 2001, 2002). Specimens of *C. tlaloci* are generally found in rocky open forest breaks and edges of cloud or humid oak-pine forest. However, we found an adult gravid female and juvenile (HINIRENA 725, 726) under logs in a clearing relatively devoid of rocky habitat.

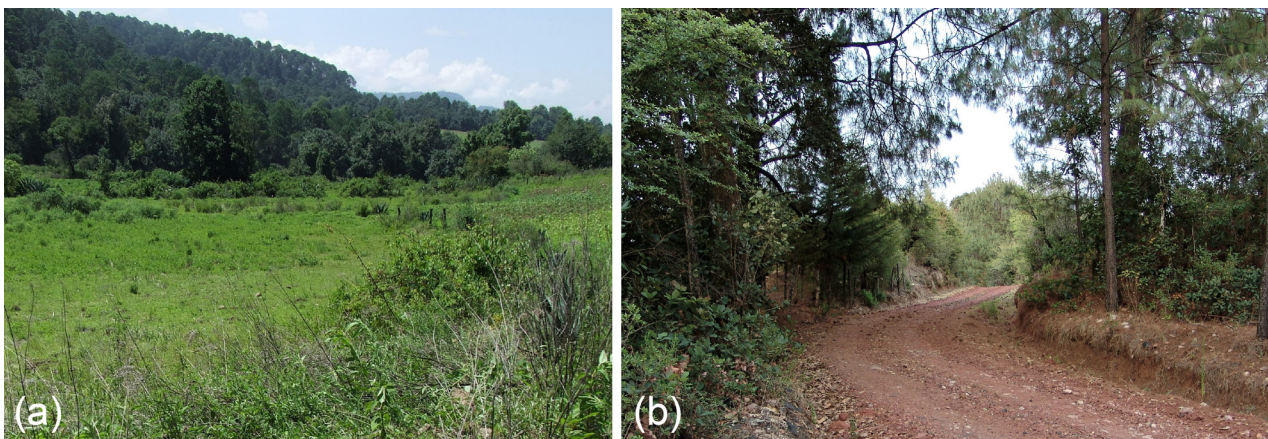


FIGURE 6. Humid oak-pine forest habitat of *Crotalus tlaloci* **sp. nov.** at the paratype localities of (a) Los Álamos, near Valle de Bravo, Estado de México; and (b) Arroyo Seco, Michoacán.

Crotalus campbelli **sp. nov.**

Figs. 7–8, Table 3

Crotalus triseriatus—Boulenger (1896): 581 (in part).

Crotalus triseriatus armstrongi—Dorcas (1992): 87 (in part).

Crotalus triseriatus armstrongi—Bryson *et al.* (2011): 699 (in part).

Crotalus triseriatus armstrongi—Reyes-Velasco *et al.* (2009): 118.

Holotype. Adult female (KU 73649) collected 25 October 1962 by Percy L. Clifton (field number PLC 3216) in the Sierra de Cuale, 9 km N El Teosinte, municipality of Talpa de Allende, state of Jalisco, Mexico.

Paratypes. 5 specimens. Mexico: JALISCO: same collection data as holotype (KU 73650). Las Playitas, Las Joyas, Sierra de Manantlán, municipality of Autlán de Navarro; collected September 1985 by E. Fanti-Echegoyen (UTA R-16352). Las Joyas, Sierra de Manantlán, municipality of Autlán de Navarro; collected September 1985 by E. Fanti-Echegoyen (UTA R-16353). ca. 25 km SE Autlán, ca. 2.1 km (by dirt road) SE Manantlán; collected 27 July 1975 by G. M. Tilger and R. G. Arndt (AMNH R-113191). Lago de Juanacatlán, Sierra de Mascota, municipality of Mascota (20°37'30.94"N, 104°43'36.30"W; 2009 m asl; WGS84); collected 10 April 2011 by R. W. Bryson Jr. and M. Torocco (MZFC 28669).

Diagnosis. *Crotalus campbelli* can be distinguished from all members of the *C. triseriatus* species group except *C. armstrongi* by the combination of the following characters: (1) presence of intercanthals, (2) infrequently divided upper preocular (9.1% of the time), (3) 150–154 ventrals in males, 147–152 in females, (4) 31–32 subcaudals in males, 22–26 in females, (5) small rattle (proximal rattle width 11.0–14.6% of head length), (6) long tail (9.1–11.0% of total body length in males, 7.5–8.9% in females), (7) pale interspaces between dorsal and lateral blotches, (8) heavy venter mottling, (9) dark proximal rattle and underside of tail, and (10) usually a single large anterior intercanthal. *Crotalus campbelli* can be distinguished from *C. armstrongi* based on higher mean number of ventrals (152 in males and 149 in females vs. 141 and 144), higher mean number of subcaudals in males (31 vs. 28), less frequently divided upper preocular (9.1% vs. 14.3%), proportionately longer tail in males (10.3% of total body length vs. 9.7%), smaller mean proximal rattle width (13.0% of head length vs. 14.0%), higher mean number of dorsal blotches (48 vs. 42), and higher number of tail bands (mode of 9 vs. 6).

Crotalus campbelli is most similar to members of the *C. triseriatus* group distributed along the Trans-Mexican Volcanic Belt, including *C. pusillus*, *C. armstrongi*, *C. triseriatus*, and *C. tlaloci*. *Crotalus campbelli* is distinguished from *C. pusillus* by possessing intercanthals and an infrequently divided upper preocular, from *C. tlaloci* by having an infrequently divided upper preocular, variable number of intercanthals, fewer ventrals (in females, 147–152 vs. 156–165; in males, mean number 152 vs. 156), lower mean number of subcaudals in females (24 vs. 28), proportionately shorter tail in females (8.2% of total length vs. 9.2%), and higher mean number of dorsal blotches (48 vs. 43), and from *C. triseriatus* by a higher number of ventrals (in males, 150–154 vs. 134–146; in females, mean number higher: 149 vs. 142), higher mean number of subcaudals in males (31 vs. 28), proportionately smaller proximal rattle (13.0% of head length vs. 15.8%), and by having pale interspaces between dorsal and lateral blotches. *Crotalus campbelli* is most similar in general appearance to *C. armstrongi*, but can be distinguished from this species by characters mentioned above. Half of the specimens of *C. campbelli* also possess a single, large anterior intercanthal. This scale arrangement is rarely seen in *C. armstrongi* and *C. triseriatus*. *Crotalus campbelli* is easily distinguished from *C. ravus* by the lack of large head plates in the parietal region.

Description of the holotype. Rostral broader than high (3.7 x 2.6 mm); two internasals, in medial contact, slightly wider than long, convex through center of scale; two canthals, large, convex, circular, separated by a single, large, convex intercanthal bordered anteriorly by three small scales; four large intersupraoculars posterior to intercanthals, followed by multiple rows of small intersupraoculars. Nostril centered between prenasal and postnasal scales, prenasal larger than postnasal and wrapping around anterior aspect of rostrum; single loreal. Loreal pit midway between eye and naris, below line from middle of eye to naris, bordered by single prelacunal, postlacunal, lower preocular; prelacunal contacting second and third supralabials and two prefovials; three prefovials; single postfoveal contacting postlacunal, lower preocular, first subocular, and fourth supralabial. Two preoculars, upper large and convex, contacting supraocular and canthal, lower preocular thin and long; three suboculars, anterior largest and in contact with fourth supralabial; three interoculabials posterior to anterior subocular; two postoculars, upper twice as large as lower. Supralabials 13/13; infralabials 11/12; first infralabials in medial contact posterior to triangular mental; genials together resemble wings. Midbody dorsal scale rows 23–25; preventral single; ventrals 152; subcaudals 22, nondivided; eight rattle fringe scales; tail bearing three rattle segments.

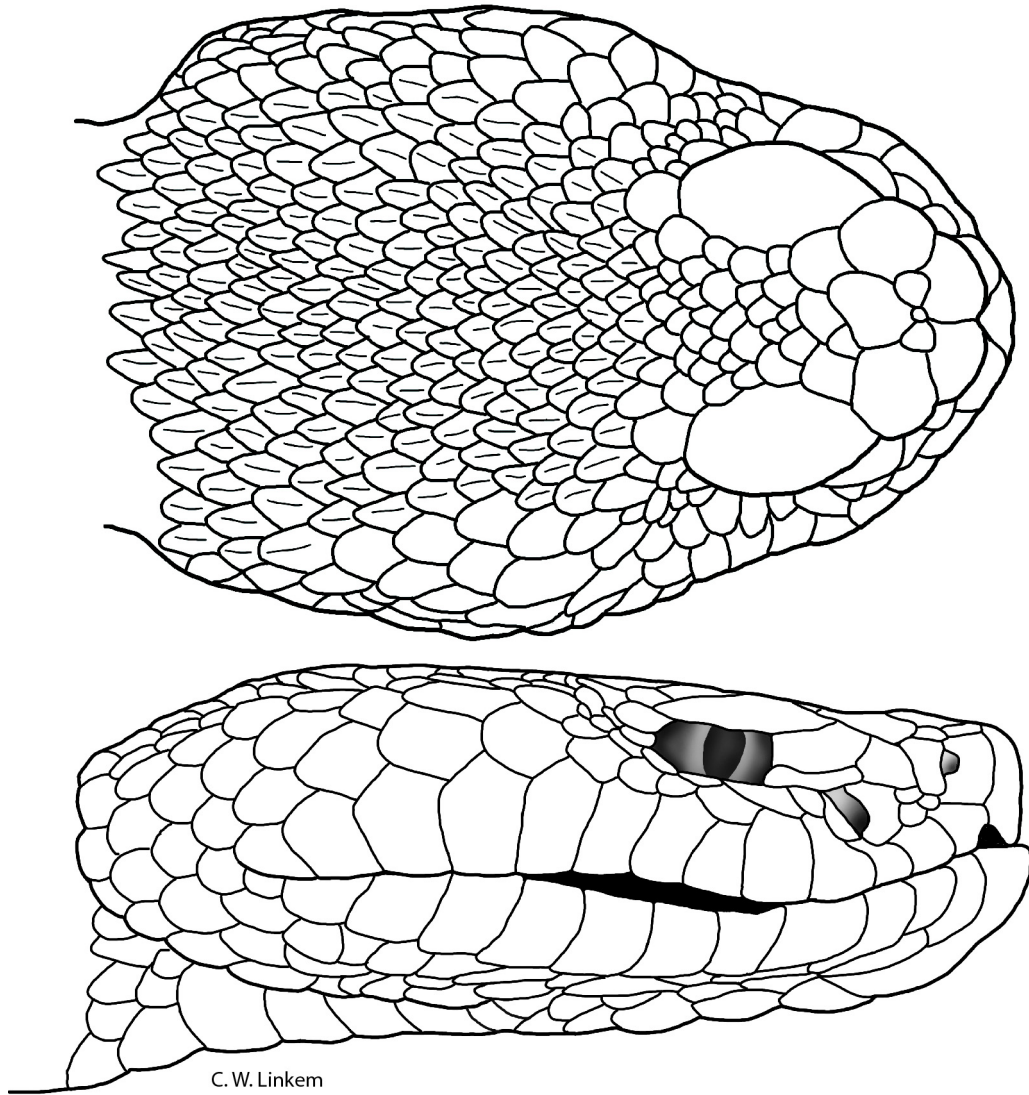


FIGURE 7. Lateral and dorsal view of the holotype of *Crotalus campbelli* sp. nov. (KU 73649).



FIGURE 8. *Crotalus campbelli* sp. nov. in life, (a) MZFC 28669, paratype from the Sierra de Mascota, Jalisco; and (b) specimen in the wild, Sierra de Manantlán, Colima.

Ground color, in preservative, light brown with 54 dark dorsal body blotches, irregularly edged in black with a very thin light outer edge on most blotches, blotches wider than long, interspaces between blotches 1–2 scales long; smaller, vertically elongated auxiliary blotches evident laterally below the dorsal blotches, often separated from the dorsal blotches by white scales. Head marked with parallel rows of small irregular blotches across prefrontals, supraoculars, and much of the occipital area, terminated by paired occipital blotches; supralabials and infralabials ground color white, stippled with dark brown spots; distinct dark postocular stripe, uniform in width, extending from posterior of eye to above the angle of the mouth and then downwards to jawline; postocular stripe bordered by a darker band on both dorsal and ventral margins, dorsally a narrow region of light brown extends from the posterior axis of the eye along the dorsal margin of the postocular band, ventral to the postocular band is a region of white with brown stippling, supralabial 8 is nearly completely white, with moderate stippling but no postocular band, supralabials 8–13 are divided by the postocular band; two large dark brown blotches are on the borders of supralabials 5–7; scale margins of infralabials with dark brown regions making for a series of dark bands, bands do not extend beyond infralabials; gular scales stippled with no pattern; ventral scutes stippled, more heavily on posterior half of scales, with total stippling intensifying past midbody; distal 1/3 ventral scutes become dark brown with a lighter band along the midline, continuing to the vent. Subcaudal scales dark brown to black; eight dark brown tail bands; proximal rattle black, distal sections brown.

Color in life. Color in life within the type series is only known for one paratype (MZFC 28669), shown in Fig. 8.

Variation. Three of the six specimens lack a single, large anterior intercanthal. The holotype, KU 73649, has a single large intercanthal bordered anteriorly by three small, seemingly anomalous scales (Fig. 7). Similarly, UTA R-16353 has paired anterior intercanthals separated by a small but elongated scale. The third specimen, AMNH R-113191, has paired anterior intercanthals. The single juvenile (AMNH R-113191) has a cream-colored proximal rattle and pale-colored tail ventrally. Variation in meristic, morphometric, and color pattern characters within the type series is listed in Table 3.

Etymology. The specific epithet is a patronym honoring Jonathan A. Campbell for his many years of field research on Mexican rattlesnakes and for his decades of unwavering support to students of Mexican herpetology.

Habitat and distribution. *Crotalus campbelli* is found in rocky open breaks within montane forest (Fig. 9) along the far western regions of the Trans-Mexican Volcanic Belt. Much of this forest is covered with remnant patches of cloud forest (Ponce-Reyes *et al.* 2012). This species is known from western Jalisco and the Sierra de Manantlán in southern Jalisco/northwestern Colima. A narrow low-elevation valley appears to separate the range of *C. campbelli* from *C. armstrongi* to the east (Fig. 3).



FIGURE 9. Humid montane forest habitat of *Crotalus campbelli* sp. nov. at the paratype localities of (a) Sierra de Mascota, Jalisco; and (b) Sierra de Manantlán, Colima.

Discussion

Taxonomy of the *Crotalus triseriatus* species group. The main goal of our study is to identify historically distinct evolutionary lineages within the *C. triseriatus* species group, a view consistent with modern taxonomy and both the evolutionary and general lineage species concepts (Wiley 1978; de Queiroz 1998, 2007). Our results suggest that the current taxonomy of the *C. triseriatus* group is in need of revision to reflect evolutionary history. Groupings based on historical taxonomic arrangements are among the lowest ranked of our 16 models (Table 1).

We suggest that the *C. triseriatus* species group is comprised of nine species. We add *C. campbelli* and *C. tlaloci* to the group. *Crotalus pusillus* and *C. aquilus* should be recognized as full species, consistent with previous suggestions (Klauber 1952; Dorcas 1992). We also recommend elevating the subspecies *C. t. triseriatus* and *C. t. armstrongi* to full species (as *C. triseriatus* and *C. armstrongi*), a taxonomic arrangement already in use (Reyes-Velasco *et al.* 2013). A previous study suggesting a close relationship between these two taxa based on morphology (Dorcas 1992) grouped *C. armstrongi* from Michoacán with *C. triseriatus* from the central and eastern regions of the Trans-Mexican Volcanic Belt as *C. t. triseriatus sensu lato*, and this grouping undoubtedly skewed cladistic analyses.

Crotalus lepidus currently contains four putative subspecies (Campbell & Lamar 2004). We tested models that split *C. l. morulus* from a lineage of *C. lepidus* containing the remaining three subspecies to capture the deep matrilineal divergence between these two lineages (Bryson *et al.* 2011). We tentatively suggest that *C. l. morulus* be elevated to full species (*C. morulus*) given the distinct phylogenetic placement of this species based on mtDNA (Fig. 1) and the Bayes factors score of our best-ranked BFD model over alternative models ($2\ln\text{BF} > 40$; Table 1) based on 3,346 base pairs of DNA from seven nuclear loci. However, we feel it is important to note that our second and third-ranked models combine the two lineages (Table 1), and the northern distribution of *C. morulus* remains uncertain (Campbell & Lamar 2004). More research involving a larger number of samples of each of the remaining subspecies of *C. lepidus* and the addition of faster-evolving nuclear genes is needed to make further taxonomic decisions about their status. For taxonomic stability, we suggest continuing to recognize the subspecies *C. l. lepidus*, *C. l. klauberi*, and *C. l. maculosus* rather than lumping them into a single taxon.

We united the subspecies *C. r. ravus*, *C. r. brunneus*, and *C. r. exiguus* into a single *C. ravus* lineage for our model testing. Genetic structure among these subspecies was less pronounced than among major mitochondrial lineages within the *C. triseriatus* group (Bryson *et al.* 2011), which were the focus of our study here. However, mitochondrial haplogroups within *C. ravus* conform to subspecies boundaries and appear to be separated by strong biogeographic barriers (Bryson *et al.* 2011). Future research using more samples of each subspecies and faster-evolving nuclear genes will probably find that each subspecies of *C. ravus* represents independent evolutionary unities. Pending this research, we suggest continuing to recognize *C. r. ravus*, *C. r. brunneus*, and *C. r. exiguus*.

Finally, although our species delimitations are robust based on BFD analyses, the species tree is poorly resolved and, thus, the relationships among species are uncertain (Fig. 2). Our analyses only recover three moderately supported clades (≥ 0.85 posterior support), including one containing *C. tlaloci*, *C. pusillus*, *C. armstrongi*, *C. triseriatus*, *C. aquilus*, *C. morulus*, and *C. lepidus*, and two additional subclades within this clade. Similar low levels of support exist for relationships within species tree analyses based mostly on mtDNA sequences for the group (Reyes-Velasco *et al.* 2013). We attribute this low support to a combination of factors, including low levels of phylogenetically informative characters in our nuclear genes (Table 2) and a probable rapid radiation during the early diversification of this group (Bryson *et al.* 2011). This radiation may have coincided with rapid uplifting of the Trans-Mexican Volcanic Belt near the end of the Neogene period (Gómez-Tuena *et al.* 2007). During a rapid radiation, coalescent patterns contained within individual gene trees may not match the true pattern of speciation because of incomplete lineage sorting and subsequent retention of ancestral polymorphisms (Degnan & Rosenberg 2006; Knowles 2009). Although we employ a coalescent-based method of species tree inference, which can accommodate gene tree discordance as a result of incomplete lineage sorting (Anderson *et al.* 2012), our seven nuclear loci may lack enough informative sites to resolve relationships for each gene, which makes estimating the species tree difficult. Sampling of more individuals and genes with more informative sites may improve the resolution of species relationships.

Conservation implications. Four of the five currently recognized species in the *C. triseriatus* group receive different levels of protection by the Mexican government (NOM-059-SEMARNAT-2010). Two are considered

threatened (*C. ravus* and *C. pusillus*) and two receive special protection (*C. aquilus* and *C. lepidus*). The fifth species, *C. triseriatus*, is not listed. Given our proposed taxonomic changes, we recommend that the conservation status of *Crotalus triseriatus sensu lato* be reevaluated. *Crotalus triseriatus sensu stricto* is restricted to populations along the Trans-Mexican Volcanic Belt in eastern Michoacán, Estado de México, Morelos, Distrito Federal, Hidalgo, Tlaxcala, Puebla, and Veracruz. Populations from central and western Michoacán and eastern Jalisco represent *C. armstrongi*. Both species occupy forested regions of the Trans-Mexican Volcanic Belt heavily impacted by destructive land-use practices (Vázquez *et al.* 2009).

Crotalus tlaloci and *C. campbelli* arguably represent two of the most threatened species in the *C. triseriatus* group. Both species have relatively small distributions (Fig. 3) and are closely associated with highly endangered remnant patches of cloud forest and humid oak-pine forest (Figueroa-Rangel *et al.* 2010; Vargas-Rodriguez *et al.* 2010; Ponce-Reyes *et al.* 2012). The entire known distribution of *C. tlaloci* lies within 125 km of one of the world's largest metropolitan areas. This region of Mexico has seen explosive urban settlement comparable to settlement rates in the mountains of Ethiopia (Galicia & García-Romero 2007). Populations of *C. tlaloci* might reside in several of the fragmented protected areas across the region, such as Corredor Biológico Chichinautzin in Morelos and Reserva de la Biosfera Mariposa Monarca in Michoacán and Estado de México. However, all specimens of *C. tlaloci* came from outside of these areas (Appendix 3). *Crotalus campbelli* is closely associated with remnant patches of cloud forest scattered across the far western regions of the Trans-Mexican Volcanic Belt. Part of their distribution lies within the Reserva de la Biosfera Sierra de Manantlán in Jalisco and Colima. The rest of the range to the west lies largely within remote yet unprotected habitat.

Future research. We cannot confidently assign several museum specimens from the mountains of southern Nayarit and adjacent Jalisco to species (KU 29500–02, USNM 46465). Although scale counts for these four specimens fall within the range of *C. armstrongi*, they are likely geographically isolated from this species and from *C. campbelli* by the low-elevation Río Ameca drainage (Fig. 3). We tentatively assign these specimens to *C. armstrongi*, but future genetic studies are needed to clarify the relationships of these snakes to *C. campbelli* and other *C. armstrongi*.

Additional species may be present within the *C. triseriatus* group. Here we focused on the most divergent mitochondrial lineages identified by Bryson *et al.* (2011). However, geographic structuring is evident within several of these lineages. *Crotalus lepidus*, for example, contains five haplogroups (Bryson *et al.* 2011), several of which may be morphologically distinct (Dorcas 1992; Campbell & Lamar 2004). Our study is the first (and overdue) quantitative revision of species limits in the *C. triseriatus* group using multilocus DNA data. We encourage other researchers to continue to explore species boundaries within this widespread group of montane rattlesnakes and update taxonomy as new data become available.

Acknowledgments

We thank the following institutions for specimen loans: American Museum of Natural History; Colección Nacional de Anfibios y Reptiles, UNAM; Field Museum of Natural History; University of Kansas; Natural History Museum of Los Angeles County; Louisiana State University; Museo de Zoología, Facultad de Ciencias, UNAM; Texas Cooperative Wildlife Collection; University of Illinois Natural History Museum; University of Michigan Museum of Zoology; United States National Museum; and University of Texas at Arlington. We thank W. Horner and C. Whitney for valuable information on an early specimen from Valle de Bravo. Additional assistance was provided by M. Torocco, U.O. Garcia, A. Nieto-Montes de Oca, O. Flores-Villela, J. Alvarado-Díaz, A. Kardon, D. Lazcano, R. Mendoza-Paz, the late F. Mendoza-Quijano, and R. W. Hansen. Special thanks to S.E. Bryson and M.A. Bryson for their support and patience. This paper is based in part upon work supported by the National Science Foundation grants DEB- 1257785 and DEB- 1258205 and by Natural Sciences and Engineering Research Council (Canada) Discovery Grant 3148.

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APPENDIX 1. Collection and voucher data for genetic samples of the *Crotalus triseriatus* species group used in this study and deposited in the Royal Ontario Museum (ROM). Names of species reflect the taxonomy proposed here.

Taxon	Locality	Voucher Number
<i>C. aquilus</i>	Mexico: Querétaro: Cerro el Zamorano	ROM 42623
<i>C. aquilus</i>	Mexico: Querétaro: Valle de Guadalupe	ROM 47042
<i>C. aquilus</i>	Mexico: Michoacán: San José de Gracia	ROM 47064
<i>C. aquilus</i>	Mexico: Michoacán: San José de Gracia	ROM 47008
<i>C. aquilus</i>	Mexico: Estado de México: Acambay	ROM 47031
<i>C. armstrongi</i>	Mexico: Jalisco: Nevado de Colima	ROM 47023
<i>C. armstrongi</i>	Mexico: Michoacán: Cerro Tancítaro	ROM 47072
<i>C. armstrongi</i>	Mexico: Michoacán: Tacámbaro	ROM 47019
<i>C. armstrongi</i>	Mexico: Michoacán: Cerro Tancítaro	ROM 48030
<i>C. lepidus klauberi</i>	Mexico: Chihuahua: Sierra Manzanillas	ROM 42414
<i>C. lepidus klauberi</i>	Mexico: Durango: Rancho Santa Barbara	ROM 47002
<i>C. lepidus klauberi</i>	Mexico: Jalisco: Volcán Tequila	ROM 47021
<i>C. lepidus lepidus</i>	USA: Texas: Davis Mountains	ROM 42415
<i>C. lepidus maculosus</i>	Mexico: Durango: Los Bancos	ROM 42404
<i>C. lepidus maculosus</i>	Mexico: Nayarit: Santa Teresa	ROM 47071
<i>C. morulus</i>	Mexico: Tamaulipas: Jaumave	ROM 42400
<i>C. morulus</i>	Mexico: Tamaulipas: Miquihuana	ROM 42401
<i>C. morulus</i>	Mexico: Nuevo León: La Huasteca	ROM 42411
<i>C. morulus</i>	Mexico: Nuevo León: Sierra Peña Nevada	ROM 42417
<i>C. morulus</i>	Mexico: Tamaulipas: Bustamante	ROM 45244
<i>C. pusillus</i>	Mexico: Michoacán: Sierra de Coalcomán	ROM 47056
<i>C. pusillus</i>	Mexico: Michoacán: Sierra de Coalcomán	ROM 47055
<i>C. pusillus</i>	Mexico: Michoacán: Tancítaro	ROM FC271
<i>C. ravus exiguus</i>	Mexico: Guerrero: Omiltemi	ROM 47053
<i>C. ravus exiguus</i>	Mexico: Guerrero: Carrizal del Bravo	ROM 47052
<i>C. ravus brunneus</i>	Mexico: Oaxaca: Mitla	ROM 47039
<i>C. ravus ravus</i>	Mexico: Puebla: Zacatepec	ROM FC228
<i>C. triseriatus</i>	Mexico: Puebla: Zacatlán	ROM 47045
<i>C. triseriatus</i>	Mexico: Puebla: Volcán Iztaccihuatl	ROM 47026
<i>C. triseriatus</i>	Mexico: Veracruz: Las Vigas	ROM 47015
<i>C. triseriatus</i>	Mexico: Michoacán: San Anganguero	ROM 47067
<i>C. triseriatus</i>	Mexico: Michoacán: SE Aporo	ROM 47034
<i>Crotalus campbelli</i> sp. nov.	Mexico: Jalisco: La Mascota highway	ROM 47028
<i>Crotalus campbelli</i> sp. nov.	Mexico: Colima: Sierra de Manantlán	ROM 47025
<i>Crotalus campbelli</i> sp. nov.	Mexico: Jalisco: Sierra de Mascota (paratype)	ROM 48027
<i>Crotalus tlaloci</i> sp. nov.	Mexico: Estado de México: Valle de Bravo (paratype)	ROM 47035
<i>Crotalus tlaloci</i> sp. nov.	Mexico: Morelos: Cuernavaca- Ocuilán highway (paratype)	ROM 48028
<i>Crotalus tlaloci</i> sp. nov.	Mexico: Estado de México: Valle de Bravo (paratype)	ROM 48029
<i>Crotalus tlaloci</i> sp. nov.	Mexico: Estado de México: Valle de Bravo (paratype)	ROM 48031

APPENDIX 2 . Information on the seven nuclear loci and primers used in this study, including annealing temperature (T_a) and reference.

Locus	Primer sequence 5'–3'	T_a (C)	Reference
TATA box-binding protein (<i>TBP</i>)	F CCTTTACCAGGAACACACC R CGAAGGGCAATGGTTTTTAG	55	Gibbs & Diaz (2010)
ETS oncogene (<i>ETS</i>)	F CCATCAACAGACACACAGG R GTCTGCTTTTACTTTGCG	50	Dolman & Phillips (2004), Friesen <i>et al.</i> unpublished
Prolactin Receptor (<i>PRLR</i>)	F GACARYGARGACCAGCAACTRATGCC R GACYTTGTGRACCTTCYACRTAATCCAT	50	Townsend <i>et al.</i> (2008)
Recombination-activating gene 2 (<i>Rag2P1Y</i>)	F CCCTGAGTTTGGATGCTGTACTT R AACTGCCTRTTGTCCCCTGGTAT	49	Gamble <i>et al.</i> (2007)
Anonymous 25 (Locus25)	F ACCTTTCTCTTTTGTTCAGCA R ATGTCTCTGTTTCCCAAATG	50	Gibbs & Diaz (2010)
Anonymous 63 (Locus63)	F ATTAGCCCAGAACTGTGCTTA R AAAGATTCTGGGAAGCCAAA	50	Gibbs & Diaz (2010)
Anonymous A (LocusA)	F AGAATTGAGCTCCCGTCCTTT R GGGAGCAATGCCTAGACCAAG	50	Gibbs & Diaz (2010)

APPENDIX 3. Specimens examined.

Crotalus armstrongi. 28 specimens. MEXICO: MICHOACÁN: Tancítaro (FMNH 39106, 39110–11, 39124, 40823, MZFC 25113). Puerto Garnica (AMNH 98846). Mil Cumbres (UINHM 26225). 5 miles S Pátzcuaro nr road to Tacambaro (UMMZ 98941). 2.3 miles N Opopeo (UMMZ 121523). Tacambaro (HINIRENA 361, 689). JALISCO: Atemajac de Brizuela, 6.4 km E (KU 106290). Tapalpa, 1.5 miles NW, Rancho San Francisco (UTA R-4909, 7232, 7937). Tapalpa, 2.4 km NW, Rancho San Francisco (UTA R-5893, 6257–60, 7739, 12589–91). Nevado de Colima, east slope (UMMZ 101550). Nevado de Colima, Refugio de Montaña (UTA R-53327). Sierra de Quila (MZFC 28668).

Crotalus cf. armstrongi. 4 specimens. MEXICO: NAYARIT: Ixtlán del Río, 6 mi S (KU 29500–02). JALISCO: Ameca (USNM 046465).

***Crotalus campbelli* sp. nov.** 6 specimens. MEXICO: JALISCO: Sierra de Cuale, municipality of Tapla de Allende (KU 73649–73650). Las Playitas, Las Joyas, Sierra de Manantlán, municipality of Autlán de Navarro (UTA R-16352). Las Joyas, Sierra de Manantlán, municipality of Autlán de Navarro (UTA R-16353). Manantlán, ca. 2.1 km SE by dirt road, ca. 25 km SE Autlán (AMNH R-113191). Lago de Juanacatlán, Sierra de Mascota, municipality of Mascota (MZFC 28669).

Crotalus pusillus. 33 specimens. MEXICO: MICHOACÁN: Acuario de las Lleguas, W of Rancho Barolosa (UMMZ 112566–67). Dos Aguas and vicinity (UMMZ 118591–99, 118601, 121512, 121513; UTA R-4530–31, 5846, 6119, 9358). Tancítaro (FMNH 37042, 37048, 39095, 39097, 39112–13, 39117, 39120–21, 39127, 40818–19, 40824–25).

***Crotalus tlaloci* sp. nov.** 12 specimens. MEXICO: GUERRERO: “Los Llanos”, 10 km by road from Taxco to Tetipac, Sierra de Taxco, municipality of Tetipac (MZFC 3666). Cerro del Huizteco, Sierra de Taxco, municipality of Taxco (MZFC 3664–3665). “Arroyo las Damas”, Sierra de Taxco, municipality of Tetipac (MZFC 3666). ESTADO DE MÉXICO: Acatitlán, municipality of Valle de Bravo (MZFC 4324). Los Álamos, municipality of Valle de Bravo (MZFC 25114–25115). Los Álamos, municipality of Valle de Bravo (HINIRENA 725–726). MORELOS: Km 12, Carr. Cuernavaca-Ocuilán, municipality of Cuernavaca (MZFC 4657). Carr. Cuernavaca-Ocuilán, near state border, municipality of Cuernavaca (MZFC 25111). MICHOACÁN: N Arroyo Seco, municipality of de Aporo (HINIRENA 724).

Crotalus triseriatus. 21 specimens. MEXICO: ESTADO DE MÉXICO: San Cayetano, municipality of Villa de Allende (CNAR 1015, 6969a, 6969b). Nevado de Toluca, carr. Toluca-Sultepec, km 10 (CNAR 3799). Rancho Viejo (CNAR 3997). Calimaya, municipality of Calimaya (CNAR 13145). Santiago Oxtotitlan El Potrero, municipality of Villa Guerrero (CNAR 13147). 0.7–2.8 mi from hwy between Valle de Bravo and Temescaltepec-Toluca Hwy, 6.4 mi NW jct Mex 134 (LACM 154837–38). MICHOACÁN: near Aporo (HINIRENA 723). MORELOS: Laguna de Zempoala (LSU 28557, UTA R-7286, 7398, 8142, 12599). environs of Huitzilac (UTA R-12600, 14519). Chapultepec, 3.9 km SSE Mexicalcingo (UTA R-12604). VERACRUZ: 4.5 mi WNW Altotonga (LSU 11014). 10 km SE Cofre de Perote (TCWC 820). Between Gutiérrez Zamora and Papanla (UIMNH 42860).