



Phylogenetic relationships and origin of the rattlesnakes of the Gulf of California islands (Viperidae: Crotalinae: *Crotalus*)

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The islands of the Gulf of California are divided into three categories – oceanic, continental, and landbridge – and were formed from the Middle Miocene to the Holocene. The species of the *Crotalus* genus are an important ecological element of the endemic fauna of these islands. This study is the first to include several island-endemic species in a phylogenetic framework. We seek to understand the phylogenetic relationships among these species, and in particular, whether these species are more related to the Baja California peninsula or continental Mexican species, and whether the divergence times for these relationships are consistent with the formation of the islands. We performed a phylogenetic analysis and estimated divergence times using Bayesian inference and two mitochondrial 12S and 16S genes. The analyses show a new relationship between *Crotalus angelensis* and *C. pyrrhus*. *Crotalus lorenzoensis* was nested with *C. ruber* individuals, making this species a paraphyletic species. A novel relationship was also detected in that *C. estebanensis* was sister to *C. basiliscus*. The divergence time for all island-endemic *Crotalus* species is consistent with the formation of these islands. In addition, the insular species are related to their congeners in the Baja California peninsula or mainland Mexico.

Keywords: Baja California Peninsula; Bayesian inference; island-endemic; mtDNA; rattlesnakes

INTRODUCTION

The Baja California peninsula began to form during the Middle Miocene due to the expansion process that occurred between the Pacific and North American tectonic plates. As far as we know, the peninsula was formed progressively at the end of the Pliocene and the beginning of the Pleistocene, approximately 2.58 million years ago (Smith, 1991; Riddle et al., 2000a; Holt et al., 2000; Oskin & Stock, 2003; Wood et al., 2008; Murphy & Aguirre-León, 2002; Murphy & Méndez de la Cruz, 2010). This also led to the formation of the Gulf of California (GC).

The Gulf of California has a complex geological history with two stages of formation: the first during the Miocene and the second in the Pliocene (Carreño & Helenes, 2002). The islands constitute one of the Gulf of California's most interesting features and are classified into three types: oceanic, continental, and landbridge (Grismer, 2002). The oceanic islands of the GC have never been connected to Baja California (BC) or mainland Mexico. The continental islands were once connected to the Baja California peninsula and/or mainland Mexico but became separated as a result of tectonic displacements along coastal fault zones, and broke off the trailing edge

of the peninsula as it moved north-west. The landbridge islands are the commonest and youngest of all the islands in the Gulf of California and were connected to Baja California or mainland Mexico, but for the most part, were cut off by a rise in the sea level (Grismer, 2002). These three types of islands can be separated into three regions: the northern, central midriff, and southern gulf areas (Carreño & Helenes, 2002).

The northern islands were formed by sedimentation of sediment delivery from the Colorado River. Some of these islands were formed in the Holocene, while the rest were the result of volcanic activity during the early Pleistocene (Carreño & Helenes, 2002). The central islands emerged during the middle to late Miocene (15-10 and 10-5 Ma). Those islands were uplifted due to the many faults in the region from the Pleistocene to the Holocene. Finally, volcanic activity, granitic rock or oceanic sediments from the Pliocene formed the southern islands. Altogether, this led to the formation of about 100 islands and islets, most of which were colonised by species of flora and fauna from Baja California and mainland Mexico. According to Grismer (2002), there are 161 species of native and non-native species of herpetofauna in the Baja California peninsula and the Pacific and GC islands. Four of these species are salamanders, 13 frogs, 4 turtles, 84 lizards,

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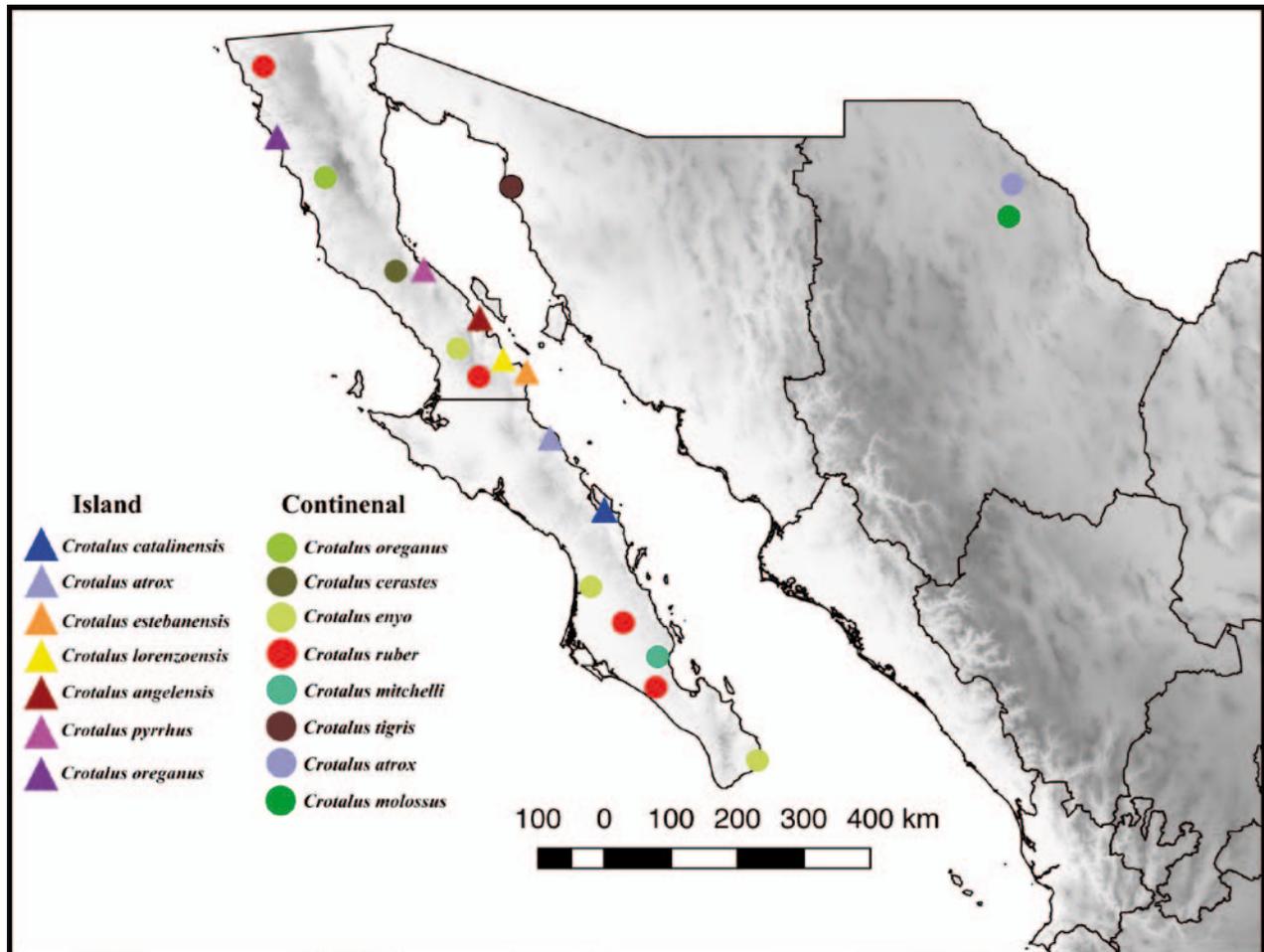


Figure 1. Distribution of species of rattlesnakes from the Gulf of California islands, the California peninsula, and mainland Mexico. Symbols represent sampling locations and indicate the corresponding species.

and 57 snakes. Among the snakes, the Viperidae family is represented by 17 *Crotalus* species (Grismer, 2002; Meik et al., 2018). Ten of these are island-endemic and one is endemic to the peninsula (Table 1; Grismer, 2002; Ernst & Ernst, 2012; Meik et al., 2018). Five species are shared between California and the Baja California peninsula (*C. atrox*, *C. cerastes*, *C. mitchellii*, *C. oreganus*, and *C. ruber*), and four island species are shared between the United States and mainland Mexico (*C. atrox*, *C. cerastes*, *C. mitchellii*, and *C. molossus*; Ernst & Ernst, 2012).

Mexico is considered the center of diversification of *Crotalus* snakes, since about 92 % of the species of this group are found in the country (Klauber, 1972; Armstrong & Murphy, 1979; Greene, 1997; Flores-Villela & Canseco-Márquez, 2004; Paredes-García et al., 2011). The *Crotalus* genus probably originated during the Middle Miocene (Bryson et al., 2010). The Sierra Madre Occidental and the Mexican Plateau have the highest diversity of *Crotalus* species (Campbell & Lamar, 2004), with the former considered the ancestral area for the genus (Place & Abramson, 2004). *Crotalus* species in mainland Mexico and Baja California may be the ancestors of the endemic island species. However, to date there has been no single phylogenetic hypothesis that includes all the *Crotalus* species endemic to the islands.

Previous molecular phylogenetic analyses using four mitochondrial genes (12S, 16S, ND4, and cytB) and 61

Table 1. *Crotalus* species present in the Baja California peninsula (BCP), Gulf de California Islands (GCI), Pacific Islands (PI), Mainland Mexico (MM), and the United States of America (USA), based on Grismer (2002), Ernst and Ernst (2012), and Meik et al. (2018).

Species	BCP	GCI	PI	MM	USA
<i>Crotalus angelensis</i>		X			
<i>Crotalus atrox</i>	X	X		X	X
<i>Crotalus catalinensis</i> ¹		X			
<i>Crotalus caliginis</i> ¹			X		
<i>Crotalus cerastes</i>	X	X		X	X
<i>Crotalus enyo</i> ²	X	X	X		
<i>Crotalus estebanensis</i> ¹		X			
<i>Crotalus lorenzoensis</i> ¹		X			
<i>Crotalus pyrrhus</i>	X	X		X	X
<i>Crotalus mitchellii</i>	X	X	X	X	X
<i>Crotalus molossus</i>		X		X	X
<i>Crotalus oreganus</i>	X				X
<i>Crotalus polisi</i> ¹		X			
<i>Crotalus ruber</i>	X	X	X		X
<i>Crotalus thalassoporus</i> ¹		X			
<i>Crotalus tortugensis</i> ¹		X			
<i>Crotalus tigris</i>		X		X	X
Total	7	15	4	6	8

¹ Island-endemic, ² BCP-endemic



Figure 2. *Crotalus* species sampled in this study. **1.** *C. oreganus*, **2.** *C. angelensis*, Ángel de la Guarda Island **3.** *C. catalinensis*, Santa Catalina Island, **4.** *C. enyo*, **5.** *C. ruber*, **6.** *C. estebanensis*, San Esteban Island, **7.** *C. lorenzoensis*, San Lorenzo Island, **8.** *C. mitchellii*, **9.** *C. enyo* (La Paz). **10.** *C. cerastes*, **11.** *C. pyrrhus*, El Muerto Island, **12.** *C. atrox*, Tortuga Island. Pictures by Gustavo Arnaud, Francisco Javier García-De León and Fernando Pozas.

Crotalinae species, of which four were *Crotalus* species, found that this genus was monophyletic and sister to the *Sistrurus* genus (Parkinson et al., 2002). Murphy et al. (2002) conducted a more extensive phylogenetic analysis using 27 *Crotalus* species. In their sampling, they included *Crotalus catalinensis* from Santa Catalina Island, *C. atrox* from Santa Cruz Island and (*C. tortugensis* = *C. atrox*) from Tortuga Island. They found sister relationships between *C. catalinensis* and *C. ruber* and (*C. tortugensis* = *C. atrox*) was nested in a clade with two *C. atrox* species, and was sister to *C. atrox* from Santa Cruz Island. Castoe et al. (2007) performed a population study of *C. atrox* and found that (*C. tortugensis* = *C. atrox*) and *C. atrox* from Santa Cruz Island are nested within mainland lineages of *C. atrox*. Finally, in a population study of *Crotalus viridis* that included *C. viridis caliginis* from Coronados Sur Island

and *C. viridis helleri* from Santa Catalina Island (USA), Ashton & de Queiroz (2001) found strong support for the separation of *C. viridis* from *C. oreganus*.

To date, not all *Crotalus* island species in the GC and the Pacific have been included in a phylogenetic study. Therefore, here we include all the insular *Crotalus* species represented by more than one individual, except the newly described *C. polisi* and *C. thalassoporus* species (Meik et al., 2018), with the aim of answering the following questions: 1) “What phylogenetic relationships exist between insular species and other *Crotalus* species?”, 2) “Are insular species more closely related to species from Baja California or species from mainland Mexico?”, and 3) “What are the divergence times of the insular species and are these correlated with the formation processes of the islands?”

METHODS

Taxon sampling and laboratory methods

Between 2008 and 2012, we collected 42 samples of 14 *Crotalus* species (Table 2) from the Baja California peninsula, its associated islands, and mainland Mexico (Figs. 1, 2). These were considered the ingroup taxa. Additionally, we used all the *Crotalus* specimens previously analysed by Murphy et al. (2002; Table 2) as outgroup taxa. Based on recent phylogenetic analyses (Murphy et al., 2002; Castoe & Parkinson, 2006; Wüster et al., 2005; Bryson et al., 2010), we used *Sistrurus catenatus*, *S. miliarius*, as a sister genus to *Crotalus* and *Agkistrodon contortrix*, *A. piscivorus* as a sister genus to the *Crotalus*+*Sistrurus* clade (Wüster et al., 2005), and *Gloydus brevicaudus* to root the tree (Wüster et al., 2005; Bryson et al., 2010).

Crotalus, *Sistrurus*, and *Agkistrodon* are genera belonging to the Crotalinae subfamily. *Crotalus* and *Sistrurus* share some morphological features such as the rattle (a corneal structure in the terminal region), which *Agkistrodon* lacks. *Sistrurus* is characterised by the presence of nine large scales, arranged symmetrically on the back of the head (Gloyd, 1940). *Sistrurus* shares this morphological feature with members of the subfamily Azemiopinae, to which *Gloydus brevicaudus* belongs (Brattstrom, 1964).

We sequenced two mitochondrial regions (12S and 16S). These gene regions have been shown to be informative at different levels of divergence within rattlesnakes (Murphy et al., 2002; Wüster et al., 2005; Bryson et al., 2010). Total genomic DNA was extracted from blood and liver, muscle or skin using the chloride salting-out method (Aljanabi & Martinez, 1997; Riera et al., 2010). All gene regions were amplified via polymerase chain reaction (PCR) in a 15 µL reaction volume containing 0.4 mM of deoxynucleoside triphosphates (dNTPs), 0.4 µM for each primer for the 12S rDNA and 0.3 µM for the 16S rDNA, buffer 1x, 0.4 mM of MgCl₂, 1.0 U Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 100 ng template DNA. Previously published primer sequences are given in Murphy et al. (2002; 12S, 16S). Initially, DNA was denatured at 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 50-52 °C for 1 min, and 72 °C for 1 min. A final extension phase of 72 °C for 5 min finalised the protocol. Sequences were amplified in both directions using the GENEWIZ facilities in New Jersey, USA. Samples were analysed with an ABI Prism 3730xl Genetic Analyser (Applied Biosystems). Forward and reverse sequences for each individual were edited and manually aligned using CodonCode Aligner 5.0.2. (Codon Code Corporation).

Phylogenetic analysis

We analysed our sequence data using Bayesian inference (BI). BI analyses were conducted using MrBayes 3.2.6 (Ronquist & Huelsenbeck, 2003) on the combined mtDNA dataset, implementing separate models for each gene region (12S and 16S). jModelTest v2.1.10 (Darriba et al., 2012) was used to select a best-fit model of evolution for each partition, based on the Akaike information criterion (AIC). Two independent runs were performed for the

partitioned data matrix. For each run, we employed one cold and three heated chains, which were set to run for 40,000,000 generations, sampling one tree every 2,000 generations. The convergence of runs was confirmed by effective sample size (ESS) >200 for all parameters, using Tracer v1.6 (Rambaut et al., 2013). Sample points collected prior to stationarity were eliminated (25 %). Posterior probabilities (PP) for supported clades were determined by a 50 % majority-rule consensus of the trees retained after burn-in. BI analyses were run in the CIPRES Science Gateway (Miller et al., 2010).

Divergence time estimates

Divergence times were estimated using a Bayesian approach as implemented in BEAST v. 1.8 (Drummond & Rambaut, 2012). The model selected was GTR + I + G, based on the results of the AIC run in jModeltest 2.1.10, under an uncorrelated lognormal relaxed clock model. We used three calibration points and treated them as minimum age constraints. Two fossil calibrations for the tree were obtained for North American pit vipers. The first was the earliest record of *A. contortrix* in the Late Miocene (Late Hemphillian; Holman, 2000). The second was the oldest fossil from the *Sistrurus* genus, from the Late Miocene (Parmley & Holman, 2007). For both nodes, we used a hard upper-bound constraint with lognormal distributions following recommendations by Ho & Phillips (2009).

The *A. contortrix* node was given a zero offset of 6 million years ago (Ma), a lognormal mean of 0.01, and a lognormal standard deviation of 0.42. The *Sistrurus* stem was constrained with a zero offset (hard upper bound) of 8 Ma, a lognormal mean of 0.01, and a lognormal standard deviation of 0.76 (Bryson et al., 2010). For the root node, we used a mean stem age of 17 Ma and a standard deviation of 1.0, with a normal distribution (Ho & Phillips, 2009) based on previous molecular estimates by Bryson et al. (2010). One independent 40,000,000-generation run was performed with random starting trees, sampling every 2,000 generations. Tracer 1.5 (Rambaut et al., 2013) was used to assess convergence and estimate effective sample sizes (ESS) for all parameters. Results were summarised in a single tree using TreeAnnotator 1.8 (Rambaut & Drummond, 2015), and visualised with FigTree 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>).

RESULTS

Phylogenetic analysis

The total number of aligned base pairs (bp) for each mtDNA marker was 511 bp for 12S and 1328 bp for 16S. The concatenated and aligned mtDNA matrix was 1839 bp.

The partitioned molecular mtDNA (12S + 16S) analysis using BI retrieved a 50 % majority-rule consensus tree in which *Crotalus* is a monophyletic genus (PP = 0.99) with five well-supported clades (hereafter named clades 1, 2, 3, 4, and 5), and sister to the *Sistrurus* genus (Fig. 3). Clade 1 is made up of *Crotalus angelensis*, *C. pyrrhus* from El Muerto Island, one *C. pyrrhus* individual from California, and *C. mitchellii* (PP = 0.96). *C. pyrrhus* was sister to *C.*

Table 2. *Crotalus* species used in this study.

Species	Individuals	Voucher	Locality	Acronym	Species	Individuals	Voucher	Locality	Acronym
<i>Crotalus adamanteus</i>		ROM 18130 *	Commercially purchased				ROM 18141-42 *	Veracruz, Mexico	
<i>Crotalus angelensis</i>	Ca1	Ca 01	Ángel de la Guarda Island, B. C.	IAG	<i>Crotalus oreganus</i>	Co1	Co UABC144	Ensenada, B. C.	ENS
<i>Crotalus aquilus</i>		ROM 18117 *	Mexico, San Luis Potosí			Co2	Co UABC124	Ensenada, B. C.	ENS
<i>Crotalus atrox</i>	Cat1	Cat D2jul2011	Ciudad Juárez, Chihuahua.	CJ		Co3	Co UABC076	Ensenada, B. C.	ENS
	Ct1	Ct1 2011	Tortuga Island, B. C. S.	IT		Cca1	Cca 060610	Coronados Sur Island, B. C.	ICS
	Ct2	Ct2 2011	Tortuga Island, B. C. S.	IT		Cca2	Cca 010610	Coronados Sur Island, B. C.	ICS
	Ct3	Ct3 2011	Tortuga Island, B. C. S.	IT		Cca3	Cca 030610	Coronados Sur Island, B. C.	ICS
	Ct4	Ct 040110	Tortuga Island, B. C. S.	IT		Cca4	Cca 040610	Coronados Sur Island, B. C.	ICS
		ROM 18192 *	Tortuga Island, B. C. S.		<i>Crotalus polystictus</i>		ROM-FC 263 *	Mexico City, Mexico	
		ROM 18224 *	Santa Cruz Island		<i>Crotalus pricei</i>		ROM-FC 2144 *	Nuevo León, Mexico	
		ROM 18144 *	Riverside Co., CA.		<i>Crotalus pusillus</i>		ROM-FC 271 *	Michoacán, Mexico	
<i>Crotalus basiliscus</i>		ROM 18188 *	Nayarit, Mexico		<i>Crotalus pyrrhus</i>	Cm1	Cm 03062010	El Muerto Island, B. C.	IM
<i>Crotalus catalinensis</i>	Cc1	Cc 11sep08	Santa Catalina Island, B.C.S.	ISC		Cm2	Cm 03032010	El Muerto Island, B. C.	IM
	Cc2	Cc 04012011	Santa Catalina Island, B.C.S.	ISC		Cm3	Cm 051109	El Muerto Island, B. C.	IM
	Cc3	Cc 04032011	Santa Catalina Island, B.C.S.	ISC		Cm4	Cm 05409	El Muerto Island, B. C.	IM
	Cc4	Cc 04092011	Santa Catalina Island, B.C.S.	ISC			ROM 18178 *	Imperial Co., CA.	
		ROM 18250 *	Santa Catalina Island, B. C. S.		<i>Crotalus ruber</i>	Cr1	Cr n=2	San Antonio, B. C. S.	SA
<i>Crotalus cerastes</i>	Cce1	Cce 09209	El Huerfanito, B. C.	EH		Cr2	Cr Ab22013	El Comitán, B. C. S.	COM
	Cce2	Cce 050109	El Huerfanito, B. C.	EH		Cr3	Cr UABC184	Ensenada, B. C.	ENS
	Cce3	Cce 09109	El Huerfanito, B. C.	EH		Cr4	Cr 09012012	San Francisquito, B. C. S.	SF
		ROM-FC 2099 *	(no data collected)				BYU 34753-54 *	Cedros Island	
<i>Crotalus enyo</i>	Ce1	Ce pocitas	Las Pocitas, B. C. S.	POC			ROM 18197-98 *	Riverside, Co., CA.	
	Ce2	Ce2 0211	Cañón de la Zorra, B. C. S.	CZ	<i>Crotalus tigris</i>	Cti1	Cti 1jul2011	Sonoyta, Sonora.	SONO
	Ce3	Ce 3	Cañón de San Dionisio, B. C. S.	CSD			ROM 18167-68 *	Sonora, Mexico	
	Ce5	Ce Ba01	Bahía de Los Angeles, B. C.	BLA	<i>Crotalus transversus</i>		KZ-shed skin*	Mexico, specific locality unknown	
		ROM 13648 *	Baja California Sur		<i>Crotalus triseriatus</i>	1	LG: ROM 18114 *	Llano Grande, Mexico City, Mexico.	
<i>Crotalus durissus</i>		ROM 18138 *	Venezuela			2	Xo: ROM 18120 *	Xochimilco ¹ , Mexico City, Mexico	
<i>Crotalus estebanensis</i>	Ces1	Ces 09022012	San Esteban Island, Sonora	ISE		3	To: ROM 18121 *	Toluca, Mexico	
<i>Crotalus horridus</i>	1	ROM 18132-33 *	New York		<i>Crotalus unicolor</i>		ROM 18150 *	Aruba Island (captive-born)	
	1	UTA R-14697 *	Arkansas		<i>Crotalus vegrandis</i>		ROM 18261 *	Venezuela (purchased from Brazil)	
<i>Crotalus intermedius</i>		ROM-FC 223 *	Veracruz, Mexico		<i>Crotalus viridis</i>		ROM 19656 *	Los Angeles Co., CA.	
<i>Crotalus lepidus klauberi</i>		ROM 18128 *	Chihuahua, Mexico		<i>Crotalus willardi</i>	1	ROM 18183 *	Sonora, Mexico	
<i>Crotalus lorenzoensis</i>	Cl1	Cl 09042012	San Lorenzo Island, B. C.	ISL		2	ROM-FC 363*	Santa Cruz Co., AZ.	
	Cl2	Cl 04012011	San Lorenzo Island, B. C.	ISL		3	HWG 2575 *	Cochise Co., AZ.	
	Cl3	Cl 09032012	San Lorenzo Island, B. C.	ISL	<i>Sistrurus catenatus</i>		ROM-FC 243, 245 *	Ontario, Canada	
	Cl4	Cl 09052012	San Lorenzo Island, B. C.	ISL	<i>Sistrurus miliarius</i>		ROM 18232 *	Florida (commercially purchased)	
<i>Crotalus mitchellii</i>	Cmi1	Cmi 9	Cañón de la Zorra, B. C. S.	CZ	<i>Agkistrodon contortrix</i>		ROM 18230 *	(commercially purchased)	
	Cmi5	Cmi 22jun10	Cañón de la Zorra, B. C. S.	CZ	<i>Agkistrodon piscivorus</i>		ROM-FC 5599 *	(commercially purchased)	
<i>Crotalus molossus</i>	Cmo1	Cmo SJ90411	Sierra Juárez, Chihuahua.	SJ	<i>Gloydus ussuriensis</i>		ROM 20459 *	Jilin Prov. China	

¹ Incorrectly referred to as "Xochomiko" in Murphy et al. (2002) and subsequent citations (e.g. Castoe et al., 2006). * Samples downloaded from GenBank and used by Murphy et al. (2002).



Figure 3. Bayesian 50 % majority consensus tree based on combined fragments of partitioned mitochondrial DNA (12S and 16S), showing the relationships among insular, California peninsula and continental US and Mexico species of *Crotalus*. The numbers below the branches are posterior probabilities.

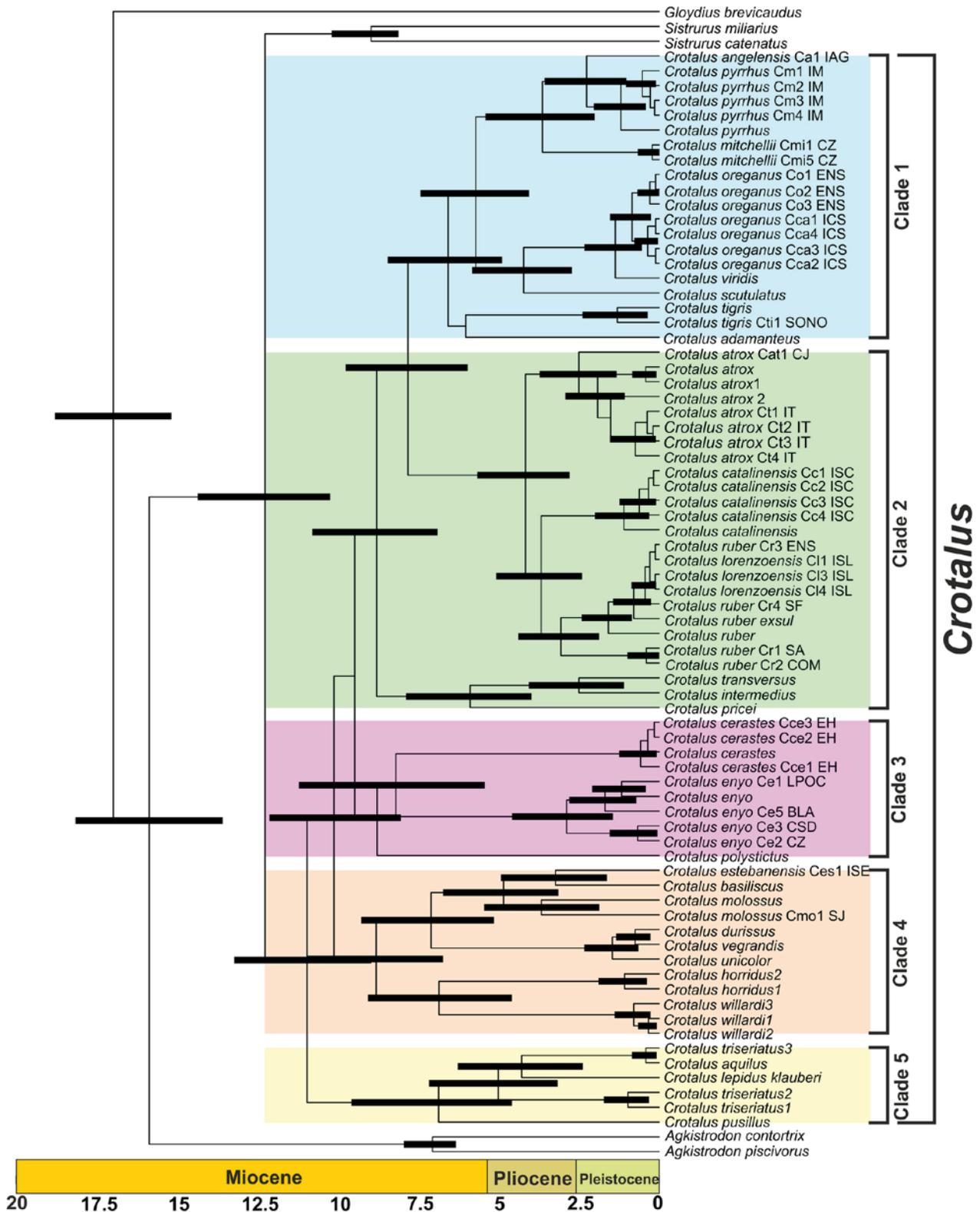


Figure 4. Chronogram based on a Bayesian approach to the *Crotalus* species of the Gulf of California, the California peninsula, and mainland USA and Mexico, from partitioned mitochondrial (12S and 16S) gene markers. Black bars indicate highest posterior density (HPD) intervals at 95 % for node age estimates.

angelensis from Ángel de la Guarda Island (PP = 0.87) and both were sister to two *C. mitchellii* individuals from Baja California Sur (PP = 0.99; Fig. 3). *Crotalus oreganus* from Ensenada formed a sister clade to *Crotalus oreganus* from Coronados Sur Island, and this species was sister to *C. viridis* (PP = 1.0; Fig. 3).

Clade 2 is made up of *C. atrox*, *C. catalinensis*, *C. lorenzoensis*, *C. ruber*, *C. polystictus*, *C. intermedius*, *C. transversus*, and *C. pricei*. All the *C. atrox* individuals from Tortuga Island were nested with the rest of the *C. atrox* individuals (PP = 1; Fig. 3). *C. catalinensis* was found to be a monophyletic species (PP = 1) sister to a clade comprising *C. lorenzoensis* from San Lorenzo Island and *C. ruber*, with low support (PP = 0.77; Fig. 3).

Clade 3 is represented by only two monophyletic sister species: *C. enyo* and *C. cerastes* (PP = 0.95). Clade 4 comprises eight species, with *C. estebanensis* from San Esteban Island sister to *C. basiliscus* (PP = 0.93) and nested in a clade with *C. molossus* (PP = 0.99; Fig. 3). Finally, Clade 5 contains the rest of the species, none of which were collected in this study (Fig. 3).

Divergence time estimates

The BEAST analyses suggest that the split between *Crotalus* and *Sistrurus* occurred 12.26 Ma (95 % High Probability Density (HPD) 12.03–8.1 Ma; Fig. 4). The stem age of *Crotalus* was 10.95 Ma (95 % HPD 13.2–8.95 Ma; Fig. 3). The speciation events occurred from the late Miocene to the Pleistocene (Fig. 4). In clade 1, the divergence between *C. pyrrhus* from El Muerto Island and the *C. pyrrhus* individual from California occurred 1.19 Ma (95 % HPD 2.02–0.41 Ma). *C. angelensis* diverged from *C. pyrrhus* 2.26 Ma (95 % HPD 3.54–1.02 Ma). The two *C. mitchellii* individuals from BCS diverged from the rest of the species 3.63 Ma (95 % HPD 5.39–2.02 Ma; Fig. 4). *C. oreganus* from Ensenada diverged from its congeners on Coronados Sur Island 0.84 Ma (95 % HPD 1.51–0.26 Ma). Finally, the divergence between *C. oreganus* and *C. viridis* occurred 1.36 Ma (95 % HPD 2.31–0.55 Ma; Fig. 4).

In clade 2, the divergence between *C. atrox* (including individuals from Tortuga Island) and *C. catalinensis*, *C. lorenzoensis*, and *C. ruber* occurred 4.16 Ma (95 % HPD 5.64–2.79 Ma). *C. atrox* first arrived on Tortuga Island 1.91 Ma (95 % HPD 2.90–1.07 Ma; Fig. 4). *Crotalus catalinensis* separated from the clade comprising *C. ruber* and *C. lorenzoensis* 3.67 Ma (95 % HPD 5.06–2.40 Ma). One *C. ruber* individual from Ensenada, BC, is nested with *C. lorenzoensis* individuals. The colonisation of San Lorenzo Island by *C. lorenzoensis* took place 0.43 Ma (95 % HPD 0.84–0.10 Ma; Fig. 3). In clade 3, the divergence between *C. cerastes* and *C. enyo* occurred 8.19 Ma (95 % HPD 11.2–5.43 Ma; Fig. 4). Finally, in clade 4, *C. estebanensis* diverged from *C. basiliscus* 3.22 Ma (95 % HPD 4.91–1.63 Ma; Fig. 4).

DISCUSSION

The islands of the Gulf of California are divided into three groups based on their origin: the northern, central midriff, and southern gulf areas (Carreño & Helenes, 2002). However, Case (2002) classified the islands into two groups: the Sonora islands (Tiburón, San Pedro Mártir,

San Esteban, and San Pedro Nolasco), which are closer to mainland Mexico; and the Baja California islands (all other islands), which are closer to the Baja California peninsula. Case (2002) also divided reptiles, including rattlesnakes, into three groups: species distributed in mainland Mexico and Baja California, species restricted to Sonora and adjacent regions, and species restricted to Baja California and the west coast of the USA. In our phylogenetic and molecular dating analyses, we included rattlesnakes with those three geographical affinities (Grismer, 2002).

We recovered the monophyly of the *Crotalus* genus with strong support (PP = 0.99), and this was sister to the *Sistrurus* genus (PP = 1). Previous molecular analyses recovered the same relationship (Parkinson et al., 2002; Murphy et al., 2002; Wüster et al., 2005). The inclusion of three rattlesnake species not previously included in any phylogenetic analysis (*C. angelensis*, *C. estebanensis*, and *C. lorenzoensis*) plus several *C. atrox* individuals from Tortuga Island, *C. mitchellii* from Baja California, *C. pyrrhus* from El Muerto Island, *C. oreganus* from BC and Coronados Sur Island, *C. ruber* from BC, *C. catalinensis* from Santa Catalina Island, and *C. enyo* from BC, yielded new phylogenetic relationships that differed from previous studies (Murphy, 2002).

The novel relationships found in clade 1 involve the phylogenetic position of *Crotalus angelensis*, which was sister to the clade comprising *C. pyrrhus* from El Muerto Island and *C. pyrrhus* from California, USA, and sister to this clade are the *C. mitchellii* individuals from Cañon de la Zorra, BCS. Meik et al. (2018) conducted a phylogenetic analysis focusing on the *C. mitchellii* complex and found sister relationships between the newly described species *C. polisi* and *C. angelensis*, with this clade being sister to *C. pyrrhus*.

In clade 2, we found *C. lorenzoensis* nested, with low support (0.85), in the same clade as *C. ruber* individuals from both BC and BCS (Fig. 3). This novel relationship makes *C. ruber* a paraphyletic species. However, *C. lorenzoensis* could be an isolated population of *C. ruber*. Murphy et al. (2002) included a sample from Cedros Island named *Crotalus "exsul"*, that sample was sister to *C. ruber*. In our phylogenetic results, the *C. "exsul"* resulted sister to the clade formed by *C. lorenzoensis* and *C. ruber* individuals. Cedros Island diamond rattlesnake (*C. exsul*) now is a subspecies of *C. ruber* (*C. ruber exsul*). These results highlight the need to use SNP (single nucleotide polymorphisms) markers and an integrative taxonomy similar to that used by Meik et al. (2018) to resolve the boundaries of the species in this complex group. The four *C. atrox* individuals from Tortuga Island included in this study were nested together but have different haplotypes to the individual previously included in the phylogeny reported by Murphy et al. (2002).

We recovered *C. catalinensis* as sister to the *C. ruber*-*C. lorenzoensis* clade, while Murphy et al. (2002) recovered *C. catalinensis* as sister to *C. ruber*. Grismer (2002) proposed taxonomic relationships between *C. angelensis* and *C. mitchellii* that were also found in our study. Additionally, Grismer (2002) proposed a sister relationship between *C. mitchellii* and *C. cerastes*, but in our phylogenetic reconstruction, the latter is sister to *C. enyo* and this relationship is well supported (Fig. 3).

These findings are consistent with Murphy et al. (2002). The close relationships between *C. atrox*, *C. catalinensis*, *C. lorenzoensis*, and *C. ruber* found here are consistent with the taxonomic relationships proposed by Grismer (2002). *C. tortugensis* was recognised as a different species from *C. atrox* (Grismer, 2002; Murphy et al., 2002). However, Castoe et al. (2007) found that *C. tortugensis* was nested with *C. atrox* individuals from the continent. Our results, which include more *C. atrox* individuals from Tortuga Island, support the findings published by Castoe et al. (2007).

Grismer (2002) proposed relationships between *C. molossus* and *C. estebanensis*. However, we found a novel relationship in that *C. estebanensis* was sister to *C. basiliscus* from Nayarit, Mexico, and nested within the same clade as *C. molossus* (Fig. 3). *Crotalus basiliscus* and *C. molossus* were found to be sister species in the phylogeny reported by Murphy et al. (2002). *Crotalus oreganus* individuals from Ensenada, BC, and individuals from Coronados Sur Island were sister species to *C. viridis* (Fig. 3), in keeping with previous results by Asthon & de Queiroz (2001).

Our phylogenetic results support the hypothesis put forward by Case (2002) with respect to invasion from areas near the islands, in which *C. estebanensis* from San Esteban Island (a Sonora island) is related to species from mainland Mexico (*C. basiliscus* and *C. molossus*). Meanwhile, the remaining island species (*C. catalinensis*, *C. angelensis*, and *C. lorenzoensis*) are related to species distributed in the Baja California peninsula and California, USA. *Crotalus atrox* from Tortuga Island differs from the rest of the island species, because individuals from mainland Mexico (Murphy & Aguirre-Léon, 2002) colonized the island.

Our phylogenetic hypothesis presented here based on two mitochondrial fragments shows a partial evolutionary history of the island endemic (some of them never studied before) and peninsular rattlesnake's species. Recent studies using UCEs (ultraconserved elements) or SNPs in continental, peninsular and island endemic *Crotalus* species (Blair et al., 2018; Meik et al., 2018) have been shown to be useful to resolve species limits in *Crotalus* lineages. Thus, the relationships found here could change with the use of nuclear markers (e.g. UCEs and SNPs).

Divergence time estimates

Vicariance has been the most recurrent hypothesis to explain the phylogeographic patterns of the flora and fauna of the Baja California peninsula. For example, the separation of the Baja California peninsula was an important factor that promoted genetic differentiation in peninsular vertebrates (Riddle et al., 2000a, 2000b; Grismer, 2002; González-Rubio et al., 2016). Another vicariance event evoked is the formation of the seaway at the Isthmus of La Paz (3 Ma; Riddle et al., 2000b). However, little is known about the invasion of the Gulf of California islands and the western part of the Baja California peninsula. Here we report results obtained from the variation of the mitochondrial DNA of rattlesnakes as a proxy to determine the temporality and origin of the invasions of the islands. Our molecular divergence time estimates indicate that the divergence

of *C. angelensis* from *C. pyrrhus* occurred at 2.26 Ma, which is consistent with the separation of the continental Ángel de la Guarda Island from Baja California during the Pleistocene (Carreño & Helenes, 2002). The colonisation of Coronados Sur Island by *C. oreganus* from BC occurred during the Pleistocene. Carreño & Helenes (2002) remarked that this island first separated from BC during the Pliocene-Pleistocene, so if our divergence times are correct, this island may have formed before 0.84 Ma.

The oceanic Tortuga Island was formed during the Pleistocene (Carreño & Helenes, 2002), which is consistent with the colonisation of this island by *C. atrox* (Murphy & Aguirre-Léon, 2002). San Lorenzo Island first separated from BC during the Pliocene (Carreño & Helenes, 2002). However, the divergence of *C. lorenzoensis* from *C. ruber* occurred during the Pleistocene. Santa Catalina Island first separated during the Pliocene (Carreño & Helenes, 2002), which is consistent with the divergence of *C. catalinensis* from *C. ruber*-*C. lorenzoensis*, 3.66 Ma. Finally, *C. estebanensis* diverged from *C. basiliscus* 3.22 Ma. This date is consistent with the origin of San Esteban Island during the Miocene-Pliocene, but not with the first separation during the Pleistocene (Carreño & Helenes, 2002). Although estimates of divergence time may vary depending on the size of the effective population and the fastest coalescence times for the case of mitochondrial genes (Arbogast et al., 2002); Blair et al. (2018) using UCE, found estimations of divergence times similar to mitochondrial genes. Therefore, the addition of nuclear genes in the study of the endemic rattlesnakes of the islands could lead to similar estimates, but with reduced variance (Edwards & Beerli, 2000).

We also found a south-north geographic pattern of separation between *C. mitchellii* individuals from BCS and CA. That separation occurred at 3.66 Ma, during the Pliocene. *C. ruber* individuals from BC also exhibited a geographical separation from individuals from BCS, which occurred 3.05 Ma, during the late Pliocene (Fig. 3). A south-north geographic pattern of separation has been found in other reptile species (Murphy & Aguirre-Léon, 2002). Additionally, an interesting pattern of genetic variation and substructure geographic was found between *C. enyo* individuals from the Cape Region and the rest of the individuals from central BCS. The separation occurred at 2.87 Ma, also during the late Pliocene. This pattern was also found with *Urosaurus nigricaudus* (Murphy & Aguirre-Léon, 2002). These findings require a more precise phylogeographic examination of these last species to determine the causative agents and temporality of the pattern of genetic variation.

CONCLUSIONS

This is the first study that includes all the insular species of rattlesnake in the Gulf of California. Our molecular tree recovered novel phylogenetic relationships, highlighting sister relationships between *Crotalus lorenzoensis* and *C. ruber*, and between *C. estebanensis* and *C. basiliscus*. With the exception of *C. atrox* from Tortuga Island and *C. estebanensis* from San Esteban Island, which are related to mainland species, the rest of the insular species are

related to species from the California peninsula. Our phylogenetic findings support most of the taxonomical relationships proposed by Grismer (2002). The Gulf of California islands were formed between the Pliocene and the Pleistocene. Our molecular divergence time estimates for the different clades in which the insular rattlesnake species are nested indicate that those endemic insular species colonized the islands after their formation, assuming realistic estimates. Finally, further phylogeographic analyses of some rattlesnake species distributed in the California peninsula are needed in order to understand whether those species were affected by climate oscillations in the Pleistocene.

ACKNOWLEDGMENTS

The third author thanks CONACyT (National Council of Science and Technology, Mexico) for the support provided by his master's scholarship, number 260590. This work constitutes fulfilment of ORCA's Master's in Science at the CIBNOR. This research was partially supported by a competitive grant (HK051) from the National Commission for the Knowledge and Use of Biodiversity (CONABIO). Gorgonio Ruiz Campos, curator of the herpetological collection of the Vertebrate Laboratory of the Autonomous University of Baja California (UABC), gave us tissues of *C. oreganus* and *C. ruber*. Collecting permits for specimens obtained for this study were issued by the government of Mexico (SEMARNAT) to GA (03943/10, 04526/11)

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Accepted: 27 March 2019