



**UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO**

**POSGRADO EN CIENCIAS BIOLÓGICAS**

**FACULTAD DE CIENCIAS**

**SISTEMÁTICA**

**TIEMPOS DE DIVERGENCIA Y PATRONES FILOGEOGRÁFICOS DE LA  
CASCABEL PIGMEA *C. RAVUS* (VIPERIDAE)**

**TESIS**

**(POR ARTÍCULO CIENTÍFICO)**

**Divergence time and integrative taxonomy of the Mexican pigmy rattlesnake *Crotalus  
ravus* (Viperidae)**

**QUE PARA OPTAR POR EL GRADO DE:**

**MAESTRO EN CIENCIAS BIOLÓGICAS**

**PRESENTA:**

**ANTONIO YOLOCALLI CISNEROS BERNAL**

**TUTOR PRINCIPAL DE TESIS: DR. OSCAR ALBERTO FLORES VILLELA  
FACULTAD DE CIENCIAS, UNAM**

**COMITÉ TUTOR: DR. HIBRAIM ADÁN PÉREZ MENDOZA  
FACULTAD DE ESTUDIOS SUPERIORES IZTACALA, UNAM**

**DRA. IRENE GOYENCHEA MEYER-GOYENCHEA  
CENTRO DE INVESTIGACIONES BIOLÓGICAS, UAEH**

**Ciudad Universitaria, CDMX., Agosto, 2020**



Universidad Nacional  
Autónoma de México



**UNAM – Dirección General de Bibliotecas**  
**Tesis Digitales**  
**Restricciones de uso**

**DERECHOS RESERVADOS ©**  
**PROHIBIDA SU REPRODUCCIÓN TOTAL O PARCIAL**

Todo el material contenido en esta tesis esta protegido por la Ley Federal del Derecho de Autor (LFDA) de los Estados Unidos Mexicanos (México).

El uso de imágenes, fragmentos de videos, y demás material que sea objeto de protección de los derechos de autor, será exclusivamente para fines educativos e informativos y deberá citar la fuente donde la obtuvo mencionando el autor o autores. Cualquier uso distinto como el lucro, reproducción, edición o modificación, será perseguido y sancionado por el respectivo titular de los Derechos de Autor.





**UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO**

**POSGRADO EN CIENCIAS BIOLÓGICAS**

**FACULTAD DE CIENCIAS**

**SISTEMÁTICA**

**TIEMPOS DE DIVERGENCIA Y PATRONES FILOGEOGRÁFICOS DE LA**

**CASCABEL PIGMEA *C. RAVUS* (VIPERIDAE)**

**TESIS**

**(POR ARTÍCULO CIENTÍFICO)**

**Divergence time and integrative taxonomy of the Mexican pigmy rattlesnake *Crotalus***

***ravus* (Viperidae)**

**QUE PARA OPTAR POR EL GRADO DE:**

**MAESTRO(A) EN CIENCIAS BIOLÓGICAS**

**PRESENTA:**

**ANTONIO YOLOCALLI CISNEROS BERNAL**

**TUTOR(A) PRINCIPAL DE TESIS: DR. OSCAR ALBERTO FLORES VILLELA**  
**FACULTAD DE CIENCIAS, UNAM**

**COMITÉ TUTOR: DR. HIBRAIM ADÁN PÉREZ MENDOZA**  
**FACULTAD DE ESTUDIOS SUPERIORES IZTACALA, UNAM**

**DRA. IRENE GOYENCHEA MEYER-GOYENCHEA**  
**CENTRO DE INVESTIGACIONES BIOLÓGICAS, UAEH**

**Ciudad Universitaria, CDMX MES (EN EL QUE SE REALIZÓ EL EXAMEN), AÑO**

COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE CIENCIAS

DIVISIÓN ACADÉMICA DE INVESTIGACIÓN Y POSGRADO

OFICIO FCIE/DAIP/0225/2020

**ASUNTO: Oficio de Jurado**

**M. en C. Ivonne Ramírez Wence**  
**Directora General de Administración Escolar, UNAM**  
Presente.

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **20 de enero de 2020** se aprobó el siguiente jurado para el examen de grado de **MAESTRO EN CIENCIAS BIOLÓGICAS** en el campo de conocimiento de **Sistemática** del alumno **CISNEROS BERNAL ANTONIO YOLOCALLI** con número de cuenta **307028978** por la modalidad de graduación de tesis por artículo científico titulado: **"Divergence time and integrative taxonomy of the Mexican pigmy rattlesnake *Crotalus ravus* (Viperidae)"**, que es producto del proyecto realizado en la maestría que lleva por título **"Tiempos de divergencia y patrones filogeográficos de la cascabel pigmea *C. ravus* (Viperidae)"**, ambos realizados bajo la dirección del **DR. OSCAR ALBERTO FLORES VILLELA**, quedando integrado de la siguiente manera:

Presidente: DR. JUAN JOSÉ MORRONE LUPI  
Vocal: DR. ALEJANDRO FRANCISCO OCEGUERA FIGUEROA  
Secretario: DRA. IRENE GOYENECHEA MAYER-GOYENECHEA  
Suplente: DR. JULIÁN ANDRES VELASCO VINASCO  
Suplente: DRA. MARISOL MONTELLANO BALLESTEROS

Sin otro particular, me es grato enviarle un cordial saludo.

**ATENTAMENTE**

**"POR MI RAZA HABLARA EL ESPÍRITU"**

Ciudad Universitaria, Cd. Mx., a 13 de marzo de 2020  
**COORDINADOR DEL PROGRAMA**



**DR. ADOLFO GERARDO NAVARRO SIGÜENZA**

AGNS/MMVA/ASR/mnm



**COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS**  
**UNIDAD DE POSGRADO**

Edificio D, 1º Piso. Circuito de Posgrados, Ciudad Universitaria  
Alcaldía Coyoacán. C. P. 04510 CDMX  
Tel. (+5255)5623 7002 <http://pcbiol.posgrado.unam.mx/>

## **AGRADECIMIENTOS**

Al posgrado en Ciencias Biológicas de la UNAM, por su apoyo académico integral para la realización de mis estudios de maestría.

Al Consejo Nacional de Ciencia y Tecnología, por el apoyo económico brindado a través de la beca No. 669772.

A la UNAM, por brindar apoyo económico para la realización de este proyecto a través del proyecto PAPIIT No. IN216218.

## **AGRADECIMIENTOS PERSONALES**

Primeramente, al Dr. Oscar Flores Villela por fungir como tutor y amigo. Por toda la confianza y apoyo durante estos años, pero sobre todo por la amistad que me ha brindado desde el inicio de mi vida académica.

A los dos miembros de mi comité tutor, la Dra. Irene Goyenechea Meyer-Goyenechea y el Dr. Hibraim Pérez Mendoza, por siempre escucharme y apoyarme en los momentos de más estrés. Por sus consejos y observaciones hacia mi investigación, que sin duda derivaron en un trabajo íntegro y de calidad.

A los miembros del jurado Dra. Irene Goyenechea, Dra. Marisol Motellano, Dr. Alejandro Ocegüera, Dr. Juan José Morrone y Dr. Julián, por sus comentarios sobre el manuscrito que sin duda ayudaron de manera sustancial a mejorarlo.

A todos mis amigos del laboratorio, pero principalmente a Atziri Ibarra, Gonzalo Medina y Ricardo Palacios, por siempre haber estado apoyándonos en las buenas y malas, más malas que buenas a veces, pero tratando de generar alianzas académicas y forjando una buena amistad en el camino.

No puedo olvidar al laboratorio de Ecología de Anfibios y Reptiles del Dr. Hibraim en la FES Iztacala, por su constante aportación de muestras para mi proyecto. Principalmente a Eduardo Bucio y Luis Jaramillo. Además de la familia Jaramillo, que cordialmente me recibieron en su casa en algún momento, de corazón gracias.

A todos los miembros del laboratorio del Dr. Matthew Fujita mientras estuve de estancia la Universidad de Texas en Arlington. A Danielle, Kyle, Kathleen y especialmente a T. J. Firreno por todo su apoyo y comprensión, en verdad los aprecio mucho. A la Dra. Flor Rodríguez por sus consejos durante todo el desarrollo del proyecto, muchas gracias.

Finalmente quisiera agradecer de manera especial a mi familia por todo el apoyo incondicional a través de los años, por ayudarme a mantenerme siguiendo mi sueño de desarrollarme en lo que me gusta. A pesar de las adversidades, han sabido, cada uno, apoyarme a su manera y con su toque especial ¡los amo mucho! Luis Cisneros, Tere Bernal y Atzin Cisneros.

## DEDICATORIA

Principalmente a mis padres, por todo ese apoyo en cada momento de mi carrera. Por siempre impulsarme a realizar lo que me gusta a toda costa, sin importar las dificultades.

A mi hermana, por siempre estar para darme un consejo o para compartir experiencias, todo este esfuerzo es para ellos.



## Índice

<b>Resumen</b> .....	9
<b>Abstract</b> .....	10
<b>Introducción</b> .....	11
<b>Artículo Sometido</b> .....	15
<b>Comprobante de requisito para titulación por artículo científico</b> .....	50
<b>Discusión</b> .....	51
<b>Conclusiones</b> .....	53
<b>Literatura Citada (No contenida en el artículo)</b> .....	54
<b>Artículos derivados de este proyecto</b> .....	56

## Resumen

Debido a su complejidad geológica, el centro y sur de México son considerados áreas de alto endemismo. Esto deriva en que los taxones que se distribuyen en esa región presenten historias evolutivas complejas. El caso de la serpiente de cascabel enana de montaña *Crotalus ravus sensu lato* no es la excepción, ya que presenta poblaciones con distribuciones alopátricas en esta zona. Por medio de marcadores moleculares (ND4, 12S y L51) se estudiaron las relaciones filogenéticas dentro del grupo, a través de análisis bayesiano y de máxima verosimilitud. Además, se elaboró una red de haplotipos, se calcularon las distancias genéticas, tiempos de divergencia, se hizo un árbol de especies y se analizaron las estructuras de morfología hemipenial de los tres taxones reconocidos dentro de *C. ravus*. En los dos análisis filogenéticos, bayesiano y máxima verosimilitud, se recuperó la monofilia de los tres linajes con valores de apoyo altos (>0.9 PP; >75 para los tres clados). El tiempo de divergencia del grupo con respecto a los demás miembros de las serpientes de cascabel de montaña, se estimó en el Mioceno-Plioceno (~5-7 Ma) y los análisis de la red de haplotipos, distancias genéticas y la estructura hemipenial proveen, de manera conjunta, evidencia de la independencia de cada linaje. Estos resultados difieren de trabajos anteriores, en cuanto a las relaciones filogenéticas dentro del grupo y a los tiempos de divergencia estimados para el clado *C. ravus sensu lato*. Se propone que la formación de la Faja Volcánica Trans-Mexicana fue el principal evento precursor vicariante que promovió la diversificación de los linajes dentro de esta serpiente de cascabel. Debido a la amplitud del muestreo y diferentes evidencias obtenidas mediante distintas aproximaciones, se propone el reconocimiento específico de cada linaje del complejo *C. ravus*, así como la correspondiente reevaluación de su estado de conservación.

## Abstract

Given its complex geography, Central and Southern Mexico are considered areas of high endemism, promoting complex evolutionary histories of the taxa distributed therein. The case of the Pygmy Mountain Rattlesnake, *Crotalus ravus sensu lato* is not an exception, having populations with allopatric distributions in this region. Using molecular markers (12S, ND4 and L51) the phylogenetic relationships of the group were estimated by both Bayesian and Maximum Likelihood approaches. Additionally, a haplotype network was constructed, genetic distances calculated, divergence times and species tree estimated, and hemipenial morphological characters analyzed in each of the three taxa within *C. ravus*. Both Bayesian and Maximum Likelihood phylogenetic analyses, recovered the monophyly of the three lineages, with high support scores (>0.9 PP; >75 BS, for the three clades). Divergence time of the group was estimated between the Miocene-Pliocene (~5-7 Mya) and the analyses of the haplotype network, genetic distances and hemipenial structures jointly provide evidence of each lineage independence. These results, differ from previous works concerning the phylogenetic relationships of the group and estimated divergence times of the *C. ravus sensu lato* clade. We propose that the formation of the Trans Mexican Volcanic Belt was the main vicariant precursor event promoting the diversification of the lineages of this rattlesnake. Due to ample sampling and different evidences obtained, the specific recognition of each lineage of the *C. ravus* complex is suggested as well as the corresponding conservation status reevaluation.

## **Introducción**

Avise et al. (1987) propusieron el término filogeografía para definir el estudio del reparto de los linajes dentro de la geografía donde se distribuyen. Esta definición vino rápidamente a situarse en el centro de la controversia sobre la relación entre los procesos macroevolutivos y microevolutivos. Se ha debatido de manera constante si es posible extrapolar procesos como mutación, deriva génica, flujo genético, historia demográfica y selección natural, a procesos a gran escala como extinciones en masa, patrones de diversificación, novedades evolutivas o disparidad morfológica (Erwin, 2010). La filogeografía utiliza conceptos y técnicas de diferentes áreas de la Biología como la genética de poblaciones, demografía, sistemática filogenética, etología y paleontología (Avise, 2000). Esto permite lograr un enfoque integrativo, que se reconoce como una línea directa entre los estudios microevolutivos y los macroevolutivos (Avise, 1998).

Algunos autores proponen que el límite entre un estudio a nivel macroevolutivo y microevolutivo está delineado por la frontera del concepto de especie (Erwin, 2010). Es decir, procesos que ocurren por debajo del nivel específico son considerados microevolutivos y por encima de este nivel, son considerados macroevolutivos. Esto nos lleva a considerar las diferentes definiciones de especie y la controversia alrededor de este concepto tan fundamental en Biología (Mayr, 1987; Rojas, 1992; Hudson & Coyne, 2002; Pigliucci, 2003). Bajo este panorama, de Queiroz (2005) definió a las especies como linajes a nivel poblacional que presentan historias evolutivas independientes y que las propiedades que definían los conceptos pasados de especie (aislamiento reproductivo, monofilia, diagnosticabilidad, etc.), son en realidad propiedades emergentes que sirven como evidencia para poder discriminar entre estas entidades de ancestría-descendencia. Bajo esta perspectiva, la identificación de linajes poblacionales que presenten historias evolutivas independientes resulta práctica, permitiendo así la proposición de especies putativas.

Por otro lado, la identificación de especies siempre ha representado un reto para los biólogos debido a que el proceso de especiación no necesariamente viene acompañado de cambios morfológicos evidentes

(Bickford et al., 2007). Esto quiere decir que existe variación derivada de las presiones selectivas a las que están siendo sometidos los linajes, que no necesariamente es apreciable a simple vista (Erwin, 2010). Por lo anterior, la diversidad conocida probablemente sea mayor a la estimada hasta este momento. La subestimación del número de linajes dentro de una especie nos lleva a concluir que existen especies crípticas, especialmente si algunos linajes se consideraban una misma especie por su parecido morfológico (Bickford et al., 2007).

La delimitación de especies se vuelve una herramienta importante para los planes de conservación. Sobre todo en especies crípticas que, por sus características naturales y distribución, son proclives a presentar complejos de especies. Además de que si estos taxones suelen encontrarse bajo alguna categoría de protección, la identificación de especies crípticas se vuelve crucial, ya que involucraría una reducción en la distribución de estas nuevas entidades identificadas, colocándolas en un riesgo mayor (Bickford et al., 2007).

Tal es el caso de los reptiles en general, se distribuyen en microhábitats específicos, y son un grupo que juega un papel importante en los ecosistemas (Raxworthy et al., 2008; Read, 1998; Böhm et al., 2013). Características como la filopatría, poca vagilidad y morfología conservada, provoca que sus distribuciones sean considerablemente más reducidas a comparación de otros vertebrados como mamíferos o aves (Böhm et al., 2013). Lo que ha derivado en un número considerable de trabajos sobre diversidad críptica dentro de este grupo de vertebrados (Poulin & Pérez-Ponce de León, 2016, trabajos ahí referidos). No hay que perder de vista que los reptiles actualmente son un grupo en peligro crítico de extinción, ya que son propensos a amenazas como el cambio de uso de suelo, contaminación y fragmentación de los hábitats, actividades que han venido en aumento actualmente por la actividad humana. (Sarukhán et al., 2009).

El caso particular de las serpientes de cascabel, presenta un panorama desalentador. Esto se debe a que no sólo se enfrentan a las amenazas antes mencionadas, sino que afrontan la caza y muerte para uso en medicina tradicional (Pérez-Gil et al., 1996), o simplemente por miedo, convirtiendo a los humanos como

sus principales amenazas (Campbell & Lamar, 2004; Monroy-Vilchis et al., 2008). En contraparte, estudios recientes sugieren que la diversidad de las serpientes de cascabel es aún desconocida (Reyes-Velasco et al., 2013; Bryson et al., 2014; Blair et al., 2018). Igualmente este desconocimiento de la verdadera diversidad existente, representa un reto para la conservación, y la correcta implementación de estrategias que busquen protegerla.

Los bosques de montaña del centro y sur de México son de las zonas más diversas en cuanto a diversidad de serpientes de cascabel, incluso se ha propuesto que podrían representar el centro de diversificación de este grupo (Blair et al., 2016). Esto se debe a que México se encuentra en la zona de contacto de fauna entre dos grandes regiones biogeográficas, la Neotropical y la Neártica (Halffter, 1987; Morrone, 2010), lo que le confiere un alto grado de endemismos, debido a su accidentada topografía (Flores-Villela, 1993; Flores-Villela & Gerez, 1994; Peterson et al., 1993; Ramamoorthy et al., 1993). Además, numerosos trabajos han evidenciado una fuerte asociación entre la complejidad geográfica y la diversidad ambiental, con el número de especies crípticas encontradas en esa zona (p. e. Bryson et al., 2014; García-Vázquez et al., 2018; Ruiz-Vega et al., 2018; Hernández-Canchola et al., 2018).

*Crotalus ravus* (Cope, 1865) es una serpiente de cascabel de montaña considerada dentro del grupo de las cascabeles enanas (Campbell & Lamar, 2004). Se distribuye entre los 1500-3000 msnm, en las montañas del centro de México, centro del estado de Guerrero y serranías del estado de Oaxaca (Harris & Simmons, 1978; Campbell & Armstrong, 1979; Campbell & Lamar, 2004). Las tres poblaciones de este taxón, presentan una distribución alopátrida (Campbell & Lamar, 2004). Esta especie ha sido declarada como amenazada por la norma mexicana NOM-059 (SEMARNAT, 2010) y de preocupación menor a nivel mundial (IUCN, 2019). A pesar de considerarse una especie tolerante al disturbio antropogénico (según nuestra experiencia en campo), el cambio de uso de suelo y la fragmentación, han reducido considerablemente el hábitat donde se distribuye y por ende el tamaño de sus poblaciones. Por todo lo anterior, por medio de un análisis filogeográfico, se buscará observar la diversidad genética y morfológica dentro de este grupo de serpientes de cascabel de montaña. El objetivo del estudio es analizar

las historias evolutivas y patrones de diversificación de *Crotalus ravus* desde una perspectiva de la taxonomía integrativa, con comentarios sobre su conservación.

## Artículo Sometido

### Divergence time and integrative taxonomy of the Mexican pigmy rattlesnake *Crotalus ravus* (Viperidae)

Cisneros-Bernal A. Yolocalli <sup>a,d,\*</sup>, Oscar A. Flores-Villela <sup>a</sup>, Mathew K. Fujita <sup>b</sup>, Ricardo Palacios-Aguilar <sup>a,d</sup> and Jesús A. Fernández <sup>c</sup>.

<sup>a</sup>Museo de Zoología, Departamento de Biología Evolutiva, Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad Universitaria, Mexico City 04510, México; <sup>b</sup>Department of Biology, University of Texas at Arlington, Arlington, Texas, 76019 USA; <sup>c</sup>Departamento de Recursos Naturales, Facultad de Zootecnia y Ecología, Universidad Autónoma de Chihuahua, Periférico Francisco R. Almada Kilómetro 1, Zootecnia, Chihuahua, Chihuahua, 31415, México; <sup>d</sup>Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México; Av. Ciudad Universitaria 3000, C.P. 04510, Coyoacán, CDMX, México.

\*Corresponding author:

E-mail address: A.Y.Cisneros@ciencias.unam.mx

#### Abstract

Central Mexico is one of the world's biodiversity hotspots given its location as a transitional zone between the Nearctic and Neotropical biogeographical regions, as well as its complex orographic and geologic history. Species distributed in this zone have been shown to have complex evolutionary histories. Such is the case for the montane rattlesnake *Crotalus ravus sensu lato*, distributed allopatrically along the mountain ranges of central and southern Mexico. Using a molecular dataset of two mitochondrial markers (ND4 and 12S) and one nuclear marker (L51), we investigate the phylogenetic relationships of the three recognized lineages within this taxon using Bayesian and Maximum Likelihood methods. Additionally, we constructed haplotype networks, calculated genetic distances, estimated divergence times of major lineages, and constructed a species tree using the molecular dataset, while an analysis of hemipenial structures of the three lineages added a morphological component. Both the Bayesian and Maximum Likelihood analyses recovered the monophyly of each lineage within *Crotalus ravus* with high support values (>0.9 PP; >75 BS, respectively). The estimated root age of the clade was dated around the Miocene-Pliocene (~5–7 Ma), and the species tree, haplotype networks, genetic distances, and hemipenial structures support the



evolutionary distinctiveness of the three lineages through an integrative approach. These results differ from previous works in respect to the phylogenetic relationships recovered and estimated divergence times. The formation of the Trans-Mexican Volcanic Belt (TMVB) likely promoted the development of independent evolutionary histories between the three lineages through vicariant processes, leading to the current distribution of *Crotalus ravus sensu lato*. As such, with the evidence presented in this study and the breadth of sampling, we propose the three disjunct populations of this Mexican pygmy rattlesnake to be recognized as separate species.

**Keywords:** Phylogeography, *Crotalus ravus*, biodiversity hotspot, lineage, rattlesnake, vicariance

## 1. Introduction

The Mexican Transition Zone (MTZ) is a biogeographic province that represents a connection between biota of the Nearctic temperate and Neotropical tropical zones (Halffter, 1987; Morrone, 2010). The geographic location of the MTZ, in addition to its complex orographic history and the convergence of climatic factors, has given rise to high species richness, diversity, and endemism that has led the region to be recognized worldwide as a ‘biodiversity hotspot’ (Flores-Villela & Gerez, 1994; Peterson et al., 1993; Ramamoorthy et al., 1993).

The relationship between geography and the distribution of lineages through time and space has been discussed extensively within the MTZ across various taxonomic groups (Morrone, 2009, 2017). These studies show that the dynamic orography of the MTZ has molded the evolutionary histories of species through different processes. Of these, both vicariance and dispersal are commonly proposed (Table 1), making it difficult to ascribe one encompassing biogeographic pattern and highlighting the importance of further studies in the regions (Flores-Villela & Martínez-Salazar, 2009). Taxa distributed in the highlands of the MTZ have been of particular interest to researchers, given that they have been shown to possess complex evolutionary histories and specific patterns of diversification. This is largely due to the asynchrony in the formation of the MTZ’s geological components, including the relatively recently formed Trans-Mexican Volcanic Belt (TMVB) of Miocene origin, and the more ancient mountain ranges of Eocene and Oligocene origin, including the Sierra Madre Occidental (SMOc), Sierra Madre Oriental (SMOr), and Sierra Madre del Sur (SMS) (Ferrusquía-Villafranca & González-Guzmán, 2005). This dynamics has caused lineages to become isolated or connected through the formation and dissolution of physical barriers through time.

One taxon that has received particular attention are rattlesnakes (Bryson et al., 2011a, 2011b; Blair & Sánchez-Ramírez, 2016; Blair et al., 2018), composed of approximately 49 species (Uetz et al.,

2019) in two genera (*Crotalus* and *Sistrurus*), broadly distributed across the American continent (Campbell and Lamar, 2004). Phylogenetic and phylogeographic studies recognize the highlands of the MTZ as a possible evolutionary area of origin for this group of reptiles (Reyes-Velasco et al., 2013; Alencar et al., 2016; Blair & Sánchez-Ramírez, 2016) given the early divergences of rattlesnakes distributed in this zone, although information regarding how these orographic events influenced the phylogenetic relationships of supraspecific lineages of *Crotalus* is still lacking. For this reason, rattlesnakes distributed in the highlands of the MTZ present a good model for understanding evolutionary patterns that have molded the present-day distributions of lineages in central Mexico. Particularly, the Mexican pygmy rattlesnake (*Crotalus ravus*) is an ideal study group for evaluating historic evolutionary patterns in the highlands of the MTZ, given that they are distributed across the montane pine-oak forests of the TMVB and the SMS. The phylogenetic placement of this rattlesnake species has been given considerable attention since its original description (Cope, 1865; Boulenger, 1896; Davis & Smith, 1953; Murphy et al., 2002; Wallack et al., 2014; Alencar et al., 2016; Blair & Sanchez-Ramirez, 2016). Once considered in the genus *Sistrurus* due to shared morphological characters such as the nine symmetrical plates of scales at the head (Boulenger, 1896; Gloyd, 1940; Davis & Smith, 1953; Klauber, 1972; Campbell & Armstrong, 1979), recent molecular data has confidently placed this taxon as the earliest divergent clade of the *Crotalus triseriatus* species group (Bryson et al., 2011a) within *Crotalus* (Murphy et al., 2002; Wallack et al., 2014; Alencar et al., 2016; Blair & Sanchez-Ramirez, 2016). Furthermore, the allopatric distribution and morphological characteristics of different populations (Table 2) have led to the recognition of distinct subspecies. The first of these to be described was *C. r. brunneus*, a population restricted to the Oaxacan highlands (Harris & Simmons, 1978). One year later, Campbell & Armstrong (1979) described *C. r. exiguus* from the Sierra Madre del Sur in Guerrero, ascribing the nominotypical form *C. r. ravus* to the central Mexican highlands of the TVMB.

Regarding the conservation perspective, *Crotalus ravus* has been declared threatened by the Mexican authorities (SEMARNAT, 2010), and internationally is considered as least concern (IUCN, 2019), since in some localities is relatively common and it seems to have tolerance to disturbed environments. This tolerance does not preclude this snake from the constant threats to which rattlesnakes are subject; as be killed by fear or to use them for traditional medicine, turning humans into the main destroyers of these snakes (Campbell & Lamar, 2004). Furthermore, the increasing deterioration of the environment caused by man, due to the change in land use and urbanization, have caused an increase in the fragmentation of many habitats in recent decades (Sarukhán et al., 2009), causing the decline in rattlesnakes populations (Campbell

& Lamar, 2009; Monroy-Vilchis et al., 2008). Insufficient conservation policies have resulted in only about 20% of the current distribution of this rattlesnake considered within a protected natural area.

This work analyzes the evolutionary history and patterns of diversification of the three recognized lineages of *Crotalus ravus* through a taxonomy approach, with comments on their conservation status.

## **2. Materials and Methods**

### **2.1 Sampling and DNA Extraction**

Thirty-six tissue samples were obtained for this study and an additional 20 sequences of *Crotalus ravus* were downloaded from GenBank. Our sampling nearly covers the entire known distribution of the three recognized lineages, *C. ravus exiguus*, *C. ravus brunneus*, and *C. ravus ravus* (Figure 1). We consider *C. molossus*, *C. scutulatus*, *Sistrurus miliarius* and *S. catenatus* as external groups for both analysis from two mitochondrial loci, NADH dehydrogenase 4 and flanking tRNA's (ND4) and the 12S ribosomal subunit (12S), as well as one nuclear locus, Locus 51 (L51) (Gibbs et al., 2010) were sequenced for this study. Primers for two of the three loci (ND4 and 12S) were taken from previous studies on montane rattlesnakes (Bryson et al., 2011a; Bryson et al., 2014) (Table 3). Genomic DNA was obtained from the muscle, liver, skin and/or skin molts following the Dneasy kit protocol from Qiagen (Qiagen Valencia, CA).

### **2.2 Sequencing Mitochondrial and Nuclear Markers**

All of the extracted DNA samples successfully amplified using the polymerase chain reaction (PCR) protocol in the Qiagen PCR kit (Qiagen Valencia, CA). Each reaction of 25 µl contained 0.5 µl of deoxynucleotide triphosphates (dNTP's) (2 mM), 0.25 U of Taq DNA polymerase, 0.5 µl each of forward and reverse primers (10 mM), 3 µl of magnesium chloride (25 mM), 2.5 µl of 10X PCR buffer, 16.75 µl of double-distilled water, and 1 µl of template DNA. The thermocycler protocol consisted of 95° C for 1 minute, followed by 37 cycles of 95° C for 30 seconds, 30 seconds of annealing (temperatures varied by gene: 52–54° C for ND4, 50° C for L51, and 61° C for 12S), and a 30 second extension at 72° C. The samples were then kept at 12° C until removal. PCR product was visualized by gel electrophoresis using a gel of 1% agarose and ethidium bromide. Purification of PCR product was done using the Qiagen quick PCR Purification Kit (Qiagen Valencia, CA), following the given protocol. Sanger sequencing was performed on a Biosystems 3730/3730XL sequencer. Forward and reverse sequences were assembled in Sequencer v.5.4.6.

## 2.3 Haplotype Networks

Genealogical relationships between the sequences of the *Crotalus ravus* (*sensu lato*) complex were calculated and visualized using Templeton's parsimony algorithm implemented in the software TCS v.2.1 (Clement et al., 2000). This algorithm constructs a haplotype network based on the number of mutational changes that separate distinct lineages. Exceeding a certain connectivity limit renders distant haplotypes as non-related. A 95% connectivity limit was implemented, and gaps were treated as missing data. Loops in the network were resolved using the method described by Pfenninger & Posada (2002).

## 2.4 Phylogenetic Analyses

Contigs were edited and aligned manually using the software PhyDE v.0.9971, while jModelTest v.2.1.10 was used to find the best-fit model of substitution for each gene according to AIC criteria with 95% confidence credibility (Darriba et al., 2015). Phylogenetic analyses were performed using Bayesian inference (BI) and maximum likelihood (ML) methods on each gene independently as well as a concatenated. BI was carried out in MrBayes v. 3.2.5 (Huelsenbeck & Ronquist, 2001) and concatenation was done in Mesquite v.3.5.1 (Maddison & Maddison, 2018). The analyses ran for 50 million generations with a sample frequency of 1000, and a 25% burn-in was implemented for each analysis. Convergence of the four chains were evaluated in Tracer v.1.4 (Rambaut & Drummond, 2007), making sure that ESS values for every parameter exceeded 200, signifying sufficient sampling of the posterior distribution. Subsequently, posterior probability (PP) support values were estimated for each clade using a majority consensus (>50%) of the results from each run, and PP values greater than 95% were considered strongly supported (Felsenstein, 2004). The resulting trees were visualized in FigTree v.1.4.3 (Rambaut, 2012), while density trees were visualized in DensyTree v.2.2.6 (Bouckaert et al., 2014).

ML analyses were performed on the online platform *Cyberinfrastructure for Phylogenetic Research* (CIPRES; Miller et al., 2010) with the same partitions used in the BI analyses. The GTRCAT model of substitution was used, and 1000 non-parametric bootstrap pseudoreplicates (BS) were implemented to assess nodal support. Bootstrap support values greater than 70 were considered strongly supported (Hillis & Bull, 1993), and trees were visualized in FigTree v.1.4.3 (Rambaut, 2012).

## 2.5 Species Tree and Genetic Distances

A species tree was estimated for *C. ravus* lineages using the coalescent algorithm of \*BEAST implemented in BEAST v.2.5.0 (Bouckaert et al., 2014). Mitochondrial loci were treated separately

using the previously inferred models of substitution, allowing for the independent evolution of each gene. Two independent runs of 50 million generations were performed, sampling every 1000 generations. The Yule speciation tree model was used, in addition to a relaxed lognormal clock model (Drummond et al., 2005). Results were visualized in Tracer v.1.4 (Rambaut & Drummond, 2007) to assure ESS values above 200 for every prior. The two runs were combined using LogCombiner (Drummond & Rambaut, 2007), and the maximum clade credibility tree was obtained using TreeAnnotator v.2.5.0 (Drummond & Rambaut, 2007). DensiTree v. 2.2.6 (Bouckaert et al., 2014) was used to visualize the density tree, while the remaining trees were visualized in FigTree v.1.4.3 (Rambaut, 2012).

Genetic distances were estimated between lineages. The analysis was performed on the concatenated dataset (ND4 + 12S + L51), using the Kimura 2-parameter model (d: Transitions + Transversions) in MEGA v.7.0.26 (Kumar et al., 2016), which establishes a gamma variation in site distribution and a shape parameter of four, per the default settings.

## 2.6 Divergence Dating

To obtain estimates of divergence dating between major lineages, we produced a phylogeny in the program BEAST v.2.5.0 (Bouckaert et al., 2014). Given the low support values found for the relationships within *C. r. ravus* (and the large number of terminal taxa), only the most divergent taxa found in the concatenated analysis were used for the BEAST analysis. Too many terminal taxa were found to make convergence of the distinct chains difficult, as well as hinder the correct phylogenetic estimation, even when runs were extended to over 1 billion generations. A relaxed lognormal clock was used with a Yule tree prior. Substitution models were taken from the previous analyses and left unlinked. Two independent analyses ran for 50 million generations each, with a sample frequency of 10,000.

Calibrations for node ages were taken from previous studies based on the fossil record or from biogeographic events of divergence (Holman, 2000; Castoe et al., 2007; Parmley & Holman, 2007). Choice of priors in BEAST to calibrated was based on previous publications that carried out similar divergence dating (Bryson et al., 2011a; Reyes-Velasco et al., 2013; Schield et al., 2018). Tracer v.1.4 (Rambaut & Drummond, 2007) was used to visualize the results and to check for sufficiently high values of effective sample size (ESS) for the priors (>200).

Fossil calibrations included the oldest fossil record known for *Sistrurus* (Clarendonian, late Miocene, ~9 mya) (Parmley & Holman, 2007), used with a lognormal prior distribution with an offset of 8, mean of 0.01 and standard deviation of 0.76, resulting in a 95% confidence interval of 8.17–12.76

mya. Additionally, as the genus *Agkistrodon* was considered as an outgroup, the fossil *A. contortrix* (late Hemphillian, ~10 mya) (Holman, 2000) was also included. This fossil was used to calibrate the *A. contortrix*-*A. piscivorus* node, set with a lognormal prior distribution with an offset of 6.0, mean of 0.01 and standard deviation of 0.42, resulting in a 95% confidence interval of 6.09–10.7 mya. In order to establish a calibration within the genus *Crotalus*, the divergence between *C. atrox* and *C. ruber* (~3.2 mya) estimated by Castoe et al. (2007) was used. For this node, a normal prior distribution with an offset of 3.2, mean of 0.01 and a standard deviation of 1.0 was used, resulting in a 95% confidence interval of 3.1–4.7 mya.

Two independent analyses were run for 50 million generations each, sampling every 10,000 generations. LogCombiner (Drummond & Rambaut, 2007) and Tracer v.1.4 (Rambaut & Drummond, 2007) were used to combine the two runs and check for adequate sampling of the posterior probabilities (ESS>200). The maximum clade credibility tree was obtained using TreeAnnotator v.2.5.0 (Drummond & Rambaut, 2007) and visualized in FigTree v.1.4.3 (Rambaut, 2012).

## **2.7 Revision of Hemipenial Structure**

Hemipenial morphology was analyzed following the methods outlined by Myers & Cadle (2003) and Zaher & Prudente (2003) on specimens collected for this study, as well as previously preserved specimens (Appendix A). Terminology follows Dowling & Savage (1960) and Savage (1997), while choice of informative characters follows Jadin et al. (2010).

## **3. Results**

### **3.1 Sequence Production**

Sequences from 61 individuals from the three subspecies of *C. ravus* were obtained by a combination of sequences generated for this study and sequences available from GenBank. Genetic samples represent the majority of the lineages' known distributions, with seven haplotypes of *C. r. brunneus* and four haplotypes of *C. r. exiguus*. *Crotalus r. ravus* was represented by 48 haplotypes, fully covering the extremes of its known distribution (GenBank numbers: MN 527363-527408, MN 527037-527037 and MN539031-MN539078).

### **3.2 Phylogenetic Reconstruction**

The phylogenetic relationships obtained from the different analyses described above all showed similar topologies for the 60 sampled individuals. The monophyly of *Crotalus ravus sensu lato* was consistently recovered with respect to the chosen outgroups in both phylogenetic inference methods

(ML and BI), and the recovered clades correspond to the geographic ranges of the subspecies previously defined by morphological characters (Figure 2).

Both the ML and BI trees recovered a well-supported clade for *C. r. ravus*, with a distribution spanning the highlands of the TMVB in central Mexico. *Crotalus r. brunneus* was recovered as sister to *C. r. ravus*, with a distribution covering northern, central, and southeastern Oaxaca. *Crotalus r. exiguus* from central Guerrero was recovered as sister to (*C. r. ravus* + *C. r. brunneus*). The only topological difference between trees was with the nuclear dataset (L51), for which little structure was observed between lineages of *C. ravus* (Appendix B, Fig. B.3).

### 3.3 Haplotype Networks

The haplotype networks from the mitochondrial loci reflect the results of the phylogenetic analyses (Figure 3). The three subspecies show distinct haplotypes that are separated by a large number of mutational events. *Crotalus r. ravus* from the highlands of central Mexico shows the highest diversity of haplotypes, while *C. r. brunneus* is shown to be related to *C. r. ravus*, although with a considerable amount of unsampled haplotypes separating the two. Lastly, the haplotypes corresponding to *C. r. exiguus* seem to be the most isolated, as they do not share any connections with the haplotypes of the other two subspecies.

### 3.4 Species Tree and Genetic Distances

The species tree recovered the same relationships obtained by the gene tree analyses. *Crotalus r. exiguus* was recovered as the earliest divergent lineage within the species complex with high support (>0.9). Within the (*C. r. ravus* + *C. r. brunneus*) clade, support values were slightly lower (0.9), perhaps due to the large genetic diversity found within *C. r. ravus*.

In concordance with the species tree, the genetic distances between different *C. ravus* lineages were found to be greater than 3%, which can be considered high according to Knight et al. (1993). The most isolated lineage was *C. r. exiguus*, showing between 4–5% divergence from the other two lineages. *Crotalus ravus brunneus* was found to have 5.4% divergence with respect to *C. r. exiguus*, and 3.4% divergence from *C. r. ravus* (Table 4).

### 3.5 Divergence Date Estimation

The results of the divergence dating analysis estimate the diversification of the subspecies of *C. ravus* to be in the late Miocene (Figure 4). The first lineage to diverge is *C. r. exiguus* from (*C. r. ravus* + *C. r. brunneus*) approximately 7 mya (11.82–3.5 mya, 95% HPD), while the split between *C. r. ravus*

and *C. r. brunneus* took place at the end of the Miocene, approximately 5 mya (9.49–2.1 mya, 95% HPD).

### 3.6 Hemipenial Structure

Five hemipenes from *C. r. ravus*, three from *C. r. brunneus*, and three from *C. r. exiguus* were examined. As in most viperids, the hemipenis of *C. r. ravus* is bifurcated, uncapitated, and with the sulcus spermaticus divided below the spinose region of the hemipenial body. Characteristics of each of the three lineages are further explained below and illustrated in Figure 5.

#### *Crotalus ravus brunneus*

The length of the retracted hemipenis (MZFC 23873) extends *in situ* to the tenth subcaudal; the retractor muscle inserts between subcaudal 27–28; the two branches of the sulcus spermaticus extend to the most distal region of each lobe; the sulcate wall has 27–36 spinules and hooks of mostly subequal size; smaller spines are found near the branches of the sulcus spermaticus, while larger hooks are found near the calyculate region; the lobes are cylindrical in shape with rounded tips; the ornamentation of each lobe consists of 25–29 rows of spinnulate calyces that become larger and more scarce towards the tip of the lobe on the asulcate side.

#### *Crotalus ravus exiguus*

The *in situ* length of the retracted hemipenis (MZFC 2893) could not be measured given that the organ had been previously everted upon fixation. The retractor muscle inserts between subcaudal 26–27; the branches of the sulcus spermaticus reach the tip of each lobe; the sulcus walls are smooth and unfolded; the sulcate face has 49 spines that get smaller in size towards the base of the sulcus spermaticus; parallel rows of enlarged spines border the branches of the sulcus spermaticus; the largest hooks are found in the center of the spinose region, approximately halfway between the base and the calyculate region; the lobes are nearly cylindrical with rounded tips; the ornamentation of the lobes consists of 19–21 rows of spinnulate calyces arranged in folds on the asulcate face. It is worth noting that the spines of the calyces in this lineage are more spread out from each other, resulting in a fewer number of total spines than observed in the other lineages.

#### *Crotalus ravus ravus*

The *in situ* length of the retracted hemipenis (MZFC 34499) is between 8–14 subcaudals; the retractor muscle inserts between subcaudal 24–26; the branches of the sulcus spermaticus reach the tip



of the lobes, where small calyces form a border; the distal side of the sulcus is covered in folds, while the proximal side is smooth; the sulcate wall has 44 spines and hooks, with the spines being smaller near the base and concentrated on the undivided region of the sulcus; parallel rows of spines border the sulcus branches; the largest hooks are located near the calyculate region; the lobes are cylindrical, decreasing in width as they approach the rounded tips; the ornamentation of the lobes consists of 25–27 rows of profusely spinnulate calyces on the asulcate face.

#### 4. Discussion

##### The TMVB's Influence on the Historical Biogeography of *Crotalus ravus*

The formation of the TMVB has been recognized as one of the principal orographic events in the creation of the MTZ and has had a significant influence on the distribution of most central and southern Mexican taxa (Míguez-Gutiérrez et al., 2013). The *C. ravus* complex is no exception, as the divergence dates estimated in this study (Figure 4) suggest that the primary influential factor on the current distribution of these three rattlesnake lineages was the formation of this volcanic range. According to Gómez-Tuena et al. (2005), the formation of the TMVB can be divided into four main episodes of orogenic activity during the mid and late Miocene. The diversification of these focal rattlesnake lineages corresponds to the third of these episodes, between ~7.5–3 mya. The uplift of the TMVB also led to the formation of low-altitude regions such as the Balsas River basin and the Papaloapan River basin, which probably have served as barriers to these rattlesnake populations (Campbell & Armstrong, 1979; Bryson et al., 2011a), effectively isolating them by limiting gene flow. Using mitochondrial data, Bryson et al. (2011a) proposed that climatic fluctuations during the Pleistocene were most influential in the diversification of montane rattlesnakes, while Blair et al. (2016) suggested that the orogenic events of the Miocene were responsible for the diversification of the group, based on both mitochondrial and nuclear data. The results of this study support the diversification of *C. ravus* during Miocene orogenic episodes. Recently, Blair et al. (2018) found support for Pliocene orogenic activity as primary factor influencing the diversification of *C. ravus* using genomic ultraconserved elements (UCE's). This conclusion is partially corroborated by the present study, as the estimated divergences were found to be associated with late Miocene for the first split of *C. r. exiguus* from (*C. r. ravus* + *C. r. brunneus*) (~7.34 mya), and the early Pliocene for the split between *C. r. ravus* and *C. r. brunneus* (~5.23 mya) (Figure 4). The discrepancies in historical biogeographic hypotheses for this group reflect the complex relationship between the evolutionary history of this region's biota and the geography in which it is evolving (Flores-Villela & Goyenechea, 2001; Flores-Villela & Martínez-Salazar, 2009). Additionally, the differences in resolution of different

hypotheses reflect the importance of considering distinct sources of variation in an integrative taxonomic view, especially when considering taxa distributed in topographically complex regions such as the MTZ.

### **Taxonomic Implications**

The phylogenetic hypotheses obtained in this study reflect the geographic distribution of the *C. rarus* complex. The concatenated ML and BI gene trees recovered the monophyly of the three lineages within *C. rarus* with high support values (Figure 2). Additionally, the \*BEAST species tree corroborated these relationships with PP values greater than 0.9. All analyses support the differentiation of the three lineages within *C. rarus*. The data suggests that *C. r. exiguus* was the earliest lineage to diverge from other populations. This corroborates the results from Bryson et al. (2011a) that used mitochondrial data, yet is inconsistent with the UCE results presented by Blair et al. (2018). These discrepancies may be attributed to the different sources of genetic data analyzed, the different phylogenetic reconstruction methods utilized, and the overall complexity of the TMVB's geological history.

The haplotype networks (Figure 3) show that *C. r. brunneus* and *C. r. rarus* have maintained genetic connectivity in the two genes examined (ND4 and 12S) although several “lost” haplotypes separate them. *Crotalus r. exiguus* retains the most private haplotypes of the three.

These haplotypic relationships are in concordance with the results of the divergence dating estimations and the phylogenetic reconstructions (Figures 2 and 4), in which *C. r. exiguus* was the first lineage to diverge in the complex. The split of this central Guerrero lineage corresponds with the formation of the Balsas Basin, which has been previously proposed as a potential barrier to gene flow in this group (Bryson et al., 2011a), caused by episodes of high volcanic activity and uplift of the TMVB (Gómez-Tuena et al., 2007).

Additionally, the genetic distances found between the lineages represent significant divergences, according the Kinght et al. (1993) who found divergence values of at least 2% between different species in the genus *Crotalus*. Within *C. rarus sensu lato*, the highest genetic distances were found separating *C. r. exiguus* from *C. r. brunneus* with 5.4% and from *C. r. rarus* with 4.0%; while *C. r. brunneus* were separated by 3.4% divergence with *C. r. rarus* (Table 4), reflecting their more recent split.

All three lineages showed a noticeable change in hemipenial ornamentation, which transitioned from spines on the body of the structure to folds on the distal region of each lobe as proposed by McCranie in 1988 (Figure 5). This condition is considered one of the principle characters

distinguishing *Crotalus* from *Sistrurus* (Gloyd, 1940; Klauber, 1972), thus supporting with morphological evidence this rattlesnake clade's phylogenetic position within *Crotalus*, despite their shared characteristic with *Sistrurus* of large symmetric plates on top of the head. Within the species complex, distinguishing characters were recognized for the three distinct lineages, included the position of the largest hooks, the size and position of the spines that border the branches of the sulcus spermaticus, and the position of the spinules on the calyces. Genitalia characters have been shown to be under rapid evolution in other Squamate lineages (Klackzco et al., 2014), as the structural differences may impede gene flow between nearby populations to avoid hybridization, eventually giving way to cladogenesis processes (Arnqvist, 1997). Other hypotheses such as sexual selection or pleiotropy have been considered as influential in the diversification of genital structures in other internally fertilizing organisms, however these have not been explored in detail in snakes. In the case of the *C. ravus* complex, we consider an isolation by vicariant process to best explain the morphological diversity of hemipenial structures, leading to the differences in observed genitalia morphotypes that result from limited gene flow between lineages, which is also shown in the phylogeographic and phylogenetic structure of the group.

The utility of morphological characters such as genitalia structure in identifying cryptic species is well-known. Campbell & Armstrong (1979) first recognized the distinctiveness of *C. r. exiguus* based on morphology and designated it as a distinct taxonomy entity at the subspecific level, distinguishable from the other *C. ravus sensu lato* populations. We suggest that future taxonomic studies on montane rattlesnake (or any other snakes) should examine morphological characters such as hemipenial structures in addition to molecular data, given that these structures may shed light on taxonomic distinctiveness as they are under heavy sexual selection.

Integrative taxonomy is a very useful tool for understanding the evolutionary histories of closely related taxa, especially in regions with a complex geological history. Considering distinct sources of variation using different methods allows a clearer understanding of the diversity present in these species complexes (Dayrat, 2005; Padial, 2010; Yeates et al., 2011). In the case of *C. ravus* whose evolutionary history is linked tightly with the complex geological history of the TMVB and the SMS, an integrative approach within this specie is necessary to reveal the evolutionary processes that have influenced the diversification of different lineages. The disjunct distributions of this species promoted by a vicariant process have facilitated the allopatric speciation, the results of this study support this hypothesis.

Considering the entirety of the available data from molecular (Bryson et al., 2011a; Blair et al., 2016 and 2018; this study) and morphological (Campbell & Armstrong, 1979; this study) sources, we

conclude that these three lineages represent independent evolutionary histories, and, therefore propose their recognition as distinct species (de Queiroz, 1999, 2007). Given that recent work using niche modeling in rattlesnakes has been shown to have limited use (Meik et al, 2015), we recommend the exploration of population dynamics models that may identify possible introgression events between the three lineages. Additionally, identifying how population sizes have interacted and fluctuated through time may shed light on their evolutionary pasts.

### **Conservation aspects**

With the proposals to modify the taxonomy within the *Crotalus ravus* complex, a reevaluation of the conservation status of each putative species becomes necessary. The habitat fragmentation may result in the decrease of genetic diversity (Roques et al., 2014; Rueda-Zozaya et al., 2016), thus increasing the risk of being subject to phenomena such as local extinction (Newman & Tallmon, 2001). In addition to the above, low vagility, high philopatry and relatively small home ranges (Böhm et al., 2013) promote that organisms do not move far from their original distribution areas in search of new habitats.

The case of these three species is an example of the aforementioned. While all three taxa have been collected in relative disturbed habitats (our field experience), owing to the possible availability of food or shelter (Bastos et al., 2006; Cortés-Ávila & Toledo, 2013). The advance of deforestation and the lack of more protected areas within their distribution, may in the future promote that populations of these rattlesnakes disappear completely or are reduced to marginal. Such is the case of *C. exiguus*, which initially was under protection when Omiltemi (Guerrero) had the status of State Park, which recently was revoked as a protected area. In the case of *C. brunneus*, 5.9% of its distribution is included in a protected area. While *C. ravus* has the greatest portion of its range within a protected area (19.6%), but also is the taxon with the largest geographic range.

Finally, we think that the development of phylogeographic studies should go hand in hand with analysis of species conservation, especially in areas of high herpetological diversity such as central and southern Mexico (Flores-Villela & Canseco-Márquez, 2004); this may warrant preservation of species over time. These studies should connect with public policies that efficiently establish conservation zones for species susceptible to negative anthropogenic activities, such as rattlesnakes are.

**Acknowledgments.**

We would like to thank the following people: the two anonymous reviewers who provided feedback on the manuscript; the members of the Fujita laboratory at UTA, Texas, for their advice and guidance in the sequencing work, especially T. J. Firneno; M. in C. Fabiola Ramírez Corona of the Laboratorio del Taller de Sistemática y Biogeografía, UNAM, for the PCR reactions and extraction of DNA; M. in F. P. Ana Isabel Bieler of the Laboratorio de Microcine, UNAM, for the high-quality photos of the hemipenis; and M. in B. María Eugenia Muñoz Díaz de León of the Taller de Biología de Plants I y II, UNAM, for assistance in quantifying DNARuDegI. Additionally, we thank all the members of the Herpetology Laboratory in the Faculty of Sciences and the Laboratory of Evolutionary Ecology of Amphibians and Reptiles at Iztacala FES for their support in collecting and analyzing the samples and data for this project. Finally, we would like to thank the Masters in Biological Sciences graduate program at UNAM, as well as CONACYT (project # 669772) and PAPIIT (project # IN216218) for funding.

## References

- Alencar L. R. V., Quental, T. B., Grazziotin, F. G., Alfaro, M. L., Martins, M., Venzon, M., Zaher, H., 2016. Diversification in vipers: Phylogenetic relationships, time of divergence and shifts in speciation rates. *Mol. Phylogenet. Evol.* 105, 50-62. <https://doi.org/10.1016/j.ympev.2016.07.029>
- Arnqvist, G., 1997. The evolution of Animalia genitalia: distinguishing between hypotheses by species studies. *Biol. J. Linn. Soc.* 60, 365-379. <https://doi.org/10.1006/bijl.1996.0109>
- Bastos, E. G. de M., de Araújo, A. F. B., da Silva, H. R. 2005. Records of rattlesnakes *Crotalus durissus terrificus* (Laurenti) (Serpentes, Viperidae) in the state of Rio de Janeiro, Brazil: a possible case of invasion facilitated by deforestation. *Rev. Bras. Zool.* 22, 812-815. <http://dx.doi.org/10.1590/S0101-81752005000300047>.
- Blair, C., Sánchez-Ramírez S., 2016. Diversity-dependent cladogenesis throughout western Mexico: evolutionary biogeography of rattlesnakes (Viperidae: Crotalinae: *Crotalus* and *Sistrurus*). *Mol. Phylogenet. Evol.* 97, 145–154. <https://doi.org/10.1016/j.ympev.2015.12.020>
- Blair, C., Bryson, R. W., Linkem, C. W., Lazcano, D., Klicka, J., McCormack, J. E., 2018. Cryptic diversity in the Mexican highlands: thousands of UCE loci help illuminate phylogenetic relationships, species limits and divergence times of montane rattlesnakes (Viperidae: *Crotalus*). *Mol. Ecol. Resour.* 2018, 1-17. <https://doi.org/10.1111/1755-0998.12970>.
- Böhm, M. et al. 2013. The conservation status of the world's reptiles. *Biol. Conserv.* 157, 372-385. <http://dx.doi.org/10.1016/j.biocon.2012.07.015>.
- Boulenger, G. A., 1896. Descriptions of new batrachians in the British Museum. *Ann. mag. nat. his.*, 17, 401–406.
- Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C-H., Xie, D., Suchard, MA., Rambaut, A., & Drummond, A. J., 2014. BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. *PLoS Comput. Biol.* 10, 1-6. <https://doi.org/10.1371/journal.pcbi.1003537>
- Bryson, R.W., Murphy, R.W., Lathrop, A., Lazcano-Villareal, D., 2011a. Evolutionary drivers of phylogeographical diversity in the highlands of Mexico: a case study of the *Crotalus triseriatus* species group of montane rattlesnakes. *J. Biogeogr.* 38, 697–710. <https://doi.org/10.1111/j.1365-2699.2010.02431.x>

- Bryson, R.W., Murphy, R.W., Graham, M.R., Lathrop, A., Lazcano, D., 2011b. Ephemeral Pleistocene woodlands connect the dots for highland rattlesnakes of the *Crotalus intermedius* group. *J. Biogeogr.* 38, 2299–2310. <https://doi.org/10.1111/j.1365-2699.2011.02565.x>
- Bryson, R. W., Linkem, C. W., Dorcas, M. E., Lathrop, A., Jones, J. M., Alvarado-Díaz, J., Gründwald, C. I., Murphy R. W., 2014. Multilocus species delimitation in the *Crotalus triseriatus* species group (Serpentes: Viperidae: Crotalinae), with the description of two new species. *Zootaxa.* 3826, 475-496. <https://doi.org/10.11646/zootaxa.3826.3.3>.
- Campbell, J.A., Armstrong, B.L., 1979. Geographic variation in the Mexican pygmy rattlesnake, *Sistrurus ravus*, with the description of a new subspecies. *Herpetologica*, 35, 304–317. <https://www.jstor.org/stable/3891962>
- Campbell, J.A., Lamar, W.W., 2004. *The Venomous Reptiles of the Western Hemisphere*. Cornell University Press, Ithaca, New York.
- Castoe, T.A., Spencer, C.L., Parkinson, C.L., 2007. Phylogeographic structure and historical demography of the western diamondback rattlesnake (*Crotalus atrox*): a perspective on North American desert biogeography. *Mol. Phylogenet. Evol.* 42, 193–212. <https://doi.org/10.1016/j.ympev.2006.07.002>
- Clement, M., Posada, D., Crandall, K., 2000. TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* 9, 1657-1660. <https://doi.org/10.1046/j.1365-294x.2000.01020.x>
- Cope, E. D. 1865. Third Contribution to the herpetology of tropical America. *Proc. Acad. Nat. Sci. Philadelphia*, 17, 185-198.
- Cortes-Ávila, L., Toledo, J. J. 2013. Study of diversity of snakes in perturbed forest and pasture areas in San Vicente del Caguán (Caquetá), Colombia. *Actual. Biol.* 35, 185-197.
- Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2015. JModelTest 2: more models, new heuristics and high-performance computing. *Nat. Methods.* 9, 6–9. <https://doi.org/10.1038/nmeth.2109>
- Davis, W. B., Smith, H. M. 1953. Snakes of the Mexican state of Morelos. *Herpetologica* 8, 133-149.
- Dayrat, B., 2005. Towards integrative taxonomy. *Biol. J. Linn. Soc.* 83, 407-415. <https://doi.org/10.1111/j.1095-8312.2005.00503.x>

- de Queiroz, K., 1999. The general lineage concept of species and the defining properties of the species category. In Wilson, R. A. (Ed.), *Species: New interdisciplinary essays*, MIT Press, Cambridge, Massachusetts. pp. 49–89.
- de Queiroz, K., 2007. Species concepts and species delimitation. *Syst. Biol.* 56, 879–886.  
<http://dx.doi.org/10.1080/10635150701701083>.
- Dowling, H. G., Savage, J. M., 1960. A guide to snakes hemipenis: a survey of basic structure and systematic characteristics. *Zool.* 45, 17-28. <http://biostor.org/reference/194094>
- Drummond, A.J., Rambaut, A., Shapiro, B., Pybus, O.G., 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. *Mol. Biol. Evol.* 22, 1185–1192.  
<https://doi.org/10.1093/molbev/msi103>
- Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol. Biol.* 7, 214. <https://doi.org/10.1186/1471-2148-7-214>
- Felsenstein, J., 2004. *Inferring phylogenies*. Sinauer Associates, Sunderland, Massachusetts.
- Ferrusquía-Villafranca, I., González-Guzmán, L.L., 2005. Northern Mexico's landscape, Part II: The biotic setting across time. In: Cartron, J.-L.E., Ceballos, G., Felger, R.S. (Eds.), *Biodiversity, Ecosystems, and Conservation in Northern Mexico*. Oxford University Press, Oxford, NY, pp. 39–51.
- Flores-Villela, O., P. Gerez., 1994. *Biodiversidad y Conservación en México: Vertebrados, Vegetación y Uso del Suelo*. CONABIO/UNAM. México.
- Flores-Villela, O., Goyenechea, I., 2001. A comparison of hypotheses of historical area relationship for Mexico and Central America, or in search for the lost pattern. pp. 171-181 In: Johnson, J., Webb, R. G., Flores-Villela, O., (Eds.). *Mesoamerican Herpetology: systematics, zoogeography and conservation*. Centennial Museum, Special Publication, University of Texas, El Paso, Texas, USA. 1, 1-200.
- Flores-Villela, O. A., Canseco-Márquez, L. 2004. Nuevas especies y cambios taxonómicos para la herpetofauna de México. *Acta. Zool. Mex. Nueva.* 20, 115–144.
- Flores-Villela, O. A., Martínez-Salazar, E. A., 2009. Historical explanation of the origin of the herpetofauna of Mexico. *Rev. Mex. Biodivers.* 80, 817-833.



- Gibbs, H. L., Díaz, J. 2010. Identification of single copy nuclear copy DNA markers for north American pit vipers. *Molecular Ecology Resources*, 10, 177-180. doi: 10.1111/j.1755-0998.2009.02707.x
- Gloyd, H.K., 1940. *The Rattlesnakes, Genera Sistrurus and Crotalus*. Special Publications of Chicago Academy of Science, Illinois.
- Gómez-Tuena, A., Orozco-Esquivel, M. T., Ferrari, L., 2005. Petrogénesis ígnea de la Faja Volcánica Transmexicana. *Bol. Soc. Geol. Mex.* 3, 227-283.
- Halffter, G., 1987. Biogeography of the montane entomofauna of Mexico and Central America. *Ann. Rev. Entomol.* 32, 95–114. <https://doi.org/10.1146/annurev.en.32.010187.000523>
- Harris, H. S., Simmons, R. S. 1978. A preliminary account of the rattlesnakes with the descriptions of four new subspecies. *Bull. Maryland Herp. Soc.* 14, 105-211.
- Hillis, D.M., Bull, J.J., 1993. An empirical-test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* 42, 182–192. <https://doi.org/10.1093/sysbio/42.2.182>
- Holman, J.A., 2000. *Fossil snakes of North America: Origin, Evolution, Distribution, Paleoecology*. Indiana University Press, Indiana.
- Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics.* 17, 754–755. <https://doi.org/10.1093/bioinformatics/17.8.754>
- Jadin, R. C., Gutberlet R. L. y Smith, E. N., 2010. Phylogeny, evolutionary morphology, and hemipenis descriptions of the Middle American jumping pitvipers (Serpentes: Crotalinae: *Atropoides*) *J. Zool. Syst. Evol. Res.* 48, 360-365. <https://doi.org/10.1111/j.1439-0469.2009.00559.x>
- Klackzco, J., Ingram, T., Losos, J. B., 2014. Genitals evolve faster other traits in *Anolis* lizards. *J. Zool.* 295, 44-48. <https://doi.org/10.1111/jzo.12178>
- Klauber, L.M., 1972. *Rattlesnakes, Their Habits, Life Histories, and Influence on Mankind*. University of California Press, Berkeley.

- Knight, A., Styler, D., Pelikan, S., Campbell, J. A., Densmore III, L. D., Mindell, D. P., 1993. Choosing among hypotheses of rattlesnake phylogeny: a best-fit rate test for DNA sequence data. *Syst. Biol.* 42, 356-367. <https://doi.org/10.1093/sysbio/42.3.356>
- Kumar, S., Stecher, G., Tamura K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 or bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. <https://doi.org/10.1093/molbev/msw054>
- Maddison, W. P., Maddison D.R., 2018. Mesquite: a modular system for evolutionary analysis. Version 3.51 <http://www.mesquiteproject.org>
- McCranie, J. R., 1988. Description of the hemipenis of *Sistrurus ravus* (Serpentes: Viperidae). *Herpetologica*, 44, 123-126. <http://www.jstor.org/stable/3892207>.
- Meik, J. M., Streicher, J. W., Lawing, A. M., Flores-Villela, O. A., Fujita, M. K., 2015. Limitations of Climatic Data for Inferring Species Boundaries: Insights from Speckled Rattlesnakes. *PLoS One.* 8, 928-934. <https://doi.org/10.1371/journal.pone.0131435>
- Miguez-Gutiérrez, A., Castillo, J., Márquez, J., Goyenechea, I., 2013. Biogeografía de la Zona de Transición Mexicana con base en un análisis de árboles reconciliados. *Rev. Mex. Biodivers.* 84, 215-224. <http://dx.doi.org/10.7550/rmb.32119>
- Miller, M. A., W. Pfeiffer, T. Schwartz., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE) 14 Octubre 2018, 1–8.
- Monroy-Vilchis O., Cabrera, L., Suárez, P., Zarco-González, M., Rodríguez-Soto, C., Urios, V. 2008. Uso tradicional de vertebrados silvestres en la Sierra Nanchititla, México. *Interciencia*, 33, 8–313 <http://www.redalyc.org/articulo.oa?id=3393341>
- Morrone, J.J., 2009. *Evolutionary biogeography: An integrative approach with case studies*. Columbia University Press, New York.
- Morrone, J.J., 2010. Fundamental biogeographic patterns across the Mexican Transition Zone: an evolutionary approach. *Ecography.* 33, 355–361. <https://doi.org/10.1111/j.1600-0587.2010.06266.x>
- Morrone, J.J., 2017. *Neotropical Biogeography. Regionalization and Evolution*. Taylor and Francis Group, Boca Ratón, Florida.

- Murphy, R. W., Fu, J., Lathrop, A., Feltham, J. V., Kovac, V. 2002. Phylogeny of the rattlesnakes (*Crotalus* and *Sistrurus*) inferred from sequences of five mitochondrial DNA genes. Pp. 69-92. *In*: Schuett, G. W., Höggren, M., Douglas, M. E., Greene, H. W., (Eds.). *Biology of vipers*, Eagle Mountain Publishing, Eagle Mountain, Utah. 580 pp.
- Myers, C., Cadle, J. E., 2003. On the snake hemipenis, with notes on *Psomophis* and techniques of eversion: a response to Dowling. *Herpetol. Rev.* 34, 295-302.
- Newman, D., Tallmon, D. A. 2001. Experimental evidence for beneficial fitness effects of gene flow in recently isolated populations. *Conserv. Biol.* 15, 1054-1063.
- Padial, J., Miralles, A., De la Riva, I., Vences, M., 2010. The integrative future of Taxonomy. *Front. Zool.* 7, 2-14. <https://doi.org/10.1186/1742-9994-7-16>
- Parmley, D., Holman, J.A., 2007. Earliest fossil record of a pigmy rattlesnake (Viperidae: *Sistrurus* Garman). *J. Herpetol.* 41, 141–144. <https://www.jstor.org/stable/4498562>
- Peterson, A., Flores-Villela, O.A., León-Paniagua, L., Llorente-Bousquets, J.E., Luis-Martinez, M.A., Navarro-Sigüenza, A., Torres-Chávez, M., Vargas-Fernandez, I., 1993. Conservation priorities in Mexico: moving up in the world. *Biodivers. Lett.* 1, 33–38. <http://dx.doi.org/10.2307/2999648>.
- Pfenninger, M., Posada, D., 2002. Phylogeographic history of the land snail *Candidula unifasciata* (Helicellinae, Stylommatophora): fragmentation, corridor migration, and secondary contact. *Evolution.* 56, 1776–1788. <https://doi.org/10.1111/j.0014-3820.2002.tb00191.x>
- Ramamoorthy, T.P., Bye, R., Lot, A., Fa, J., 1993. *Biological Diversity of Mexico: Origins and Distribution*. Oxford University Press, New York.
- Rambaut, A., Drummond A., 2007. Tracer v1. 4.
- Rambaut, A., 2012. FigTree v1.1.2. Available via <http://tree.bio.ed.ac.uk/software/figtree/>. Accessed August 19, 2018.
- Reyes-Velasco, J., Meik J. M., Smith E. N., Castoe T. A., 2013. Phylogenetic relationships of the enigmatic longtailed rattlesnakes (*Crotalus ericsmithi*, *C. lannomi*, and *C. stejnegeri*). *Mol. Phylogenet. Evol.* 69, 524–534. <https://doi.org/10.1016/j.ympev.2013.07.025>

- Roques, S., Furtado, M., Jácomo, A. T. A., Silveira, L., Sollmann, R., Torres, N. M., Godoy, J. A., Palomares, F. 2014. Monitoring jaguar populations *Panthera onca* with non-invasive genetics: a pilot study in Brazilian ecosystems. *Oryx*. 1-9. <https://doi.org/10.1017/S0030605312001640>.
- Rueda-Zozaya, P., Mendoza-Martínez, G. D., Martínez-Gómez, D., Monroy-Vilchis, O., Godoy, J. A., Sunny, A., Palomares, F., Chávez, C., Herrera-Haro, J. 2016. Genetic variability and structure of jaguar (*Panthera onca*) in Mexican zoos. *Genetica*, 144, 59-69. <https://doi.org/10.1007/s10709-015-9878-6>
- Sarukhán J., Koleff, P., Carabias, J., Soberón, J., Dirzo, R., Llorente-Bousquets, J. *et al.* 2009. Capital Natural de México. Síntesis Conocimiento actual, evaluación y perspectivas de la sustentabilidad. Comisión Nacional para el Uso y Conocimiento de la Biodiversidad. México. [https://www.biodiversidad.gob.mx/pais/pdf/CapNatMex/Capital%20Natural%20de%20Mexico\\_Sintesis.pdf](https://www.biodiversidad.gob.mx/pais/pdf/CapNatMex/Capital%20Natural%20de%20Mexico_Sintesis.pdf) (Accessed 17 de Agosto de 2019).
- Savage, J. M., 1997. On terminology for the description of the hemipenes of Squamata reptiles. *Herpetol. J.* 7, 23-25.
- Schild, R. D., Adams, R. H., Card, D. C., Corbin, A. B., Jezkova, T., Hales, N. R., Meik, J. M., Perry, B. W., Spencer, C. L., Smith L. L., García, G., Bouzid, N. M., Strickland, J. L., Parkinson, C. L., Borja, M., Castañeda-Gaytán, G., Bryson, R. W., Flores-Villela, O. A., Mackessy, S. P., Castoe, T. A., 2018. Cryptic genetic diversity, population structure, and gene flow in the Mojave rattlesnake (*Crotalus scutulatus*). *Mol. Phylogenet. Evol.* 127, 669-681. <https://doi.org/10.1016/j.ympev.2018.06.013>.
- Uetz, P., Freed, P., Hošek, J., (eds.). 2019. The Reptile Database, <http://www.reptile-database.org>, (Accessed 10 de Febrero de 2019).
- Wallach, V., Williams K. L., Boundy, J. 2014. Snakes of the World: A Catalogue of Living and Extinct Species. Taylor and Francis, CRC Press, 1237 pp.
- Yeates, D., Seago, A., Nelson, L., Caameron, S., Joseph, L., Trueman, J., 2011. Integrative taxonomy or Iterative taxonomy? *Syst. Entomol.* 36, 209-217. <https://doi.org/10.1111/j.1365-3113.2010.00558.x>
- Zaher, H., Prudente, A. L. C., 2003. Hemipenes of *Siphlophis* (Serpentes, Xenodontinae) and techniques of hemipenial preparation in snakes: A response to Dowling. *Herpetol. Rev.* 34, 302-307.

**Figure 1.** Map displaying the present-day distribution of *Crotalus ravus* (per Campbell & Lamar, 2004) and the representative samples obtained for this study. Blue dots represent *C. r. ravus*, green dots represent *C. r. brunneus*, and orange dots represent *C. r. exiguus*.

**Figure 2.** Phylogenetic trees of the *Crotalus ravus* complex using the concatenated dataset of the three loci examined in this study (ND4, 12S, and L51). A) Density tree from the Bayesian analysis; B) The phylogenetic relationships obtained from the maximum likelihood and Bayesian analyses, which recovered the same topology; C) Species tree obtained from \*BEAST. Numbers above branches represent posterior probability support values, while numbers below branches reflect bootstrap support values. Asterisks denote significant support values (>0.95 PP; >75 BS).

**Figure 3.** Haplotype networks of the mitochondrial ND4 (A) and 12S (B) loci, showing a correlation between haplotypes and geographic distribution of distinct rattlesnake lineages.

**Figure 4.** Time-calibrated relaxed clock tree of *Crotalus ravus sensu lato*. Black arrows denote calibrations used in the analysis, and values above nodes represent the 95% confidence interval of estimated node age in millions of years.

**Figure 5.** Hemipenial structures of the three lineages comprising the *C. ravus* complex. Panel A shows the sulcate and asulcate parts of the hemipenial structures characteristic of the *Crotalus ravus brunneus* (MZFC 23873) lineage from southwestern Oaxaca; Panel B displays both sides of the hemipenis characteristic of the *Crotalus ravus exiguus* lineage (MZFC 2893); and panel C shows both sides of the hemipenis characteristic of the *Crotalus ravus ravus* lineage (MZFC 34499) from the eastern part of this taxon's range in central Mexico.

Tables.

**Table 1.** References of works carried out in the ZTM where the estimated geological time and the possible speciation events giving rise to the divergence of the taxa studied.

Author	Taxa	Geological time	Possible speciation event
<b>Anphibians</b>			
Mulcahy et al., 2006	<i>Bufo</i> (now <i>Rhinella</i> ) <i>marina</i> , <i>Ollotis</i> (now <i>Incillius</i> ) <i>valliceps</i> and <i>nebulifer</i>	Pliocene and Pleistocene	Dispersal and Vicariance
Bryson et al., 2010	<i>Hyla arenicolor</i>	Miocene-Pliocene	Dispersal and Vicariance
Rovito & Parra-Olea, 2016	Neotropic salamanders	Pliocene and Pleistocene	Vicariance
Bryson et al., 2018	<i>Isthmura belli</i>	Miocene	Dispersal
<b>Reptiles</b>			
Daza et al., 2009	<i>Leptodeira</i>	Miocene	Vicariance
Castoe et al., 2009	<i>Atropoides</i> , <i>Cerrophidion</i> and <i>Botriechis</i>	Miocene-Pliocene and Pleistocene	Dispersal and Vicariance
Bryson et al., 2011	<i>Crotalus triseriatus</i> (group)	Miocene-Pliocene and Pleistocene	Dispersal and Vicariance
Bryson et al., 2011	<i>Pituophis catenifer</i> , <i>Pituophis deppei</i> y <i>Pituophis lineaticollis</i>	Miocene-Pliocene and Pleistocene	Dispersal and Vicariance
Bryson et al., 2011	<i>Crotalus intermeidus</i>	Pleistocene	Dispersal and Vicariance
Bryson et al., 2012	<i>Sceloporus scalaris</i> (group)	Miocene-Pliocene	Vicariance
Blair et al., 2016	<i>Crotalus</i> y <i>Sistrurus</i>	Miocene	Vicariance
García-Vázquez et al., 2018	<i>Gherronotus</i>	Miocene-Pliocene	Vicariance
<b>Birds</b>			
Rodríguez-Gómez & Ornelas, 2015	<i>Amazilia violiceps</i> and <i>Amazilia viridifrons</i>	Pleistocene	Vicariance
Hernández-Soto et al., 2018	<i>Colibri thalassinus</i>	Pleistocene	Dispersal and Vicariance
Venkatraman et al., 2018	<i>Aphelocoma unicolor</i>	Pliocene and Pleistocene	Dispersal and Vicariance

**Mammals**

Edwards y Bradley, 2002	<i>Neotoma mexicana</i> (group)	Plioceno and Pleistocene	Dispersal and Vicariance
León-Paniagua et al., 2007	<i>Habromys</i>	Pleistocene	Dispersion
Hernández-Canchola & León-Paniagua, 2017	<i>Sturnira parvidens</i>	Pleistocene	Dispersal and Vicariance
Ruiz-Vega et al., 2018	<i>Osgoodomys banderanus</i>	Pliocene	Dispersal and Vicariance

**Table 2.** Morphological characteristics that distinguish the *Crotalus ravus* subspecies proposed by Campbell and Armstrong (1979). Taken and modified from Campbell & Lamar (2004).

Subspecies	Parietal condition	Dorsal scale rows at midbody	Prefoveals	Rattle size (dorsoventral width of proximal rattle segment of tail length)		Number of Tail Bands	
<i>Crotalus ravus brunneus</i>	Usually undivided	23	3-6	Large Males <10%	Females >13%	Males 5-8	Females 4-6
<i>Crotalus ravus exiguus</i>	Usually divided transversely	23	6 or more	Small Males <10%	Females <13%	Males 3-6	Females 2-4
<i>Crotalus ravus ravus</i>	Highly variable	21	< 3	Small Males <10%	Females <13%	2-4 both sexes	

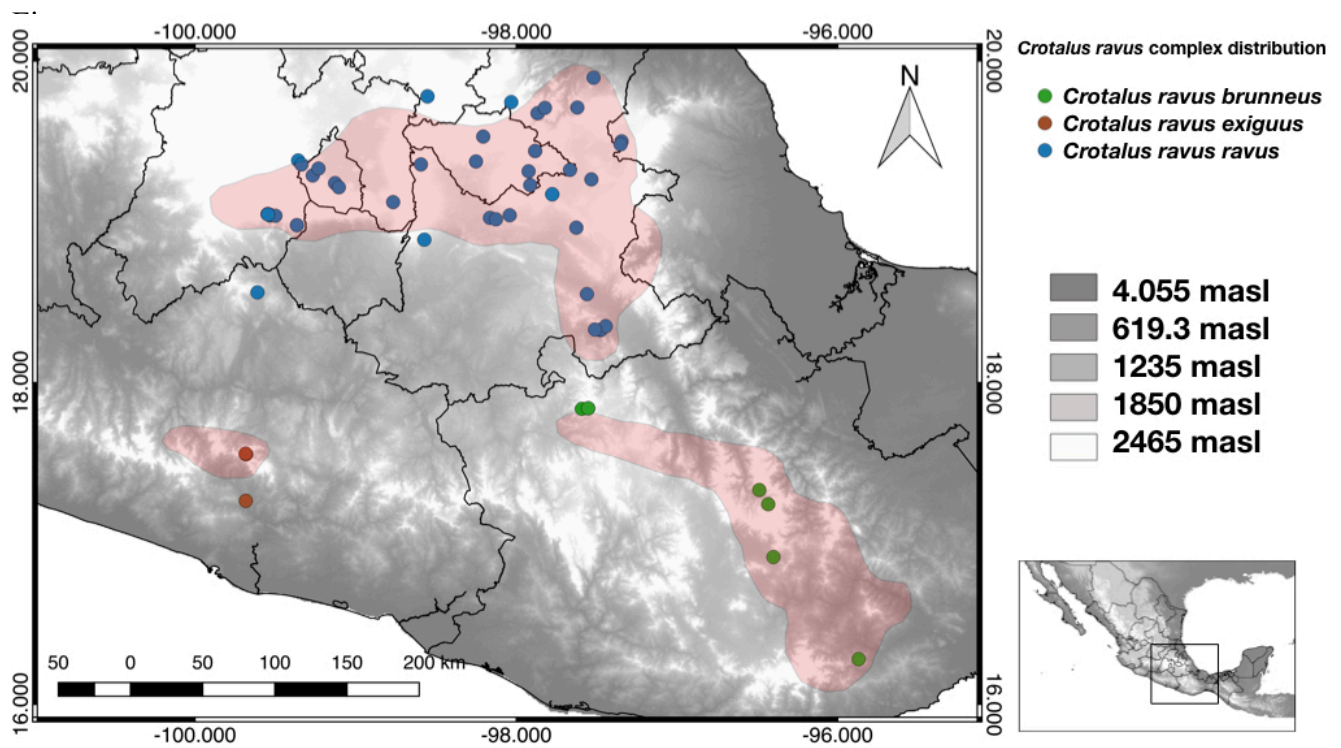
**Table 3.** Primers used for each locus considered in this work.

Locus	Primers	Nucleotide sequences	Bibliography
<b>ND4, tRNA H and tRNA S</b> (768 pb)	ND4 (F)	5' - CACCTATGACTACCAAAGCTCATGTAGAA GC	Arévalo et al., 1994
	LEU (R)	5' -CATTACTTTTACTTGGATTTCACCA	Arévalo et al., 1994
<b>L51</b> (373 pb)	L51 (F)	5' -ACTTGCCTTCAGAAATCATG	Gibbs & Díaz, 2010
	L51 (R)	5' -GGGATCAAAGGTTTAAAGAA	Gibbs & Díaz, 2010
<b>12S</b> (526 pb)	L1091 (F)	5' -CAAAGTGGGATTAGATACCCCACTAT	Arévalo et al., 1994
	H1478 (R)	5' -AGGGTGACGGGCGGTGTGT	Arévalo et al., 1994

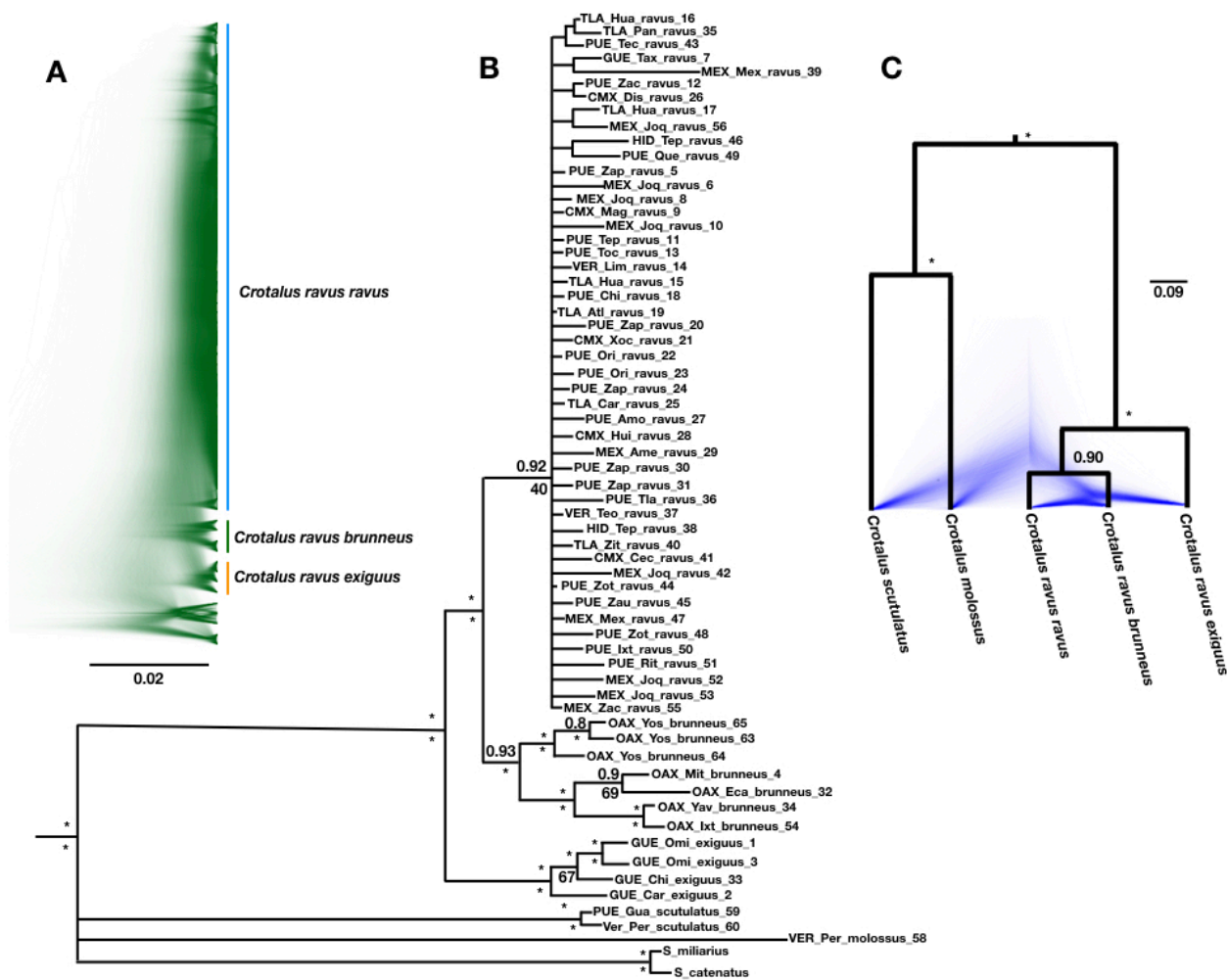
**Table 4.** Genetic distances calculated for the three lineages of *Crotalus ravus sensu lato*. Values greater than 2% in the intersections between lineages of this rattlesnake are highlighted in bold (see 3.4 section for details). The values denote percentage of genetic divergence.

	<i>C. r. exiguus</i>	<i>C. r. brunneus</i>	<i>C. r. ravus</i>
<i>C. r. exiguus</i>	-		
<i>C. r. brunneus</i>	<b>0.054</b>	-	
<i>C. r. ravus</i>	<b>0.0404</b>	<b>0.0341</b>	-

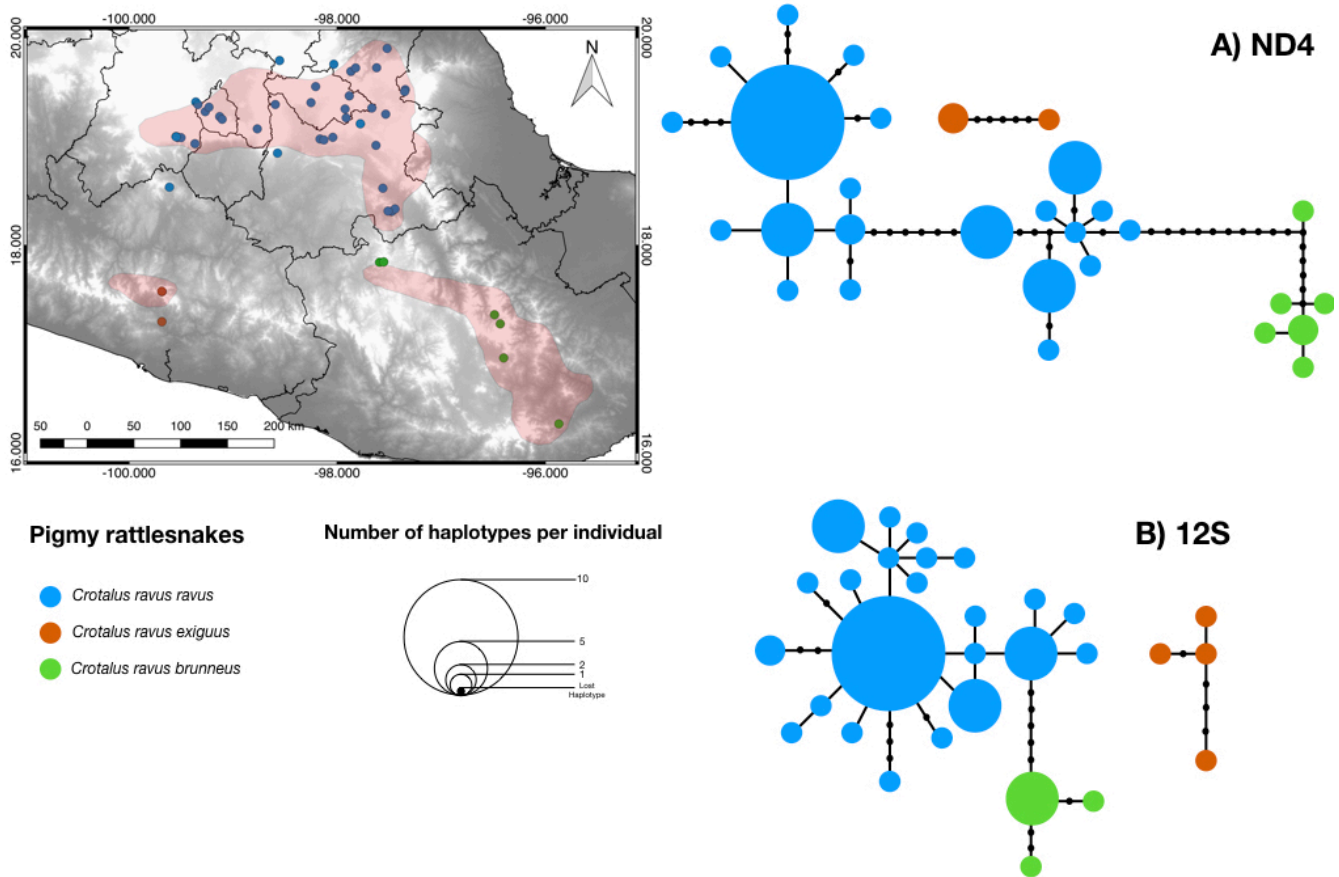




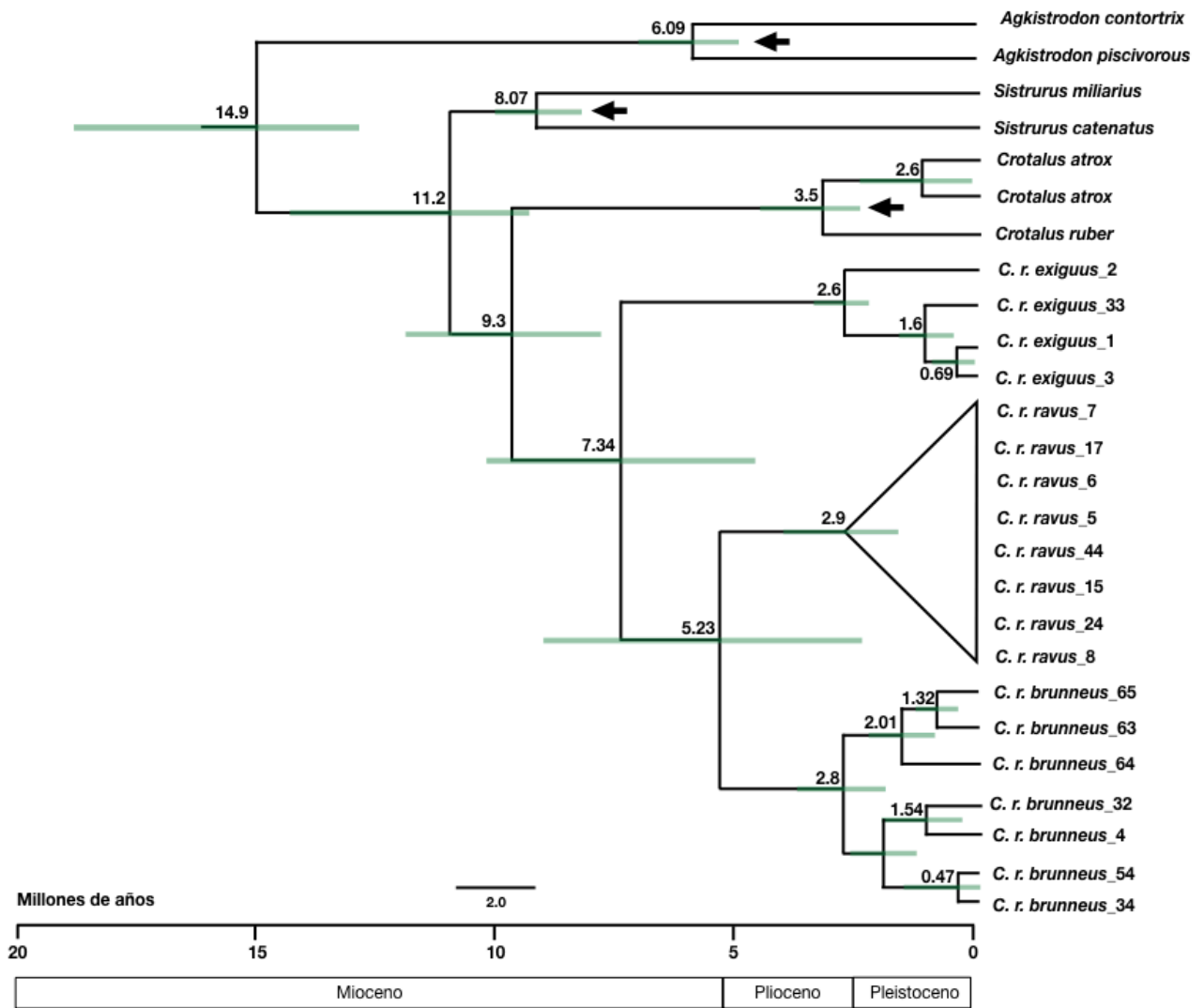
**Figure 1.** Map displaying the present-day distribution of *Crotalus ravus* (per Campbell & Lamar, 2004) and the representative samples obtained for this study. Blue dots represent *C. r. ravus*, green dots represent *C. r. brunneus*, and orange dots represent *C. r. exiguus*.



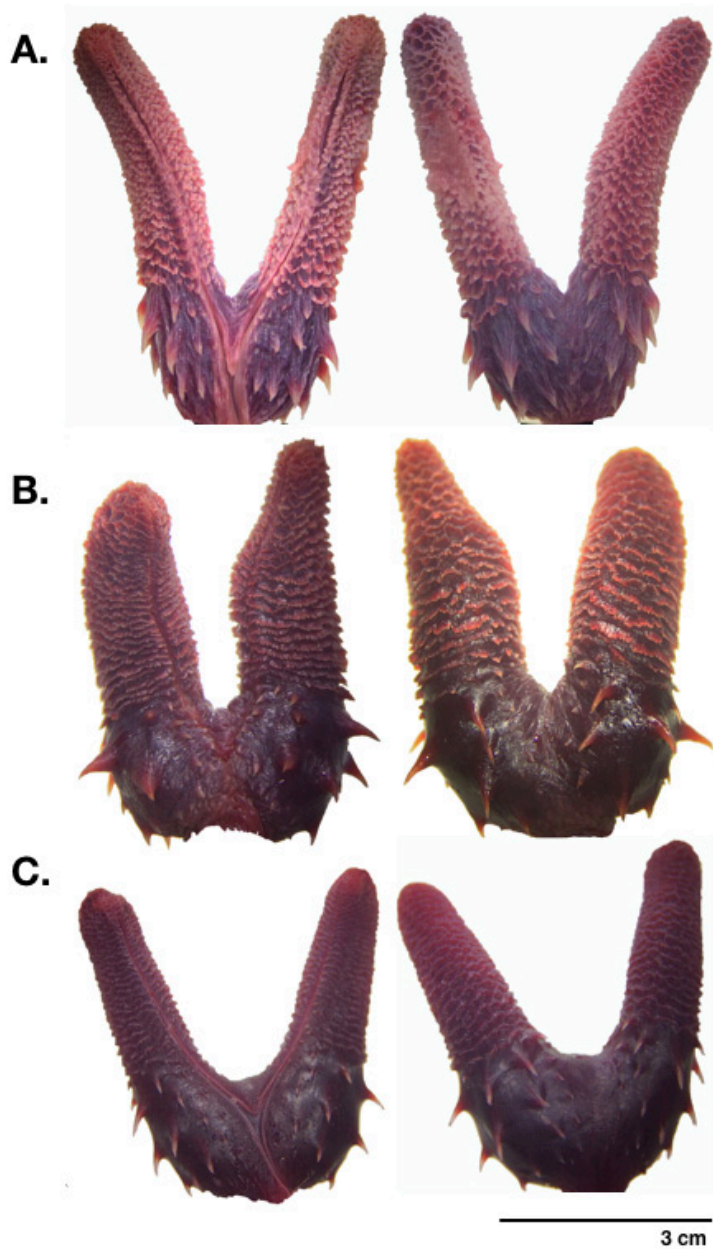
**Figure 2.** Phylogenetic trees of the *Crotalus ravus* complex using the concatenated dataset of the three loci examined in this study (ND4, 12S, and L51). A) Density tree from the Bayesian analysis; B) The phylogenetic relationships obtained from the maximum likelihood and Bayesian analyses, which recovered the same topology; C) Species tree obtained from \*BEAST. Numbers above branches represent posterior probability support values, while numbers below branches reflect bootstrap support values. Asterisks denote significant support values (>0.95 PP; >75 BS).



**Figure 3.** Haplotype networks of the mitochondrial ND4 (A) and 12S (B) loci, showing a correlation between haplotypes and geographic distribution of distinct rattlesnake lineages.



**Figure 4.** Time-calibrated relaxed clock tree of *Crotalus ravus sensu lato*. Black arrows denote calibrations used in the analysis, and values above nodes represent the 95% confidence interval of estimated node age in millions of years.



**Figure 5.** Hemipenial structures of the three lineages comprising the *C. ravus* complex. Panel A shows the sulcate and asulcate parts of the hemipenial structures characteristic of the *Crotalus ravus brunneus* (MZFC 23873) lineage from southwestern Oaxaca; Panel B displays both sides of the hemipenis characteristic of the *Crotalus ravus exiguus* lineage (MZFC 2893); and panel C shows both sides of the hemipenis characteristic of the *Crotalus ravus ravus* lineage (MZFC 34499) from the eastern part of this taxon's range in central Mexico.

## Appendix

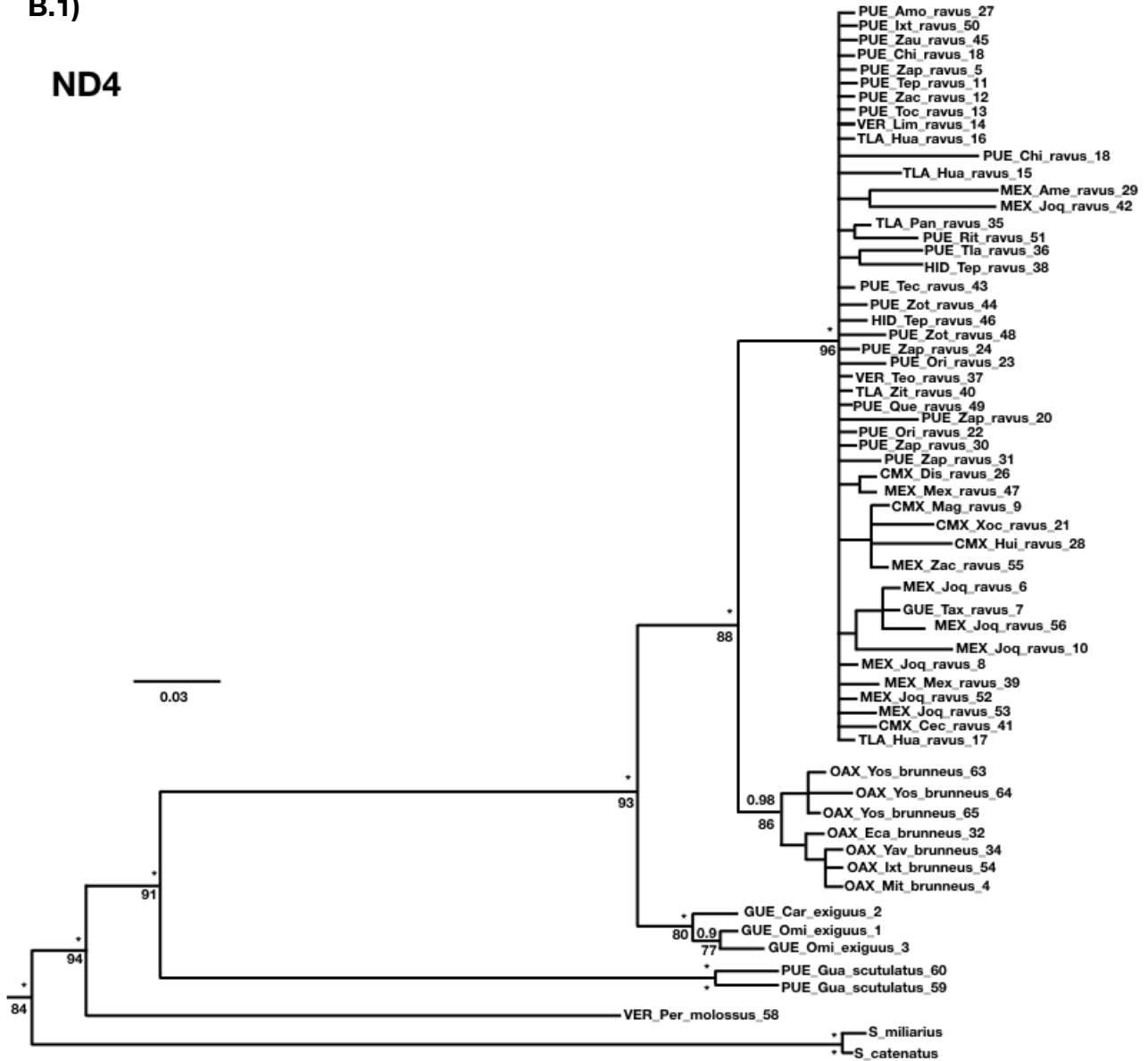
**Appendix A.** Specimens considered for the analyzing of the hemipenes structure.

<b>Linage</b>	<b>Collection Id</b>	<b>Collector id</b>	<b>Country</b>	<b>State</b>	<b>Minicipality</b>
<i>C. r. brunneus</i>	MZFC 23873	RVT 138	Mexico	Oaxaca	Santa María Yavesia
<i>C. r. exiguus</i>	MZFC 2893	AMA 267	Mexico	Guerrero	Chilpancingo de los Bravo
<i>C. r. ravus</i>	MZFC 34499	AYCB 14	Mexico	Puebla	Zotoltepec

**Appendix B.** Phylogenetic relationships of the different sets of sequences per gene considered in this study. Subsection B.1) represents the tree obtained by analyzing only the ND4 locus; subsection B.2) contains the resulting tree for the 12S locus; subparagraph B.3) is the resulting tree with the set of the L51 gene; while subsection B.4) represents the tree resulting from the combination of the ND4-12S genes.

**B.1)**

**ND4**



B.2)

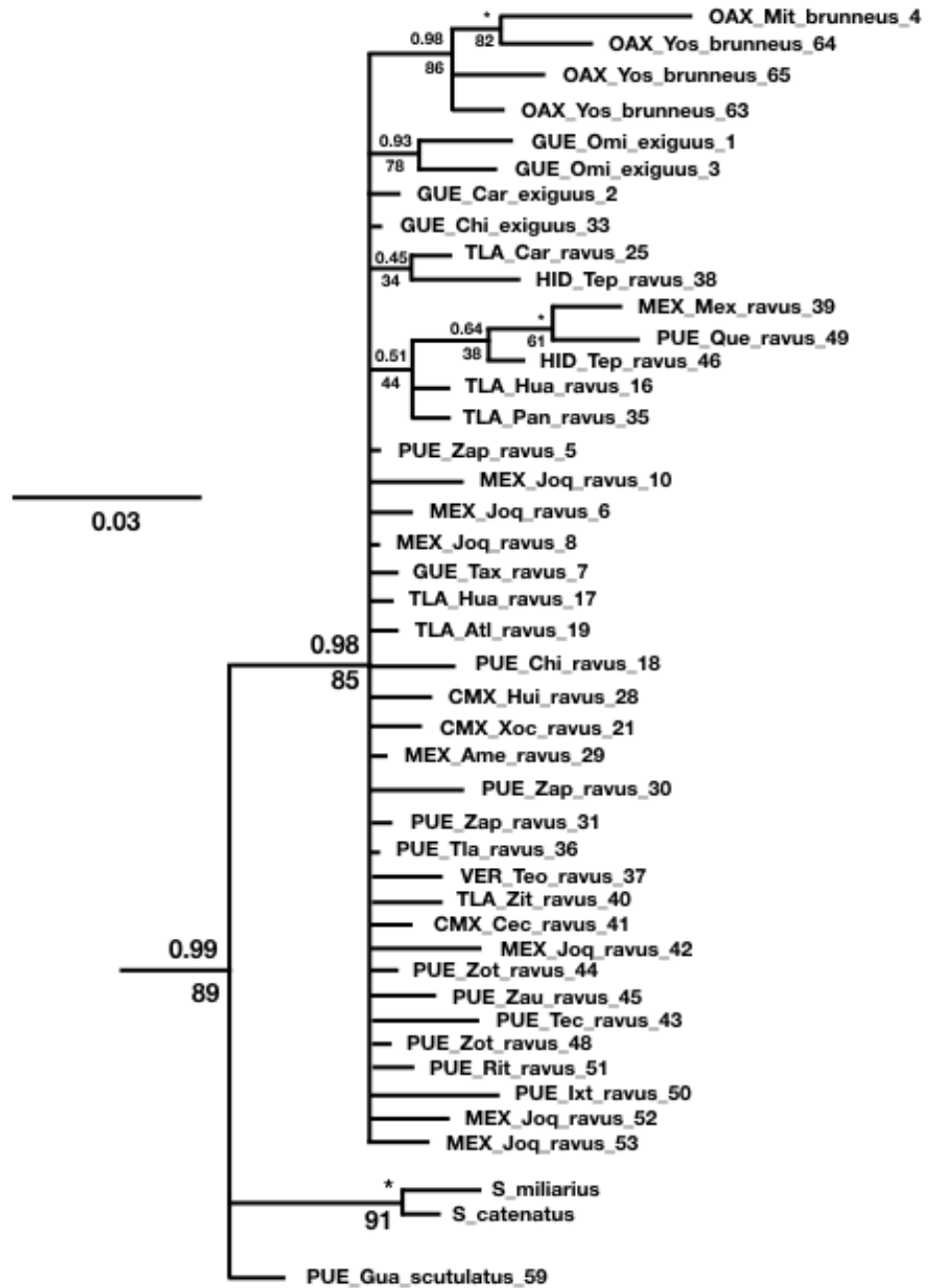
12S





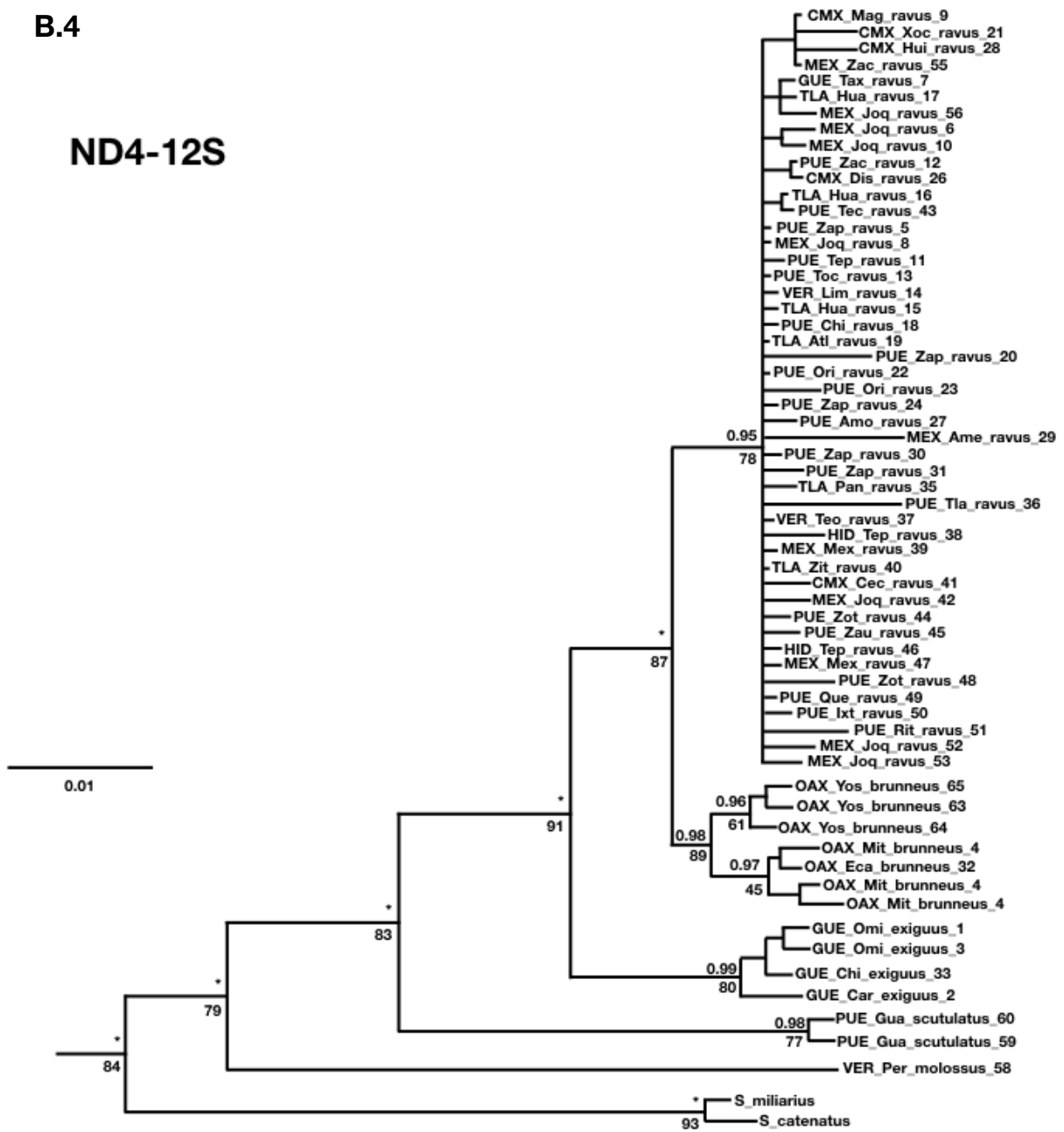
B.3)

L51



B.4

ND4-12S



## Comprobante de requisito para titulación por artículo científico

Correo de UNAM Facultad de Ciencias - Successfully received: submissi... Crotalus ravus (Viperidae) for Molecular Phylogenetics and Evolution 09/10/19 17:41



Antonio Yolocalli Cisneros Bernal <a.y.cisneros@ciencias.unam.mx>

---

### Successfully received: submission Divergence time and integrative taxonomy of the Mexican pigmy rattlesnake *Crotalus ravus* (Viperidae) for Molecular Phylogenetics and Evolution

1 mensaje

---

**Molecular Phylogenetics and Evolution** <EvisSupport@elsevier.com>  
Responder a: mpe@elsevier.com  
Para: a.y.cisneros@ciencias.unam.mx

9 de octubre de 2019, 17:34

*This message was sent automatically.*

Ref: MPE\_2019\_578

Title: Divergence time and integrative taxonomy of the Mexican pigmy rattlesnake *Crotalus ravus* (Viperidae)

Journal: Molecular Phylogenetics and Evolution

Dear Professor. Cisneros,

Thank you for submitting your manuscript for consideration for publication in Molecular Phylogenetics and Evolution. Your submission was received in good order.

To track the status of your manuscript, please log into EVISE® at: [http://www.evise.com/evise/faces/pages/navigation/NavController.jspx?JRNL\\_ACR=MPE](http://www.evise.com/evise/faces/pages/navigation/NavController.jspx?JRNL_ACR=MPE) and locate your submission under the header 'My Submissions with Journal' on your 'My Author Tasks' view.

Thank you for submitting your work to this journal.

Kind regards,

Molecular Phylogenetics and Evolution

#### **Have questions or need assistance?**

For further assistance, please visit our [Customer Support](#) site. Here you can search for solutions on a range of topics, find answers to frequently asked questions, and learn more about EVISE® via interactive tutorials. You can also talk 24/5 to our customer support team by phone and 24/7 by live chat and email.

---

Copyright © 2018 Elsevier B.V. | [Privacy Policy](#)

Elsevier B.V., Radarweg 29, 1043 NX Amsterdam, The Netherlands, Reg. No. 33156677.

## Discusión

La independencia de las historias evolutivas en los linajes de *C. ravus sensu lato*, según los resultados del estudio, ha sido influenciada por la formación de la Faja Volcánica Trans-Mexicana (FVTM). Esta formación fisiográfica de origen relativamente reciente ha moldeado de manera directa a varios linajes de las especies que se distribuyen ahí (Míguez-Gutiérrez et al., 2013). Al tratarse de una serpiente con poca vagilidad y de hábitos montanos (Campbell & Lamar, 2004), tiene sentido que la reciente formación de un sistema montañosos constituido principalmente por volcanes, haya tenido influencia en el reparto de los linajes dentro de este complejo. Nuestros resultados sugieren una divergencia dentro del grupo de alrededor de  $\sim 7.5$ -3 Ma, lo cual concuerda con periodos de actividad volcánica intensa en la zona (Gómez-Tuena et al. 2005). Se ha propuesto que las contracciones y expansiones de los bosques de pino-encino han influido en la formación de los linajes de *C. ravus* (Bryson et al., 2014), conectando a las poblaciones y permitiendo el flujo genético, para después contraerse y aislarlas entre sí. Los resultados obtenidos con nDNA (L51) (Apéndice 2, B3), revelan una baja resolución dentro del complejo, mientras que los dos genes mitocondriales recuperan de manera sustancial la monofilia de los tres clados, *C. ravus*, *C. brunneus* y *C. exiguus*. Parece ser que la divergencia del grupo es un poco más antigua que lo propuesto por Bryson et al. (2011) (Pleistoceno). Además, estos autores sugieren que los cambios en la extensión del bosque de pino-encino permitieron cierto contacto entre las poblaciones (flujo genético), aunque no lo suficiente como para que los linajes perdieran locus privados. Por ejemplo, *C. exiguus*, que fue el linaje que divergió primero, posee más loci privados. Asimismo, parece ser que la causa que limitó el flujo genético fue la formación de la cuenca del Balsas, separando las poblaciones de *C. ravus* y de *C. brunneus*; mientras que, entre *C. ravus* y *C. exiguus*, la formación de la cuenca del río Papaloapan, fue la causal de esta interrupción en el flujo genético, lo que concuerda con Bryson et al. (2011) en cuanto a que la formación de las cuencas de los ríos, afectaron el flujo génico entre poblaciones. Estas cuencas funcionaron como barreras geográficas para organismos montanos como estas serpientes. En resumen, parece ser que la alta actividad tectónica que existía en la zona (en el Mioceno-Plioceno) fue el principal

factor responsable del acomodo de los tres linajes en la geografía, efecto que demuestra la compleja influencia que guarda la geografía del centro y sur de México con los grupos biológicos que exhiben distribuciones en esas zonas.

En cuanto a las implicaciones taxonómicas, podemos decir que existe una clara correspondencia geográfica y filogenética en los linajes de *C. ravus sensu lato*. Es decir, se había propuesto la existencia de subespecies dentro de este complejo por la forma de la distribución de sus poblaciones y por ciertas características morfológicas (Harris & Simmons, 1978; Campbell & Armstrong, 1979; Campbell & Lamar, 2004), pero no se había realizado un estudio integral que evaluara la independencia de las historias evolutivas de los linajes de esta serpiente de cascabel.

Recientemente, Blair et al. (2018), con base en elementos ultra conservados del genoma (UCE) reconocieron que existen tres linajes dentro del complejo *C. ravus*, pero su trabajo se limitó a evaluar pocos miembros de cada subespecie. En este trabajo se respalda lo propuesto por Blair y colaboradores, pero con mayor evidencia, con un muestreo más amplio y con fuentes de información distintas. Por lo tanto, aquí se propone reconocer a los tres linajes establecidos, dentro de este complejo, a nivel específico. Se plantea que la identificación se lleve a cabo a partir de las características morfológicas de escutelación propuestas por trabajos previos (Campbell & Armstrong, 1979; Campbell & Lamar, 2004), sin perder de vista la variación en la arquitectura hemipenial presentada en este trabajo.

Finalmente, se propone llevar a cabo una reevaluación del estado de conservación de cada especie, ya que la situación de dos de las tres resulta crítica. En el caso de la distribución de *C. brunneus*, sólo el 5.9% del territorio se encuentra protegida bajo alguna categoría en el estado de Oaxaca. Para *C. exiguus* la situación se torna aún peor, al no contar con protección en ninguna de las zonas donde se distribuye, considerando la reciente pérdida del nombramiento de Parque Estatal en la localidad de Omiltemi, la cual abarca gran parte de la distribución de esta serpiente de cascabel en el centro del estado de Guerrero. El caso de *C. ravus*, que es la que tiene el área de distribución de mayor extensión, la preocupación es menor, ya que cerca del 20% de su distribución se encuentra bajo alguna área protegida. Tomando en

consideración los datos actuales en cuanto a la distribución de estas serpientes, es importante resaltar que la FVTM y la SMS se han visto afectadas actualmente por actividades antropogénicas como la fragmentación del hábitat y el cambio de uso de suelo (Sunny et al. 2018). Estos procesos pueden derivar en un mayor riesgo de extinción biológica (Newman & Tallmon, 2001) al no permitir que existan zonas de flujo genético entre poblaciones o que preserven el hábitat natural. Por lo anterior, es conveniente que al realizar estudios que deriven en el reconocimiento de especies nuevas, se lleve a cabo una reconsideración de su estado de conservación, para promover la protección de la diversidad que ya se conoce y la que se potencialmente puede descubrirse.

### **Conclusiones**

El llevar a cabo un análisis filogeográfico debe de ser un proceso que involucre de manera explícita el mayor número de datos disponibles sobre el taxón en cuestión, desde la perspectiva de la taxonomía y la biogeografía. El trabajo aquí presentado muestra que la serpiente de cascabel de montaña *C. ravus sensu lato* está compuesto por tres linajes con historias evolutivas independientes, por lo cual deben de elevarse las subespecies a nivel específico. Esta independencia se originó debido a procesos de especiación alopátrida promovidos por eventos vicariantes en la zona de distribución de estas especies, principalmente influenciados por la formación de la Faja Volcánica Trans-mexicana. Este proceso de cladogenesis comenzó a finales del Mioceno y principios del Plioceno, alrededor de hace 7.5 Ma cuando las poblaciones, ahora especies, comenzaron a aislarse entre ellas impidiendo el flujo genético entre ellas. Por todo lo anterior, se sugiere llevar a cabo una reevaluación de los estados de conservación de las especies reconocidas en este estudio debido a su distribución en zonas propensas a actividades humanas como la fragmentación, cambio de uso de suelo y contaminación.

## Literatura Citada (No contenida en el artículo)

Avise, J. C., 1998. The history and preview of phylogeography: A personal reflection. *Molecular Ecology*, 7, 371-379. doi.org/10.1046/j.1365-294x.1998.00391.x

Avise, J. C., 2000. *Phylogeography*. Harvard Univ. Press, Cambridge. Pp. 447

Avise, J.C., J. Arnold, R. M. Ball, E. Bermingham, T. Lamb, J. E. Neigel, C. A. Reeb & N. C. Saunders. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology, Evolution and Systematics* 18, 489–522. doi.org/10.1146/annurev.es.18.110187.002421

Bickford, D., Lohman, D. J., Sodhi, S., Ng, P. K. L., Meier, Winker, K., Ingram, K. K., Das, I. 2006. Cryptic species as a window on diversity and conservation. *TRENDS in Ecology and Evolution*, 22, 148-155. doi:10.1016/j.tree.2006.11.004

de Queiroz, K. 2005. A unified concept of species and its consequences for the future of taxonomy. *Proceedings of the the California Academy of Science*, 56, 196-215.

García-Vázquez, U. O., Nieto-Montes de Oca, A., Bryson, R. W., Schmit-Ballardo, W., Pavón-Vázquez, C. J. 2018. Molecular systematics and historical biogeography of the genus *Gherronotus* (Squamata: Anguidae). *Journal of Biogeography*, 45, 1640-1652. doi.org/10.1111/jbi.13241

Hernández-Canchola, G., León-Paniagua, L. 2017. Genetic and ecological processes promoting early diversification in the lowland Mesoamerican bat *Sturnira parvidens* (Chiroptera: Phyllostomidae). *Molecular Phylogenetics and Evolution*, 114, 334-345.

Hudson, R., R. Coyne. 2002. Mathematical consequence of the genealogical species concept. *Evolution* 8, 1557-1565. doi.org/10.1111/j.0014-3820.2002.tb01467.x

- Mayr, E. 1987. The ontological status of species: Scientific progress and philosophical terminology. *Biology and Philosophy*, 2, 145-166. doi:10.1007/BF00057959
- Pigliucci, M. 2003. Species as family resemblance Concept: the (dis-) solution of the species problem? *BioEssays* 6, 596-602. doi:10.1002/bies.10284
- Pérez-Gil R, Jaramillo, F., Muñiz, A., Torres, M. 1996. *Importancia Económica de los Vertebrados Silvestres de México*. PG7 Consultores y CONABIO. México. 170 pp.
- Poulin, R., Pérez-Ponce de León, G. 2016. Global analysis reveals that cryptic diversity is linked with habitat but not mode of life. *Journal of Evolutionary Biology*, 30, 641-649. doi: 10.1111/jeb.13034
- Raxworthy, C. J., Pearson, R. G., Zimkus, B. M., Reddy, S., Deo, A. J., Nussbaum, R.A., Ingram, C. M. 2008. Continental speciation in the tropics: contrasting biogeographic patterns of divergence in the *Uroplatus* leaf-tailed gecko radiation of Madagascar. *Journal of Zoology*, 275, 423-440. doi.org/10.1111/j.1469-7998.2008.00460.x
- Read, J. L. 1998. Are geckos useful bioindicators of air pollution? *Oecologia* 114, 180-187. doi:10.1007/s004420050434
- Rojas, M. 1992. The species problem and conservation: What are we protecting? *Conservation Biology* 2, 170-178.
- Ruiz-Vega, M. L., Hernández-Canchola, G., León-Paniagua, L. 2018. Molecular systematics and phylogeography of the endemic Osgood's deer mouse *Osgoodomys banderanus* (Rodentia: Cricetidae) in the lowlands of western Mexico. *Molecular Phylogenetics and Evolution*, 127, 867-877. doi.org/10.1016/j.ympev.2018.06.034
- Sunny, A., Monroy-Vilchis, O., Zarco-González, M. M. 2018. Genetic diversity and structure of *Crotalus triseriatus*, a rattlesnake of central Mexico. *Journal of Genetics*, 97, 1119-1130.



## Artículos derivados de este proyecto

**Cisneros-Bernal, A. Y.,** Palacios-Aguilar, R., Medina-Rangel, Gonzalo, Campillo-G, Gustavo, Rivera-Reyes, Ricardo. 2019. New distributional records of amphibians and reptiles from the Mixteca Region of Oaxaca, Mexico. *North-Western journal of Zoology*. **Accepted.**



**NORTH-WESTERN JOURNAL OF ZOOLOGY**  
*International scientific research journal of zoology and animal ecology*  
of the Herpetological Club - Oradea  
University of Oradea, Faculty of Sciences, Department of Biology  
Universitatii str. No.1, Oradea – 410087, Romania  
Publisher: University of Oradea Publishing House  
Contact e-mail: [sas.steve@gmail.com](mailto:sas.steve@gmail.com)

### **NORTH – WESTERN JOURNAL OF ZOOLOGY** *(International journal of zoology and animal ecology)*

#### **MANUSCRIPTS SUBMISSION**

The manuscript and artwork should be submitted with the following letter to editors:

Dear Editor-in-Chief (or Corresponding Editor) Mr./Mrs.

..... Yurii Kornilev.....

Type of the paper (according to the author's guidelines)

Full-length paper  Short paper

Natural history note  Fauna note  Taxonomical note  Other

Please find enclosed our manuscript entitled: “**NEW DISTRIBUTIONAL RECORDS OF AMPHIBIANS AND REPTILES FROM THE MIXTECA REGION OF OAXACA, MEXICO**”, intended as submission for *North-Western Journal of Zoology*.

Authors: Antonio Yolocalli CISNEROS-BERNAL, Ricardo PALACIOS-AGUILAR, Gonzalo MEDINA-RANGEL, Gustavo CAMPILLO-G., Ricardo RIVERA-REYES.

Corresponding author: Antonio Yolocalli CISNEROS-BERNAL, Ricardo PALACIOS-AGUILAR

Affiliation/Correspondence address: **Museum of Zoology “Alfonso L. Herrera”, Faculty of Science, National Autonomous University of Mexico, A.P. 70-399, Ciudad de México CP 04510, México. AYCB ([A.Y.Cisneros@ciencias.unam.mx](mailto:A.Y.Cisneros@ciencias.unam.mx)), RPA ([ricardopalaciosaguilar@gmail.com](mailto:ricardopalaciosaguilar@gmail.com)).**

Short description of the subject (max: 100 words)

We present a short contribution reporting new records of amphibians and reptiles from the Mixteca region, one of the most speciose biogeographic provinces of southern Mexico

Short explanation (no more than 100 words) of why the manuscript suits the NWJZ.

Oaxaca is the state with the richest herpetofauna of Mexico and the Mixteca region is the largest biogeographic region of it, also harboring a great proportion of endemic species. Here we provide new data on a poorly explored zone in the northern Mixteca region, that include first records for the province and for the state.

Palacios-Aguilar, R., **Cisneros-Bernal, A. Y.**, Parra-Olea, G., Arias-Montiel, J. D. 2019. A new species of the *Bolitoglossa macrinii* species group (Amphibia: Plethodontidae) from the central highlands of Guerrero Mexico. *Canadian Journal of Zoology*. 98: 359-365.



## Canadian Journal of Zoology

### **A new species of the *Bolitoglossa macrinii* species group (Amphibia: Plethodontidae) from the central highlands of Guerrero, Mexico.**

Journal:	<i>Canadian Journal of Zoology</i>
Manuscript ID	cjz-2019-0244
Manuscript Type:	Article
Date Submitted by the Author:	16-Oct-2019
Complete List of Authors:	Palacios Aguilar, Ricardo; Museo de Zoología Alfonso L Herrera Cisneros, Antonio; Universidad Nacional Autónoma de México, Parra-Olea, Gabriela; Universidad Nacional Autónoma de México Instituto de Biología Arias-Montiel, Juan Diego; Universidad Nacional Autónoma de México Facultad de Estudios Superiores Iztacala
Is your manuscript invited for consideration in a Special Issue?:	Not applicable (regular submission)
Keyword:	Salamander, Sierra Madre del Sur, TAXONOMY-SYSTEMATICS < Discipline

SCHOLARONE™  
Manuscripts