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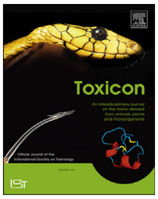

Venom phenotypes of the Rock Rattlesnake (*Crotalus lepidus*) and the Ridge-nosed Rattlesnake (*Crotalus willardi*) from México and the United States

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ABSTRACT

Although the Mexican Highlands has the highest diversity of small-bodied rattlesnakes in the world, studies on the species found throughout this region have been relatively scarce. This has led to challenges with examining venom phenotypic characteristics, as well as species misidentifications and misclassifications. In the current study we investigated venom variation among four subspecies of *Crotalus lepidus* (*C. l. klaluberi*, *C. l. lepidus*, *C. l. maculosus*, *C. l. morulus*) and four subspecies of *C. willardi* (*C. w. amabilis*, *C. w. obscurus*, *C. w. silus*, and *C. w. willardi*) that inhabit regions of southwestern United States and central México. SDS-PAGE patterns show the presence of many of the major compounds found in other rattlesnake venoms, although minor variations in protein banding patterns and intensity are recognizable. Most notably, PI-metalloproteinase (SVMP) bands appear to be very faint to absent in northern *C. l. lepidus* and *C. l. klauberi* subspecies, but are fairly prominent in all other *C. lepidus* and *C. willardi* subspecies. Enzyme activity assays revealed that *C. lepidus* subspecies exhibit higher SVMP and thrombin-like activities when compared to *C. willardi* subspecies. Significant differences between subspecies were also observed for kallikrein-like serine protease, L-amino acid oxidase, and phosphodiesterase activities, although these differences appear to be random and fail to follow a geographical or phylogenetic trend. The same relationship was also observed for fibrinogenolytic and coagulation assays. Toxicity assays conducted on lab mice (*Mus musculus*), house geckos (*Hemidactylus frenatus*), and house crickets (*Acheta domestica*) revealed varying toxicities between subspecies, with *C. l klauberi* being the most toxic towards mice ($LD_{50} = 1.36$ μg/g) and house geckos ($LD_{50} = 0.17$ μg/g), and *C. w. silus* being most toxic to house crickets ($LD_{50} = 1.94$ μg/g). These results provide additional evidence that geographical isolation, natural selection, and adaptive evolution in response to diets may be driving forces contributing to population-level variation in venom composition.

1. Introduction

An array of environmental factors can influence evolutionary diversification, and recognizing how a species adapts, and the mechanisms by which species arise, are fundamental areas of biological research. Large-scale landscape reconfiguration, such as the uplifting of mountains, can cause geographical isolation of previously continuous populations, interfere with gene flow, and drive patterns of phenotypic change (Scott and Reynolds, 1984; McCormack et al., 2008; Fjeldså et al., 2012; Noutsos et al., 2014). Spanning México, the Mexican highlands extend from the southern Rocky Mountains of the United States to the northern edge of Central America, comprising four major mountain ranges consisting of hundreds of kilometers of mountains and isolated peaks (Ferrusquía-Villafranca, 1990, 1993). These mountain ranges consist of the north-south trending Sierra Madre Occidental and Sierra Madre Oriental in north-central México, and the west-east trending Sierra Madre del Sur and the Trans-Mexican Volcanic Belt in south-central México (Ferrusquía-Villafranca, 1990, 1993). Vast

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intervening lowlands formed by the Chihuahuan Desert and Central Mexican Plateau have established complex but isolated assemblages of montane biotas (“sky islands”), creating a significant biodiversity hotspot for temperate, often endemic taxa (Ramamoorthy et al., 1993; Campbell, 1999; Mittermeier et al., 2005; Mastretta-Yanes et al., 2015). The Mexican highlands have therefore become a major landscape for exploring species adaptation and diversification in habitats with limited opportunities for biological dispersal (e.g., Bryson et al., 2011a).

Reptiles, and specifically rattlesnakes, represent ideal model organisms for investigating phenotypic adaptations driven by the geographical barriers of the Mexican highlands. In fact, this region is known to have the highest number of small rattlesnake species in the world (Alvarado-Díaz and Campbell, 2004), and most authors suggest that rattlesnakes originated in México and diversified in these mountainous areas (Gloyd, 1940; Klauber, 1956; Place and Abramson, 2004; Blair and Sanchez-Ramirez, 2016). Of these, the Rock Rattlesnake (*Crotalus lepidus*) is a diminutive species often found at high elevations (up to 3000 m) in central México and the southwestern United States (Campbell and Lamar, 1989; Lemos-Espinal et al., 2016, 2017). There are four subspecies of *C. lepidus* (*C. l. lepidus*, *C. l. klauberi*, *C. l. maculosus* and *C. l. morulus*; Campbell and Lamar, 1989) found throughout varying habitats that are often separated by large regions of unsuitable matrix. For instance, *C. l. lepidus* are found from southeastern New México and western Texas, south into the eastern Central Mexican Plateau in generally rocky habitat from 300 m to over 2000 m in elevation (Campbell and Lamar, 1989, 2004). *Crotalus l. klauberi* also occurs throughout the southwestern United States into northern and central México, primarily in rocky outcrops of desert grasslands and woodlands at elevations ranging from 1200 to 2500 m (Campbell and Lamar, 1989; Lowe et al., 1986). *Crotalus l. morulus*, recently elevated to a full species (Bryson et al., 2014) but retained here as a subspecies for simplicity, inhabits humid pine-oak forest in the Mexican states of Coahuila, Nuevo Leon, and Tamaulipas at elevations from 1200 to 2748 m (Campbell and Lamar, 2004), and *C. l. maculosus* is found in humid pine-oak forest in southwestern México in the states of Durango, Sinaloa, and Nayarit (Campbell and Lamar, 1989, 2004).

The Ridge-nosed Rattlesnake (*Crotalus willardi*) is a similar small-bodied rattlesnake, with adult males reaching an average length of 500 mm (Barker, 1992*)*. *Crotalus willardi* is distributed from the sky islands of southwestern United States south into northwestern México, occurring mainly in the pine-oak woodlands of the Sierra Madre Occidental. *Crotalus willardi* is distinguishable from other species of rattlesnakes by the (usual) presence of a distinct facial pattern of pale stripes that converge dorsally to the canthus rostralis, and the tip of the snout that is distinctively raised, giving the species the common name of the Ridge-nosed Rattlesnake. Although all members of the *C. willardi* complex have this distinctive pattern of stripes, there is considerable variation in the color, breadth, and length of the stripe between populations (Campbell and Lamar, 2004), resulting in five recognized subspecies (or species, as recently proposed by Barker (2016)). *Crotalus w. willardi* is found in the Huachuca, Patagonia, Santa Rita, and Whetstone Mountains of Arizona and adjacent sky islands in northern Sonora, México (Campbell and Lamar, 1989, 2004). *Crotalus w. obscurus* has the lightest colored facial stripe (nearly absent in some individuals), is the largest of the five subspecies, and occurs in the Animas and Peloncillo Mountains of New México and in the Sierra de San Luis in northern Sonora and Chihuahua (Campbell and Lamar, 1989, 2004). *Crotalus w. silus* has the largest distribution of the five subspecies and can be found throughout the northern portion of the Sierra Madre Occidental in Sonora and Chihuahua. *Crotalus w. amabilis* appears to have the smallest known range and has only been documented from the canyons of the Sierra del Nido in north-central Chihuahua. *Crotalus w. meridionalis* is the southernmost subspecies and is found in the Mexican states of Durango and Zacatecas.

The high endemism of the Mexican highlands suggests a strong role for these mountains in driving divergence and phenotypic adaptations among venomous snakes. Although several studies have addressed the phylogenetic relationship of rattlesnakes (Bryson et al., 2011a,b, 2014) and other pitvipers (Castoe et al., 2005, 2009) in this region, little is known about venom compositional patterns of these rattlesnakes. Recently, Martínez-Romero et al. (2013) explored the biochemical and toxicological properties of *C. l. lepidus*, *C. l. klauberi*, and *C. l. morulus* venoms, providing a profile of venom composition among these three subspecies (see also Forstner et al., 1997). However, an examination of the venom composition of *C. l. maculosus*, as well as the *C. willardi* subspecies complex, has not been completed. Examining the venom profiles of closely related but geographically isolated rattlesnake subspecies can provide insight into the mechanisms driving venom evolution, and provide a deeper understanding of the natural history and evolutionary relationships among venomous snakes. Therefore, the current study explored the protein compositional patterns and biological activities of the venoms from all four C. *lepidus* subspecies as well as four subspecies of the *C. willardi* complex (*C. w. amabilis, C. w. obscurus, C. w. silus,* and *C. w. willardi*).

2. Materials and methods

2.1. Venoms and reagents

All venoms (*C. l. klauberi* n = 4, *C. l. lepidus* n = 5, *C. l. maculosus* n = 5, *C. l. morulus* n = 5, *C. w. amabilis* n = 6, *C. w. obscurus* n = 7, *C. w. silus* n = 4, and *C. w. willardi* n = 6; see Appendix) were extracted from adult captive snakes using standard techniques (Mackessy, 1988), briefly centrifuged, lyophilized, and stored at −20 °C until used. Lyophilized samples were reconstituted in 18.3 MΩ Millipore-filtered water and prepared at a concentration of 4.0 mg/mL for experimental use. Protein gels, mass standards, and electrophoretic reagents were obtained from Invitrogen-Life Technologies (Grand Island, NY, USA), and all additional buffers, substrates, and reagents (analytical grade or better) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). *Crotalus lepidus klauberi* (United States) was sampled under permits from the Arizona Game and Fish Department (SPM: MCKSY000221). Collecting and research in México were conducted under permits issued by SEMARNAT (SEMARNAP D00.02.-2546, D00.02.-6390; SEMARNAT OFICIO NÚM/SGPA/DGVS/3394, 4267, 5431, 1643, 2847); the USFWS approved importation of the *C. w. obscurus* (MA053885-0).

2.2. Gel electrophoresis

Individual *C. lepidus* and *C. willardi* crude venom samples were assessed for the relative number and molecular masses of venom components by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under dithiothreitol (DTT) reducing conditions. Briefly, 20 μg of venom or 5 μL of Mark 12 standards were loaded onto precast NuPage bis-tris 12% acrylamide gels and run in MES SDS running buffer for 50 min at 175 V. Gels were stained in 0.1% Coomassie brilliant blue R-250, destained with 30% methanol/7% acetic acid, and photographed using a Bio-Rad gel imaging system (Munekiyo and Mackessy, 1998).