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Venomomics of New World pit vipers: Genus-wide comparisons of venom proteomes across *Agkistrodon*

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Abstract

We report a genus-wide comparison of venom proteome variation across New World pit vipers in the genus *Agkistrodon*. Despite the wide variety of habitats occupied by this genus and that all its taxa feed on diverse species of vertebrates and invertebrate prey, the venom proteomes of copperheads, cottonmouths, and cantils are remarkably similar, both in the type and relative abundance of their different toxin families. The venoms from all the eleven species and subspecies sampled showed relatively similar proteolytic and PLA₂ activities. In contrast, quantitative differences were observed in hemorrhagic and myotoxic activities in mice. The highest myotoxic activity was observed with the venoms of *A. b. bilineatus*, followed by *A. p. piscivorus*, whereas the venoms of *A. c. contortrix* and *A. p. leucostoma* induced the lowest myotoxic activity. The venoms of *Agkistrodon bilineatus* subspecies showed the highest hemorrhagic activity and *A. c. contortrix* the lowest. Compositional and toxicological analyses agree with clinical observations of envenomations by *Agkistrodon* in the USA and Central America. A comparative analysis of *Agkistrodon* shows that venom divergence tracks phylogeny of this genus to a greater extent than in *Sistrurus* rattlesnakes, suggesting that the distinct natural histories of *Agkistrodon* and *Sistrurus*

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2013.10.036>.

clades may have played a key role in molding the patterns of evolution of their venom protein genes.

Biological significance—A deep understanding of the structural and functional profiles of venoms and of the principles governing the evolution of venomous systems is a goal of venomics. Isolated proteomics analyses have been conducted on venoms from many species of vipers and pit vipers. However, making sense of these large inventories of data requires the integration of this information across multiple species to identify evolutionary and ecological trends. Our genus-wide venomics study provides a comprehensive overview of the toxic arsenal across *Agkistrodon* and a ground for understanding the natural histories of, and clinical observations of envenomations by, species of this genus.

Keywords

Snake venomics; Genus *Agkistrodon*; Mass spectrometry

1. Introduction

The subfamily Crotalinae contains 220 species of venomous pit vipers distributed throughout the Old and New Worlds (<http://reptile-database.org/>). Anatomical and molecular evidence provides strong support for the monophyly of all New World pit vipers [1-6], and suggests a historical biogeographic scenario involving a single Holoartic dispersal event of an ancestral Asian lineage into the New World across Beringia in the late Oligocene/Early Miocene (24–22 Mya) [6,7] or the late Cretaceous to early Tertiary (18–14 Mya) [8] (Fig. 1A), when this land bridge was mostly composed of deciduous and coniferous forests; this stock might have divided early into a Nearctic clade and a predominantly Neotropical branch [9,10]. The southern branch gave rise to Central and South American genera, and the Nearctic lineage differentiated into the common ancestors of *Agkistrodon* and rattlesnakes (*Crotalus* and *Sistrurus*) [10]. Four Old World genera (*Protophops*, *Ovophis*, *Trimeresurus*, and *Gloydius*) have been proposed as potential sister taxa to the New World clade [2,3,11-13]. Fossil records of *Agkistrodon contortrix* date from the Late Miocene (Hemphillian from Nebraska, 12–10 Mya), indicating that the ancestral landscape of *Agkistrodon* was Tertiary [14,15]. Molecular (DNA sequence) evidence also indicates a relatively recent origin of *Agkistrodon* [11].

Agkistrodon is a genus of venomous New World pit vipers found in North and Central America, from the eastern United States to northern Costa Rica [16-18] (Fig. 1B). Currently, this genus comprises four species, namely *A. contortrix* (Linnaeus, 1766) [19], *Agkistrodon piscivorus* (Lacépède, 1789) [20], *Agkistrodon bilineatus* (Günther, 1863) [21], and *Agkistrodon taylori* [10]. Several subspecies of copperheads (*A. c. contortrix*, southern copperhead; *A. c. laticinctus*, broad-banded copperhead; *A. c. mokasen*, northern copperhead; *A. c. phaeogaster*, Osage copperhead; *A. c. pictigaster*, Trans-Pecos copperhead), cottonmouths (*A. p. conanti*, Florida cottonmouth; *A. p. leucostoma*, western cottonmouth; *A. p. piscivorus*, eastern cottonmouth), and cantils (derived from the Tzeltal language, “kan” and “till”, meaning “yellow lips” [22]) (*A. b. bilineatus*, common or Mexican cantil; *A. b. howardgloydi*, Castellana cantil; *A. b. russeolus*, *A. b. lemosespinali*,

Yucatecan cantil) are currently recognized ([10,17]; <http://reptile-database.org>). *A. b. lemosespinali*, a patronim honoring Prof. Julio Alberto Lemos-Espinal, was described by Smith and Chiszar [23] based on a single specimen from near Palma Sola, Veracruz, Mexico. Originally classified as a subspecies of *A. bilineatus* by Burger and Robinson, 1951) [24], *A. taylori*, Taylor's cantil, named in honor of the American herpetologist Edward Harrison Taylor (1889–1978) [25,26], was elevated to species status by Parkinson et al. based on mtDNA sequence analysis [10]. Very recently, Porras and co-workers revisited the taxonomic status for *A. bilineatus* subspecies [27]. Based on morphological assessment, biogeographical evidence, and DNA-based studies, these authors have elevated the three subspecies of *A. bilineatus* to full species [27].

The phylogeny of *Agkistrodon* has a long history of instability. The ancestral habitats of *Agkistrodon* were temperate, as are those of its most basal living lineages (*A. contortrix* and *A. piscivorus*). Divergence dating (Fig. 1A) indicates that the origin of *A. contortrix* occurred in the Late Miocene (~6.6 Mya) and lineage diversification in the early Pleistocene (~1.4–1.0 Mya) [28]. The origin of *A. piscivorus* has been dated to the Late Miocene/Early Pliocene (~5.3 Mya), and the separation between the Florida and the Continental lineages took place during the Late Pliocene (~2.5 Mya) [28], when rising sea levels separated Peninsular Florida into isolated islands, restricting gene flow between Florida and mainland populations [29]. *A. bilineatus* and *A. taylori* occupied increasingly tropical regions, following divergence from a common ancestor with *A. piscivorus* (~2.6 Mya) [10,30]. Copperheads and cottonmouths are found throughout much of eastern United States (Fig. 1B), inhabiting deciduous forests and mixed woodlands. Despite evidence for hybridization [17], *A. contortrix* and *A. piscivorus* exhibit unique habitat preferences [16-18]: *A. piscivorus* tends to be restricted to lowland shaded swamps, whereas *A. contortrix* is much more of a habitat generalist, living in swamps, deciduous hardwoods, pine forests, scrub desert, and high elevation mountain regions [16-18]. The Trans-Pecos copperhead, *A. c. pictigaster*, is also found in riparian habitats of the Chihuahuan Desert of west Texas and northern Mexico [16,17] (Fig. 1B). The Taylor's cantil exhibits a highly restricted geographical distribution in the northeastern Mexican states of Nuevo León, San Luis Potosí and Tamaulipas [16,17] (Fig. 1B). According to Gloyd and Conant [17], cantils are greatly feared throughout its range, on the Atlantic and Pacific sides of Mexico and Central America.

Because of the dramatic size differences of slender neonates (17–30 cm long) and stout-bodied adult snakes (*A. contortrix* and *A. bilineatus* range from ~0.6 to 1.3 m and *A. piscivorus* can attain lengths greater than 1.6 m), ontogenetic shifts in diet are particularly important to these snakes. Adult specimens of *A. contortrix* and *A. bilineatus* usually prey on vertebrates, including mammals (rodents), reptiles (small turtles, lizards, and snakes), birds, and amphibians (anurans and salamanders), whereas juveniles primarily prey on invertebrates (insects, millipedes, and spiders), small lizards, and frogs [16-18,31]. However, at least in *A. b. howardgloydi* [31], the ontogenetic change in diet does not seem to be accompanied with changes in venom composition. The cottonmouths are opportunistic feeders that eat a wide variety of foods; fish and amphibians are the most frequent prey [16-18]. The diet of *A. taylori* consists of grasshoppers and pocket and white-footed mice

[16]. Adults spend considerable time actively foraging, and neonates employ caudal luring to attract prey within striking distance [16,32,33].

Copperheads and cottonmouths are able to deliver, respectively, 40–75 mg and 80–170 mg of venom [34]. Toxicological analyses of *A. contortrix*, *A. piscivorus* and *A. bilineatus* venoms showed higher lethal and hemorrhagic activities in the venom of *A. bilineatus* [32,35,36]. However, no systematic attempts have been made to perform a comparative analysis of the toxicity of *Agkistrodon* venoms at different taxonomic levels. Envenomations by copperheads and cottonmouths produce severe local pain, edema, erythema, and thrombocytopenia, in addition to nausea, vomiting, and occasionally hypotension and shock [16]. Copperhead bites are common, but fatalities are almost nonexistent [16–18]. Cottonmouths can be aggressive snakes when cornered or provoked, and fatalities have been recorded [16–18]. The irritable and aggressive nature of *A. bilineatus* has also been noted by many authors ([15], and references cited). In Nicaragua, *A. b. howardgloydi* is regarded as a highly dangerous snake. Bites from *A. bilineatus* have caused human fatalities [16,37], but the clinical features of envenomations by this species have not been reported in detail.

Venom represents a critical adaptive innovation in ophidian evolution that has played an important role in their natural history, allowing them to be ecologically successful by way of being efficient predators. However, despite occupying an important phylogenetic position for understanding the current biodiversity within New World Crotalinae, the study of *Agkistrodon* venoms has received little attention. For example, the UniProtKB/Swiss-Prot database (<http://www.uniprot.org>) contains just a handful of full-length sequences of toxin molecules from *A. contortrix*, *A. piscivorus*, and *A. bilineatus* venoms, including snake venom metalloproteinases of classes PI (B7U492, P28891, Q92031, Q92032), PII (P0C6E3, Q805F4, C9E1S1, Q805F6, Q9IAB0), and PIII (C9E1S0, O42138); RDG-disintegrin molecules (Q805F5, P16338, C9E1S2, Q805F7); protein C activator (P09872) and thrombin-like (Q9PSN3, P82981) serine proteinases; K49 (P04361, P49121) and D49 (P51972) phospholipases A₂; vascular endothelial growth factor-like proteins (C0K3N2, C0K3N4); potential vasoactive peptides (P85025, P0C7R6), and a cysteine-rich secretory protein, CRISP (Q7ZTA0). More importantly, a holistic and comparative view of the venoms of the different clades of the genus is not available. Filling this gap was the aim of this genus-wide comparative venomomics work. Finally, following Gibbs et al. [38], we conducted a phylogeny-based comparative analysis of venom variation to assess the degree to which venom variation and phylogeny covaried. Gibbs et al. [38] found little evidence for a close association between the venom divergence and phylogeny in *Sistrurus* rattlesnakes. We conducted a similar analysis on the *Agkistrodon* taxa in this study as a test of the generality of this result for closely-related venomous snakes because *Agkistrodon* shows similar levels of phylogenetic diversity as compared to *Sistrurus* [28,39].

2. Materials and methods

2.1. Venoms

Venom from *A. c. contortrix* was a generous gift of private snake dealer César Olmos (Cullera, Valencia). We collected venoms from specimens of *A. c. laticinctus* (Tarrant County, TX), *A. c. mokasen* (Powell County, KY), *A. c. pictagaster* (Pecos County, TX), *A.*

p. leucostoma (Harris County, TX), and *A. c. conanti* (Liberty County, FLA) that were maintained in the serpentarium of the National Natural Toxins Research Center at Texas A&M University-Kingsville, USA (<http://ntrc.tamuk.edu>). Venoms from *A. c. phaeogaster* and *A. taylori* were collected from specimens caught in Cole County, Missouri (USA), and Sota La Marina, Tamaulipas (Mexico), respectively. Venom from *A. b. howardgloydi* was pooled from specimens collected in the Costa Rican Province of Guanacaste and maintained in captivity in the serpentarium of the Instituto Clodomiro Picado (University of Costa Rica). Venoms from *A. p. piscivorus* (USA) and *A. b. bilineatus* (Mexico) were purchased from Latoxan (Valence, France, <http://www.latoxan.com>).

2.2. Proteomic profiling

Venom samples (2–2.5 mg) were dissolved in 200 μ L of water containing 0.1% trifluoroacetic acid (TFA), centrifuged to remove debris, and fractionated by RP-HPLC on a C18 column (4.6 \times 250 mm, 5 μ m particle; Teknokroma) using an Agilent 1200 chromatograph. Elution was performed at 1 mL/min by applying a gradient towards solution B (acetonitrile, containing 0.1% TFA), as follows: 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min. Fractions were collected manually, dried in a vacuum centrifuge (Savant), redissolved in water, and further submitted to SDS-PAGE separation in 12% gels, under reducing conditions. After staining with Coomassie blue R-250, protein bands were excised and subjected to in-gel reduction (10 mM dithiothreitol) and alkylation (50 mM iodacetamide), followed by overnight trypsin digestion (66 ng/ μ L in 25 mM ammonium bicarbonate, 10% acetonitrile; 0.25 μ g/sample) in an automated processor (ProGest Digilab) following manufacturer's instructions. The resulting tryptic peptides were analyzed by MALDI-TOF-TOF mass spectrometry (MS) on a Proteomics Analyzer 4800-Plus instrument (Applied Biosystems). Peptides were mixed with an equal volume of saturated α -CHCA matrix (in 50% acetonitrile, 0.1% TFA), spotted (1 μ L) onto Opti-TOF 384-well plates, and dried. TOF spectra were acquired in reflector positive mode, using 1500 shots and a laser intensity of 3000. The ten most intense precursor ions were automatically selected for fragmentation and their TOF/TOF spectra were acquired using 500 shots at a laser intensity of 3900. External calibration of the instrument in each run was performed with CalMix standards (ABSciex) spotted onto the same plate. Spectra were analyzed with the aid of ProteinPilot v.4 and the Paragon algorithm (ABSciex), against the UniProt/SwissProt database, at 95% confidence, for the assignment of proteins to known families. Few peptide sequences with lower confidence scores were manually searched using BLAST (<http://blast.ncbi.nlm.nih.gov>).

Venom chromatographic peaks lacking protein bands in SDS-PAGE, but possibly containing low molecular weight peptides, were redissolved in 50% acetonitrile containing 0.1% formic acid, and loaded into metal-coated capillaries (Proxeon) for direct infusion into a nano-electrospray II ion source coupled to a QTrap 3200 mass spectrometer (Applied Biosystems). Samples were scanned in enhanced multicharge mode and selected ion precursors were fragmented for MS/MS spectra acquisition using the enhanced product ion mode and collision energies of 20–45 eV. Spectra were manually interpreted to obtain de novo amino acid sequences.

Finally, the relative abundance of each protein identified by mass spectrometry was estimated by integrating the HPLC peak signals at 215 nm using ChemStation B.04.01 (Agilent). When more than one protein band appeared in SDS-PAGE, their proportions were estimated by densitometry, using ImageLab (Bio-Rad). Protein family abundances were expressed as percentages, and represented in pie charts [40,41].

2.3. Venom activities

2.3.1. Myotoxic activity—Each venom (50 µg, in 50 µL of phosphate-buffered saline [0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2]; PBS) was injected into the gastrocnemius muscle of groups of four CD-1 mice (18–21 g body weight). A control group received an identical injection of PBS only. After 3 h, blood was collected from the tip of the tail into a heparinized capillary, centrifuged, and the plasma creatine kinase (CK) activity was determined by a commercial kinetic UV assay (CK-Nac, Biocon Diagnostik, Germany). CK activity values were expressed in Units/L. After 24 h, mice were sacrificed by CO₂ inhalation, and injected muscle samples were obtained, fixed in formaldehyde, and processed for histological evaluation of hematoxylin–eosin stained sections [42].

2.3.2. Hemorrhagic activity—Each venom (5 µg/100 µL PBS) was injected intradermally into groups of four CD-1 mice (18–21 g). Two hours later, mice were sacrificed by CO₂ inhalation, their skin was removed, and the total area of the hemorrhagic spot was drawn on a transparent film and measured in mm². Hemorrhage was expressed as the diameter of the measured area [43].

2.3.3. Phospholipase A₂ activity—Each venom (25 µg/25 µL water) was added to 200 µL of 10 mM Tris, 10 mM CaCl₂, 0.1 M NaCl, pH 8.0, followed by 25 µL of 4-nitro-3-octanoyloxy-benzoic acid substrate (1 mg/mL acetonitrile), in triplicate microplate wells [44]. After 60 min at 37 °C, absorbances were recorded at 405 nm, and PLA₂ activity was expressed as the net change in absorbance, after subtracting the values of control wells containing substrate only.

2.3.4. Proteinase activity—Each venom (20 µg/20 µL PBS) was added to 100 µL of 10 mg/mL azocasein (Sigma-Aldrich, MO, USA) dissolved in 50 mM Tris, 0.15 M NaCl, 5 mM CaCl₂, pH 8.0, in triplicate microplate wells. After 90 min of incubation at 37 °C, the reaction was stopped by the addition of 200 µL of 5% trichloroacetic acid, the mixture was centrifuged, and 150 µL of supernatant was added to 150 µL of 0.5 M NaOH. Absorbances were recorded at 490 nm. The absorbance of azocasein incubated with PBS alone was subtracted from the absorbances of venom-containing samples [45].

2.3.5. Coagulant activity—Twenty micrograms of each venom, dissolved in 100 µL PBS, was added to 200 µL of citrated human plasma, previously incubated at 37 °C, in duplicate tubes. Clotting times were recorded and, if coagulation occurred, the Minimum Coagulant Dose was estimated, corresponding to the dose that induced plasma clotting in 60 s. Tubes were observed for a maximum period of 10 min [46].

2.3.6. Phylogeny-based comparative analyses—We analyzed changes in venom in a phylogenetic context following the methods outlined by Gibbs et al. [38]. For a phylogeny, we used mtDNA cytochrome b data from Guiher and Burbrink [28] and the program BEAST [47] to generate a Bayesian Inference mtDNA-based gene tree for *A. contortrix*, *A. piscivorus*, and *A. bilineatus* subspecies with *Sistrurus miliarius* as an outgroup. Details of methods are described in [28]. A key finding of the original phylogenetic analysis was that several named subspecies within *A. contortrix* and *A. piscivorus* were not phylogenetically distinct and were assigned to the same mtDNA clade or had geographic distributions that spanned different clades. Because comparative analyses are based on comparisons among distinct taxa, we used the mtDNA clade designations described in Guiher and Burbrink [28] for our analyses and assigned individual samples to clades based on the location from which the snake was sampled. The following clades were recognized: Abi, *A. bilineatus* (*A. b. howardgloidi* and *A. b. bilineatus*); Ata, *A. taylori*; ApC, *A. piscivorus* Central mtDNA clade (*A. p. piscivorus* and *A. p. leucostoma*); ApF, *A. piscivorus* Florida mtDNA clade (*A. p. conanti*); AcE, *A. contortrix* Eastern mtDNA clade (*A. c. contortrix* and *A. c. mokasen*); AcC, *A. contortrix* Central mtDNA clade (*A. c. phaeogaster*); and AcW, *A. contortrix* Western mtDNA clade (*A. c. pictigaster* and *A. c. laticinctus*) (Figs. 1A and S1). Venom composition for each taxa was measured as the relative percent of total venom composition made up by proteins from distinct families using data from the venomics portion of this study. We averaged values for specific proteins where multiple samples existed for a single mtDNA clade and for the two samples from different subspecies of *A. bilineatus*. Percent values for individual proteins for each taxon were arcsine square root transformed to normalize the data. We also analyzed composite variables in the form of Principle Components 1 and 2 that summarized variation across all venom proteins.

We then used the subroutine multiPhylosignal in the R-package Picante [48] to determine if there was evidence for phylogenetic signal in venom composition across taxa. Phylogenetic signal refers to a tendency for evolutionary related organisms to resemble each other (pattern), with no implication as to the mechanism that might cause such resemblance (process) [49]. To estimate the amount of signal present in a given trait the multiPhylosignal program generates a K value for each trait being analyzed [49]. K can vary from 0 (no correspondence between phylogeny and trait variation) to 1 (evolution by Brownian motion, wherein trait differences are correlated with amount of phylogenetic divergence), to greater than 1 (closely related species have diverged in phenotype less than expected based on the levels of divergence). A test of whether the observed K is greater than random expectation is carried out by randomly assigning trait values to tree tips and calculating a “random” K value 1000 times and comparing the observed value to the distribution of random K values.

2.3.7. Animal ethics—The protocols of experiments performed in mice were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA), Universidad de Costa Rica.

3. Results and discussion

3.1. Venom proteome profiling across *Agkistrodon*

The venom protein compositions of 11 taxonomic lineages within the genus *Agkistrodon* were characterized by venomomics analysis, which included reverse-phase HPLC separation of venom components (Figs. 2-4), SDS-PAGE of the chromatographic fractions (insets in the corresponding panels of Figs. 2-4), and tandem mass spectrometric sequencing of tryptic peptides derived by in-gel digestion of Coomassie Blue-stained electrophoretic bands (Supplementary Tables S1-S10). With the exception of the Yucatecan cantil, *A. b. russeolus*, our proteomics survey included the venoms of all the recognized species and subspecies within *Agkistrodon*. Despite the wide variety of habitats currently occupied by the genus *Agkistrodon* over its extensive range (Fig. 1B), and the fact that all its taxa feed on many different species of prey [16,17], the venom proteomes of copperheads, cottonmouths, and cantils are remarkably similar, both in the type of toxin class present and in the relative abundance of their different toxin families (compare the pie charts in Figs. 2-4). Specifically, the venom proteomes of *Agkistrodon* taxa comprise three major toxin families, which are (in decreasing order of relative abundance): PLA₂ (38 ± 8% of the total venom proteome), SVMP (30 ± 3%; chiefly of the P-I type, although the P-II SVMP bilitoxin is abundant in the venoms of the subspecies of *A. bilineatus*), and serine proteinase (16 ± 6%). All the venoms sampled also contain a number of peptides (SVMP tripeptide inhibitors, bradykinin-inhibitory and potentiating peptides, proteolytic peptides) that account for ~5–9% of the venom proteome; L-amino acid oxidase (0.8–9%); medium-sized and dimeric disintegrin molecules (0.8–3%); and C-type lectin-like proteins (0.1–3%). CRISP molecules, the venoms (Figs. 2-4 and Supplementary Tables S1-S10). However, the apparent lack of uniformity for the presence of some of these minor venom components in the taxa here studied should be viewed with caution, as their concentrations might be close to the sensitivity limits of the venomomics methodology employed [40].

The possible evolutionary reasons for the striking uniformity in venom composition across *Agkistrodon* are unclear and are discussed below. Regardless, mapping high-resolution venomomics data onto existing phylogenetic hypothesis can yield insights into specific evolutionary innovations in the venom arsenal that define specific groups of venomous snakes. In particular, a phylogenetic-based analysis of venomomics data that includes both New World and Old World species can be used to assess major changes in venom composition along the branch leading to New World pit vipers. Unfortunately, proteomics data on venoms from the major sister clades of Old World pit viper genera are still scarce and in most cases fragmentary[50-53], thus precluding a deep venomomics-based comparative analysis. On the other hand, our venomomics data across *Agkistrodon* lay the foundations for identifying the origin of toxin innovations evolved in derived genera, such as crotamine [P01475] [54], myotoxin a [P01476] [55,56], Mojavetoxin [P18998, P62023]/crotoxin [P08878, P62022] [57-60], and the multi-BPP precursor protein [Q27J49] [61-63], which are presently known only in venoms of New World pit vipers.

3.2. *Agkistrodon* venoms have similarities and differences in their toxic and enzymatic profiles

Fig. 5 depicts the analysis of proteolytic, PLA₂, hemorrhagic and myotoxic activities of the venoms of species and subspecies of *Agkistrodon* whose proteomes were studied. The venoms from all species and subspecies induced these four effects, albeit with different potencies; in general, venoms showed relatively similar proteolytic and PLA₂ activities (Fig. 5, panels A and B). In contrast, evident quantitative differences were observed regarding hemorrhagic and myotoxic activities in mice. The venom of *A. bilineatus* from Costa Rica and Guatemala exhibited the highest hemorrhagic activity (Figs. 5 and 6). This agrees with previous observations indicating that the venoms of *A. bilineatus* from Costa Rica and Guatemala have some of the highest hemorrhagic activities among Central American viperid venoms [33,35]. Our observations on hemorrhagic activity are also in agreement with the Minimum Hemorrhagic Doses of the venoms of *A. b. howardgloydi*, *A. piscivorus*, and *A. contortrix*, which are 0.12 µg, 2.8 µg, and 6.4 µg, respectively [33,36]. Moreover, the mouse Median Lethal Doses (LD₅₀) of the venoms of these species, as estimated by the intraperitoneal route, are 1.25 µg/g (*A. b. howardgloydi*), 6.24 µg/g (*A. piscivorus*), and 9.17 µg/g (*A. contortrix*) [33,36]. Although further detailed composition–activity correlation studies should provide the molecular basis of such difference in toxicity, we hypothesize that the higher relative abundance of PII and PIII-SVMP, as compared to PI-SVMP, determines the hemorrhagic and toxic potencies of the venoms [64]. In particular, the potent dimeric P-II hemorrhagic SVMP, bilitoxin-I, identified in the venoms of *A. b. howardgloydi* (fractions 24–26 in Fig. 3C and Table S7) and *A. b. bilineatus* [65,66], possibly plays a relevant role in the overall toxicity.

Regarding myotoxicity, highest activity was observed with the venoms of *A. b. bilineatus*, followed by *A. p. piscivorus*, whereas the venoms of *A. c. contortrix* and *A. p. leucostoma* induced the lowest myotoxic activity (Fig. 5D). Histological assessment demonstrated a widespread myonecrosis in the gastrocnemius muscle after injection of the venom of *A. p. leucostoma* (Fig. 6). Myotoxic activity of viperid venoms is largely due to the direct action of basic Asp49 PLA₂s and Lys49 PLA₂ homologues [67-69]. These toxins exert myotoxicity by directly damaging the integrity of the skeletal muscle cell plasma membrane, either by enzymatic degradation of phospholipids, in the case of Asp49 PLA₂s or by a catalytically-independent membrane perturbation, in the case of Lys49 PLA₂ homologues [68-71]. The majority of the venoms studied presented conspicuous peaks in the HPLC profile which correspond to Asp49 PLA₂s or Lys49 PLA₂ homologues, thus constituting rich sources of myotoxic proteins. Myotoxic PLA₂s and PLA₂ homologues have been isolated from the venoms of *A. p. piscivorus* [72], *A. c. laticinctus* [73], *A. bilineatus*, *A. c. contortrix*, *A. c. mokasen*, and *A. p. piscivorus* [74]. The lack of correlation between PLA₂ activity and myotoxicity in these venoms can be explained on the grounds that enzymatically-inactive Lys49 PLA₂ homologues are responsible for a high extent of the myotoxic action, and also because it is likely that these venoms contain acidic PLA₂s, which are largely devoid of myotoxicity [75].

Significant differences were observed in the myotoxic and hemorrhagic activities of the various subspecies of *A. contortrix* and of *A. piscivorus*, thus highlighting variability in

toxicity not only between but also within species. On the other hand, when tested at a dose of 20 µg, none of the venoms induced coagulation of human plasma within 10 min at 37 °C. This agrees with previous observations reporting lack of coagulant activity in the venoms of *A. contortrix* and *A. piscivorus* [36]. The presence of serine proteinases in these venoms, as shown by proteomic analysis, in the context of lack of coagulant activity, strongly suggests that these enzymes do not have thrombin-like activity. Snake venom serine proteinases display a wide pharmacological spectrum since, in addition to thrombin-like activity, they cause increases in vascular permeability and platelet aggregation, among other effects [76]. The toxicity of serine proteinases present in *Agkistrodon* venoms deserves further investigation. On the other hand, the potential toxicological role of less abundant components in these venoms, such as small peptides, L-amino acid oxidase, disintegrins, C-type lectin-like proteins and CRISPs is likely to be minor.

Taken together, our toxicological analysis indicates that *Agkistrodon* venoms induce significant local tissue damage, i.e. edema, hemorrhage and myonecrosis. This is in agreement with clinical observations of envenomings by *Agkistrodon* in the USA, where the main clinical manifestations involve local effects, associated with permanent limb dysfunction in some cases, whereas systemic alterations are largely absent [77,78]. In contrast, owing to the potent hemorrhagic activity and high lethal potency of venoms of *A. bilineatus*, systemic bleeding in envenomings by this species may occur, together with prominent local pathological alterations. Indeed, severe cases inflicted by *A. bilineatus*, involving systemic bleeding and local tissue damage, have been reported in Central America [79].

3.3. Comparative analysis and possible evolutionary causes of venom variation in *Agkistrodon*

The most striking finding of our study is that venom variation is limited across different *Agkistrodon*. This is unusual in that variation in venom composition at different levels is widespread [80,81], and has long been argued to represent a trophic adaptive trait [82-84]. Other have proposed that, due to the high toxicity and large doses of venom that are injected, much of the compositional venom variation may be largely a by-product of neutral evolutionary processes [85-87], and as consequence interspecific differences in venom variation could covary with phylogenetic divergence. However, a recent study [38] showed no evidence of significant phylogenetic signal in the relative abundances of major venom proteins across *Sistrurus* species. This suggests that venom variation among these closely-related rattlesnakes is an evolutionary highly labile trait, and that natural selection acting through diet variation may have played a role in molding the relative abundance of specific venom proteins in this group of snakes.

In a neutral evolutionary scenario, interspecific differences in venom composition should be closely related to the degree of phylogenetic divergence between species. As outlined in [38], Blomberg et al.'s K-statistics [49] is a measure of the amount of phylogenetic signal for any trait in any tree. Here we estimated K for proteomics data for all clades of genus *Agkistrodon* using *Sistrurus* sp. as outgroup. K values for seven classes of snake venom proteins as well as for the composite variables PC1 and 2 (see Table 1 for factor loadings of

individual proteins on each PC variable) ranged from 0.19 (CRISP) to 0.92 (PEP) with a mean K value of 0.50 ± 0.28 (Table 1). None of the K values were significantly different from zero (all $P > 0.06$). The small number of taxa ($n = 7$) included in this analysis likely plays an important role in the non-significance of individual K values. A K less than one implies that relatives resemble each other less than expected under Brownian motion evolution along the specified topology and branch lengths of a phylogenetic tree. This could be caused by departure from Brownian motion evolution, such as when adaptation to a particular environmental factor occurs in some but not all members of a set of species. In any case, our results provide evidence consistent with the emerging view that venom is a relatively labile evolutionary trait since K is substantially less than 1, which is the predicted value if divergence in venom matched divergence in phylogeny [38]. Nonetheless, a comparison of K values between *Agkistrodon* (this study) and *Sistrurus* [38] argues that the phylogenetic signal in venom variation across taxa is higher in *Agkistrodon*, and this is consistent with an increased similarity in venom composition among members of this genus. Based on a protein-by-protein comparison, K values for *Agkistrodon* are significantly greater than those for *Sistrurus* (Wilcoxon signed rank test, $P = 0.009$) with the mean K value for proteins in *Agkistrodon* (0.50) being almost five times greater than the mean K for *Sistrurus* venom proteins (0.11). Unlike *Sistrurus*, the *Agkistrodon* K values are similar in magnitude to those found for a wide range of behavioral, morphological, reproductive and physiological traits in other species [49].

The reason for the increased covariation between phylogeny and venom variation in *Agkistrodon* relative to *Sistrurus* is unclear. Increased phylogenetic similarity among taxa within *Agkistrodon* does not explain this pattern because diversification within each group has occurred over a similar time period (<15 Mya) [28,39] and a number of internal nodes have similar divergence dates. One untested possibility is that diet varies less across different *Agkistrodon* taxa and so the increased similarity in venom is more a reflection of less intense similar selection pressures acting on venom variation in *Agkistrodon* than a direct effect of phylogenetic similarity. However, this relies on the assumption that diet variation drives evolutionary changes in venom composition for which there is mixed evidence (see Gibbs et al. [38] for recent discussion). This hypothesis could be tested by estimating the degree to which positive selection acts on homologous genes coding for venom proteins across different *Agkistrodon* and *Sistrurus*. The prediction is that if this hypothesis is true then the magnitude of positive selection should be substantially less among *Agkistrodon*. As a consequence, neutral processes such as drift, the magnitude of which are related to phylogenetic divergence, would play a greater role in causing changes in venom composition accounting for the relatively higher K values for *Agkistrodon*. Regardless of the cause, the difference between *Agkistrodon* and *Sistrurus* demonstrates that the degree to which venom composition is evolutionarily labile varies among clades of closely-related venomous snakes.

4. Concluding remarks and perspectives

Here we report a genus-wide proteomics survey across *Agkistrodon*. The only *Agkistrodon* taxon that could not be analyzed is the Yucatecan cantil, *A. b. russeolus*. Among the subspecies of *A. bilineatus*, *A. howardgloydi* and *A. russeolus* are the more closely related

[17], even though they are currently widely separated geographically (Fig. 1B). A former, much broader range of the *russeolus*–*howardgloydi* complex has been proposed, suggesting that the present distribution is a relictual pattern due to changing environmental conditions during the Pleistocene [17]. Although it is tempting to hypothesize that *A. b. howardgloydi* and *A. b. russeolus* may express very similar venoms, how vicariance has shaped the composition of *A. b. russeolus* venom requires detailed venomomics analysis. The presence of abundant Asp49 PLA₂s and Lys49 PLA₂ homologues, together with SVMs, explains a toxicological profile characterized by myotoxic and hemorrhagic activities, and the lack of coagulant activity in *Agkistrodon* venoms. The higher toxicity in the venoms of subspecies of *A. bilineatus* is likely to depend on the action of highly hemorrhagic SVMs. The close similarity across *Agkistrodon*, in the type and relative abundance of their different toxin families, and the qualitative pharmacological profile of their venoms, is also reflected in the increased co-variation between phylogeny and venom composition among the closely-related *Agkistrodon* ($K = 0.5$) relative to the more labile *Sistrurus* clade ($K = 0.1$). This could mean that neutral evolutionary processes play a greater role in determining venom variation in *Agkistrodon* or that the generalized diet of most species results in less variable selection pressures on venom genes in *Agkistrodon* compared to *Sistrurus*. Although tests for phylogenetic signal do not offer any formal insight as to how evolution may have deviated from stochastic variation, the low amount of signal in *Sistrurus* may suggest a high rate of evolution through key innovations [88] that allow different *Sistrurus* to consume a variety of distinct prey, whereas in *Agkistrodon* the uniformity of diet would mean that such innovations have not arisen. In terms of practical applications of our results, while the low correlation between phylogeny and venom variation in *Sistrurus* argues against phylogeny as the sole criterion for selecting a venom mixture in antivenom production strategies [89,90], the remarkably similar structural and functional profiles across *Agkistrodon* venoms suggest that monospecific antivenoms may exhibit a high degree of paraspecificity against the other congeneric species. Comparative antivenomics studies are underway in our laboratories to assess this possibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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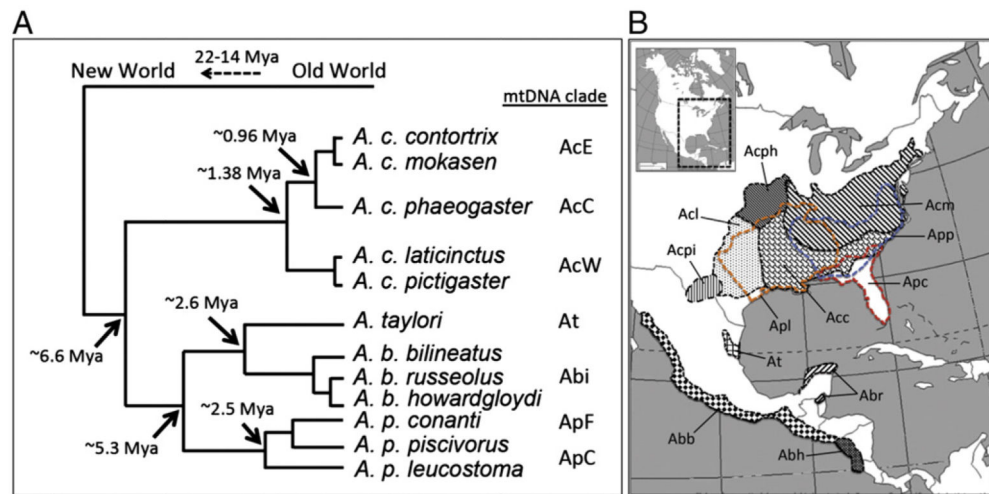
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**Fig. 1.**

Cladogenesis and present distribution of *Agkistrodon*. Panel A, Proposed cladogenesis of genus *Agkistrodon* following the Holoartic dispersal of an ancestral Asian lineage into the New World across Beringia (adapted from [7,10,24,28,30]), showing phylogenetic relationships and approximate dates of divergence for all *Agkistrodon* taxa. mtDNA clades [28] used for phylogeny-based comparative analyses of venom variation are indicated at the right. Panel B, approximate ranges of the different general taxa of genus *Agkistrodon* in North and Middle America (adapted from [16,17]; consult also <http://ntrc.tamuk.edu/distribution.html>): *A. c. laticinctus* (Acl) ranges from south-central Texas, north through central Oklahoma to the extreme south of Cowley County in Texas; *A. c. pictigaster* (Acpi) is found in western Texas and in northern Chihuahua and Coahuila (Mexico); *A. c. phaeogaster* (Acph) occurs in eastern Kansas, extreme southeastern Nebraska and a large part of Missouri; *A. c. contortrix* (Acc) occurs in the lower Mississippi Valley and the states bordering the Gulf of Mexico, from eastern Texas and southeastern Oklahoma to southern Illinois, and from the Florida panhandle north to Massachusetts and west to Nebraska to South Carolina; *A. c. mokasen* (Acm) is distributed in southern Illinois, extreme northeastern Mississippi, northern Alabama, northern Georgia, and the Appalachian Mountain region and associated plateaus. The range of *A. p. conanti* (Apc) includes extreme southern Georgia and virtually all of the states of Florida, including many of the islands off the coast; *A. p. piscivorus* (App) ranges in southeastern Virginia, the Atlantic Coastal Plain and lower Piedmont of North and South Carolina; *A. p. leucostoma* (Apl) occurs from southern Alabama along coast of the Gulf of Mexico, including many offshore islands, to southeastern and central Texas, and north to Oklahoma, Missouri, Illinois and Indiana. *A. taylori* (At) is restricted to the Mexican northeastern states of Nuevo Leon, San Luis Potosí and Tamaulipas; *A. b. bilineatus* (Abb) inhabits the Pacific Coastal Plain from southern Sonora in Mexico, southeast to Guatemala and El Salvador. It also occurs in the Mexican state of Morelos; *A. b. russeolus* (Abr) is found in the northern arid portions of the Yucatan Peninsula (Mexico) and in northern Belize; *A. b. howardgloydi* (Abh) occupies the dry Pacific tropical lowlands from the Golfo de Fonseca in Honduras to northwestern Costa Rica, Guanacaste province.

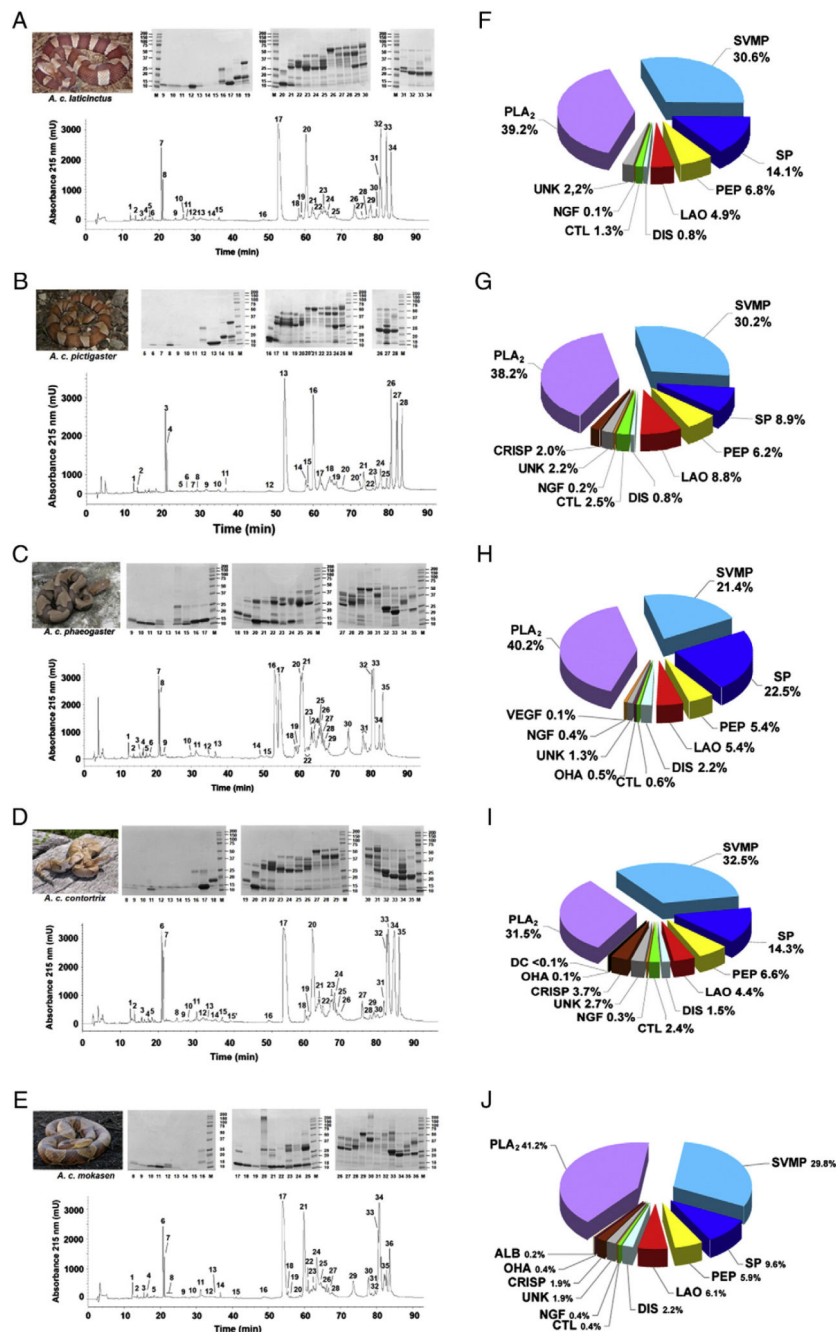


Fig. 2. Venomics of copperhead subspecies. The venom proteins of *A. c. laticinctus*, broad-banded copperhead (panel A), picture, ©2000 John White); *A. c. pictigaster*, Trans-Pecos copperhead (panel B); *A. c. phaeogaster*, Osage copperhead (panel C), picture, ©2009 Mike Pingleton); *A. c. contortrix*, southern copperhead (panel D); and *A. c. mokasen*, northern copperhead (panel E) were fractionated on a C₁₈ column as described in the Materials and methods section. HPLC fractions were collected manually and analyzed by SDS-PAGE (insets) under reducing conditions). Protein bands excised from Coomassie-stained SDS-

polyacrylamide gels were identified by tryptic peptide mass fingerprinting and CID-MS/MS. The peptide sequencing results are listed in Supplementary Tables S1–S5. Panels F–J display pie charts showing the relative occurrence of proteins from different toxin families in the venoms of copperheads fractionated in panels A–E, respectively. SVMP, snake venom Zn²⁺-metalloproteinase; SP, serine proteinase; PEP, peptides (including tripeptide SVMP inhibitors and bradykinin-potentiating and bradykinin-inhibitory peptides); LAO, L-amino acid oxidase; DIS, disintegrin; CTL, C-type lectin-like proteins; NGF, nerve growth factor; UNK, unknown; CRISP, cysteine-rich secretory protein; PLA₂, phospholipase A₂; OHA, ohanin-like; VEGF, vascular endothelial growth factor; DC, disintegrin-like/cysteine-rich fragment; ALB, albumin.

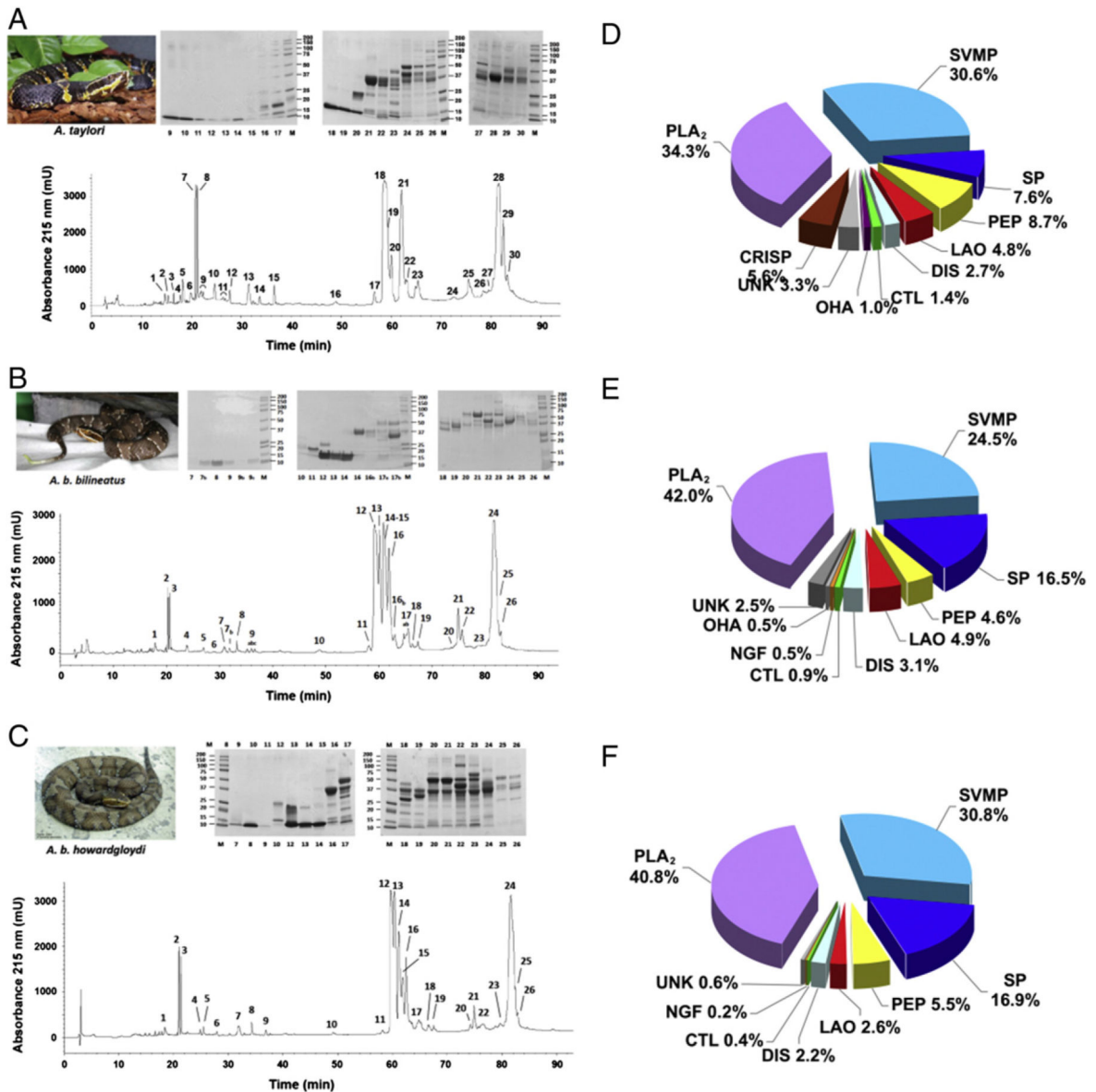


Fig. 3. Venomics of Mesoamerican cantils. The venom proteins of *A. taylori*, Taylor's cantil (panel A); *A. b. bilineatus*, common or Mexican cantil (panel B); and *A. b. howardgloydi*, Castellana or Yucatecan cantil, (panel C) were fractionated and analyzed as described in Fig. 2. The results are listed in Supplementary Tables S6-S7. Panels D–F display pie charts showing the relative occurrence of proteins from different toxin families in the venoms of cantils fractionated in panels A–C, respectively. Acronyms as in the legend of Fig. 2.

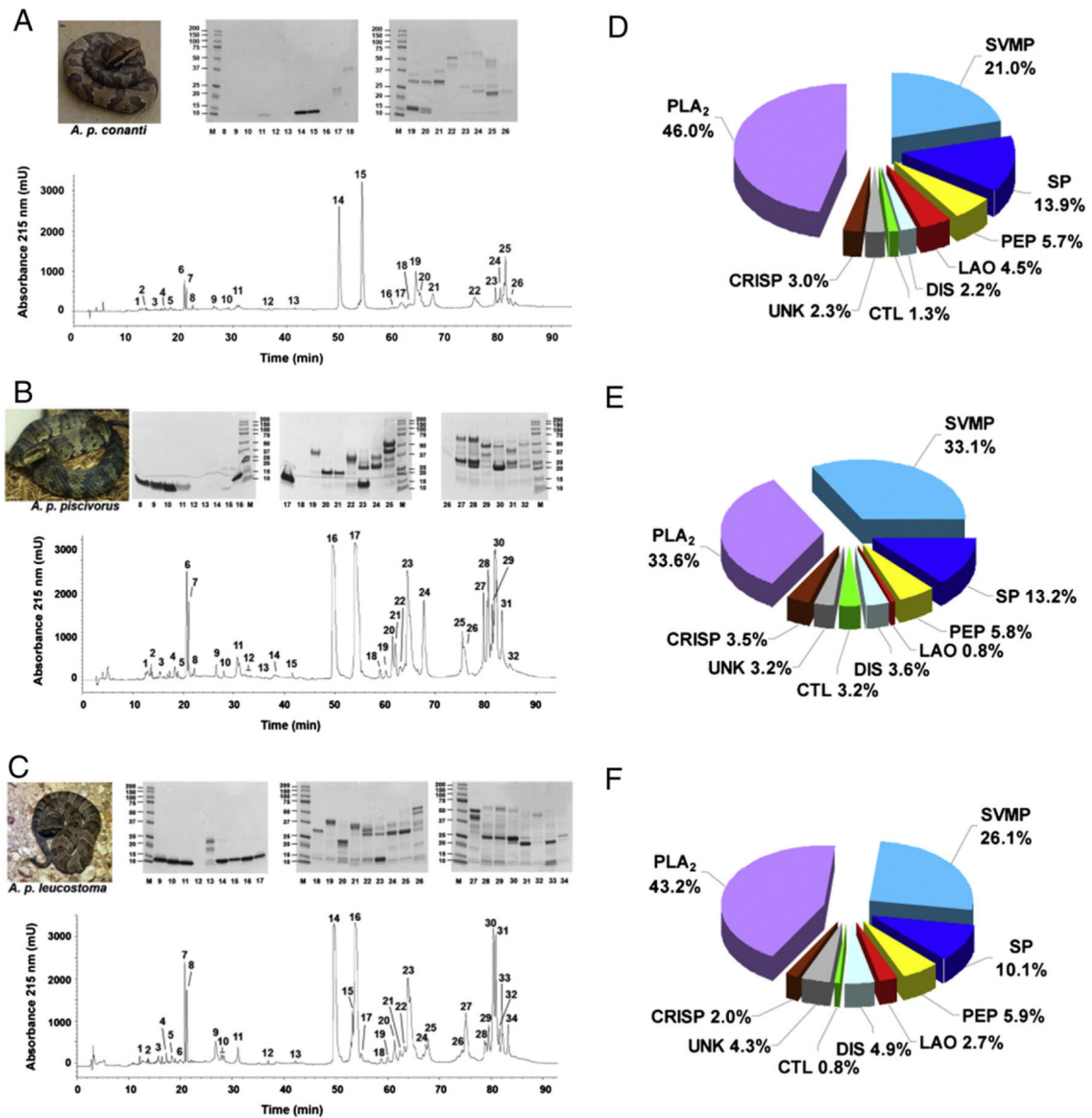


Fig. 4. Venomics of cottonmouth subspecies. The venom proteins of *A. p. conanti*, Florida cottonmouth, (panel A); *A. p. leucostoma*, western cottonmouth, (panel B); and *A. p. piscivorus*, eastern cottonmouth, (panel C) were fractionated and analyzed as described in the legend of Fig. 2. The results are listed in Supplementary Tables S8–S10. Panels D–F display pie charts showing the relative occurrence of proteins from different toxin families in the venoms of cottonmouths fractionated in panels A–C, respectively. Acronyms as in the legend of Fig. 2.

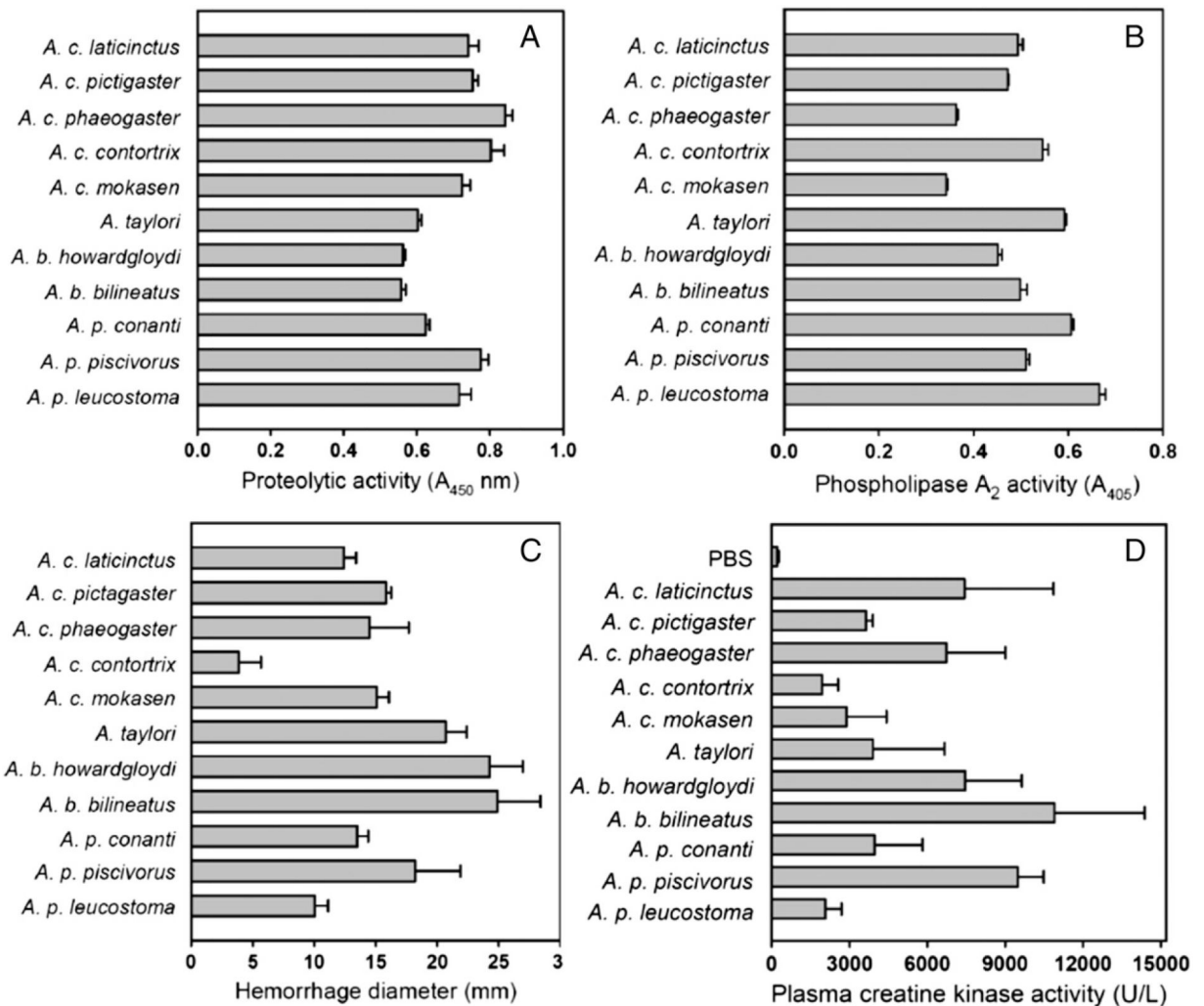


Fig. 5. Enzymatic and toxic activities of venoms of *Agkistrodon* species and subspecies. Venoms were compared for proteinase activity on azocasein (panel A), PLA₂ activity (panel B), hemorrhagic activity in mice (panel C), and myotoxicity in mice (panel D). Details of experimental protocols are included in the Materials and methods section. Results are presented as mean \pm S.D. ($n = 3$ for proteinase and PLA₂ activities and $n = 4$ for hemorrhagic and myotoxic activities).

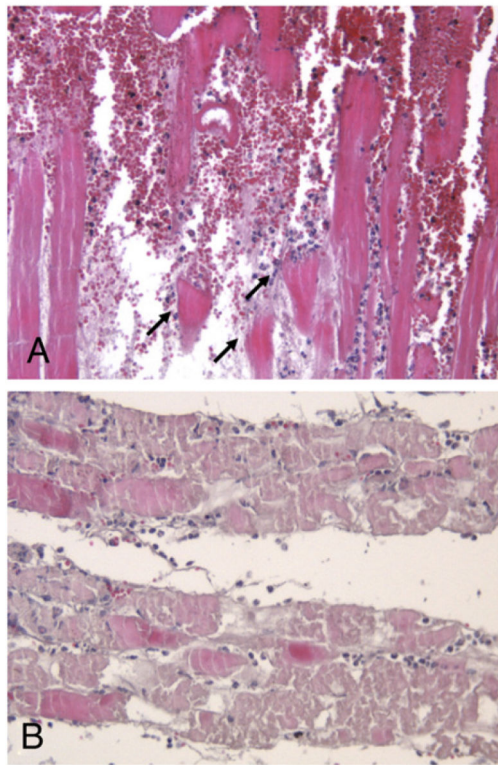


Fig. 6. Light micrographs of sections of skeletal muscle tissue after injection of *Agkistrodon* venoms. Mice received an intramuscular injection, in the gastrocnemius, of 50 μ g of venoms of either *A. b. howardgloydi* (A) or *A. p. leucostoma* (B). After 24 h, mice were euthanized, and a sample of injected muscle was obtained and processed for embedding in paraffin. Sections were then stained with hematoxylin–eosin. Prominent hemorrhage is observed in panel A, as evidenced by abundant erythrocytes located between muscle fibers; in addition, the occurrence of few necrotic fibers is indicated by arrows. Widespread myonecrosis is observed in panel B, together with an inflammatory infiltrate. Micrographs were taken at a magnification of 200 \times .

Table 1

Measures of phylogenetic signal (estimated as K values) for venom across *Agkistrodon* taxa defined by molecular phylogenetic analysis as shown in Fig. S1. P-value represents the probability that the observed value is greater than the expected value if trait values were randomly assigned to tip taxa. Acronyms for venom proteins are as in the legend of Fig. 2. PC 1 and 2 — PC scores for composite venom variables. PC 1 and 2 explained 53% and 31% of total variation in venom protein abundance, respectively, and had these variable loadings (PC1: DISI (0.12); LAO (−0.34); CRISP (0.33); PLA2 (−0.51); SP (−0.15); SVMP (0.69); PEP (−0.10); PC2: DISI (0.58); LAO (−0.17); CRISP (−0.69); PLA2 (−0.16); SP (0.55); SVMP (0.21); CTL (−0.40); PEP (−0.27).

Venom proteins	K-value	P-value	K-value (<i>Sistrurus</i>) ^a
DISI	0.37	0.30	0.052
LAO	0.93	0.06	0.26
CRISP	0.193	0.65	0.04
PLA ₂	0.524	0.24	0.08
SP	0.204	0.63	0.07
SVMP	0.709	0.17	0.11
CTL	0.223	0.59	n/a
PEP	0.922	0.07	0.15
PC 1 — venom	0.508	0.29	0.09
PC 2 — venom	0.310	0.34	0.12

^aK-values for *Sistrurus* are from Gibbs et al. [38].