Review

Proteomics in Venom Research: a Focus on PLA_2 Molecules

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Dedicated to the memory of Professor Franc Gubenšek

Abstract

This paper focuses on the application of proteomic tools to study the composition and natural history of snake venoms, and their crossreactivity with current homologous and heterologous antivenoms. Proteomic analyses on *Bothrops* indicated the suitability of using PLA_2 molecules as taxonomic and population-specific markers. The lack of phylogenetic clustering among Neotropical and Neartic rattlesnakes with neurotoxic PLA_2 molecules in their venoms suggests that phylogeny may not be an important consideration in venom evolution. Proteomic-guided identification of evolutionary and immunological trends among venoms may aid replacing the traditional geographic- and phylogenetic-driven hypotheses for antivenom production strategies by a more rationale approach based on venom proteome phenotyping and immunological profile similarities. Recent proteomic and antivenomic surveys on *Bothrops*, *Crotalus*, and *Bothriechis* illustrate the feasibility of this view.

Keywords: Snake venomics, mass spectrometry, venom toxins, PLA_2 molecule, taxonomic marker, population marker

1. Introduction

Venom represents an adaptive trophic trait that allowed snakes to transition from a mechanical (constriction) to a chemical means of subduing and digesting prey. Venoms comprise unique mixtures of deadly toxins tailored by Natural Selection in a prey-predator co-evolutionary arms race.[1] Venoms also represent a sophisticated natural source of chemical and pharmacological novelty. The parallelism between the biological systems impaired by envenomation and dysregulated in certain pathological conditions, such as hypertension, thrombosis, ischemia, chronic pain, type-II diabetes, tumor angiogenesis, etc., has opened up new exciting opportunities for the modern pharmaceutical industry for the development of new bioactive compounds for innovative therapeutic intervention based on venom toxin activities.[2–4] While research on venoms ("venomics") has emerged as a discovery science, snake envenoming constitutes a highly relevant public health issue in many tropical and subtropical countries,[5,6] and has been recognised by the World Health Organization as a "neglected tropical disease" (http://www.who.int/neglected_diseases/diseases_en). Adequate treatment of envenoming is critically dependent on the ability of antivenoms to neutralize the symptoms of systemic envenoming.[7] A robust knowledge of the toxin composition and pathophysiological activities of venom proteomes is instrumental for the treatment of envenomed victims and for the selection of specimens for the generation of improved antidotes.[6,8]

Research on venoms has been continuously enhanced by advances in technology. Technological developments in classical protein chemistry and molecular biology protocols, such as high-performance protein separation techniques (i.e., reverse-phase HPLC and two-dimensional electrophoresis using isoelectric focusing in immobilized pH-gradients) and high-sensitivity protein and DNA automated sequencers, catalyzed an explosion of knowledge on structure-function correlations of individual toxins during the last quarter of the 20th century. The emergence of "omic" technologies (genomics, transcriptomics, and proteomics) in the field of toxinology at the

turn of the 21st century offered the unprecedented possibility to explore global biological trends and expand our understanding of the clinical correlation of the global toxin composition of venoms.[9] In particular, the development of hyphenated mass spectrometric techniques has been crucial for unraveling the complexity of venoms.[10,11] Our approaches using proteomic tools ("*venomics*", "*antivenomics*", and "*venom phenotyping*") to study the composition and natural history of snake venoms, and the crossreactivity of antivenoms with homologous and heterologous venoms, has been extensively reviewed[8,9,12–14] and will not be repeated here. The focus of this paper is the application of mass spectrometry-based phospholipase A_2 (PLA_2) profiling. PLA_2 molecules are among the most common and abundant toxins in viperid and elapid snake venoms.[15] The adaptive role PLA_2s play in the radiation of venomous snakes is highlighted by the demonstration of this multigene toxin family being subjected to neofunctionalization through accelerated evolution.[16–21] Gene gain and loss and protein sequence evolution via positive selection are important evolutionary forces driving adaptive divergence in venom proteins in closely related species of venomous snakes.[21] A goal of snake venomics of applied importance in human therapy is to understand the molecular mechanisms and evolutionary forces that underlie venom variation.[22–24] A comprehensive understanding of the evolutionary diversification of venoms may aid in taxonomy.[25–27] In addition, a robust knowledge of the onset of ontogenetic, individual, and geographic venom variability may have an impact in the treatment of envenomed victims and in the selection of specimens for the generation of improved antidotes.[6,8,28] The following examples will illustrate these views.

1. 1. A PLA_2 Molecule as a Taxonomy Signature of *B. Fonsecai*

The genus Bothrops (subfamily Crotalinae of Viperidae) comprises 32 (http://www.reptile-database.org) or 37 species[29] of primarily South and Central American pitvipers, commonly referred as lanceheads. Except for southwestern South America, the extreme highlands of the Andes, and southernmost Patagonia, this genus is widely distributed in tropical Latin America, from northeastern Mexico to Argentina, and the southern parts of the lower Caribbean islands.[29] *Bothrops* are diverse in their morphology and natural history, and represent a particularly interesting group because of the wide array of bioto-

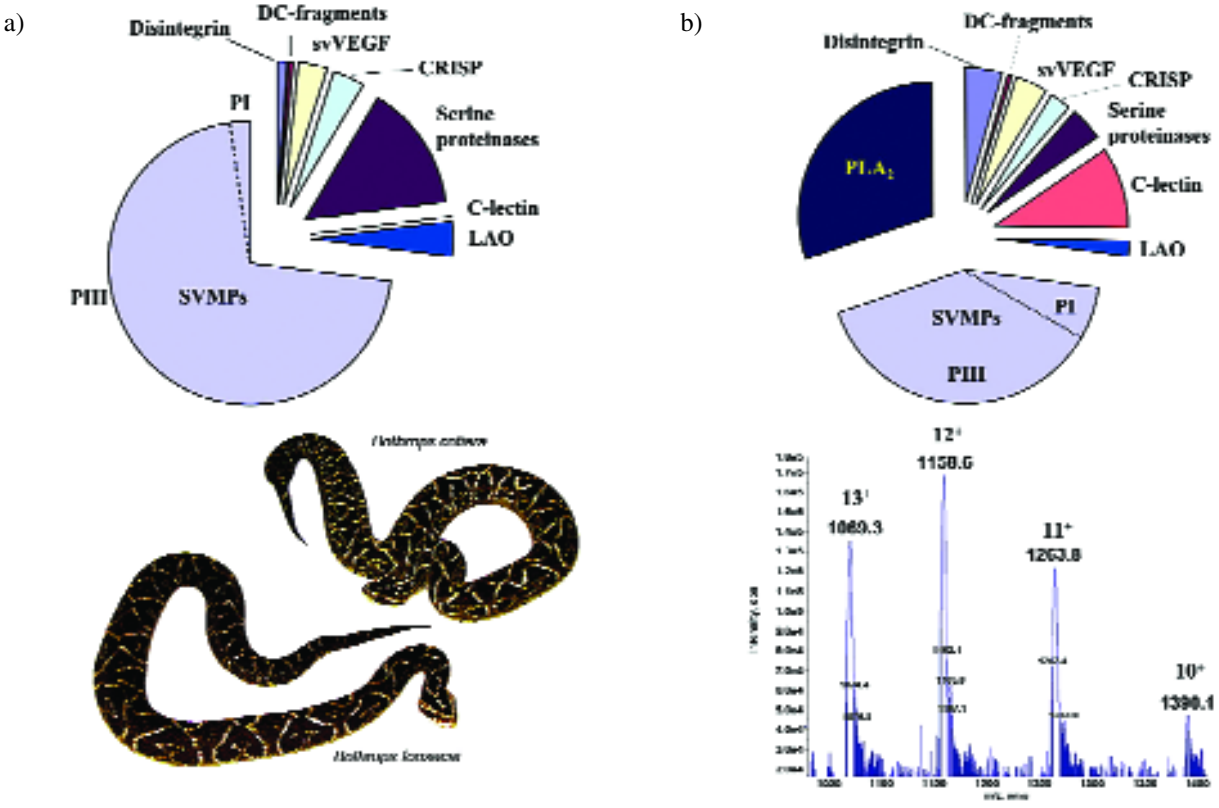

Figure 1. The venom proteomes of *B. cotiara* (**a**) and *B. fonsecai* (**b**). Both species are moderately heavy-bodied snake (adult length usually 0. 7–1. 0 m) that inhabit very specialized biotopes (*Araucaria angustifolia* pine forests on highlands). Although *B. cotiara* and *B. fonsecai* are not sympatric, they are morphologically extremely similar (lower left panel). A comparative proteomic analysis[26] showed that compositional differences between their venoms, particularly the unique presence in *B. fonsecai* venom of a G6-D49-PLA_2 molecule of isotope-averaged molecular mass 13980 Da (lower right panel) and N-terminal sequence NLWQFGMMIQHTTRENPLFKYFSYGCYCGWGGGGPLDATDRCCFVHDCCYG can be employed as a taxonomic signature for unambiguous species identification independently of geographic origin and morphological characteristics.

pes they inhabit, such as lowland evergreen forests, montane semideciduous forests, savannas, and montane open formations. *B. cotiara* and *B. fonsecai* inhabit similar, highly specialized habitats (*Araucaria angustifolia* pine forests), in different geographical regions of Brazil. *Bothrops cotiara*'s habitat include the Araucaria forests of southern Brazil in the states of São Paulo, Paraná, Santa Catarina and Rio Grande do Sul. It is also found sporadically in northeastern Argentina in the province of Misiones, with a vertical distribution from sea level to at least 1,800 m. *B. fonsecai* is endemic to Southeastern Brazil (northeastern São Paulo, southern Rio de Janeiro and extreme southern Minas Gerais). Its elevational distribution ranges from 1000 to 1600 m. Both species are mammal specialists and are morphologically very difficult to distinguish. A comparative proteomic analysis shows the overall composition of *B. cotiara* and *B. fonsecai* venoms highlighting compositional differences. In particular, *B. fonsecai* expresses a high abundance PLA_2 molecule (13890 Da) whereas *B. cotiara´*s venom is devoid of PLA_2 proteins. The absence of PLA_2 proteins is a unique feature among all viperid venoms characterized to date and defines a taxonomy signature that can be employed for the unambiguous differentiation of *B. cotiara* and *B. fonsecai* independently of geographical and morphological factors.

1. 2. Population-specific PLA_2 Molecules Provide Clues to Trace the Dispersal Pattern of *B. atrox* in Northern South America

Bothrops atrox (Viperidae: Crotalinae), the Common Lancehead, is a terrestrial, generally nocturnal, and highly adaptable pitviper found in tropical lowlands and rainforest up to 1200 m of northern South America east of the Andes, including southern and eastern Venezuela, southeastern Colombia, eastern Ecuador, eastern Perú, northern Bolivia, the northern half of Brazil, and throughout Guyana, Suriname, and French Guiana.[29] Despite its wide range of ecological and geographical habitats, no subspecies are currently recognized.[28] *B. atrox* is a very dangerous species being notorious as the leading cause of more human fatalities than any other South American reptile.[30] Comparative proteomic investigations of *B. atrox* venoms from 19 localities from Colombia, Ecuador, Perú, Venezuela, and Brazil (highlighted in Figure 2)[31,32] showed that PLA_2 molecules exhibit large interpopulational

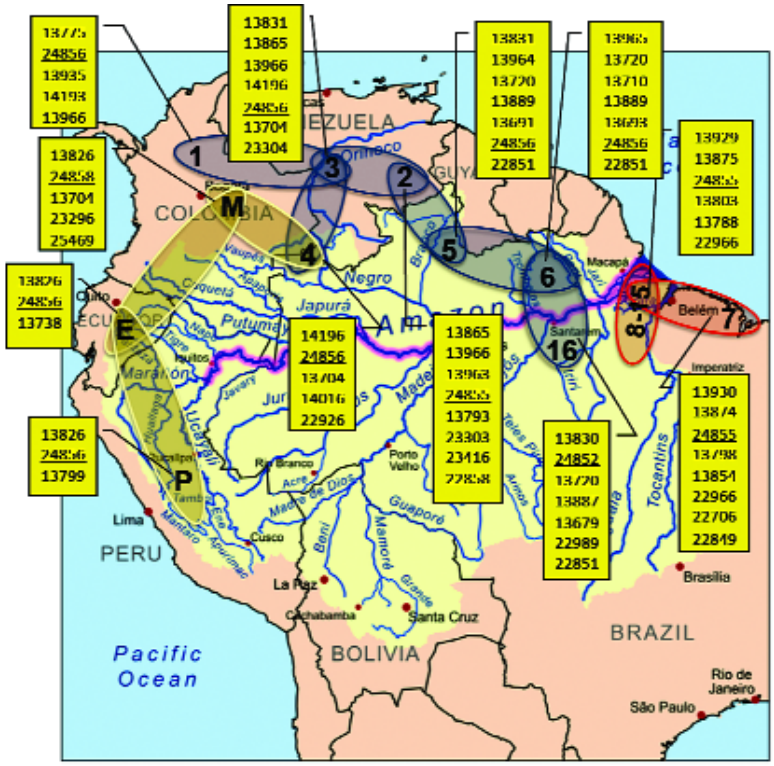

Figure 2. Physical map of northern South America highlighting the Amazon River basin and the sampling localities for the *B. atrox* venoms investigated by Núñez *et al.*[31] and Calvete and co-workers,[32] including specimens from Colombia: Meta (M), Magdalena Medio (Antioquia Department) (1); Ecuador (E), Perú (P), Venezuela: El Paují (2) (Orinoquia) and Puerto Ayacucho (3) (Amazonia); and Brazil: São Gabriel de Cachoeira (4) and Presidente Figueiredo (5) (Amazonas), São Bento (7) (Maranhão), and and Monte Alegre (6), Ananindeua (8), Santa Isabel (9), Tucuruí (10), Icoaraci (11), Barcarena (12), Acara (13), Belém (14), Ilha de Mosqueiro (15), and Santarém (16) (Pará). The isotope-averaged molecular masses of PLA_2 molecules (13–15 kDa), a conserved CRISP protein (24854 ± 2 Da), and PI-SVMPs (22–25 kDa) found in the different venoms are indicated. Neighbour populations sharing identical toxins are grouped by ovals.

variation, with the venoms from neighboring locations expressing common and variable molecules (Figure 2). This pattern of geographic intraspecific variability of PLA_2 loci has been reported in other species, i.e. *Vipera palestinae*,[33] *B. asper*,[34] *Trimeresurus* (*Protobothrops*) *flavoviridis*,[35–37] and *Lachesis muta*.[38] This phenomenon is often linked to differences in diet among populations.[39] Snake venom PLA_2 genes are members of a large, rapidly-evolving multigene family with many diverse functions.[15,20,21] Positive Darwinian selection is common in group-II viperid snake venom PLA_2 genes and is associated with the evolution of new toxin functions and speciation events,[20] suggesting adaptation of the PLA_2 arsenal to novel prey species after niche shifts. Mapping the molecular diversity between conspecific populations onto a physical map (Figure 2) provides clues for tracing dispersal routes that account for the current biogeographic distribution of the species. The emerging phylogeographic hypothesis summarized in Figure 2 is consistent with an intricate model of southeast and southwest dispersal and allopatric fragmentation northern of the Amazon Basin, and trans-Amazonian expansion through the Andean Corridor and across the Amazon river. On the other hand, venoms from São Bento (Maranhão State), and Ananindeua, Santa Isabel, Tucuruí, Icoaraci, Barcarena, Acara, Belém, and Ilha de Mosqueiro (Pará State), located south of the mouth of the Amazon river (Figure 2), share two PLA_2 molecules (13930 and 13875 Da), but have no common molecules with the venoms from specimens inhabiting regions north of the Amazon river. These populations may have been established in Pará and Maranhão by ancient vicariance of a *B. atrox* population which managed to cross the Amazon river or from a dispersal event involving *B. atrox* populations not sampled in the proteomic survey.

Venoms from snakes collected northern of the Amazon Basin (Magdalena Medio Valley and Orinoquia) exhibited the ontogenetic phenotype reported in adult specimens of Venezuelan *B. colombiensis*,[40] and Costa Rican *B. asper*,[41] (characterized by %PI-SVMP (snake venom metalloproteinase) > %PIII-SVMP and %K49-PLA_2 > %D49-PLA_2), whereas Amazonian *B. atrox* venoms showed a paedomorphic phenotype comprised predominantly of PIII-SVMPs and %D49-PLA_2 > %K49-PLA_2. Antivenoms raised in Costa Rica and Brazil using different *Bothrops* venoms in the immunization mixtures immunodepleted very efficiently the major toxins (PIII-SVMPs, serine proteinases, CRISP, LAO) of paedomorphic venoms, but had impaired reactivity towards PLA_2 and P-I SVMP molecules abundantly present in the ontogenetic venoms. These results clearly illustrate that knowledge of evolutionary and immunological trends among conspecific populations may aid replacing the traditional geographic- and phylogenetic-driven hypotheses for antivenom production strategies by a more rationale approach based on proteome phenotype and immunological profile similarities.

1. 3. Neurotoxic PLA_2 Molecules in Rattlesnake Type II Venoms. Clues for the Management of Rattlesnake Envenomings

The monophyletic clade of the rattlesnakes (genera *Crotalus* and *Sistrurus*) had its origin ~20 Mya in the Sierra Madre Occidental in the north-central Mexican Plateau.[42] Rattlesnakes dispersed northward into North America and southward into South America, and today genus *Crotalus* groups approximately 70 species and subspecies (http://www.reptile-database.org) of venomous pitvipers widely and discontinuosly distributed from southern Canada to northern Argentina.[29,43] Rattlesnake venoms belong to one of two distinct phenotypes, which broadly correspond to type I (high levels of SVMPs and low toxicity, LD_{50} >1 mg/g mouse body weight) and type II (low metalloproteinase activity and high toxicity, LD_{50} <1 mg/g mouse body weight) defined by Mackessy.[44] The high toxicity of type II venoms and the characteristic systemic neuro- and myotoxic effects observed in envenomations appear to be directly related to the expression of the presynaptic β-neurotoxic heterodimeric PLA_2 molecules, crotoxin (in Central and South American rattlesnake venoms),[45–47] Mojave toxin (in Neartic rattlesnakes),[48] and sistruxin (in *Sistrurus catenatus catenaus* and *S.c. tergeminus* venoms).[49,50] Crotoxin, Mojave toxin, and sistruxin are composed of two subunits, a non-toxic acidic subunit (CA) (named crotapotin in crotoxin), which lacks PLA_2 activity, and a weakly toxic basic subunit (CB), which exhibits PLA_2 activity. The acidic subunit undergoes proteolytic processing to form three polypeptides held together by disulphide bridges. Although the CB subunit exhibits neurotoxic activity, the native crotoxin complex is at least one order of magnitude more potent than CB alone. The increase of toxicity in mice, rats and rabbits, due to crotapotin acting as a chaperone blocking the binding to non-specific sites and guiding the CB subunit to its specific target site, is the crucial feature of crotoxin.[51]

The New World pitvipers represent a monophyletic radiation of a single unidirectional invasion by an ancestral crotaline form of Old World origin across the Bering Land Bridge in the late Cretaceous to early Tertiary.[52] Although PLA_2 enzymes mostly exist as monomers, both covalent and noncovalent oligomeric complexes with other PLA_2 (or PLA_2-like molecules) or with other proteins have been described in a number of Old World and New World *Elapidae* and *Viperidae* taxa.[53] Venoms of *Vipera* include postsynaptic (e.g., vipoxin from *Vipera ammodytes meridionalis*)[54,55] and presynaptic acting toxins (*e.g.*, ammodytoxin from *V. a. ammodytes*,[56] and vipertoxin F from *Daboia russellii formosensis*[57]), where the neurotoxic PLA_2 either works alone, or is complexed with an inhibitor or a chaperone.[53] Within the Old World crotaline taxa, monomeric or dimeric neurotoxic PLA_2s have been described in the genera *Gloydius* and *Protobothrops*. However, the fact

that few Old World species possess neurotoxic components, and none to date are found with β-neurotoxic heterodimeric crotoxin-like PLA_2s, indicates that the emergence of these presynaptic-acting complexes must be dated after the derivation of rattlesnakes in the New World.

The increased concentration of crotoxin in South American rattlesnake venoms represents a paedomorphic trend.[58,59] Gain of neurotoxicity and lethal venom activities to mammals (rodents) have represented the key axis along which overall venom toxicity has evolved during *Crotalus* radiation in South America.[58,59] Mojave toxin positive Neartic type II venoms, such as *C. tigris* (Tiger rattlesnake), *C. horridus* (Timber rattlesnake), *C. scutulatus scutulatus* type A (Mohave rattlesnake), the midget-faded rattlesnake (*C. oreganus concolor*), and *C. oreganus helleri* (Southern Pacific rattlesnake), exhibit toxin venom phenotypes closely resembling those of neurotoxic South American *Crotalus durissus* subspecies (*terrificus, cascavella, collilineatus*) (Figure 3), and the subunits of the Mojave toxin (SwissProt accession codes P18998 and P62023) share respectively 95% and 100% amino acid sequence identity with the acidic (A, P08878) and the basic (CB1, P62022) subunits of crotoxin. Powell and Lieb[60] have predicted that the extremely high neurotoxicity exhibited by North American rattlesnakes represents a transitory populational phenomenon associated with novel prey bases. The phylogeny and evolution of β-neurotoxic PLA_2s present in the venoms of rattlesnakes has been investigated by Werman.[61] Maximum parsimony phylogenetic reconstructions support the view of gene duplication and subsequent independent evolution for the origin of the two subunits of the heterodimeric sistruxin, crotoxin, and Mojave toxin from pre-existing PLA_2 sequences already present in non-neurotoxic species.[61] However, the distribution of PLA_2 β-neurotoxins among rattlesnakes shows very little phylogenetic structure, as there are no clades that have neurotoxic PLA_2 complexes in all members. Only terminal clades of recently divergent taxa (*e.g.*, subspecific taxa within *Sistrurus catenatus*, *Crotalus durissus*, *C. simus*, and *C. scutulatus*) appear to express neurotoxin in adult venoms.[61]

The lack of phylogenetic clustering among rattlesnakes with neurotoxin indicates that phylogeny may not be an important consideration in venom evolution. Wer-

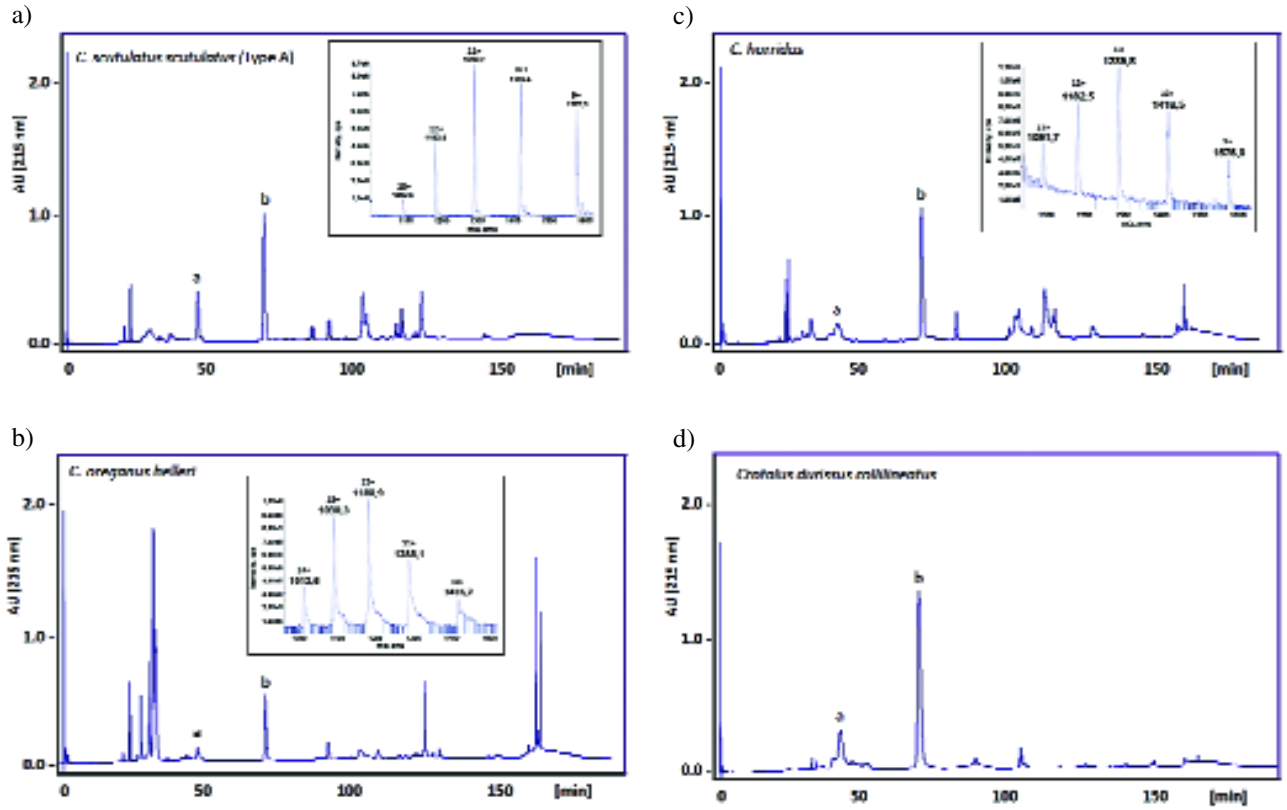

Figure 3. Comparison of the reverse-phase HPLC separations of the venom proteins of (**a**) *C. scutullatus scutulatus* (type A), (**b**) *C. oreganus helleri*, (**c**) *C. horridus*, and (**d**) *C. durissus collilineatus*. Crotoxin/Mojave toxin acidic and basic subunits are labelled "a" and "b", respectively. Inserts in panels A-C, ESI-MS spectra of the basic subunits of Mojave toxin from *C. scutullatus scutullatus* (type A) (N-terminal sequence, HLLQFNKMIKFETRK, isotope-averaged mass, 14186 Da), *C. oreganus helleri* (HLLQFNKMIKFETRK, 14177 Da), and *C. horridus* (HLLQFNKMIKFETRK, 14156 Da), respectively. The homologous crotoxin B-subunit from *C. durissus collilineatus* has N-terminal sequence HLLQFNKMIKFETRK and an ESI-MS of 14187 Da. [59]

man[61] has speculated that the presence of neurotoxins among rattlesnakes may have resulted from the influence of a combination of factors, including paedomorphosis in terminally-derived clades and gene transfer through ancestral hybridization events. According to this author, *S. catenatus* venom containing both heterodimeric neurotoxin (sistruxin) and other PLA_2 molecules could represent the archetype of rattlesnake type I venoms. The archetype for neurotoxic type II venoms may be represented by *C. simus*, where juveniles are highly neurotoxic, but adults have hemorrhagic type I venoms.[61]

An anti-crotalic antivenom produced at Instituto Butantan against *C. d. terrificus* venom showed a very high effectiveness in the neutralization of the lethal, myotoxic, and neurotoxic effects of venoms of *C. durissus* subspecies and newborn *C. simus*.[62] However, this antivenom failed to neutralize the hemorrhagic activity of adult *C. simus* and *C. d. cumanensis* and *C. d. ruruima* venoms. In contrast, a polyvalent antivenom produced by Instituto Clodomiro Picado (San José, Costa Rica) effectively neutralized the hemorrhagic activity of *Crotalus* venoms but not against *C. durissus* venoms, and showed a very low neutralizing activity against newborn *C. simus* envenoming.[63] Such neutralizing profile is fully explained by the proteomic finding that adult *C. simus* and *C.d. cumanensis* synthesize type I venoms while the venoms of juvenile *C. simus* and C.d. *durissus*, *C.d. ruruima*, *C.d. collilineatus*, *C.d. cascavella*, and *C.d. terrificus* belong to the type II class. The evolutionary trend observed in *Crotalus* suggests that an effective pan-American anti-*Crotalus* antivenom should primarily neutralize the toxic actions of four major toxin groups, PIII-SVMPs, crotoxin, crotamine, and thrombin-like serine proteinases. Such antivenom might be achievable by hyperimmunizing with a mixture of type I and type II venoms comprising conserved antigenic determinants for each of the major toxin families of the genus.

1. 4. Extreme Venom Variabilty Among Palm Viper Venoms. A Vrotoxin-like PLA_2 Molecule in B. *Nigroviridis* Venom

The genus *Bothriechis* comprises 9 species (*B. aurifer*, *B. bicolor*, *B. lateralis*, *B. marchi*, *B. nigroviridis*, *B. rowleyi*, *B. schlegelii*, *B. supraciliaris*, *B. thalassinus*) of relatively slender to medially robust, arboreal, prehensile-tailed, New World pitvipers[29] (Figure 4). Documentation of human accidents by *Bothriechis* snakebites is scarce, and although *Bothriechis* venoms investigated seem to be of moderate toxicity, bites may have dire consequences due to the arboreal nature of these snakes which results in

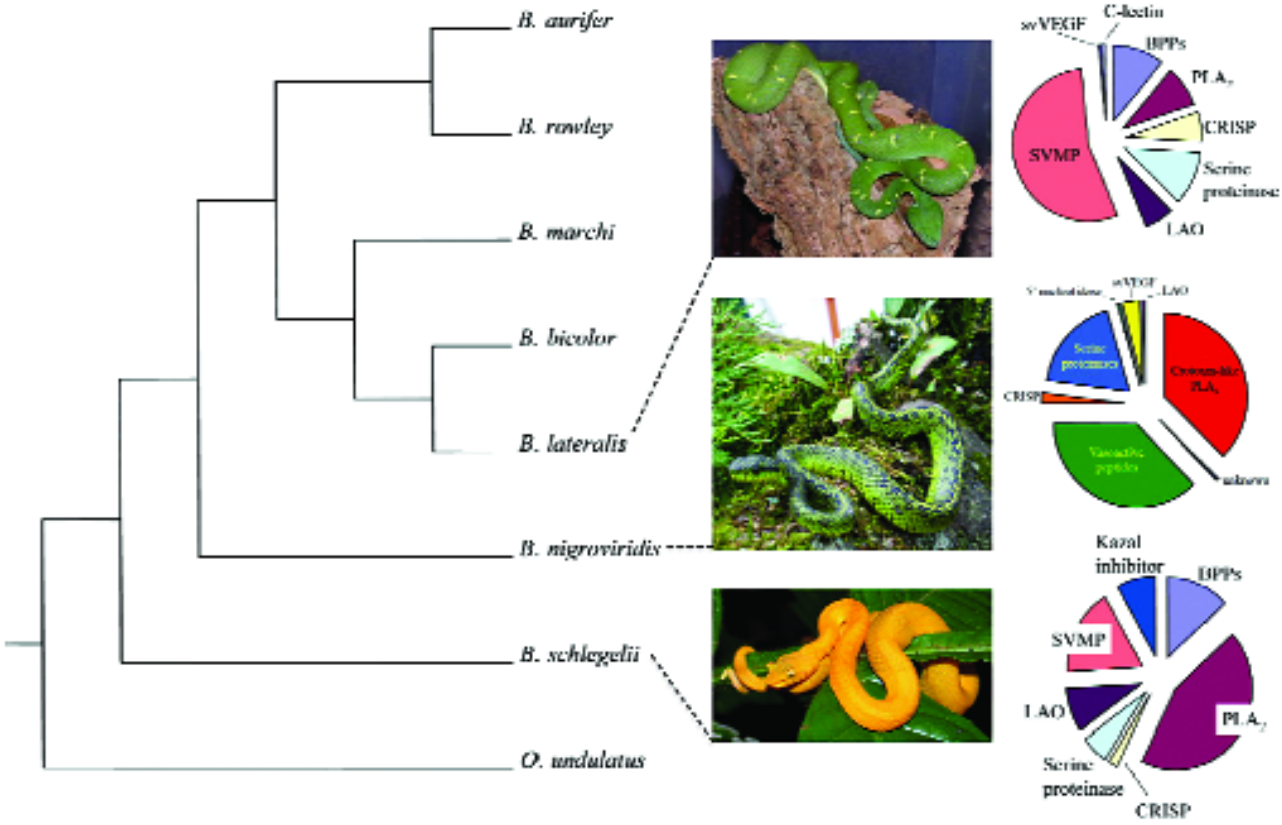

Figure 4. Mapping the venom toxin profiles of *B. lateralis*, *B. schlegelii*, and *B. nigroviridis* onto the phylogenetic tree of *Bothriechis*, reveals the extreme venom composition divergence among congeneric taxa. CRISP, cysteine-rich secretory protein; C-lectin, C-type lectin-like molecule; BPP, bradykinin-potentiating peptide; svVEGF, snake venom vascular endothelial growth factor; LAO, L-amino acid oxidase; SVMP, snake venom metalloproteinase; PLA_2, phospholipase A_2.

many of the bites being inflicted in the head, neck, and shoulder regions. Venomic studies have revealed a high divergence in the venom compositions of *B. lateralis*, *B. schlegelii*, and *B. nigroviridis*[64,65] (Figure 4), in spite of the fact that these species have evolved to adapt to arboreal habits and seem to have similar generalist-type diets.

The major toxin families of *B. lateralis* and *B. schlegelii* venoms are SVMP (55% of the total proteins) and PLA_2 (44%), respectively (Figure 4). Their different venom toxin compositions provide clues for rationalizing the distinct signs of envenomation in experimental animals caused by *B. schlegelii* and *B. lateralis*.[64] The venom from *B. nigroviridis* is devoid of hemorrhagic activity, has low edematogenic and coagulant effects, presents modest myotoxic and phospholipase A_2 activities, but has higher lethality than the venoms of other *Bothriechis* species. Strikingly, the venom proteome of *B. nigroviridis* does not possess detectable Zn^{2+}-dependent SVMPs, and is uniquely characterized by a high content of crotoxin-like PLA_2 subunits and vasoactive peptides, each of these groups of toxins representing as much as 38% of total venom proteins[65] (Figure 4). These data support the view that different evolutionary solutions have evolved within the arboreal genus *Bothriechis* for the same trophic purpose, and underscore the versatility of venoms as adaptive traits in these viperid snakes. On the other hand, the presence of crotoxin-like PLA_2 subunits in the venom of *B. nigroviridis* could not have been guessed through a phylogenetic hypothesis. However, neutralization of the lethal activity of *B. nigroviridis* venom by an anti-crotalic antivenom,[65] manufactured by Instituto Butantan using venom of *C. d. terrificus* as immunogen, points to a major role of crotoxin-like PLA_2 in *B. nigroviridis* venom-induced lethality, and highlights the relevance of *in vivo* neutralization assays and antivenomic profiling for expanding the clinical use of heterologous antivenoms on an immunologically sound basis.

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Povzetek

Članek je osredotočen na uporabo proteomskih orodij pri raziskavah sestave in razvoja kačjih strupov ter njihove navskrižne reaktivnosti s trenutno razpoložljivimi homolognimi in heterolognimi protistrupi. Proteomske analize strupov kač iz družine *Bothrops* so izpostavile primernost uporabe PLA_2 molekul kot taksonomskih in populacijsko-specifičnih označevalcev. Izostanek filogenetskega združevanja neotropskh in nearktičnih klopotač, ki v strupih vsebujejo nevrotoksične PLA_2, nakazuje, da filogenija živali ni pomembna pri obravnavi evolucije njihovih strupov. Na proteomski analizi osnovana identifikacija evolucijskih in imunoloških smeri razvoja kačjih strupov bi utegnila nadomestiti tradicionalne geografsko- in filogenetsko-pogojene hipoteze za oblikovanje strategij za pripravo protistrupov z bolj racionalnim pristopom, ki bi temeljil na primerjavi proteomov in imunoloških profilov strupov. Najnovejše analize protistrupov in proteomske analize strupov kač iz družin *Bothrops*, *Crotalus* in *Bothriechis* kažejo na obetaven potencial takega pristopa.