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Venom Proteomes of Closely Related *Sistrurus* Rattlesnakes with Divergent Diets

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The protein composition of the venoms of the three subspecies of *Sistrurus catenatus* (*S. c. catenatus*, *tergeminus*, and *edwardsii*) and a basal species, *Sistrurus miliarius barbouri*, were analyzed by RP-HPLC, N-terminal sequencing, MALDI-TOF peptide mass fingerprinting, and CID-MS/MS. The venoms of the four *Sistrurus* taxa contain proteins from 11 families. The protein family profile and the relative abundance of each protein group in the different venoms are not conserved. Myotoxins and 2-chain PLA_2s were detected only in *S.c. catenatus* and *S.c. tergeminus*, whereas C-type BPP and Kunitz-type inhibitors were exclusively found in *S.c. edwardsii* and *Sistrurus miliarius barbouri*. Among major protein families, taxa were most similar in their metalloproteases (protein similarity coefficient value: 34%) and most divergent in PLA_2s (12%), with values for disintegrins and serine proteases lying between these extremes (25 and 20%, respectively). The patterns of venom diversity points to either a gain in complexity in *S. catenatus* taxa or a loss of venom diversity occurring early on in the evolution of the group involving the lineage connecting *S. milarius* to the other taxa. The high degree of differentiation in the venom proteome among recently evolved congeneric taxa emphasizes the uniqueness of the venom composition of even closely related species that have different diets. Comparative proteomic analysis of *Sistrurus* venoms provides a comprehensive catalog of secreted proteins, which may contribute to a deeper understanding of the biology and ecology of these North American snakes and may also serve as a starting point for studying structure−function correlations of individual toxins.

Keywords: *Sistrurus* • Snake venom protein families • proteomics • snake venomics • N-terminal sequencing • mass spectrometry

Introduction

Venoms produced by snakes in the families *Viperidae* and *Elapidae* possess the most widely studied types of animal toxins.[1−3] Snakes of the family *Viperidae* (vipers and pitvipers) produce a complex mixture of a large number of distinct proteins[4,5] in paired specialized venom glands located ventral and posterior to the eyes;[6] venom is introduced deeply into prey tissues via elongated, rotatable fangs. These venoms contain numerous proteins that interfere with the coagulation cascade, the normal haemostatic system and tissue repair, and human envenomations are often characterized by clotting disorders, hypofibrinogenemia, and local tissue necrosis.[3,7,8] Although viperid venoms may contain well over 100 protein components,[5] venom proteins belong to only a few major protein families, including enzymes (serine proteinases, Zn^{2+}-metalloproteases, L-amino acid oxidase, and group II PLA_2) and proteins without enzymatic activity (disintegrins, C-type lectins, natriuretic peptides, myotoxins, CRISP toxins, nerve and vascular endothelium growth factors, cystatin, and Kunitz-type protease inhibitors).[5,7,9−11] Venom toxins likely evolved from proteins with a normal physiological function and appear to have been recruited into the venom proteome before the diversification of the advanced snakes, at the base of the Colubroidea radiation.[10−13] Venoms represent the critical innovation that allowed advanced snakes to transition from a mechanical (constriction) to a chemical (venom) means of subduing and digesting prey larger than themselves, and as such, venom proteins have multiple functions including immobilizing, paralyzing, killing, and digesting prey. Given the central role that diet has played in the adaptive radiation of snakes,[14] venom thus represents a key adaptation that has played an important role in the diversification of these animals.

There is evidence that suggests the high level of diversity in venom proteins is a result of strong diversifying selection on venom genes, possibly due to differences in prey consumed. First, studies of the molecular evolution of venom genes have repeatedly shown strong signals at DNA level for positive

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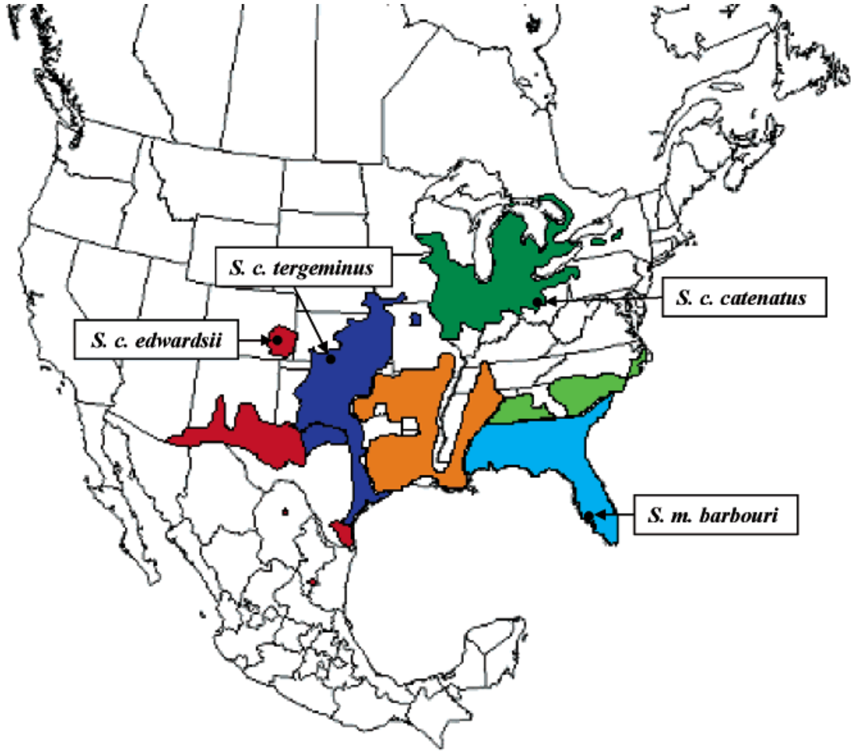

Figure 1. Geographic distribution of *Sistrurus catenatus* and *Sistrurus miliarius* in North America. Dots represent geographic locations where samples from particular taxa were collected. Dark green = *S. c. catenatus*; dark blue = *S. c. tergeminus*; red = *S. c. edwardsii*. Light green = *S. m. miliarius*; light blue = *S. m. barbouri*; orange = *S. m. streckeri*. Modified from Mackessy[73] and Campbell and Lamar.[74]

selection for functional diversity both within and between species levels.[15−27] Second, analysis of venom proteins using gel electrophoresis has consistently shown relatively high levels of intra- and interspecific variation.[28] Third, there is a small but increasing number of studies that strongly support the idea that this variation reflects adaptation for differential utilization of distinct prey types.[29−32] However, although the notion that evolutionary interactions between snakes and their prey may be responsible for variation in venom composition has been controversial,[33,34] the recent demonstration of taxa-specific effects of some venoms supports the hypothesis that evolutionary adjustments in venom composition have occurred.[32] The loss of a functional major toxin (α-neurotoxin) from the venom of *Aipysurus eydouxii*, which appears to have occurred following a shift from feeding on fish to fish eggs,[35] further supports a link between diet and venom composition. Of particular value in addressing this issue would be studies that adopt a comprehensive approach of assessing variation in venom proteins[4,5,36] in closely related species that show clear, significant differences in diet. The use of phylogenetically similar species would allow the strong inference that differences in venom characteristics are most likely due to differences in selection pressures alone rather than an unknown combination of the effects of selection and divergence due to phylogenetic relationships among the species being compared.

To address the need for detailed proteomic studies of venoms of closely related venomous snakes, we have initiated a project whose long-term goal is a detailed analysis of venom proteins and genes among *Sistrurus* rattlesnakes that specialize on different prey. These are small rattlesnakes that are found in different regions of North America (Figure 1), and here we report on venom variation in four taxa: *Sistrurus miliarius barbouri* (Pygmy Rattlesnake) (see also 9) and *S. catenatus catenatus*, *S. c. tergeminus*, and *S. c. edwardsii* (Eastern, Western, and Desert Massasauga rattlesnakes, respectively). Recent phylogenetic analyses based on mitochondrial and nuclear DNA indicate that *miliarius* is basal to all three *catenatus* subspecies, whereas the named *catenatus* subspecies fall into two distinct clades: one consisting of *S. c. catenatus* alone and the other consisting of both *tergeminus* and *edwardsii*[37] (Gibbs et al., unpublished data). A third species previously included in this genus (*Sistrurus ravus*, Mexican Pygmy Rattlesnake) has been placed in the genus *Crotalus* and may be the sister taxon to this clade.[38]

Diet studies show that different taxa of *Sistrurus* rattlesnakes vary in the degree to which they specialize on endothermic versus ectothermic prey.[39,40] Specifically, there are snakes that largely specialize on mammals (*S. c. catenatus*) versus frogs and lizards (*S. miliaris barbouri*) as well as snakes that bridge this dietary transition by utilizing both mammals and ectotherms (*S. c. tergeminus* and *S. c. edwardsii*). If venom composition is strongly related to aspects of diet,[29,30,32,41] then variation in venom biochemistry should be observed among these related taxa.

Despite its potential value, little is known about the venom protein composition of *Sistrurus* species. Indeed, a survey in the Swiss-Prot/TrEMBL database (http://us.expasy.org/sprot/) (release of 16 May 2006; 3 134 187 entries) matched only the following 6 full-length venom toxin sequences (accession numbers are indicated): disintegrin barbourin (Southeastern pigmy rattlesnake) (P22827); disintegrin tergeminin (Western massasauga) (P22828); phospholipase A_2 (Western massasauga) (Q6EAN6); N6a and N6b basic phospholipase A_2 (Western

Table 1. Assignment of the Reversed-Phase Isolated Fractions of *Sistrurus catenatus catenatus* (SCC) Venom to Protein Families by N-Terminal Edman Sequencing, MALDI-TOF Mass Spectrometry, and Collision-Induced Fragmentation by nESI-MS/MS of Selected Peptide Ions from In-Gel Digested Protein Bands[a]

HPLC fraction SCC-		N-terminal sequencing	Isotope-averaged MALDI-TOF mass (± 0.2%)	peptide ion *m*/*z*	*z*	MS/MS-derived sequence	protein family
1		n.p.					
2		n.p.					
3		EAGEECDCGSPANPCCDAAT					disintegrin
		EECDCGSPANPCCDAATCKL	[7380, 7578]				disintegrin
4		Blocked	4738 (5386*)	621.9	3	XCSPPYSDVGQXDCR	myotoxin
5		EECDCGSPANPCCDAATCKL	7069 (8340*)	743.6	3	XRPGAQCADGLCCDQCR	disintegrin
6		EAGEECDCGSPANPCCDAAT	[7069, 7187,				disintegrin
		AGEECDCGSPANPCCDAATC	7316, 7470]				disintegrin
		EECDCGSPANPCCDAATCKL					disintegrin
7		N. D.	22806				
		GCYCGTGGQGWPQDASDRCCFE		1147.6	2	(575.2)VVYEAEDSCFESNQK	DC-fragment
8		[ ]	9212				2-chain PLA_2
		SLENCQGESQPC					
		GCYCGTGGQGWPQDASDRCCFE	[9044, 9309,				
9		[ ]	9577]				2-chain PLA_2
		SLENCQGESQPC					
10		N. D.	12961	744.7	3	GKPXDATDRCCFVHDCCGK	PLA_2
11		N. D.	15, 17 kDa§	711.9	2	AXTMEDNEASWR	nerve growth factor
				640.3	2	XDSACVCVXSR	
				556.3	2	NPNPVPTGCR	
				983.8	3	GNXVTVMVDVNXNNNVYKQY-FFETK	
12		NLLQFNKMIKIMTKK	13814				N6−PLA_2
13		NLLQFNKMIKIMTKK	13880				N6−PLA_2
14		HLLQFNKMIKFETNK	14120				N6−PLA_2
15		SVDFDSESPPKPEIQ	24831				CRISP
16	M)	HLIQFETLIMKIAGR	13967				PLA_2
	m)	Blocked	22206	753.8	2	CCFVHDCCYGK	PLA_2
17		HLIQFETLIMKIAGR	13952				PLA_2
18	M)	N. D.	13941	655.3	2	SXVQFETXXMK	PLA_2
				837.3	2	XTGCDPXTDVYTYR	
	m)	N. D.	28−30 kDa§	582.8	2	XMGWGTXSATK	Ser-proteinase
				595.9	2	WDKDXMXXR	
				648.3	2	NNXXDYEVCR	
19	m)	N. D.	13941	655.3	2	SXVQFETXXMK	PLA_2
				837.3	2	XTGCDPXTDVYTYR	
	M)	IIGGDECNINEHRFL	28−30 kDa§	582.8	2	XMGWGTXSATK	Ser-proteinase
				595.9	2	WDKDXMXXR	
				648.3	2	NNXXDYEVCR	
20		VIGGDECNINEHRSL	27090				Ser-proteinase
21		IIGGDECNINEHRFL	30918				Ser-proteinase
22		VVGGDECNINEHRFL	[26545, 28556]				Ser-proteinase
23		VVGGDECNINEHRSL	[27115, 27323]				Ser-proteinase
24		APEHQRYVELFIVVD	23053	555.8	2	TXNSFGEWR	PI-metalloprotease
				874.9	2	VAVTMTHEXGHNXGNR	
				900.9	2	YVEFVVVXDHGMYTK	
25		Blocked	33 kDa§	664.8	2	YXEXVXVADHR	metalloprotease
				840.8	2	VHEXVNFXNEFYR	
				821.4	2	HDNAQXXTAXDFQR	
				660.9	3	(233.2)EQHNPQCXXNKPXR	
			52 kDa§	627.3	2	SAGQXYEESXR	L-amino acid oxidase
				647.8	2	EGWYANXGPMR	
				744.8	2	EDTYEEFXEXAK	
				881.4	2	EGWYANXGPMRLPEK	
26		LTPEQQAYLDAKKYV	32 kDa§	605.3	2	VTXNSFGEWR	metalloprotease
				737.1	3	XYEXVNTMNEXYXPXNXR	
			35 kDa§	605.3	2	VTXNSFGEWR	metalloprotease
				737.1	3	XYEXVNTMNEXYXPXNXR	
			48 kDa§	605.3	2	VTXNSFGEWR	PIII-metalloprotease
				555.8	2	TXNSFGEWR	
				737.1	3	XYEXVNTMNEXYXPXNXR	
				900.9	2	YVEFVVVXDHGMYTK	
				643.9	3	KYVEFVVVXDHGMYTK	
				691.4	2	(263.2)EFXXVVDQR	
				814.4	2	NQCXSFFGPSATVAK	Cys-rich domain
				640.8	2	NXCNVXYMPR	Cys-rich domain
27	M)	LTPEQQAYLDAKKYV	[33394, 33669]				metalloprotease
	m)	Blocked	31 kDa§	605.3	2	VTXNSFGEWR	PII-metalloprotease
				737.1	3	XYEXVNTMNEXYXPXNXR	
				900.9	2	YVEFVVVXDHGMYTK	
				978.4	2	GDWNDDTCTGQSADCPR	disintegrin
			47 kDa§	659.3	2	YTXNEFGEWR	PIII-metalloprotease
				527.2	2	GNYYGYCR	Cys-rich domain
				640.8	2	NXCNEXYMPR	Cys-rich domain
28		APTPQQQAYLDAKKY	33478				metalloprotease
29		Blocked	48877	659.3	2	YTXNEFGEWR	PIII-metalloprotease
				527.2	2	GNYYGYCR	Cys-rich domain
30		APNPYRYIELVIVAD	32 kDa§	672.9	2	YXEXVVVTDHR	metalloprotease
				844.4	2	SYFSDCSMDEYR	

Table 1 (Continued)

HPLC fraction SCC-	N-terminal sequencing	Isotope-averaged MALDI-TOF mass (± 0.2%)	peptide ion *m*/*z*	*z*	MS/MS-derived sequence	protein family
		72 kDa[§]	677.8	2	FEEXVXVXVADYR	PIII-metalloprotease
			957.8	2	TWVYEXVNTXNEXYR	
			829.7	2	NCRDPCCDATTCK	disintegrin
			964.3	2	SNEXXEAGEECDCGSPR	
			527.2	2	GNYYGYCR	Cys-rich domain
31	N. D.	13 kDa[§]	695.8	3	TNDNQSXSRPC(797.9)	C-type lectin
			567.3	2	VXDDQWXSR	
		16 kDa[§]	720.8	2	QNKYYVWXGXR	C-type lectin
		23 kDa[§]	626.3	2	(SG)VTXNSFGEWR	metalloprotease
		33 kDa[§]	605.4	2	VTXNSFGEWR	metalloprotease
			746.4	3	(KS)HDNAQXXTAVNXN-GDTXGR	
			657.9	2	YXEXVVVADHR	
		52−58 kDa[§]	647.9	2	EGWYANXGPMR	L-AMINO acid oxidase
			665.4	2	VTVXEASQXVNR	

[a] X, Ile or Leu; Unless other stated, for MS/MS analyses, cysteine residues were carbamidomethylated; n.p.: not peptidic material. M: major species; m: minor species; *: MALDI-TOF mass of the reduced and pyridylethylated molecule; [§]: apparent molecular mass determined by SDS−PAGE after sample reduction with β-mercaptoethanol; N. D.: not determined.

massasauga) (Q6EER2 and Q6EER3, respectively); and an N6 basic phospholipase A2 from *Sistrurus miliarius streckeri* (Q6EER6).

In a previous paper, we reported a proteomic approach for characterizing the major protein families of *S. m. barbouri* venom.[9] Here, we present an exhaustive comparative proteomic characterization of the protein composition of the venom proteomes of the three subspecies of *S. catenatus*, combined with a more exhaustive analysis of *S. m. barbouri*. We have the dual goals of determining the relative abundances of different toxin families in the venoms and identifying novel proteins for structural and functional investigations. In additon to understanding how venoms evolve, characterization of the protein/peptide content of snake venoms also has a number of potential benefits for basic research, clinical diagnosis, development of new research tools and drugs of potential clinical use, and antivenom production strategies. Within-species heterogeneity of venoms may also account for differences in the clinical symptoms observed in accidental invenomations.

Experimental Section

Venom Samples. Venoms were extracted manually from adult snakes of the three subspecies of *Sistrurus catenatus* using standard methods.[29] Snakes were from the following locations: *S. c. catenatus* (SCC), Killdeer Plains Wildlife Area, Wyandot County, Ohio; *S. c. tergeminus* (SCT), Cheyenne Bottoms Wildlife Area, Barton County, Kansas; *S. c. edwardsii* (SCE), Lincoln County, Colorado. Additional analyses were conducted on a single venom sample of *S. m. barbouri* (SMB) from Florida (Latoxan Serpentarium, Rosans, France) as described in Juarez et al.[9]

Isolation of Proteins. For reverse-phase HPLC separations, 2−5 mg of crude venom were dissolved in 100 μL of 0.05% trifluoroacetic acid (TFA) and 5% acetonitrile, and insoluble material was removed by centrifugation in an Eppendorff centrifuge at 13 000 × g for 10 min at room temperature. Proteins in the soluble material were separated using an ETTAN LC HPLC system (Amersham Biosciences) and a Lichrosphere RP100 C_{18} column (250 × 4 mm, 5 μm particle size) eluted at 1 mL/min with a linear gradient of 0.1% TFA in water (solution A) and acetonitrile (solution B) (5% B for 5 min, followed by 5−15% B over 20 min, 15−45% B over 120 min, and 45−70% B over 20 min). Protein detection was at 215 nm, and peaks were collected manually and dried in a Speed-Vac (Savant). The relative abundances (% of the total venom proteins) of the different protein families in a given venom were estimated from the relation of the sum of the areas of the reverse-phase chromatographic peaks containing proteins from the same family to the total area of venom protein peaks.

Characterization of HPLC-Isolated Proteins. Isolated protein fractions (2−5 mg/mL in 100 mM ammonium bicarbonate, pH 8.3, containing 5 M guanidinium hydrochloride) were reduced with 1% (v/v) 2-mercaptoethanol for 15 min at 85 °C and alkylated by addition of 4-vinylpyridine at 5% (v/v) final concentration and incubation for 15 min at room temperature. Pyridylethylated (PE) proteins were freed from reagents using a C18 Zip-Tip pipet tip (Millipore) after activation with 70% acetonitrile and equilibration in 0.1% TFA. Following protein adsorption and washing with 0.1% TFA, the PE-proteins were eluted with 10 μL of 70% ACN and 0.1% TFA and subjected to N-terminal sequence analysis (using a Procise instrument, Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Amino acid sequence similarity searches were performed against the available databanks using the BLAST program[42] implemented in the WU−BLAST2 search engine at http://www.bork.embl-heidelberg.de. The molecular masses of the purified proteins were determined by SDS−PAGE (on 12−15% polyacrylamide gels) and by MALDI-TOF mass spectrometry using an Applied Biosystems Voyager-DE Pro mass spectrometer operated in linear mode. To this end, equal volumes (0.5 μL) of the protein solution and the matrix (sinapinic acid, Sigma, saturated in 50% acetonitrile and 0.1% TFA) were mixed on the MALDI-TOF plate. The mass calibration standard consisted of a mixture of the following proteins (isotope-averaged molecular masses in Daltons): bovine insulin (5734.6), *Escherichia coli* thioredoxin (11 674.5), horse apomyoglobin (16 952.6), *E. coli N*-acetyl-L-glutamate kinase (NAGK) (27 159.5), *Pyrococcus furiosus* carbamoyl-phosphate synthetase (PFU) (34 297.4), *Parkia platycephala* seed lectin (PPL) (47 946), and bovine serum albumin (66 431). NAGK, PFU and PPL were generous gifts of Dr. Vicente Rubio (Instituto de Biomedicina de Valencia, Valencia, Spain) and Dr. Benildo S. Cavada (Universidade Federal de Ceará, Fortaleza, Brazil), respectively. The other proteins were purchased from Applied Biosystems.

In-Gel Enzymatic Digestion and Mass Fingerprinting. Protein bands of interest were excised from a Coomassie Brilliant Blue-stained SDS−PAGE and subjected to automated reduction with DTT, alkylation with iodoacetamide, and diges-

Table 2. Assignment of the Reversed-Phase Isolated Fractions of *Sistrurus catenatus tergeminus* (SCT) Venom to Protein Families by N-Terminal Edman Sequencing, MALDI-TOF Mass Spectrometry, and Collision-Induced Fragmentation by nESI-MS/MS of Selected Peptide Ions from In-Gel Digested Protein Bands[a]

HPLC fraction SCT-	N-terminal sequencing	isotope-averaged MALDI-TOF mass (± 0.2%)	peptide ion *m*/*z*	*z*	MS/MS-derived sequence	protein family
1−4	n.p.					
5	EECDCGSPANPCCDAAT	7579				disintegrin
6	EECDCGSPANPCCDAAT	7185				disintegrin tergeminin (P22828) 4−71
	M: SGMFSYSAYGCYCGWGG	11648				PLA_2
	m: Blocked	4737 (5384*)	621.9	3	XCSPPYSDVGQXDCR	myotoxin
7	GEECDCGSPANPCCDA	[7658, 7459]				disintegrin
	EECDCGSPANPCCDAA					tergeminin (P22828)
8	GEECDCGSPANPCCDA	[7073, 7346,				disintegrin tergeminin
	EECDCGSPANPCCDAA	7402]				(P22828) 4−70, 4−72, 3−72
9	EAGEECDCGSPANPCCDA	7438				disintegrin tergeminin (P22828) 1−71
10	SPNECGNNFVDLGEECDCGLPANP	8495				disintegrin
11	SVGEECDCGTPENDQ	11352				disintegrin-like
		23 kDa§	527.2	2	GNYYGYCR	DC-fragment
			761.2	3	(608.5)YEAEDSCFEPTXR	
	GCYCGTGVQGWPQDASD					
12	[ ]	[8628, 8703]				2-chain PLA_2
	SLENCQGESQP					
	GCYCGTGVQGWPQDASD					
13	[ ]	9245				2-chain PLA_2
	SLENCQIESQPC					
		23 kDa§	527.2	2	GNYYGYCR	DC-fragment
	GCYCGTGGQGWPQDASD					
14	[ ]	9025				2-chain PLA_2 (Q6EAN6)
	SLENCQGESQPC					(41−112)-S−S−(127−138)
15	N. D.	13 kDa§	744.7	3	GKPXDATDRCCFVHDCCGK	PLA_2
16	N. D.	16 kDa§	556.3	2	NPNPVPTGCR	nerve growth factor
			682.8	2	AXTMEGNQASWR	
17	NLLQFNKMIKIMTKK	13741				N6−PLA_2 (Q6EER3)
18	NLLQFNKMIKIMTKK	13887				N6−PLA_2
19	NLLQFNKMIKFETNA	14119				N6−PLA_2 (Q6EER2)
20	m:SVDFDSESPRKPEIQ	24794				CRISP
	M:NLIQFETLILKVAKK	13826				PLA_2
21	VIGGDECNINEHRSL	27847	677.3	2	FXVAXYHSR	Ser-proteinase
22	VIGGDECN(I+V)NEHRFL	[26976, 27186]				Ser-proteinase
23	VIGGDECNVNEHRSL	27015				Ser-proteinase
24	IIGGDECN(I+V)NEHRFL	[27392, 28557]				Ser-proteinase
25	VVGGDECNINEHRFL	[28672, 29092]				Ser-proteinase
26	VIGGDECNINEHRSL	[27621, 27723]				Ser-proteinase
27	NPEHQRYVELFIVVD	23082				PI-metalloprotease
28	Blocked	56 kDa§	783.0	3	XYFAGEYTAQFHGWXDSTXK	L-amino acid oxidase
		48 kDa§	588.2	2	NFPCAQPDVK	cysteine-rich
			530.6	2	QGDNFYCR	cysteine-rich
29−31	Heterogeneous	23 kDa§	657.8	2	YXEXVVVADHR	metalloprotease
			874.9	2	VAVTMTHEXGHNGNR	
			564.3	3	HSTGVVEDHSEXNXR	
			899.8	2	YVEXFNVVDHGMFTK	
		33 kDa§	559.8	2	TXCAGXXEGGK	Ser-proteinase
		36 kDa§	664.3	2	YXEXVXVADHR	metalloprotease
			878.6	3	(807)VTXSADDTXES-FGEWR	
		46 kDa§	784.3	2	SAAADTXQEFGDWR	metalloprotease
			527.2	2	GNYYGYCR	Cys-rich
		53 kDa§	766.9	2	(SV)PNDPDFGMVTVR	metalloprotease
			784.3	2	SAAADTXQEFGDWR	
			679.3	2	(228.2)QGQGDFYCR	Cys-rich
32	Blocked	46 kDa§	629.3	2	YTXNAFGEWR	metalloprotease
			807.9	2	MYEXANXVNDXYR	
			527.2	2	GNYYGYCR	Cys-rich
33	Blocked	48861	629.3	2	YTXNAFGEWR	metalloprotease
			807.9	2	MYEXANXVNDXYR	
			979.6	3	ASMSECDPAEHCTGQ-SSECPADVFHK	disintegrin-like
			527.2	2	GNYYGYCR	Cys-rich
			615.8	2	DNSPGQNNPCK	
			776.8	2	VCSNGHCVDVATAY	
		14 kDa§	849.2	3	DCPSGWSSYEGHCYKPF NEPK	C-type lectin
34, 35	Heterogeneous	72 kDa§	653.4	2	YVEXVXVADQR	metalloprotease
			745.3	2	DNQKGNDYGYCR	Cys-rich
		56 kDa§	627.3	2	QAWXYEESXR	L-amino acid oxidase
			647.8	2	EGWYANXGPMR	
			881.4	2	EGWYANXGPMRLPEK	
			486.2	2	VQVHFNAR	
		46 kDa§	629.3	2	YTXNAFGEWR	metalloprotease
			677.8	2	YEDTMQYEFK	
			807.9	2	MYEXANXVNDXYR	
			957.4	2	TWVYEXVNTXNEXYR	

Table 2 (Continued)

HPLC fraction SCT-	N-terminal sequencing	isotope-averaged MALDI-TOF mass (± 0.2%)	peptide ion *m*/*z*	z	MS/MS-derived sequence	protein family
			843.9	3	XHSWVECESGECCEQCRFR	disintegrin-like
			527.2	2	GNYYGYCR	Cys-rich
		36 kDa§	657.8	2	YXEXVVVADHR	metalloprotease
			629.3	2	YTXNAFGEWR	
			807.9	2	MYEXANXVNDXYR	
			912.8	2	VTXSADDTXQAFAEWR	
			851.6	2	VAXXGXEXWSSGEXSK	
			527.2	2	GNYYGYCR	Cys-rich
		23 kDa§	657.8	2	YXEXVVVADHR	metalloprotease
			807.9	2	MYEXANXVNDXYR	
			874.9	2	VAVTMTHEXGHNGNR	
			846.8	2	HSTGVVEDHSEXNXR	
			899.8	2	YVEXFNVVDHGMFTK	
		14 kDa§	849.2	3	DCPSGWSSYEGHCYKPFNEPK	C-type lectin

[a] X, Ile or Leu; Unless other stated, for MS/MS analyses, cysteine residues were carbamidomethylated; n.p.: not peptidic material. M: major species; m: minor species; *: MALDI-TOF mass of the reduced and pyridylethylated molecule; §: apparent molecular mass determined by SDS−PAGE after sample reduction with β-mercaptoethanol; N. D.: not determined. If available, Swiss-Prot or GenBank accession codes are given between square brackets.

tion with sequencing grade bovine pancreas trypsin (Roche) using a ProGest digestor (Genomic Solutions) following the manufacturer's instructions. The tryptic peptide mixtures were dried in a Speed-Vac and redissolved in 5 μL of 70% acetonitrile and 0.1% TFA. Digests (0.65 μL) were spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile containing 0.1% TFA, dried, and analyzed with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer, operated in delayed extraction and reflector modes. A tryptic peptide mixture of *Cratylia* floribunda seed lectin (Swiss-Prot accession code P81517), prepared and previously characterized in our laboratory, was used as mass calibration standard (mass range, 450−3300 Da).

CID-MS/MS. For peptide sequencing, the protein digest mixture was loaded in a nanospray capillary column and subjected to electrospray ionization mass spectrometric analysis using a QTrap mass spectrometer (Applied Biosystems)[43] equipped with a nanospray source (Protana, Denmark). Doubly or triply charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were analyzed in enhanced resolution MS mode, and the monoisotopic ions were fragmented using the enhanced product ion tool with Q_0 trapping. Enhanced resolution was performed at 250 amu/s across the entire mass range. Settings for MS/MS experiments were as follows: Q1, unit resolution; Q1-to-Q2 collision energy, 30−40 eV; Q3 entry barrier, 8 V; LIT (linear ion trap) Q3 fill time, 250 ms; and Q3 scan rate, 1000 amu/s. CID spectra were interpreted manually or using the on-line form of the MASCOT program at http://www.matrixscience.com.

Variation in Venom within and between *Sistrurus* Taxa. We used similarity coefficients to estimate the similarity of venom proteins between individuals within and between taxa. These coefficients are similar to the bandsharing coefficients used to compare individual genetic profiles based on multilocus DNA fingerprints.[44] We defined the protein similarity coefficient (PSC) between two individuals "a" and "b" in the following way: PSC_{ab} = 2(individual proteins shared between a and b)/(total number of distinct proteins in a + total number of distinct proteins in b). For intraspecific comparisons, we carried out this comparison using the proteomics data for each of the two individuals sampled for *S. c. catenatus*, *S. c. tergeminus*, and *S. c. edwardsii*. For interspecific comparisons, we used all pairwise comparisons between individuals from different taxa and then averaged the values for particular combinations of taxa. We judged two proteins (listed in Tables 1−4) as being different when they met one or more of these criteria: (1) Had different N-terminal sequences and/or distinct internal peptides sequences (derived from MS/MS data) corresponding to homologous regions; (2) had different peptide mass fingerprints; (3) were of different sizes (judged by MALDI-TOF MS or SDS−PAGE). For these comparisons, two proteins were judged to differ in size if they differed by more than our estimate of the 95% confidence interval for particular sizing techniques (0.4% for MALDI-TOF MS derived masses and ± 1.4 kDa for SDS−PAGE-determined masses). (4) Eluted in different reverse-phase HPLC peaks. Because of the difficulty of comparing HPLC profiles across different taxa, we restricted the use of this criterion to within taxon comparisons. We emphasize that these measures will give only minimum estimates of the similarities between the venom profiles of two individual. We suspect that a number of the proteins that we judge to be the same using the above criteria would be found to differ at one or more of these criteria if more complete information were available.

Finally, we also estimated one metric of overall venom diversity for each taxa by determining the numbers of distinct proteins across all families that were present in the venom of each taxa, using the data presented in Tables 1−5. For each taxa, proteins were judged as different using the criteria described above, and the mean total number of distinct proteins were calculated. Values estimated for SCC, SCT, and SCE (see Figure 7) represent averages across the data for two individuals presented in Tables 1−4, whereas the results for SMB are for the single individual analyzed in Table 5.

Results and Discusion

Proteomic Characterization of Sistrurus Venoms. Venoms of *Sistrurus* snakes were fractionated by reversed-phase HPLC (Figures 2−5), followed by analysis of each chromatographic fraction by SDS−PAGE, N-terminal sequencing, and MALDI-TOF mass spectrometry. Many reversed-phase peaks corresponded to essentially pure proteins (i.e., fraction 7, 22, and 29 in Figure 2B). Fractions containing mixtures of components were either subjected to rechromatography using a flatter acetonitrile gradient, or their constitutent SDS−PAGE-separated protein bands were excised and identified by by MALDI-TOF mass fingerprinting and CID-MS/MS.

Table 3. Assignment of the Reversed-Phase Isolated Fractions of *Sistrurus catenatus edwardsii* (SCE) Venoms to Protein Families by N-Terminal Edman Sequencing, MALDI-TOF Mass Spectrometry, and Collision-Induced Fragmentation by nESI-MS/MS of Selected Peptide Ions from In-Gel Digested Protein Bands[a]

HPLC fraction SCE-	N-terminal sequencing	isotope-averaged MALDI-TOF mass (± 0.2%)	peptide ion *m/z*	*z*	MS/MS-derived sequence	protein family
1−6	n. p.					
7	GEECDCGSPANPCCD	[7423, 7631]				disintegrin
8	SPPVCGNKILEQGEDCDCGSP -ANCQDRCCNAATCKLTPGSQ	8960				disintegrin (bitistatin-like)
9,10	NLIQFETLILKVAKK	13832				PLA_2
11	N. D.	8960	534.3	2	FETPEECR	Kunitz protease inhibitor
12	GSGCFGLKLDRIGSMSGLGC	1956.0[¶]				C-type BPP
		27 + 30 kDa[§]	534.7	2	GXCCDQCR	disintegrin-like
			527.7	2	NGHPCXNNK	
			813.8	2	NQCXSFFGPSATVAK	cysteine-rich
			823.0	3	NNCNVXYTPTDEDXGmVXPGTK	
			777.3	2	VCSNGHCVDVATAY	
13−17					SVGEECDCGSPNTE	disintegrin-like
		23 kDa[§]	588.2	2	NFPCAQPDVK	cysteine-rich
			530.6	2	QGDNFYCR	cysteine-rich
		25 kDa[§]	679.3	2	VQKGQGVYYCR	cysteine-rich
			774.9	2	SPPNDPDFGFVSR	cysteine-rich
19	N. D.	33 kDa[§]	583.8	2	XGNYYGYCR	cysteine-rich
20,21	Blocked	16 kDa[§]	556.8	2	NPNPVPTGCR	nerve growth factor
			690.9	2	AXTmEGNQASWR	
			640.9	2	XDSACVCVXSR	
22	N. D.	23211	588.2	2	NFPCAQPDVK	cysteine-rich
			530.6	2	QGDNFYCR	
			626.3	2	XYNDNXNPCK	
23	NLLQFET	13856				PLA_2
		16 kDa[§]	708.2	2	(217)XPFmEVYQR	nerve growth factor
24	SVDFDSESPRKPEIQ	24829				CRISP
25	NLIQFETLILKVAKK	13842				PLA_2
26	IIGGEECNINEHRFL	[26202, 28742]				Ser-proteinase
27	VVGGEECNINEHRSL	29 kDa[§]				Ser-proteinase
28	(V+I)IGGDECN(I+V)NEHR(F+S)L	[27957, 28497]				Ser-proteinase
29	VVGGDECNINEHRFL	[28561, 28813]				Ser-proteinase
30	VIGGDECNINEHRSL	[29090, 30332]				Ser-proteinase
31	VVGGEECNINEHRSL	[27260, 27491]				Ser-proteinase
32	VVGGEECNINEHRSLV	[27547, 27769]				Ser-proteinase
33	N. D.	30406 (15 kDa[§])	730.3	2	GXDCXSDWSSYR	C-type lectin
			483.6	2	YSAWXGXR	
34−36	Blocked	46 kDa[§]	851.9	2	HDNAQXXTADEFDGR	metalloprotease
			588.2	2	NFPCAQPDVK	cysteine-rich
			530.6	2	QGDNFYCR	cysteine-rich
		15 kDa[§]	730.3	2	GXDCXSDWSSYR	C-type lectin
			483.6	2	YSAWXGXR	
37	Blocked	52 kDa[§]	584.2	3	SHQXPSEFSDCSEK	metalloprotease
			742.2	3	XYEXVNTmNEXYXPXNXR	
			881.3	2	TTTDFDGDTVGXAFFR	
			535.2	2	GXCCDQCR	disintegrin-like
			475.7	2	GSSYGYCR	cysteine-rich
			604.8	2	XFCFPNKPGK	
			814.3	2	NQCXSFFGPSATVAK	
		43 kDa[§]	843.9	2	HDNAQXXTAXDFDGR	metalloprotease
			774.8	2	FAXVGXQXWSTGQK	
			827.8	3	TWVmQFVNTXNEXYXPXNXR	
		15 kDa[§]	730.3	2	GXDCXSDWSSYR	C-type lectin
			483.6	2	YSAWXGXR	
38	Blocked	52 kDa[§]	584.2	3	SHQXPSEFSDCSEK	metalloprotease
			742.2	3	XYEXVNTmNEXYXPXNXR	
			881.3	2	TTTDFDGDTVGXAFFR	
			535.2	2	GXCCDQCR	disintegrin-like
			475.7	2	GSSYGYCR	cysteine-rich
			604.8	2	XFCFPNKPGK	
			814.3	2	NQCXSFFGPSATVAK	
		15 kDa[§]	730.3	2	GXDCXSDWSSYR	C-type lectin
			483.6	2	YSAWXGXR	
39	Blocked	46 kDa[§]	584.2	3	SHQXPSEFSDCSEK	metalloprotease
			742.2	3	XYEXVNTmNEXYXPXNXR	
			774.8	2	FAXVGXQXWSTGQK	
			822.6	3	NNCNVXYTPTDEDXGmVXPGTK	
			535.2	2	GXCCDQCR	disintegrin-like
			475.7	2	GSSYGYCR	cysteine-rich
			604.8	2	XFCFPNKPGK	
			814.3	2	NQCXSFFGPSATVAK	
		15 kDa[§]	730.3	2	GXDCXSDWSSYR	C-type lectin
			483.6	2	YSAWXGXR	
40	Blocked	52 kDa[§]	627.3	2	QAWXYEESXR	L-amino acid oxidase
			486.2	2	VQVHFNAR	
			558.4	2	VXEXQQNDR	
			583.8	2	XKFEPPXPPK	
		42 kDa[§]	677.9	2	YXEXVXVADYR	metalloprotease
			718.0	2	ASQXYXTPEQQR	

Table 3 (Continued)

HPLC fraction SCE-	N-terminal sequencing	isotope-averaged MALDI-TOF mass (± 0.2%)	peptide ion *m*/*z*	*z*	MS/MS-derived sequence	protein family
		33 kDa§	664.9	2	YXEXVXVADHR	metalloprotease
		24 kDa§	646.3	2	YXNVXVVADQR	metalloprotease
		15 kDa§	730.3	2	GXDCXSDWSSYR	C-type lectin
			483.6	2	YSAWXGXR	
41	Blocked	52 kDa§	627.3	2	QAWXYEESXR	L-amino acid oxidase
			486.2	2	VQVHFNAR	
		36 kDa§	630.3	2	QPYXTPEQQR	metalloprotease
			652.3	2	SHXTYXPQCXXNEPXR	
		15 kDa§	730.3	2	GXDCXSDWSSYR	C-type lectin
			483.6	2	YSAWXGXR	
42	Blocked	56 kDa§	677.8	2	YEDTMQYEFK	metalloprotease
			957.4	2	TWVYEXVNTXNEXYR	
			843.9	3	XHSWVECESGECCEQCRFR	disintegrin-like
			514.7	2	IPCAPEDVK	cysteine-rich
			583.8	2	XGDYYGYCR	cysteine-rich
43	N. D.	52 kDa§	627.3	2	QAWXYEESXR	L-amino acid oxidase
			486.2	2	VQVHFNAR	
		36 kDa§	657.8	2	YXEXVVVADHR	metalloprotease
			912.8	2	VTXSADDTXQAFAEWR	
		15 kDa§	730.3	2	GXDCXSDWSSYR	C-type lectin
			483.6	2	YSAWXGXR	

[a] X, Ile or Leu; m, methionine sulfoxide. Unless other stated, for MS/MS analyses, cysteine residues were carbamidomethylated; n.p.: not peptidic material found; *: MALDI-TOF mass of the reduced and pyridylethylated molecule; ¶: monoisotopic mass; §: apparent molecular mass determined by SDS−PAGE after sample reduction with β-mercaptoethanol; N. D.: not determined.

Despite the fact that only six *Sistrurus* venom protein entries are annotated in the Swiss-Prot/TrEMBL nonredundant database, HPLC fractions that yielded unambiguous N-terminal sequences could be classified into known protein families using a BLAST amino acid similarity search (Tables 1−4), indicating that representative members of each of these families are present in the protein sequence banks. Protein fractions with ambiguous or blocked N-termini were digested with trypsin (in-gel or in solution) and the resulting peptides were analyzed by MALDI-TOF mass fingerprinting followed by CID-MS/MS. As expected from the rapid amino acid sequence divergence of venom proteins by accelerated evolution,[2,18,31,45,46] with a few exceptions, the product ion spectra did not match to any known protein using the MASCOT search program. The CID-MS/MS spectra were therefore manually interpreted and the deduced peptide ion sequences submitted to BLAST sequence similarity searches. This approach allowed us to assign unambiguously all of the isolated venom fractions to protein families present in the nonredundant databases (Tables 1−4).

Recent reports surveyed gene transcriptional activity (transcriptome) of the snake venom glands of *Bothrops insularis,*[47] *Bothrops jararacussu,*[48] *Bitis gabonica,*[49] and *Deinagkistrodon acutus*[50] by generation of expressed sequence tags (ESTs) or construction of a cDNA library followed by sequencing of the clones. These works have provided catalogs of full-length venom gland mRNAs. Our proteomic approach complements these studies by showing the relative abundance of the various protein families that are actually secreted into the venoms.

Supporting the view that venom proteomes are mainly composed of proteins belonging to a few protein families,[9−11,36] the proteins found in the venoms of the four *Sistrurus* snakes cluster in 11 different families (disintegrins, myotoxins, C-type bradikinin potentiating peptides (BPP), Kunitz-type inhibitors, PLA_2, nerve growth factors, cysteine-rich secretory proteins (CRISP), serine proteinases, C-type lectins, L-AMINO acid oxidase, Zn^{2+}-dependent metalloproteases, and released disintegrin-like/cysteine-rich (DC) fragments) (Table 5). However, the protein family expression profile and the relative abundance of each group of proteins (calculated from the combined areas of the reverse-phase chromatographic peaks corresponding to proteins of the same family) in the different venoms are not conserved. Myotoxins and 2-chain PLA_2 molecules were detected only in the venoms of *S. c. catenatus* (SCC) and *S. c. tergeminus* (SCT), whereas C-type BPP and Kunitz-type inhibitors were only found (at low abundance) in *S. c. edwardsii* (SCE) and *S. m. barbouri* (SMB) venoms (Table 5). The small basic protein, myotoxin a, is known to cause muscle necrosis and has been reported to be widely distributed among rattlesnake species in the New World (including *Sistrurus catenatus*) but varies qualitatively by geographical region in several species and subspecies.[51−54] Also notable is the lack of N6−PLA_2 in SCE and the high relative abundance of disintegrins and CRISP in SMB and SCE, respectively. The N6−PLA_2s SCC-12−14 (Table 1) and SCT-17−19 (Table 2) are homologues of myotoxic or neurotoxic basic phospholipases that exist as either monomers or as the B-subunits of sistruxin-like heterodimers in some Asian and New World pit vipers, including *S. m. streckeri*, *S. m. barbouri*, and *S. c. tergeminus*).[55] The 2-chain PLA_2 SCT-14 (Table 2) appears to be identical to sistruxin-A (Swiss-Prot Q6EAN6) residues 41−112 and 127−138, which are linked by two disulfide bonds (calculated M_{av} = 9028.8 Da). Sistruxin A is the acidic subunit of the heterodimeric PLA_2 sistruxin, a homologue of the well-characterized presynaptic-acting neurotoxic Mojave toxin from *Crotalus scutulatus scutulatus*.[56,57] Thus, the existence in *S. c. catenatus* and *S. c. tergeminus* of 2−3 A-type and 3 B-type sistruxin subunits suggest the existence in these snakes of neurotoxic PLA_2 isoforms. Similarly, analyses of venoms from *Crotalus scutulatus scutulatus* individuals also indicated that each snake produced multiple isoforms of the neurotoxin.[58]

Intra- and Interspecies Accelerated Evolution of Venom Protein Families. Though the four *Sistrurus* venoms contain similar amounts of major proteins (Zn^{2+}-metalloproteases (43 ± 6%), serine proteinases (20 ± 3%), and PLA_2s (15 ± 3%)), the degree of structural diversity within each protein family varies among the venoms. The occurrence in the different venoms of both, apparently identical proteins and distinct sets of isoforms and multigene products of each major protein family, is particularly evident when the HPLC separation

Table 4. Assignment of the Reversed-Phase Isolated Fractions of *Sistrurus miliarius barbouri* (SMB) Venom to Protein Families by N-Terminal Edman Sequencing, MALDI-TOF Mass Spectrometry, and Collision-Induced Fragmentation by nESI-MS/MS of Selected Peptide Ions from In-Gel Digested Protein Bands[a]

HPLC fraction SMB-	N-terminal sequencing	isotope-averaged MALDI-TOF mass (± 0.2%)	peptide ion *m*/*z*	*z*	MS/MS-derived sequence	protein family
1−5	n.p.					
6−8	AGEECDCGSP					
	GEECDCGSPE					
	EECDCGSPEN					
	ECDCGSPENP	7 kDa§				disintegrin
9	EECDCGSPENPCCDA	7098				barbourin [P22827] 4−70
	GEEDCDGSPENPCCD	7156				barbourin 3−70
10	GSGCFGLKLDRIGSMSGLGC	1956.0¶				C-type BPP
11, 12	SPPVCGNKILEVGEECDCGTPEN	22866				DC-fragment
13	DIISPPVCGNELLEVGEECDCGEPE	23268				DC-fragment
14	N. D.	16 kDa§	556.3	2	NPNPVPTGCR	nerve growth factor
			682.8	2	AXTMEGNQASWR	
15	NLLQFNKMIKIMT	13952				N6−PLA$_2$
16	NLLQFNKMIKIMTKKNAIP	13956				N6−PLA$_2$
17	SVNFDSESPPKPEIQ	24842				CRISP
18	NLIQFETLIM	13963				PLA$_2$
19	HLIQFETLIMKIAGRSGVFW	13980				PLA$_2$
20−25	VIGGNECNINEHRSL	27 kDa§				serine proteases
26	NPEHQRYVELFIVVDHGM	23 kDa§				PI-metalloprotease
27	NPEHQRYVELFIVVD	23 kDa§	518.6	2	STGVVQDHSEXXXR	PI-metalloprotease
		59 kDa§	619.7	2	SAGQXYEDSXR	L-AMINO acid oxidase
			688.7	3	DCADXVXNDXSXXHQXPK	
			557.7	2	VXEXQQNDR	
			436.7	2	KVVEEXR	
			503.9	3	YXXDKYDTYSTK	
			783.3	3	XYFAGEYTAQFHGWXDSTXK	
28	BLOCKED	48.5 kDa§	615.3	2	LPDSEAHAVYK	PIII-metalloprotease
			932.9	2	IQNDADSTASXSACNGLK	(CAD29055)
			744.8	3	XSHQPSTQFSDCSEEYCR	
			737.0	3	XYEXVNTMNEXYXPXNXR	
			653.0	3	AAKDDCDMADXCTGQSAK	disintegrin-like
			755.0	3	NQCXSFFGPSATVAKDSCFK	cysteine-rich
			818.0	3	NNCNVXYTPTDEDXGMVXPGTK	
			776.8	2	VCSNGHCVDVATAY	
			543.2	2	XPCEPQDVK	
29, 30	Blocked	46 kDa§	743.8	2	ETDYEEFXEXAK	L-AMINO acid oxidase
			619.7	2	SAGQXYEDSXR	
			688.7	3	DCADXVXNDXSXXHQXPK	
			783.3	3	XYFAGEYTAQFHGWXDSTXK	
		43 kDa§	555.8	3	(320)MAHEXGHNXGXR	metalloprotease
			590.3	3	SGSQCGHGDCCEQCK	disintegrin-like
			526.7	2	GNYYGYCR	
			514.8	2	IPCAPEDVK	cysteine-rich
			776.7	2	VCSNGHCVDVATAY	
		23 kDa§	672.8	2	YXEXVVVTDHR	PI-metalloprotease
			631.2	3	TWVHEXVNTXNVFYR	
			547.8	2	YNSNXNTXR	
		17 kDa§	611.8	3	DCPSGWSSYEGHCYK	C-type lectin
31	blocked	46 kDa§	743.8	2	ETDYEEFXEXAK	L-AMINO acid oxidase
			619.7	2	SAGQXYEDSXR	
			688.7	3	DCADXVXNDXSXXHQXPK	
			783.3	3	XYFAGEYTAQFHGWXDSTXK	
		43 kDa§	555.8	3	(320)MAHEXGHNXGXR	metalloprotease
			590.3	3	SGSQCGHGDCCEQCK	disintegrin-like
			514.8	2	IPCAPEDVK	cysteine-rich
			776.7	2	VCSNGHCVDVATAY	
		36 kDa§	657.8	2	YXEXVVVADHR	metalloprotease
			807.9	2	MYEXANXVNDXYR	
			912.8	2	VTXSADDTXQAFAEWR	
			851.6	2	VAXXGXEXWSSGEXSK	
		23 kDa§	605.1	3	YVEXFXVVDQEMVTK	PI-metalloprotease
			518.6	2	STGVVQDHSEXXXR	
			588.6	3	VAVTmTHEXGHNXGXR	
		17 kDa§	611.8	3	DCPSGWSSYEGHCYK	C-type lectin

[a] X, Ile, or Leu; m, methionine sulfoxide. Unless other stated, for MS/MS analyses, cysteine residues were carbamidomethylated; n.p.: not peptidic material found; ¶: monoisotopic mass; §: apparent molecular mass determined by SDS−PAGE after sample reduction with β-mercaptoethanol; N. D.: not determined. If available, Swiss-Prot or GenBank accession codes are given between square brackets.

profiles (Figure 6) and the MALDI-TOF masses of proteins from the same family (Tables 1−4) are compared.

The largest intraspecies structural divergence occurs among the serine proteinases and the Zn^{2+}-metalloproteases. Accelerated evolution of genes encoding serine proteinases and metalloproteases has been reported in other crotaline species.[21,59] The large variability in their chromatographic elution, molecular masses, and tryptic peptide mass fingerprinting (Tables 1−4) supports the existence of multiple isoforms of serine proteinases and Zn^{2+}-metalloproteases in venoms of the four *Sistrurus* taxa. The finding of nonidentical internal peptide sequences corresponding to homologous regions of Zn^{2+}-metalloproteases eluting in different chromatographic peaks of the same venom (i.e., ions 605.3^{2+} (VTXNSFGEWR) and

Table 5. Overview of the Relative Occurrence of Proteins[a] of the Different Families in the Venoms of *Sistrurus catenatus catenatus* (SCC), *Sistrurus catenatus tergeminus* (SCT), *Sistrurus catenatus edwardsii* (SCE), and *Sistrurus miliarius barbouri* (SMB)

	venom			
protein family	SCC	SCT	SCE	SMB
disintegrin	2.5	4.2	0.9	7.7
myotoxin	0.4	<0.1	-	-
C-type BPP	-	–	<0.1	< 0.1
Kunitz-type inhibitor	-	–	<0.1	0.1
DC-fragment	<0.1	<0.1	<0.1	1.3
2-chain PLA_2	2.5	1.9	-	-
nerve growth factor	<0.1	<0.1	<0.1	<0.1
N6–PLA_2	12.8	14.9	–	13.9
PLA_2	14.6	14.8	13.7	18.6
CRISP	0.8	1.3	10.7	2.9
serine proteinase	18.2	20.4	24.4	17.1
C-type lectin	<0.1	<0.1	<0.1	<0.1
L-AMINO acid oxidase	4.2	1.6	2.5	2.1
Zn^{2+}-metalloproteinase	43.8	40.6	48.6	36.1

[a] In percentage of the total HPLC-separated proteins.

659.3^{2+} (YTXNSFGEWR) in different protein bands of fractions SCC-26, 27, 29, and 31; and 629.3^{2+} (YTXNAFGEWR), 784.3^{2+} (SAAADTXQEFGDWR), and 912.8^{2+} (VTXSADDTXQAFAEWR) in SCT-29–32, 34 and 35) further indicates that the corresponding parent molecules were isoforms. It is also noteworthy that among 93 unique Zn^{2+}-metalloprotease-derived tryptic peptide ions sequenced by MS/MS, very few are common to any pair of the *Sistrurus* snakes under study. SCC and SCT share the largest number (6 out of 45) of identical tryptic peptide ions, but this value is small (~13%). The data thus indicate that Zn^{2+}-metalloproteases have evolved intra- and interspecifically in an accelerated manner such that each venom contains a distinct set of metalloprotease isoenzymes.

Table 6 shows conservation and divergence among PLA_2 molecules identified in the different *Sistrurus* venoms. The fact that a number of *Sistrurus* share apparently identical PLA_2 molecules could reflect recent common ancestry and/or convergence in aspects of their diets (Figure 1). However, the occurrence of distinct PLA_2 molecules in each *Sistrurus* venom is consistent with an accelerated and regional evolution of PLA_2 isozymes, similar to the diversity documented for PLA_2 isoforms of *Trimeresurus* species inhabiting the south-western islands of Japan[24,31,60] and Taiwan,[61] and the birth-and death model for the evolution of elapid three-finger toxins.[62]

The venoms of SCC and SCT also appear to be rather similar, based on the observations that (i) myotoxins and 2-chain PLA_2 molecules are detected only in SCC and SCT venoms and (ii) the major PIII-metalloproteases of SCC (HPLC peak 29; 48877 Da) (Table 1) and SCT (HPLC peak 33; 48861 Da) (Table 2) and the PI-metalloproteases SCC-24 (23053 Da) and SCT-27 (23082 Da) exhibit almost identical chromatographic behavior (Figure 6) and mass tryptic peptide fingerprinting. Again, this similarity could be due to either convergent selection or common ancestry—convergence appears more likely, because if common ancestry was of overriding importance, then the venom of SCT should be more similar to a member of the same clade (SCE) than to SCC, but this relationship does not hold. Alternatively, divergence of SCE metalloproteases from a shared ancestral condition could give rise to the observed similarities in metalloproteases.

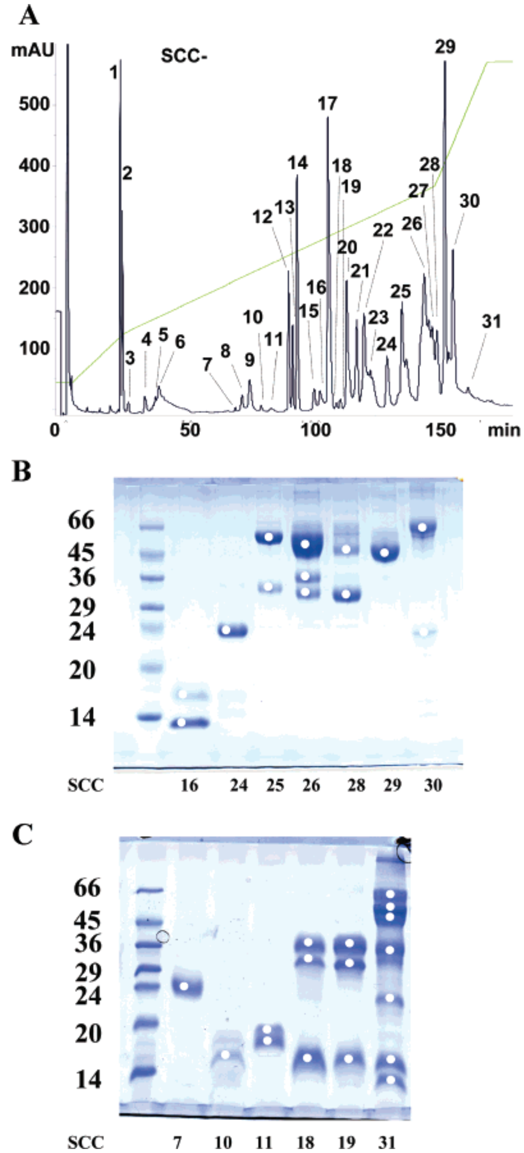

Figure 2. (A) Reversed-phase HPLC separation of the *Sistrurus catenatus catenatus* venom proteins. Chromatographic conditions were: isocratically (5% B) for 10 min, followed by 5–15% B for 10 min, 15–50% B for 140 min, and 50–70% B for 10 min. Fractions were collected manually and characterized by N-terminal sequencing, MALDI-TOF mass spectrometry, tryptic peptide mass fingerprinting, and CID-MS/MS of selected doubly or triply charged peptide ions. The results are shown in Table 1. (B) and (C) SDS–PAGE showing the protein composition of major and minor fractions, respectively, of the reversed-phase HPLC separation of *Sistrurus catenatus catenatus* venom shown in (A). The holes contained those pieces of the SDS–PAGE bands excised for characterizing the proteins by mass fingerprinting and CID-MS/MS.

Broad-Scale Patterns of Venom Diversity and Differentiation. The availability of detailed proteomic information on individual proteins described above make possible detailed estimates of the similarity and differentiation of the venom proteomes of different taxa that are based on comparisons of individual proteins which are then useful in revealing broad-scale evolutionary patterns. Using one measure of overall venom protein diversity (numbers of distinct proteins; see Table 7), the *Sistrurus* taxa sampled fall roughly into two groups: three species with relatively diverse venoms (SCC, 41.5; SCT,

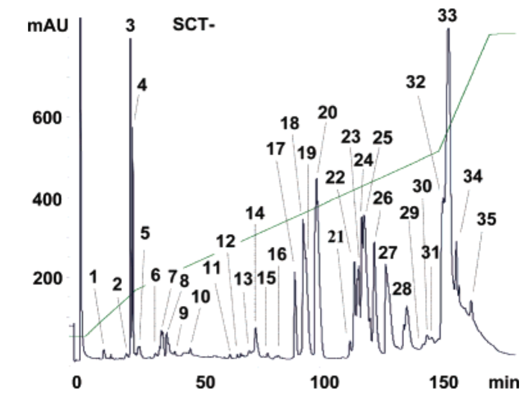

Figure 3. Reversed-phase HPLC separation of the *Sistrurus catenatus tergeminus* venom proteins. Chromatographic conditions were as in Figure 2. Chromatographic fractions were collected manually and characterized by N-terminal sequencing, MALDI-TOF mass spectrometry, tryptic peptide mass fingerprinting, and CID-MS/MS of selected doubly or triply charged peptide ions. The results are shown in Table 2.

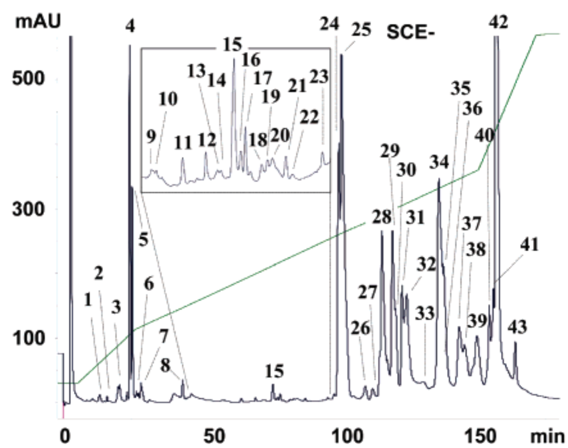

Figure 4. Reversed-phase HPLC separation of the *Sistrurus catenatus edwardsii* venom proteins. Chromatographic conditions were as in Figure 2. Chromatographic fractions were collected manually and characterized by N-terminal sequencing, MALDI-TOF mass spectrometry, tryptic peptide mass fingerprinting, and CID-MS/MS of selected doubly or triply charged peptide ions. The results are shown in Table 3.

44.0; and SCE, 35.5 distinct proteins, respectively) and SMB, which is substantially less variable[24] (Figure 7). When mapped onto a phylogeny of these taxa, this pattern points to a key event occurring early on in the evolution of the group involving the lineage connecting *S. milarius* to the other taxa (Figure 7). Specifically, depending on the level of diversity present in the outgroup (*Crotalus sp.*), the most parsimonious interpretation of how venom diversity evolved was either through a loss of venom diversity in the lineage leading to the other *Sistrurus* taxa or a significant increase along the lineage containing the *S. catenatus* taxa. The first scenario would be supported if the venom variation in *Crotalus* was at a similar level or more diverse than found in taxa within the *S. catenatus* group while if *Crotalus* showed low levels of diversity similar to *S.* miliarius, then the "diversity gain" scenario would be most likely. Although venom protein diversity is not well-known for most *Crotalus* species, based on a two-dimensional gel electrophoretic evaluation of the venom proteome of *Crotalus atrox*,[5] it appears that *Crotalus* venoms contain at least as many

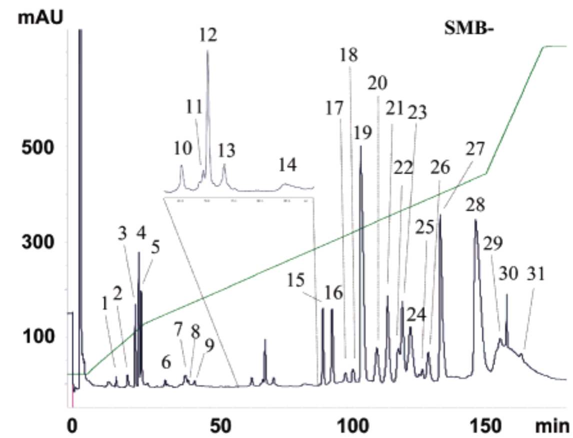

Figure 5. Reversed-phase HPLC separation of the *Sistrurus miliarius barbouri* venom proteins. Chromatographic conditions were as in Figure 2. Chromatographic fractions were collected manually and characterized by N-terminal sequencing, MALDI-TOF mass spectrometry, tryptic peptide mass fingerprinting, and CID-MS/MS of selected doubly or triply charged peptide ions. The results are shown in Table 4.

proteins as *Sistrurus*, consistent with a "diversity loss" scenario in the venom of SMB. However, absolute compositional variation, leading to high or low protein diversity, is likely dependent on numerous factors besides phylogeny. In some species of *Crotalus*, such as *C. oreganus concolor*, venoms with apparently low protein diversity are highly toxic.[41] Therefore, to distinguish between these possibilities requires a comparable detailed analysis of the venom proteome of a *Crotalus* species, which we are currently undertaking.

In terms of within taxon differentiation, comparisons for SCA, SCT, and SCE show that the two individuals analyzed for each taxa shared an average of 83% of their overall venom proteins (Table 7), with the mean proportion of shared proteins being similar for disintegrins, serine proteases, and Zn^{2+}-metalloproteases (83−94%) but substantially lower for PLA_2 proteins (60%). There are also high levels of differentiation between taxa. On the basis of overall protein similarity coefficients (Table 7), on average, less than 30% of the venom proteins identified within each taxon are shared. There is also variation among the most abundant proteins in the level of differentiation between taxa (Table 7): taxa were most similar for metalloproteases (34%) and most divergent in PLA_2 proteins (12%), with values for disintegrins and serine proteases lying between these extremes (25 and 20%, respectively). Thus, among the major proteins found in *Sistrurus* venoms, PLA_2 proteins appear to be exceptionally divergent at both the intra and the interspecific level (also see above). This may be because they have been subject to exceptionally strong balancing selection within, and diversifying selection between, taxa. Other studies have also shown that the genes which underlie these proteins show high levels of divergence between species and high levels of protein diversity at the population level.[15−17,23,24,46,60,61]

Overall, our results emphasize the uniqueness of the venom composition of even closely related species of venomous snakes and point to a strong role for adaptive diversification via natural selection as a cause of this distinctiveness.

Concluding Remarks

Our analysis represents the most detailed analysis of venom composition and variation yet completed among a set of closely

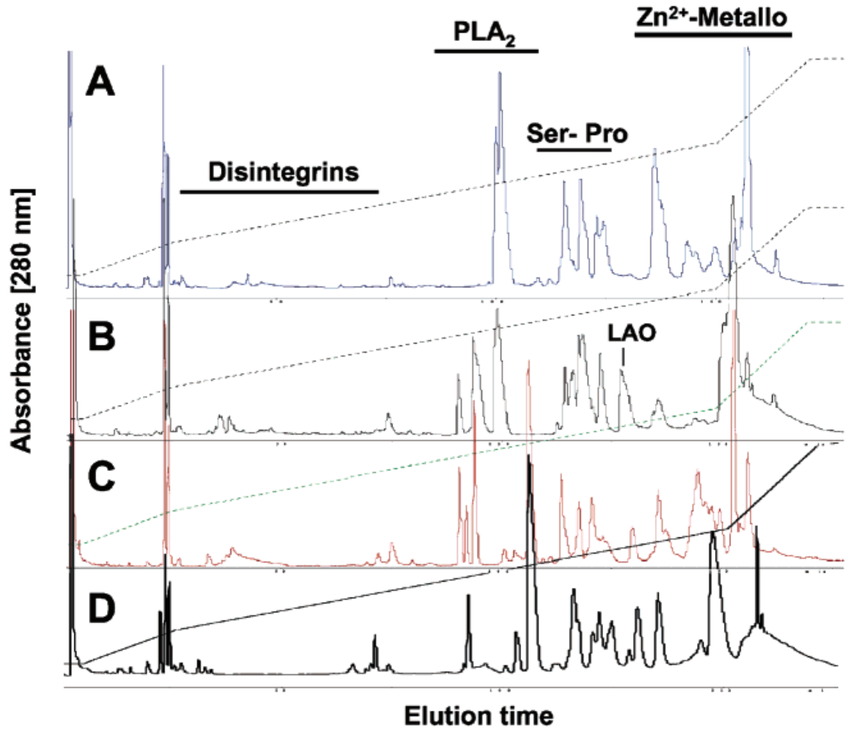

Figure 6. Comparison of the separation profiles of the venom protein from (A) *Sistrurus catenatus edwardsii*, (B) *Sistrurus catenatus tergeminus*, (C) *Sistrurus catenatus catenatus*, and (D) *Sistrurus miliarius barbouri*. The elution positions of major proteins are indicated.

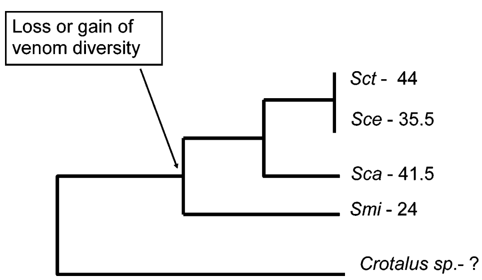

Figure 7. Phylogenetic relationships in the genus *Sistrurus* and the overall venom protein diversity of each taxon branch. Numbers at the tip of each branch represent the mean number of distinct proteins (see above) across all protein families for each taxa. The most parsimonious interpretation of how venom diversity evolved, either through a loss of venom diversity in the lineage leading to the other *Sistrurus* taxa or a significant increase along the lineage containing the *catenatus* taxa, depends on the level of diversity in the outgroup (*Crotalus sp.*).

related yet ecologically distinct venomous snakes. If we assume a link between structural and functional variation in terms of effectiveness at killing and processing different prey,[29,32,41] then our results have implications for how venom has evolved as an adaptation in these snakes.

First, the finding of the high degree of differentiation in the venom proteome among recently evolved, congeneric taxa emphasizes unique aspects of venom composition of even closely related species of venomous snakes and points to a strong role for adaptive diversification via natural selection as a cause of this distinctiveness. Moreover, the high level of within-taxon variation in almost all proteins suggests an important role for balancing selection[63] in maintaining high levels of functional variation in venom proteins within populations. The mechanism leading to this mode of selection is unclear, but we speculate that it may be related to unpredict-

Table 6. Comparison of PLA_2 Molecules Characterized in the Venom Proteomes of of *Sistrurus catenatus catenatus* (SCC), *Sistrurus catenatus tergeminus* (SCT), *Sistrurus catenatus edwardsii* (SCE), and *Sistrurus miliarius barbouri* (SMB)[a]

N-terminal sequence	SCC	SCT	SCE	SMB
NLLQFNKMIKIM-TKK	12 (13 814)	17 (13 741)		15 (13 952)
NLLQFNKMIKIM-TKK	13 (13 880)*	18 (13 887)*		16 (13 956)
HLLQFNKMIKFE-TNK	14 (14 120)$^{\Phi}$	19 (14 119)$^{\Phi}$		
HLIQFETLIMKIAGR	16 (13 967)$^{\beta}$			18 (13 963)$^{\beta}$
HLIQFETLIMKIAGR	17 (13 952)$^{\Theta}$			19 (13 980)$^{\Theta}$
NLLQFET			23 (13 856)	
N. D.	18 (13 941)			
NLIQFETLILKVAKK		20 (13826)$^{\blacklozenge}$	25 (13 842)	

[a] Molecules thought to correspond to the same protein by N-terminal sequencing, reversed-phase HPLC retention time, and MALDI-TOF mass spectrometry are labeled with the same symbol. SCT-17 and SCT-19 correspond, respectively, to Swiss-Prot entries Q6EER3 and Q6EER2 (calculated isotope-averaged molecular masses of 13 749.8 and 14 123.1 Da, respectively). SMB-15 and 16 may correspond to isoforms of the monomeric myotoxic N6−PLA_2 from the same species reported by Chen et al.[55] (13 948 Da).

ability with which a sit-and-wait predator like a rattlesnake encounters different types of prey, each of which are most efficiently subdued with different venom proteins. Thus, to deal with this uncertainty, snakes are required to have a variety of proteins "available" in their venom at all times to deal with different prey. Differential effects of venom to specific prey types (= taxa-specific toxicity) can be extreme,[32] demonstrating the clear functional link between venom composition and effects on prey. However, prey physiological responses are not static, and among mammalian prey in particular, selection for resistance mechanisms may be profound.[64,65] In a sense, the selection pressure leading to high levels of variation in venom genes, namely the capacity for the evolution of detoxifying responses by prey, may parallel the selection pressures acting

Table 7. Protein Similarity Coefficients (PSCs) (Mean ± SD) Based on Comparisons Within and Between *Sistrurus* Taxa[a]

protein	within taxa	between taxa
total	0.83 ± 0.07	0.28 ± 0.19
disintegrins	0.94 ± 0.06	0.25 ± 0.31
PLA2s	0.60 ± 0.09	0.12 ± 0.15
serine proteases	0.86 ± 0.17	0.20 ± 0.18
Zn^{2+}-metalloproteases	0.84 ± 0.09	0.34 ± 0.17

[a] Within taxon comparisons are averages of pairwise comparisons of the venom data for each of two individuals of species *S. c. catenatus*, *S. c. termgeminus*, and *S. c. edwardsii* as presented in Tables 1−3. Between taxon values are means of the four possible comparisons between each of two individuals of *S. c. catenatus*, *S. c. tergeminus*, and *S. c. edwardsii* or two comparisons between these taxa and *S. m. barbouri*.

to promote high levels of variation in the genes involved in the vertebrate immune system, such as those which encode major histocompatibility complex proteins,[66] or in plant host defense genes.[67,68] Various aspects of this hypothesis could be tested by directly examining patterns of allelic variation in specific venom genes to see if they show molecular signatures of balancing selection at the DNA level,[67,69] by assaying purified components to determine if different venom components of the same protein type act more efficiently on different prey, and by assessing the predictability of the diets of individual snakes through time.

A final implication of our results is that there does not appear to be a simple relationship between levels of venom variation and diet diversity. Because species-specific effects of venom components are largely unknown, it is difficult to assign a functional role unequivocally to the variation we observed in *Sistrurus* venoms. However, if one considers relative importance of mammals in the diet of *Sistrurus*, diet trends and complexity trends are parallel and show the following order, from high to low: SCC > SCT > SCE > SMB. Both SCC and SCT include a much greater proportion of mammals in their diets than does SCE,[39] and SMB rarely feed on mammals in the wild.[40,70] It is possible that increased reliance on mammalian prey has driven selection for greater venom protein diversity, and the presence of myotoxin-a homologues and 2-chain PLA_2s *only* detected in SCC and SCT venoms is consistent with this hypothesis. The very high level of CRISP proteins in the venom of SCE, relative to the other taxa, is intriguing, as this toxin appears to be a component of virtually *all* venoms and therefore became incorporated into the venom proteome early in the evolution of venom systems;[13] unfortunately, the biological activity of most venom CRISPs are currently unknown.[71] It is clear that venom composition and diet are related, but because various species have mixed diets, including both endotherms and ectotherms, invertebrates and vertebrates, the diet/composition relationship is likely rather complex. We feel the key to understanding the relationship between diet and venom diversity lies in a better understanding of the action of specific venom components on particular prey. Other measures of diet that rely on techniques such as stable isotope ratio analyses[72] may also give a different more fine-scaled assessment of diet than has been previously possible, because these analyses sample diet over a longer time frame than static gut content studies. Combined with detailed proteomic analysis of venom composition, stable isotope analysis may lead to a clearer picture of the link between venom variation and diet diversity.

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