

## Snake Venomics of *Bitis* Species Reveals Large Intra-genus Venom Toxin Composition Variation: Application to Taxonomy of Congeneric Taxa

Juan J. Calvete,\* José Escolano, and Libia Sanz

Instituto de Biomedicina de Valencia, C.S.I.C., Jaume Roig 11, 46010 Valencia, Spain

Received March 27, 2007

The protein composition of the venoms of the West African Gaboon viper (*Bitis gabonica rhinoceros*), the rhinoceros viper (*Bitis nasicornis*), and the horned puff adder (*Bitis caudalis*) were analyzed by RP-HPLC, N-terminal sequencing, SDS-PAGE, MALDI-TOF peptide mass fingerprinting, and CID-MS/MS. In line with previous proteomic and transcriptomic analyses showing that snake venom proteins belong to only a few major protein families, the venom proteomes of *Bitis gabonica rhinoceros*, *Bitis nasicornis*, and *Bitis caudalis* comprise, respectively, toxins from 11, 9, and 8 toxin families. Dimeric disintegrins, PLA<sub>2</sub> molecules, serine proteinases, a CRISP, C-type lectin-like proteins, L-amino acid oxidases, and snake venom metalloproteases are present in the three *Bitis* snake venoms, though they depart from each other in the composition and the relative abundance of their toxins. The venom composition appears to keep information on the evolutionary history of congeneric taxa. Protein similarity coefficients used to estimate the similarity of venom proteins of the *Bitis* taxa sampled here and in previous studies (eg. *Bitis arietans* and *Bitis gabonica gabonica*) support the monophyly of the three West African taxa (*B.g. gabonica*, *B.g. rhinoceros*, and *B. nasicornis*) based on genetic distance reconstructions, the lack of alliances between *B. arietans* and any other *Bitis* species, and are consistent with the taxonomic association of *Bitis caudalis* within the differentiated group of small *Bitis* species. The low level of venom toxin composition similarity between the two conventionally recognized subspecies of *Bitis gabonica*, *B. g. gabonica* and *B. g. rhinoceros*, supports the consideration by some authors of *B. g. rhinoceros* as a separate species, *Bitis rhinoceros*. Moreover, our proteomic data fit better to a weighted phylogram based on overall genetic distances than to an unweighted maximum-parsimony tree.

**Keywords:** *Bitis gabonica rhinoceros* • *Bitis nasicornis* • *Bitis caudalis* • West African Gaboon viper • rhinoceros viper • horned puff adder • snake venom protein families • proteomics • viperid toxins • snake venomics • N-terminal sequencing • mass spectrometry

### Introduction

The suborder of snakes (*Serpentes*) of the reptilian order Squamata, named for their scaly skin, includes about 3000 extant species placed in approximately 400 genera and 18 families (<http://www.reptile-database.org>). The timing of major events in snake evolution is not well understood, however, owing in part to a relatively patchy and incomplete fossil record.<sup>1,2</sup> The most generalized phylogenetic view is that the group evolved from a family of terrestrial lizards during the time of the dinosaurs in the Jurassic period, about 200 million years (Myr) ago. After the end of the non-avian dinosaurs reign on the Earth, around the Cretaceous-Tertiary boundary 65 Myr ago,<sup>3</sup> the boids (the ancestors of boas, pythons, and anacondas) were the dominant snake family on earth. Within the Cenozoic era that followed, advanced snakes (Caenophidia) arose in the

Oligocene epoch 35–25 Myr ago. Colubrids, the family which we regard today as typical snakes, remained a small taxon until the tectonic plates drifted apart from the equator and the cool climate pushed boids to disappear from many ecological niches. Colubrids quickly colonized these empty habitats, and this family today comprises over two-thirds of all the living snake species.<sup>4</sup> Higher-level relationships of snakes inferred from nuclear and mitochondrial genes indicate that vipers diverged from the ancestral boid stock, underwent a rapid radiation in their initial burst of evolution, and developed afterward independently and in parallel.<sup>5,6</sup>

Evolutionary relationships among the true (pitless) vipers (subfamily Viperinae of Viperidae), inferred from mitochondrial DNA sequences, identify consistently five major monophyletic groups: *Bitis*, *Cerastes*, *Echis*, the Atherini (*Adenorhinos*, *Atheris*, *Protoatheris*, and *Montatheris*), and the Eurasian viperines (*Macrovipera*, *Daboia*, *Vipera*, *Pseudocerastes*, and *Eristicophis*).<sup>7</sup> The genus *Bitis* is comprised of 16 currently recognized species

\* To whom correspondence should be addressed. Juan J. Calvete, Instituto de Biomedicina de Valencia, C.S.I.C., Jaime Roig 11, 46010 Valencia, Spain. Phone, +34 96 339 1778; Fax, +34 96 369 0800; E-mail, [jcalvete@ibv.csic.es](mailto:jcalvete@ibv.csic.es).

**Table 1.** Assignment of the Reverse-Phase Fractions of *Bitis gabonica rhinoceros* Venom, Isolated as in Figure 1A, to Protein Families by N-Terminal Edman Sequencing, Mass Spectrometry, and Collision-Induced Fragmentation by nESI-MS/MS of Selected Peptide Ions from In-Gel Digested Protein Bands (Separated by SDS-PAGE as in Figure 1B)

HPLC fraction	N-terminal Bgr-sequencing	isotope-averaged molecular mass	peptide ion <i>m/z</i>	<i>z</i>	MS/MS-derived sequence	protein family
1	Blocked	826.9	413.7	2	ZEDXSPR	Unknown
2			440.1	2	(249.1)RPYVP	Metalloprotease fragment
3	n.p.					
4	KKRPLFCNLPADTGP	6901.7, 7003.4, 7158.7				Kunitz inhibitor
5	n.p.					
6	N.D. Blocked	973.6	487.1 4338.1	2	ZRPGPEXP	Bradykinin-potentiating peptide (BPP) N.D.
7	KKRPNFCYLPADPGP	7 kDa ▼	615.7 658.3 794.1	3	FTYGGCHGNANNFETR KFTYGGCHGNANNFETR RPNFCYLPADPGPCMANFPR	Kunitz inhibitor-2
8	KKRPNFCYLPADPGP	7023.5	615.7	3	FTYGGCHGNANNFETR	Kunitz inhibitor-2
9	KKRPNFCYLPADPGP N.D.	7096.1, 7155.9 1286.8				Kunitz inhibitor-2 BPP
10	NSAHPCCDPVTCKPK	M: 15183.5	644.2	2	ZWERPGPEXP	Bitisgabonin-1 Gabonin-1 [Q6T6T3]
			650.8 553.4 671.9 766.9 598.9 636.7 791.9 702.6 962.1	3 2 2 2 3 3 2 3 3	GDSLHDYCTGVTPDCPR FLRPGTVCR GDWDDDFCTGR AWEHCISGPCCR SSECESNPWNFWNH ZNSPHCCDPVTCKPK NFCESVDNXGXCR LTPGSQCDYGECCDQCR AANGCEDVADLCTGQSAE-CPLDVFQR	Metalloprotease-4 [Q6T271]
		m: 22 kDa ■				DC-fragment Metalloprotease BGR-25
11	NSAHPCCDPVTCKPK	15110.7	667.6 512.8 728.8 553.6 671.8 767.0 646.6	2 2 3 2 2 2 2	GEHCISGPCCR FLNAGTICK TMLDGLNDYCTGVTPDCPR FLRPGTVCR GDWDDDFCTGR AWEHCISGPCCR PSVTVAPDACFK	Bitisgabonin-2 Gabonin-2 [Q6T6T2]
			958.7	3	CPIMTDQCISLFDPSVTVAP-DACFK	Metalloprotease 4 [Q6T271]
12	SPAVCGNYFVEMGEE	M: 23 kDa ■	671.3 666.8 774.8 886.4 650.8 553.4 671.9 766.9 636.7	3 2 2 2 3 2 2 2 3	SYSSQDDPDYGMVDFGTK GEHCISGPCCR RGEHCISGPCCR NSAHPCCDPVTCKPK GDSLHDYCTGVTPDCPR FLRPGTVCR GDWDDDFCTGR AWEHCISGPCCR ZNSPHCCDPVTCKPK	DC-fragment Metalloproteinase-3
	NSAHPCCDPVTCKPK	m: 14926.8				Gabonin-1 [Q6T6T3]
			553.4 671.9 766.9 636.7	2 2 2 3	FLRPGTVCR GDWDDDFCTGR AWEHCISGPCCR ZNSPHCCDPVTCKPK	Metalloprotease-4 [Q6T271]
13	QLLEFGKMIKKETGF	13812.4				PLA <sub>2</sub>
14	SLLEFAKMIKEETGF	13891.3				PLA <sub>2</sub>
15	SVDFDSESDRKPEIQ	24973.5				CRISP
16	VIGGDECDINEHPFL	28350.3				Serine proteinase
17	KVGGLYPRKVMDEPV	13222.3				Cystatin
	VIGGAECNINEHRSL	33–36 kDa ■				Serine proteinase
18	VIGGAECNINEHRSL	38 kDa				Serine proteinase
	DQGCLPDWSSHQHICY	29337.6				C-type lectin-like α-chain <sup>b</sup>
	SDCASGWTAYGWHCY					C-type lectin-like β-chain <sup>b</sup>
19	DQGCLPDWSSHQHICY	29337.6				C-type lectin-like α-chain <sup>b</sup>
	SDCASGWTAYGWHCY					C-type lectin-like β-chain <sup>b</sup>
			587.9 846.7 667.8	2 2 3	(171.3)NFVWXGXR EEADFVAQLISDNK TWNILCGDDYPFVCK	
20	VIGGAECKIDGHRSL	35 kDa ■				Serine proteinase
21	VIGGAECDINEHPSL	30830.9				Serine proteinase
22	DFQCPFGWSAYGQHICY	90 kDa ■	587.9	2	GXNFVWSGXR	C-type lectin-like
		(14 kDa ▼)	655.6	3	GSHLLSLHNI AEA DFLK	
	DFECPSEWRPFQHCY	(16 kDa ▼)	940.1	2	FCSEQGNSGHLVSIQSK	C-type lectin-2 [AAR06852.1]
			774.1	2	NCFGLEKETEYR	
			656.1	2	KWTDGSNVITYK	
			870.1	2	SSPDYVVMGLWNQR	
			846.6	2	EEADFVAQLISDNK	

**Table 1.** (Continued)

HPLC fraction	Bgr-	N-terminal sequencing	isotope-averaged molecular mass	peptide ion <i>m/z</i>	<i>z</i>	MS/MS-derived sequence	protein family
23	N.D.		58 kDa <sup>■</sup>	667.8	3	TWLNILCGDDYPFVCK	l-amino acid oxidase
				534.2	2	NPLEECFR	
				743.6	2	EADYEEFLEIAR	
				618.6	2	SAEGLFEESLR	
				748.1	3	IFFAGEYTANAHGWIDSTIK	
24	N.D.		29 kDa <sup>■</sup>	587.9	2	GXNFVWSGXR	C-type lectin-like
				667.8	3	TWLNILCGDDYPFVCK	C-type lectin-2 [AAR06852.1]
				538.3	2	YPVKPSEAGK	l-amino acid oxidase
				502.3	2	VTVLEASER	
				618.8	2	SAEGLFEESLR	
24	N.D.		56 kDa <sup>■</sup>	748.1	3	IFFAGEYTANAHGWIDSTIK	C-type lectin-3 [Q6T7B5]
				874.6	3	KFGLQLNEFVQETENAWYYIK	
				475.3	2	VIYVNR	
				731.1	3	DFQCPSSEWSAYGQHICYR	
				995.6	3	AGHLVSIQSIQEANFVAQ-LVSGFISGSPK	
25	Blocked		52 kDa <sup>■</sup>	479.5	3	LYCFDNLPEHK	PIII-metalloproteinase
26	DQGCLPDWSSHQHCY SDCASGWTAYGWHCY		29 kDa <sup>■</sup>	515.1	2	IPCAPQDVK	
27	Blocked		56 kDa <sup>■</sup>	587.9	2	GXNFVWSGXR	C-type lectin-like a-chain
				667.8	3	TWLNILCGDDYPFVCK	C-type lectin-like b-chain
				792.1	2	NFCESVDNXGXCR	C-type lectin-like
				702.6	3	LTPGSQCDYGECCDQCR	C-type lectin-2 [AAR06852.1]
				962.1	3	AANGCEDVADLCTGQSAE-CPLDVFQR	PIII-metalloproteinase
28	N.D.		115 kDa <sup>■</sup>	900.0	2	TDIVSPPVCGSPTAXER	PIII-metalloproteinase
				514.9	2	IPCAPQDVK	
				792.1	2	NFCESVDNXGXCR	
29,30	Blocked		110 kDa <sup>■</sup> (52 kDa <sup>▼</sup> )	962.1	3	AANGCEDVADLCTGQSAE-CPLDVFQR	PIII-metalloprotease-3 [Q6T270]
				870.0	2	LFCVEPSTGNSIKCK	
				646.3	2	PSVTVAPDACFK	
				671.3	3	SYSSQDDPDYGMVDFGTK	
				591.8	2	WGXXQDXPK	
				724.9	2	SECDLLEHCTGK	
				693.3	3	LTPGSQCNHGECCDQCR	
				487.8	2	NGYCYNGK	
				472.7	2	NGQPCQNK	
				492.4	2	FXVDEHPK	
502.7	2	IKTAGTVCR					

<sup>a</sup> X, Ile or Leu; Z, pyrrolidone carboxylic acid. Unless other stated, for MS/MS analyses, cysteine residues were carbamidomethylated; molecular masses of native proteins were determined by electrospray-ionization ( $\pm 0.02\%$ ) or MALDI-TOF (<sup>\*</sup>) ( $\pm 0.2\%$ ) mass spectrometry. Apparent molecular mass determined by SDS-PAGE of non-reduced (<sup>■</sup>) and reduced (<sup>▼</sup>) samples; n.p., non peptidic material found. M and m, major and minor products within the same HPLC fraction, respectively. Previously reported proteins are identified by their databank accession codes. <sup>b</sup> N-terminal sequence similarity to an  $\alpha$ - or a  $\beta$ -subunit of C-type lectin-like proteins.

(<http://en.wikipedia.org/wiki/Bitis>) confined exclusively to the continent of Africa. *Bitis* species (Puff adders) are known for their behavior of inflating and deflating their bodies while hissing and puffing loudly. Size variation within this genus is extreme, ranging from the very small *B. schneideri*, which grows to a maximum of 28 cm and is perhaps the world's smallest viperid, to the very large *B. gabonica*, which can attain a length of over 2 m and is the heaviest viper in the world.

Lenk and co-workers<sup>7,8</sup> used molecular data (immunological distances and mitochondrial DNA sequences) to estimate phylogenetic relationships among species of *Bitis*. They identified four monophyletic groups for which they created four subgenera: *Bitis* (*B. arietans*); *Calechidna* (*B. albanica*, *B. armata*, *B. atropos*, *B. caudalis*, *B. cornuta*, *B. heraldica*, *B. inorata*, *B. peringueyi*, *B. rubida*, *B. schneideri*, *B. xeropaga*); *Macrocerastes* (*B. gabonica*, *B. nasicornis*, *B. parviocula*); and *Keniabitis* (*B. worthingtoni*). The emergence of *B. worthingtoni* (Kenya horned viper) as a distinct taxon reflects its

isolated distribution in Kenya and is in congruence with morphological<sup>9</sup> and immunological<sup>10</sup> findings. An affinity of *B. arietans* to the other large-bodied species of the *B. gabonica* clade is not supported by significant bootstrap values.<sup>7</sup> The two conventionally recognized subspecies of *Bitis gabonica*: *B. g. gabonica* and *B. g. rhinoceros* are as differentiated from each other as each is from *B. nasicornis*, and some authors<sup>8</sup> consider *B. g. rhinoceros* to represent a separate species, *Bitis rhinoceros*.

Venoms represent the critical innovation in ophidian evolution 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. Venom toxins likely evolved from proteins with normal physiological functions and appear to have been recruited into the venom proteome before the diversification of the advanced snakes, at the base of the Colubroid radiation.<sup>11-14</sup> Given

**Table 2.** Assignment of the Reverse-Phase Fractions of *Bitis nasicornis* Venom, Isolated as in Figure 2A, to Protein Families by N-Terminal Edman Sequencing, Mass Spectrometry, and Collision-Induced Fragmentation by nESI-MS/MS of Selected Peptide Ions from In-Gel Digested Protein Bands (separated by SDS-PAGE as in Figure 2B)<sup>a</sup>

HPLC fraction Bn-	N-terminal sequencing	isotope-averaged molecular mass	peptide ion <i>m/z</i>	<i>z</i>	MS/MS-derived sequence	protein family
1	Blocked	827.0	413.7	2	ZEDXSPR	Unknown
2–4	n.p.					
5	Blocked	4058.1				N.D.
6	N.D.	527.3			PKDAP	Fragment of PLA <sub>2</sub>
7	Blocked	672.6		2	(216.2)TAXQQDXSPR	Similar to Bn-1
8, 9	n.p.					
10, 11	N.D.	26 kDa■	616.3	2	SAECPXDHFR	DC-fragment
12	NSAHPCCDPVTCKPK	15192.8	650.6	2	GDSLHDYCTGVTPDCPR	Dimeric disintegrin
		15061.6	496.9	2	RGEHCISGPCCR	~ Bitisgabinin-1
			520.3	2	FXNSGTXCK	
			628.0	2	ZNSAHPCCDPVTCKPK	
			553.3	2	FLRPGTVCR	Metalloprotease-4 [Q6T271]
			671.9	2	GDWNDDFCTGR	
			636.7	3	ZNSPHSCPEVTCKPK	
13	N.D.	24 kDa■	616.6	2	SAECPXDHFR	DC-fragment
			713.3	2	NFCEXDQNPKC	
14	SLLEFAKMIKEETGF	13828.1				PLA <sub>2</sub>
15	HLTQFGNMIDKMGQS	13472.1				PLA <sub>2</sub>
16 M:	SVDFDSESPRKPEIQ	24973.5	635.8	2	KPEIQNEIVDLHNSLR	CRISP
			569.3	2	SVDFDSESPR	
			581.4	2	SVNPTASNMLK	
			768.8	2	MEWYPEAANAER	
m:	KVGGLYPRKVM DPEV	13216.6				Cystatin
17	VIGGDECNINEHR(S/F)L	58 kDa■	749.4	2	SLPSSPPRVGSVCR	(Serine proteinase) <sub>2</sub>
			688.3	3	VPHCANNNXXDHTXCER	
		29612.5	749.4	2	SLPSSPPRVGSVCR	Serine proteinase
			610.3	3	SFETVPWXHSXXAGDR	
18	KVGGLYPRKVM DPEV	13204.8	672.6	2	IVEAQSQVVSGVK	Cystatin
		29 kDa■	749.4	2	SLPSSPPRVGSVCR	Serine proteinase
			610.3	3	SFETVPWXHSXXAGDR	
	VIGGDECNINEHPFLV	27 kDa■	797.3	2	TLCAGILEGGIDSCK	Serine proteinase-1 [Q6T6S7]
			530.3	2	LFDYSVCR	
			597.3	2	IMGWGSITTTK	
			756.8	2	VTYDPVPHCANIK	
19	VIGGDECNINEHR	38 kDa■	504.8	3	VIGGDECNINEHR	Serine proteinase
		31 kDa■	414.8	2	NPCGQPR	Serine proteinase
			487.2	2	AAYPQHCK	
			622.3	2	VYDYNWXR	
	DEGCLP(D)WSSHRHCY	29594.1				C-type lectin-like α-chain <sup>b</sup>
	SDCASGWTAYGWHCL					C-type lectin-like β-chain <sup>b</sup>
20	IIGGAECNINEHRFL	31 kDa■				Serine proteinase
21	DQGCLPDWSSHQHCY	29337.6				C-type lectin-like α-chain <sup>b</sup>
	SDCASGWTAYGWHCY					C-type lectin-like β-chain <sup>b</sup>
22	VIGGDECNINEHPSL	31 kDa■				Serine proteinase
23	DFECPFGWSAYGQHCY	92151				[C-type lectin-like] × 3
		CM-[16479, (16366) × 2, 15190, 15293, 15235] (14 kDa▼)	587.9	2	GXNFVWSGXR	C-type lectin-like α-chain <sup>b</sup>
			655.6	3	GSHLLSLHNIAEADFVLK	
	DFECPSEWRPFDQHCY	(16 kDa▼)	776.9	3	DFECPSEWRPFDQHCYR	C-type lectin-2 [AAR06852.1]
			774.1	2	NCFGLEKETEYR	
			656.1	2	KWTDGNSVIYK	
			869.9	2	SSPDYVWMGLWNQR	
			846.6	2	EEADFVAQLISDNK	
			667.8	3	TWLNILCGDDYPFVCK	
			591.8	2	WTDGNSVIYK	
24,25	DDGKNPLEECFREAD	56 kDa■	512.2	2	TFKPPLPK	L-amino acid oxidase
			743.6	2	EADYEEFLEIAR	
			618.6	2	SAEGLFEESLR	
26	Blocked	120 kDa■	724.8	2	(192.2)AYTFDSE(GX)VR	PIII-Metalloprotease
			479.3	3	LYCFDNLPEHK	
			514.3	2	IPCAPQDVK	
			566.3	2	XANDYGYCR	
			766.3	3	GNATVAEDACFEFNR	
		15 kDa▼	517.2	2	TTDNQWXR	C-type lectin-like
27	Blocked	52 kDa■	613.3	2	GVVQDHSQVTR	PIII-Metalloprotease
			742.3	3	LHSWVECESGECCEQCR	
			566.3	2	XANDYGYCR	

Table 2. (Continued)

HPLC fraction Bn-	N-terminal sequencing	isotope-averaged molecular mass	peptide ion <i>m/z</i>	<i>z</i>	MS/MS-derived sequence	protein family	
28	Blocked	15 kDa▼	517.2	2	TTDNQWXR	C-type lectin-like	
		52 kDa■	613.3	2	GVVQDHSQVTR	PIII-Metalloprotease	
			742.3	3	LHSWVECESGECCEQCR		
29	Heterogeneous	15 kDa▼	517.2	2	TTDNQWXR	C-type lectin-like	
		52 kDa▼	613.3	2	GVVQDHSQVTR	PIII-Metalloprotease	
			742.3	3	LHSWVECESGECCEQCR		
			566.3	2	XANDYGICR		
			517.2	2	TTDNQWXR	C-type lectin-like	
30	Heterogeneous	110 kDa■	870.0	2	LFCVEPSTGNSIKCK	PIII-metalloprotease-3	
		(52 kDa▼)	671.3	3	SYSSQDDPDYGMVDFGTK	[Q6T270]	
			570.2	2	SAECPDVFVK		
			724.9	2	SECDLLEHCTGK		
			487.8	2	NGYCYNGK		
			472.7	2	NGQPCQNK		
			517.2	2	TTDNQWXR	C-type lectin-like	
			15 kDa▼	517.2	2	TTDNQWXR	C-type lectin-like

<sup>a</sup> X, Ile or Leu; Z, pyrrolidone carboxylic acid. Unless other stated, for MS/MS analyses, cysteine residues were carbamidomethylated; molecular masses of native proteins were determined by electrospray-ionization ( $\pm 0.02\%$ ) or MALDI-TOF (<sup>\*</sup>) ( $\pm 0.2\%$ ) mass spectrometry. Apparent molecular mass determined by SDS-PAGE of non-reduced (■) and reduced (▼) samples; CM-, carbamidomethylated; n.p., non-peptidic material found. M and m, major and minor products, respectively, within the same HPLC fraction. Previously reported proteins are identified by their databank accession codes. <sup>b</sup> N-terminal sequence similarity to an  $\alpha$ - or a  $\beta$ -subunit of C-type lectin-like proteins.

the central role that diet has played in the adaptive radiation of snakes,<sup>15</sup> venom thus represents a key adaptation that has played an important role in the diversification of these animals. On the other hand, venom composition may retain information on its evolutionary history, and may thus have a potential taxonomical value. In addition to understanding how venoms evolve, characterization of the protein (toxin) 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 for antivenom production strategies.<sup>16</sup> To address the need for detailed proteomic studies of snake venoms, we have initiated a project whose long-term goal is a detailed analysis of viperid venomes. To date, we have reported detailed analysis of the protein composition of the venoms from the North American rattlesnakes *Sistrurus miliarius barbouri*,<sup>17,18</sup> *Sistrurus catenatus* subspecies *catenatus*, *tergeminus*, and *edwardsii*,<sup>18</sup> the Tunisian vipers *Cerastes cerastes*, *Cerastes vipera*, and *Macrovipera lebetina*,<sup>19</sup> and the Afrotropical species *Bitis arietans* (Ghana)<sup>20</sup> and *Bitis gabonica gabonica*.<sup>21</sup> Here, we report the proteomic characterization of the venoms of *Bitis gabonica rhinoceros* (West African gaboon viper), *Bitis nasicornis* (rhinoceros viper), and *Bitis caudalis* (horned puff adder).

## Experimental Section

**Isolation of Venom Proteins.** *Bitis gabonica rhinoceros* (West African gaboon viper) venom was a generous gift of César Olmos Jiménez (EntomoZoo Fauna Arcana, S.L., Cullera, Valencia, Spain). Lyophilized venoms of *Bitis nasicornis* (rhinoceros viper) and *Bitis caudalis* (horned puff adder) were purchased from Latoxan (Valence, France). For reverse-phase HPLC separations, 2–5 mg of crude, lyophilized venom were dissolved in 100  $\mu$ L of 0.05% trifluoroacetic acid (TFA) and 5% acetonitrile, and insoluble material was removed by centrifugation in an Eppendorf centrifuge at 13 000 $\times$  g for 10 min at room temperature. Pelleted material was devoid of proteins as judged by SDS-PAGE. Proteins in the soluble material were separated using an ETTAN LC HPLC system (Amersham Biosciences) and a Lichrosphere RP100 C<sub>18</sub> column (250  $\times$  4 mm, 5  $\mu$ m 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 10 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 the venoms 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 were 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<sup>22</sup> 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 15% polyacrylamide gels) and by electrospray ionization (ESI) mass spectrometry using an Applied Biosystems QTrap mass spectrometer<sup>23</sup> operated in Enhanced Multiple Charge mode in the range *m/z* 600–1700.

**In-Gel Enzymatic Digestion and Mass Fingerprinting.** Reverse-phase HPLC-separated venom fractions were analyzed on SDS 15% (non-reduced) or (reduced) polyacrylamide gels using the Laemmli tris-glycine buffering system.<sup>24</sup> Protein bands of interest were excised from a Coomassie Brilliant Blue-stained SDS-PAGE and subjected to automated reduction with DTT and alkylation with iodoacetamide, and in-gel digestion with sequencing grade bovine pancreas trypsin (Roche) using a ProGest digester (Genomic Solutions) following the manufacturer's instructions. Tryptic peptide mixtures (0.65  $\mu$ L) (total volume of  $\sim 20$   $\mu$ L) were spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of  $\alpha$ -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 (SwissProt

**Table 3.** Assignment of the Reverse-Phase Fractions of *Bitis caudalis* Venom, Isolated as in Figure 3A, to Protein Families by N-Terminal Edman Sequencing, Mass Spectrometry, and Collision-Induced Fragmentation by nESI–MS/MS of Selected Peptide Ions from In-Gel Digested Protein Bands (Separated by SDS-PAGE as in Figure 1B)<sup>a</sup>

HPLC fraction Bc-	N-terminal sequencing	isotope-averaged molecular mass	peptide ion <i>m/z</i>	<i>z</i>	MS/MS-derived sequence	protein family
1	n.p.					
2	n.p.					
3	KNRPEFCNLPADTGP	6950.8, 7081.0				Kunitz inhibitor
4	Blocked		441.3	2	ZXDXSP(QP)	Unknown
5	n.p.					
6	KKRPDFCYLPADTGP	6969.3, 6840.6 14.5 kDa <sup>■</sup>	582.3	2	FQTWFECEK	Kunitz inhibitor
			637.4	2	FTYGGCGGNANR	
			701.4	2	KFTYGGCGGNANR	
		7.5 kDa <sup>■</sup>	494.8	2	KFTYGGCR	
			504.8	2	FYYDSVSK	
			777.4	2	GNANNFETXECEK	
			637.4	2	FTYGGCGGNANR	
7, 8	n.p.					
9	NSAHPCCDPVTCKP	14812.8 <sup>■</sup> / 7 kDa <sup>▼</sup>	512.8	2	FLNAGTICK	Dimeric disintegrin
			666.3	2	GEHCISGPCCR	
10	NSAHPCCDPVTCKP	14870.8 <sup>■</sup> / 7 kDa <sup>▼</sup>	512.8	2	FLNAGTICK	Dimeric disintegrin
			666.3	2	GEHCISGPCCR	
11	NSAHPCCDPVTCKP	15127.8 <sup>■</sup> / 7 kDa <sup>▼</sup>	512.8	2	FLNAGTICK	Dimeric disintegrin
12	NLIQFGNMISAMTGKSSLAY	13350.6 (23 kDa <sup>■</sup> )	459.2	2	MILYSYK	PLA <sub>2</sub> Caudoxin [P00622]
			463.3	2	CTGTAEK	
			483.5	2	KVCECDR	
			519.3	2	VAAICFAASK	
			575.8	2	NLWRYPSSK	
			609.3	2	HSYNKNLWR	
			631.2	2	CFNGDIVCGDK	
			753.2	2	CCFVHDCCYK	
			812.8	2	NLIQFGNMISAMTGK	
			629.2	3	SSLAYASYGCYCGWGGK	
			702.8	3	DDTDRCCFVHDCCYK	
13	NL(I/y)QF(G/a)(K/n)MI(S/n)(A/h)M	13350.2, 13366.1 (38 kDa <sup>■</sup> )	483.5	2	KVCECDR	PLA <sub>2</sub>
			547.8	2	KKVCECDR	
			519.3	2	VAAICFAASK	
			575.8	2	NLWRYPSSK	
			631.2	2	CFNGDIVCGDK	
			753.2	2	CCFVHDCCYK	
			629.2	3	SSLAYASYGCYCGWGGK	
			789.3	2	CCFVHDCCYEK	
14	NL(I/y)QFG(K/n)MISA(K/M)TGK	13366.3, 13916.3				PLA <sub>2</sub>
15	NLYQFGKMISYMAKS	13966.4 (48 kDa <sup>■</sup> )	483.5	2	KVCECDR	PLA <sub>2</sub>
			547.8	2	KKVCECDR	
			753.2	2	CCFVHDCCYK	
			629.2	3	SSLAYASYGCYCGWGGK	
16	NLYQFGKMISYMAKR	46 kDa <sup>■</sup> / 14 kDa <sup>▼</sup>	483.5	2	KVCECDR	PLA <sub>2</sub>
			575.8	2	NLWRYPSSK	
			629.2	3	SSLAYASYGCYCGWGGK	
17	NLYQFAKMISHVTKR	Het <sup>■</sup> / 14 kDa <sup>▼</sup>	483.5	2	KVCECDR	PLA <sub>2</sub>
			575.8	2	NLWRYPSSK	
			629.2	3	SSLAYASYGCYCGWGGK	
			789.3	2	CCFVHDCCYEK	
18	NLYQFAKMISHVTKR	Het <sup>■</sup> / 14 kDa <sup>▼</sup>	483.5	2	KVCECDR	PLA <sub>2</sub>
			575.8	2	NLWRYPSSK	
			629.2	3	SSLAYASYGCYCGWGGK	
			789.3	2	CCFVHDCCYEK	
19	NLYQFAKMISHMTRK	14 kDa <sup>▼</sup>				PLA <sub>2</sub>
20	NLIQFGNMI(S/R)AMTGK	14 kDa <sup>▼</sup>				PLA <sub>2</sub>
21	SSLAYASYGCYCGWG	14 kDa <sup>▼</sup>				PLA <sub>2</sub> [P00622]
22	NLNQFREMIDHVSQK	16 kDa <sup>▼</sup>				PLA <sub>2</sub> ~ [AY429476]
23	SVDFDESSEPRKPEIQ	26 kDa <sup>■▼</sup>				CRISP
24	VIGGAECNIKEHRSL	32 kDa <sup>▼</sup>				Serine proteinase
25	VIGG(D/A)ECNINEHR(S/F)L	38 kDa <sup>■▼</sup>	756.7	2	VIGGDECNINEHR	Serine proteinase
			734.7	2	VIGGAECNINEHR	
			567.6	2	VDFTFCXXR	
			468.7	2	FXCXNNR	
		32 kDa <sup>■▼</sup>	459.7	2	DVPGXYTR	Serine proteinase
			593.7	2	VFDYTDWIK	
			552.2	2	VXNEDEQTR	
			567.6	2	VDFTFCXXR	
		29 kDa <sup>■▼</sup>	459.7	2	DVPGXYTR	Serine proteinase
			551.2	2	VXCAGVXQK	
			552.2	2	VXNEDEQTR	

**Table 3.** (Continued)

HPLC fraction Bc-	N-terminal sequencing	isotope-averaged molecular mass	peptide ion <i>m/z</i>	<i>z</i>	MS/MS-derived sequence	protein family
26	DFECPF(G/E)W(S/T)AYGQHGY	90 kDa■ 16 kDa▼	773.3	2	NCFGLEKETEYR	C-type lectin-like ~ C-type lectin-2 [Q6T7B6]
			860.9	2	SSPDYVWXGXWNQR	
		12 kDa▼	667.6	3	TWLNILCGDDYPFVCK	
			753.3	2	GQHGYQA(237.8)YXX	
			668.3	2	GSHXXSXHDXAEAHYVXX	
			474.6	2	FCTKQHK	
26–34	ADDKNPLEECFREAD	48 kDa■▼	460.2	2	THQFVCK	L-amino acid oxidase
			742.7	2	EADYEEFLEIAR	
			747.2	2	ADDKNPLEECFR	
			626.2	2	SAGQLYQESLR	
			502.2	2	VTVXEASER	
			455.7	2	XFXVCMK	
28	N.D.	14.6 kDa▼ 28 kDa■/16 kDa▼	589.2	2	(157.3)DYVWXWPR	C-type lectin-like C-type lectin-like
			517.3	2	IYIWIGLR	
			500.7	2	FDYNANTR	
30	N.D.	72 kDa▼	773.3	2	NCFGLEKETEYR	PIII-metalloprotease PIII-metalloprotease
			860.9	2	SSPDYVWXGXWNQR	
			566.3	2	XANDYGYCR	
30–34	Heterogeneous	95 kDa■/42 kDa▼	724.9	2	(263.3)YTFDSEGXVR	PIII-metalloprotease PIII-metalloprotease
			566.1	2	XANDYGYCR	
			540.7	2	(224.3)NXTPEQR	
		56 kDa■▼	657.1	2	NPCQXYTVR	
			718.1	2	XYCFDNXSTFR	
			764.1	2	ATVAQDACFQFNR	
35, 36	Blocked	98 kDa■/ 48 kDa▼	494.7	2	GNGDFYCR	PIII-metalloprotease C-type lectin-like PI-metalloprotease
			558.7	2	KNGDFYCR	
			646.9	2	(252.2)TNHNPQCXXNQPSR	
		28 kDa■/16 kDa▼	755.8	2	XFCESVANXGXCK	
			517.3	2	IYIWIGLR	
			500.7	2	FDYNANTR	
26 kDa■/23 kDa▼	562.3	2	TXNSFAEWR			
	641.3	2	AFXSGMCQPDR			
	712.8	2	SVGXVEDYSQTAR			
	517.2	2	TTDNQWXR			
775.3	2	(417.1)YQSCXXNEP				
	561.1	2	SYQFSDCSK			
483.8	2	HNGDXASPR				

<sup>a</sup> X, Ile or Leu; Z, pyrrolidone carboxylic acid. Amino acid sequence heterogeneity is indicated by “(X/y)”, where upper and lower case letters indicate major and minor residue, respectively. Unless other stated, for MS/MS analyses, cysteine residues were carbamidomethylated; molecular masses of native proteins were determined by electrospray-ionization (±0.02%) or MALDI-TOF (\*) (±0.2%) mass spectrometry. Apparent molecular mass determined by SDS-PAGE of non-reduced (■) and reduced (▼) samples; n.p., non peptidic material found. M and m, major and minor products, respectively, within the same HPLC fraction. Het, heterogeneous. Previously reported proteins are identified by their databank accession codes.

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)<sup>23</sup> 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<sub>0</sub> 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 Composition between *Bitis* Taxa.** We used similarity coefficients to estimate the similarity of

venom proteins between taxa. These coefficients are similar to the bandsharing coefficients used to compare individual genetic profiles based on multilocus DNA fingerprints.<sup>25</sup> We defined the protein similarity coefficient (PSC) between two species “a” and “b” in the following way:  $PSC_{ab} = [2 \times (n^\circ \text{ of proteins shared between a and b}) / (\text{total number of distinct proteins in a} + \text{total number of distinct proteins in b})] \times 100$ . We judged two proteins (listed in Tables 1–3) 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 (0.01% for ESI-QTrap MS; 0.4% for MALDI-TOF MS derived masses; and ±1.4 kDa for SDS-PAGE-determined masses); or (4) eluted in different reverse-phase HPLC peaks. We emphasize that these measures will give only minimum estimates of the

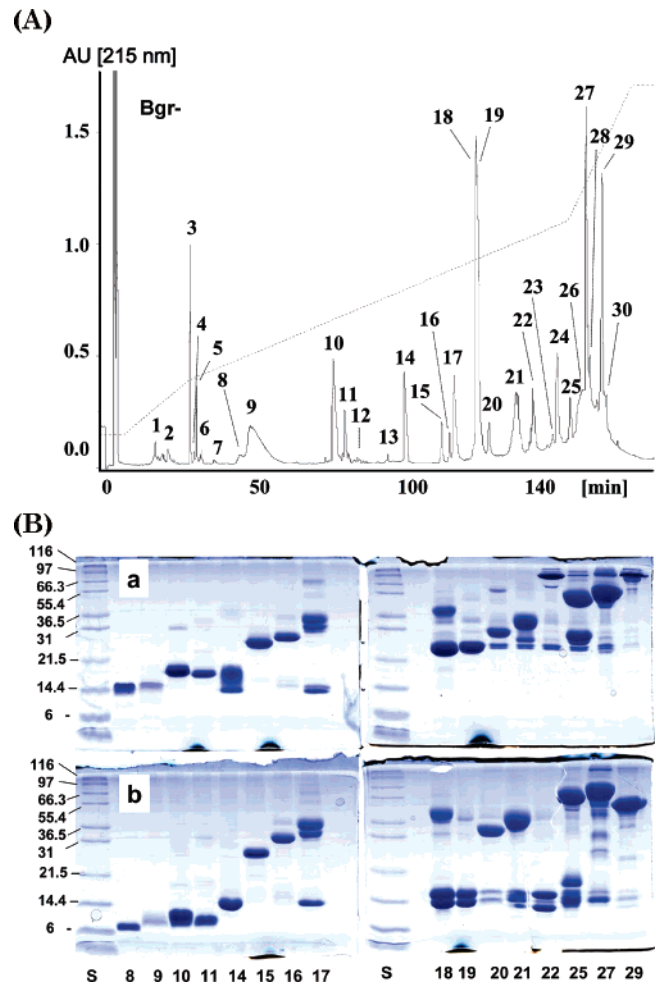
similarities between the venom profiles. We suspect that a number of the proteins that we judge to be the same using the above criteria would be found to differ at one or more of these criteria if more complete information were available.

## Results and Discussion

**Proteomic Characterization of *Bitis* Venoms.** The crude venoms of *Bitis gabonica rhinoceros* (West African gaboon viper), native to high rainfall areas of West, Central, and East Africa, *Bitis nasicornis* (rhinoceros viper, occurring in forested areas of West and Central Africa), and *Bitis caudalis*, (horned adder), a short and stout little viper mostly found in arid regions of South-West Africa, were fractionated by reverse-phase HPLC (Figures 1A, 2A, and 3A), followed by analysis of each chromatographic fraction by SDS-PAGE, N-terminal sequencing, and nanospray-ESI-QTrap mass spectrometry. Several reverse-phase peaks corresponded to essentially pure proteins (i.e., fractions 8–16 in Figure 1B; 12, 14, 15, 22, 25 in Figure 2B; and 9, 10, 22–24 in Figure 3B). However, more commonly, fractions contained mixtures of components and were thus subjected to rechromatography using a flatter acetonitrile gradient, or their constituents separated by SDS-PAGE followed by excision of the protein bands, in-gel tryptic digestion, and protein identification by combination of MALDI-TOF mass fingerprinting and CID-MS/MS.

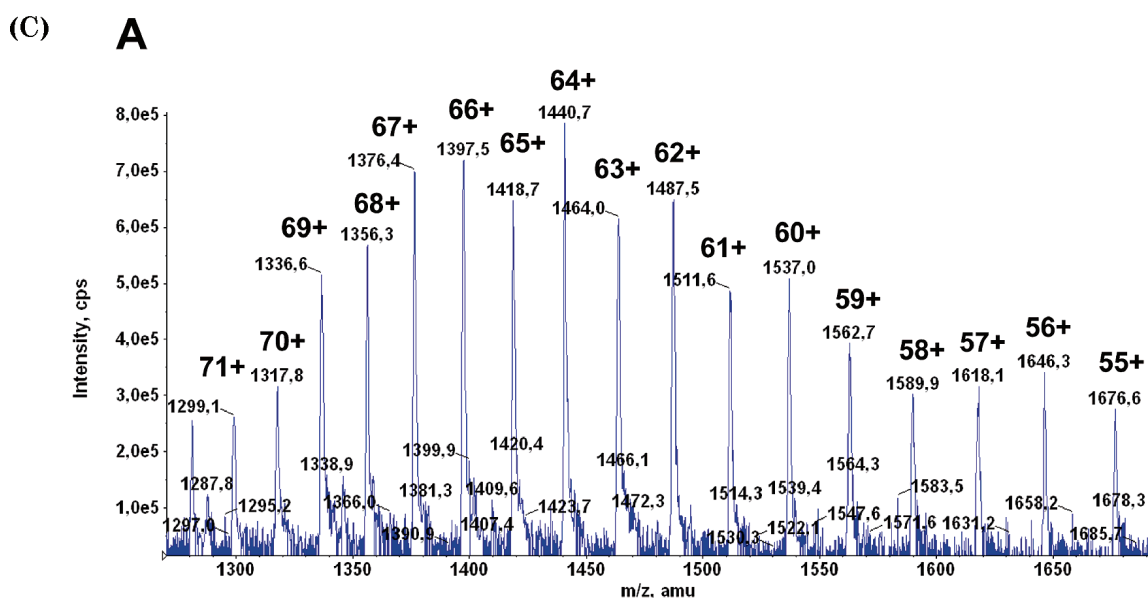
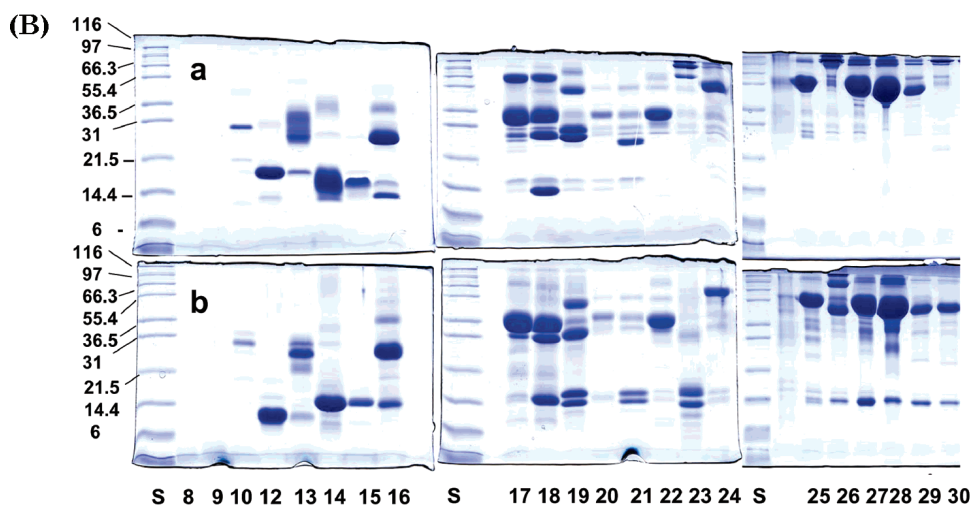
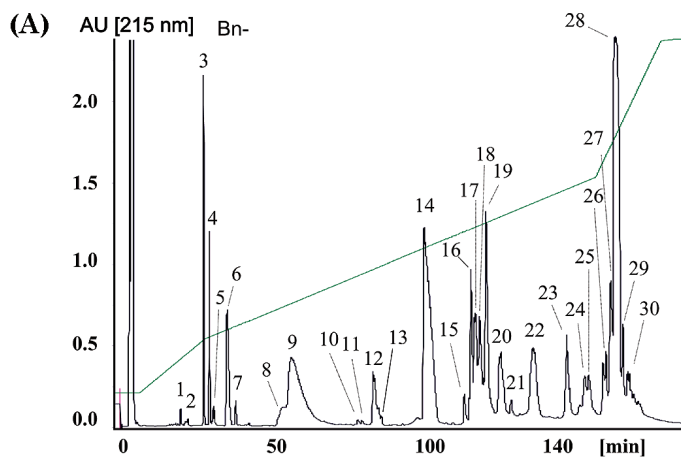
Despite the fact that only a few venom toxins from *Bitis* species are annotated in the SwissProt/TrEMBL non-redundant database (Knowledgebase Release 10.0 of March 2007 comprising 23 partial and full-length EST-derived sequences from *Bitis gabonica gabonica*<sup>26</sup>, 1 full-length PLA<sub>2</sub> sequence from each, *Bitis nasicornis* (P00621)<sup>27</sup> and *Bitis caudalis* (P00622),<sup>28</sup> and no entry from *Bitis gabonica rhinoceros*), all HPLC fractions yielding unambiguous N-terminal sequences could be classified into known protein families using a BLAST amino acid similarity search (Tables 1–3), indicating that representative members of each snake venom toxin families are present among the 923 viperid protein sequences deposited in the SwissProt/TrEMBL databank. Protein fractions showing heterogeneous or blocked N-termini were analyzed by SDS-PAGE and the bands of interest were digested in-gel. The resulting tryptic 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,<sup>12,16,29–34</sup> with a few exceptions, neither the tryptic mass fingerprints nor the product ion spectra matched any known protein using the ProteinProspector (<http://prospector.ucsf.edu>) or the MASCOT search programs. The CID-MS/MS spectra were therefore interpreted manually and the deduced peptide ion sequences were submitted to BLAST sequence similarity searches. This approach allowed us to assign unambiguously all the isolated venom toxins representing over 0.05% of the total venom proteins to protein families present in the non-redundant databases (Tables 1–3).

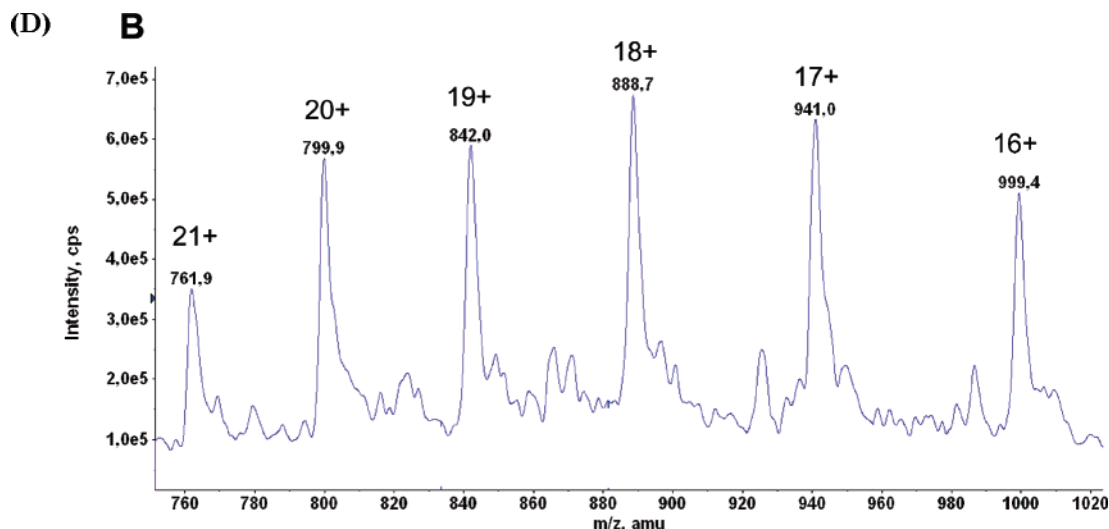
In line with previous proteomic<sup>17–21</sup> and transcriptomic analyses of the venom glands of viperid (*Bitis gabonica*,<sup>26</sup> *Bothrops insularis*,<sup>35</sup> *Bothrops jararacussu*,<sup>36</sup> *Bothrops jararaca*,<sup>37</sup> *Agkistrodon acutus*,<sup>38</sup> *Echis ocellatus*,<sup>39</sup> and *Lachesis muta*<sup>40</sup>) and colubrid (*Philodryas olfersii*<sup>41</sup>) snake species, showing that snake venom proteins belong to only a few major protein families, the venomes of *Bitis gabonica rhinoceros*, *Bitis nasi-*



**Figure 1.** *Bitis gabonica rhinoceros* venom proteome. (A) Reverse-phase separation of the *Bitis gabonica rhinoceros* venom proteins. Chromatographic conditions were: isocratically (5% B) for 10 min, followed by 5–15% B for 20 min, 15–45% B for 120 min, and 45–70% for 20 min. HPLC fractions were collected manually and characterized by N-terminal sequencing, ESI-QTrap mass spectrometry, and SDS-PAGE. (B) SDS-PAGE of reverse-phase separated *Bitis gabonica rhinoceros* venom fractions. Protein fractions were run on SDS-(15%) polyacrylamide gels<sup>24</sup> under non-reduced (upper panels, a) and reduced (lower panels, b) conditions. Lanes S, molecular mass markers:  $\beta$ -galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), BSA (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic dehydrogenase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.0 kDa). Insulin A- (3.5 kDa) and B- (2.5 kDa) chains run unresolved with the front in this gel system. Protein bands were excised and characterized by mass fingerprinting and CID-MS/MS of selected doubly or triply charged peptide ions. The combined results are shown in Table 1.

*cornis*, and *Bitis caudalis* comprise, respectively, toxins from 11, 9, and 8 toxin families (Table 4). However, these *Bitis* snake venoms depart from each other in the composition and the relative abundance of their toxins (Table 4, Figure 4). These results appear to be consequence of the recruitment of a restricted set of proteins into the venom proteome before the diversification of the advanced snakes, at the base of the radiation of Colubroideae.<sup>11–14</sup> The occurrence of multiple isoforms within each major toxin family in *Bitis* species (Tables





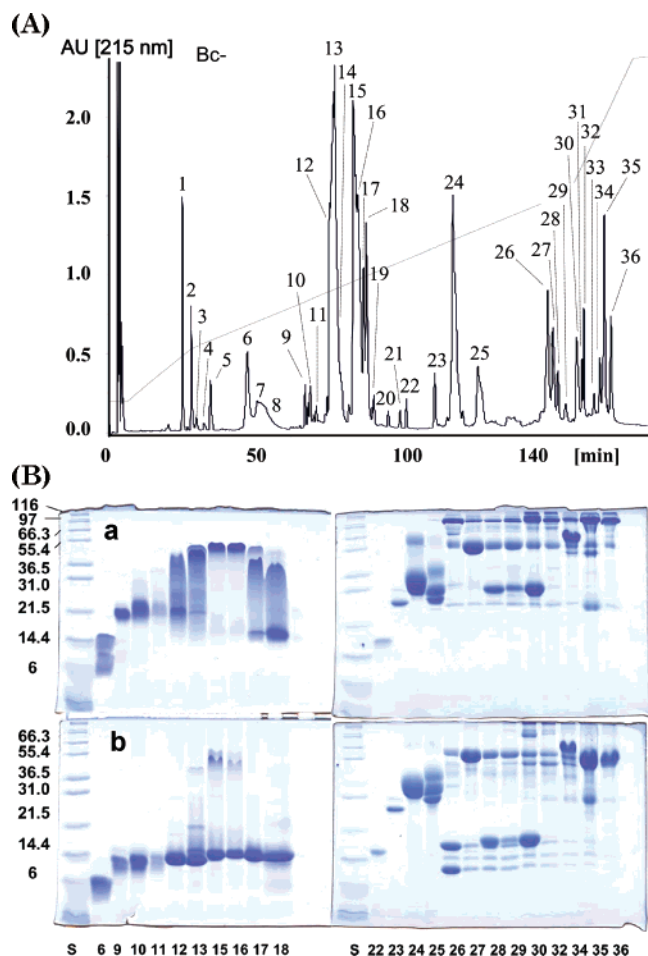
**Figure 2.** *Bitis nasicornis* venom proteome. (A) Reverse-phase separation of the *Bitis nasicornis* venom proteins. Chromatographic conditions were as in Figure 1A. HPLC fractions were collected manually and characterized by N-terminal sequencing, ESI-QTrap mass spectrometry, and SDS-PAGE. (B) SDS-PAGE of reverse-phase separated *Bitis nasicornis* venom fractions. Protein fractions were run on SDS-(15%) polyacrylamide gels<sup>24</sup> under non-reduced (upper panels, a) and reduced (lower panels, b) conditions. Lanes S, molecular mass markers as in Figure 1B. Protein bands were excised and characterized by mass fingerprinting and CID-MS/MS of selected doubly or triply charged peptide ions. The combined results are shown in Table 2. (C) Electrospray-ionization mass spectrum of protein Bn-23 (C-type lectin-like,  $92151 \pm 18$  Da). (D) Electrospray-ionization mass spectrum of one of the subunits of protein Bn-23 isolated by reverse-phase HPLC after reduction. Deconvolution of the spectrum yields an isotope-averaged molecular mass of  $15978 \pm 2$  Da).

1–3), as well as in all other snake “venomes” investigated,<sup>17–21,26,35–40</sup> evidences the emergence of paralogous groups of genes across taxonomic lineages where gene duplication events occurred prior to their divergence, and suggests an important role for balancing selection<sup>42</sup> in maintaining high levels of functional variation in venom proteins within populations. The mechanism leading to this mode of selection is unclear but we have speculated<sup>18</sup> that it may be related to unpredictability 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. The selection pressure leading to high levels of variation in venom genes may parallel the birth-and-death model of protein evolution<sup>43</sup> acting to promote high levels of variation (neofunctionalization) in the genes involved in the vertebrate’s adaptative immune response,<sup>43</sup> such as those which encode major histocompatibility complex proteins,<sup>44</sup> or in plant host defense genes.<sup>45–47</sup>

**Comparison of the Toxin Composition of *Bitis* Venoms.** The venom proteomes of *Bitis gabonica rhinoceros* (Table 1), *Bitis nasicornis* (Table 2), and *Bitis caudalis* (Table 3) contain, respectively, at least 33, 28, and 30 proteins. Dimeric disintegrins, PLA<sub>2</sub> molecules, serine proteinases, a CRISP, C-type lectin-like proteins, L-amino acid oxidases, and snake venom metalloproteases are present in all these three venoms, albeit in quite distinct relative abundances (Figure 4, Table 4). It is worth to notice the presence in each venom of a highly conserved multimeric (92 kDa) C-type lectin-like molecules, i.e., Bgr-22, Bn-23, and Bc-26. The molecular mass of the native multimeric C-type lectin-like protein of *Bitis caudalis* (Bn-23, Table 2) was accurately measured by electrospray-ionization MS ( $92151$  Da, Figure 2C). The quaternary structure of Bn-23, determined by mass spectrometry after

reduction and separation of subunits by reverse-phase HPLC (Figure 2D), was built by the association of polypeptides of molecular masses (carbamidomethylated): 16 479, 16 366, 15 190, 15 293, and 15 235 Da. All C-type lectin-like molecules characterized to date in snake venoms are built by different associations of  $\alpha\beta$  dimers. Alfa ( $\sim 16$  kDa) and beta ( $\sim 14$  kDa) subunits are homologous polypeptides each containing 7 conserved cysteine residues engaged in the formation of 1 intersubunit disulfide bond and 3 intrasubunit disulfides. The  $\alpha\beta$  dimer is the basic unit from which the different structures of the snake C-type lectin-like family are assembled. Higher order oligomerization is driven by the evolutionary appearance of an extra cysteine residues at both, the C-terminus of the  $\alpha$ -chain and the N-terminus of the  $\beta$ -subunit, enabling multimerization of  $\alpha\beta$ -heterodimers via inter-dimer disulfide linkages into a cyclic “head-to-tail” arrangement  $[\alpha\text{-SS-}\beta]_1\text{-Cys}_{\text{Cter}}\alpha\text{-Cys}_{\text{Nter}}\beta\text{-}[\alpha\text{-SS-}\beta]_n\text{-Cys}_{\text{Cter}}\alpha\text{-Cys}_{\text{Nter}}\beta\text{-}[\alpha\text{-SS-}\beta]_1$ , where  $n$  is the number of intervening  $\alpha\beta$  units linked between the C-terminal cysteine residue of the  $\alpha$ -subunit and the N-terminal cysteine of the  $\beta$ -subunit of the same  $\alpha\beta$  heterodimer. C-type lectin-like proteins isolated to date from a large number of viperid and crotalid venoms occur in a variety of oligomeric forms, including  $\alpha\beta$  ( $n = 0$ ),  $(\alpha\beta)_2$  ( $n = 1$ ), and  $(\alpha\beta)_4$  ( $n = 3$ ).<sup>48,49</sup> Assuming that each Bn-23 subunit may contain 8 cysteine residues engaged in 1 intersubunit, 3 intrasubunit, and 1 interdimer disulfide linkages, the best fitting subunit combination is  $[16\ 479 + (16\ 366)_2$  ( $\alpha$ -type subunits) +  $15\ 190 + 15\ 293 + 15\ 235$  ( $\beta$ -type subunits)], indicating that Bn-23 may represent a trimer of  $\alpha\beta$  dimers. Hence, Bn-23 represents the first reported  $(\alpha\beta)_3$  ( $n = 2$ ) C-type lectin-like structure.

Another molecular feature conserved in the *Bitis* venoms studied is the presence of dimeric snake venom PIII-metalloproteases (Bgr-29, Figure 1A and Table 1; Bn-30, Figure 2A and Table 2; Bc-36, Figure 3A and Table 3). In *Bitis g. rhinoceros* and *B. nasicornis*, the dimeric PIII-SVMP are highly homologous



**Figure 3.** *Bitis caudalis* venom proteome. (A) Reverse-phase separation of the *Bitis caudalis* venom proteins. Chromatographic conditions were as in Figure 1A HPLC fractions were collected manually and characterized by N-terminal sequencing, ESI-QTrap mass spectrometry, and SDS-PAGE. (B) SDS-PAGE of reverse-phase separated *Bitis caudalis* venom fractions. Protein fractions were run on SDS-(15%) polyacrylamide gels<sup>24</sup> under non-reduced (upper panels, a) and reduced (lower panels, b) conditions. Lanes S, molecular mass markers as in Figure 1B. Protein bands were excised and characterized by mass fingerprinting and CID-MS/MS of selected doubly or triply charged peptide ions. The combined results are shown in Table 3.

to metalloprotease 3 (Q6T270) described in the *Bitis gabonica gabonica* venom transcriptome<sup>26</sup> and proteome.<sup>21</sup> On the other hand, among the nonconserved protein families, bradykinin-potentiating peptides, hypotensive peptides acting as highly site-specific inhibitors of the somatic angiotensin-converting enzyme, were only found in *B.g. rhinoceros*, whereas cystatin (a cysteine protease inhibitor) and disintegrin/cysteine-rich (DC) fragments were present in *B. nasicornis* and *B.g. rhinoceros*, and serine-type endopeptidase Kunitz-type inhibitors occur in *B.g. rhinoceros* and *B. caudalis*. In both species, the Kunitz-type inhibitors exhibited apparent molecular masses of 14–16 kDa and 7–8 kDa when analyzed in non-reduced or reduced SDS-PAGE, respectively (Bgr, Figure 1B, lanes 8 and 9; Bc, Figure 3B, lane 6). However, electrospray ionization mass spectrometry showed that these Kunitz-type inhibitors had native (non-reduced) molecular masses of about 7 kDa (Tables 1 and 3). Hence, the distinct electro-

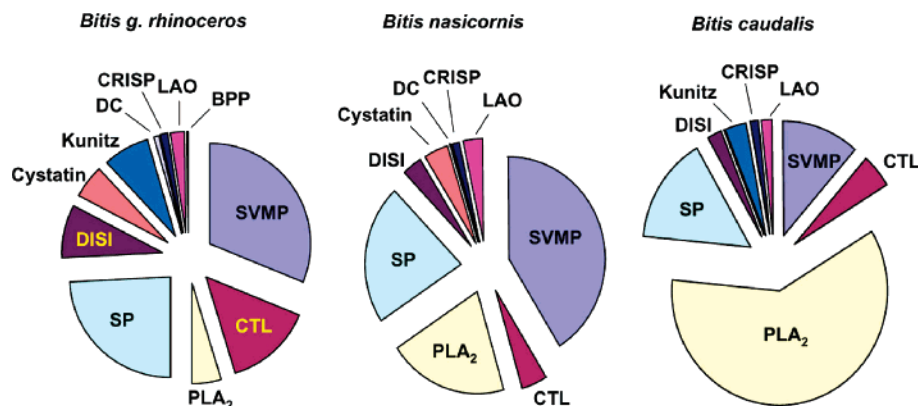
phoretic behavior of the Kunitz-type inhibitors in non-reduced (migrating as ~14 kDa proteins) versus reduced gels (apparent molecular masses of ~7 kDa) may be regarded as artifactual or as a indicating the existence of noncovalent dimers. Similar electrophoretic behavior has been previously reported for Kunitz-type inhibitors 1 and 2 from *Bitis gabonica gabonica*.<sup>21</sup>

The venom of the small *B. caudalis* departs from those of the large-bodied species of the *gabonica* clade in the large amount and diversity of PLA<sub>2</sub> molecules (59.8% of the total venom proteins versus 20.1% in *B. nasicornis* and 4.8% in *B.g. rhinoceros*) (Table 4) and in the tendency of these phosphatidylcholine 2-acylhydrolases to form high molecular mass aggregates. Hence, PLA<sub>2</sub>s isolated from the venoms of *B.g. rhinoceros* and *B. nasicornis* run as monomeric proteins in both, non-reduced and reduced SDS-PAGE (Bgr-13 in Figure 1B, and Bn-14 and Bn-15 in Figure 2B, respectively), whereas those from *B. caudalis* venom show a pattern of polydisperse molecules in non-reduced SDS-PAGE separation (Bc-12 to Bc-18 in Figure 3B). The fact that in reducing gels the *Bitis caudalis* PLA<sub>2</sub> smears yielded sharp protein bands of apparent molecular masses of 14–15 kDa (Figure 3B panel b), whereas electrospray ionization mass spectrometry showed molecular masses in the range of 13 kDa (Table 3, Bc12-Bc15), indicated that the apparent polydispersity of the *Bitis caudalis* PLA<sub>2</sub> molecules might not be due to covalent (disulfide) linkages, but rather to their tendency to form high molecular mass aggregates that resist dissociation in the conditions employed for SDS-PAGE separation.

Overall, as judged by their N-terminal sequences, molecular masses, and MS/MS-derived tryptic peptide sequences, the three *Bitis* species investigated exhibit extreme intragenus venom toxin composition variation.

Table 5 show adults average length and venom features of *Bitis* species. In line with a previous study on *Sistrurus catenatus* subspecies,<sup>18</sup> an implication of our results is that here does not appear to be a simple relationship between levels of venom complexity and toxicity. *Bitis gabonica rhinoceros* (West African gaboon viper) is native to high rainfall areas of West, Central, and East Africa; *Bitis nasicornis* (rhinoceros viper) occurs in forested areas of West and Central Africa, rarely venturing into woodlands, and *Bitis caudalis*, (horned puff adder) is mostly found in arid regions of South-West Africa. The high degree of differentiation in the venom proteome among congeneric taxa emphasizes unique aspects of venom composition of related species of *Bitis* snakes and points to a strong role for adaptive diversification via natural selection as a cause of this distinctiveness.

**Snake Venomics May Aid in Taxonomy.** Morphology-based and DNA-inferred molecular trees commonly differ in the branching orders proposed, making taxonomy and phylogeny of viperine snakes controversial. Given the central role that diet has played in ophidian evolution,<sup>15</sup> we argue that comparison of venom composition may aid in recognizing phylogenetic relationships. The availability of detailed proteomic information make possible estimates of the similarity and differentiation of the venom proteomes of different *Bitis* species, which are then useful in revealing broad-scale evolutionary patterns. Using protein similarity coefficients (PSC) to estimate the similarity of venom proteins of the *Bitis* taxa sampled here and in previous studies (*Bitis gabonica gabonica*<sup>20</sup> and *Bitis arietans*<sup>21</sup>), we estimate that *B.g. gabonica* and *B.g. rhinoceros* venoms share 10 venom proteins (PSC = 28), *B.g. gabonica* and



**Figure 4.** Comparison of the protein composition of the venoms of *Bitis gabonica rhinoceros*, *Bitis nasicornis*, and *Bitis caudalis*. SVMP, snake venom metalloproteinase; CTL, C-type lectin-like; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; SP, serine proteinase; DISI, disintegrin; Cystatin, cysteine proteinase inhibitor; Kunitz, Kunitz-type serine proteinase inhibitor; DC-fragment, disintegrin-like and cysteine-rich fragment derived from PIII–SVMP; CRISP, cysteine-rich secretory protein; LAO, L-amino acid oxidase; BPP, bradykinin-potentiating peptide.

**Table 4.** Overview of the Relative Occurrence of Proteins (in Percentage of the Total HPLC-Separated Proteins) of the Different Families in the Venom of *Bitis gabonica rhinoceros* and *Bitis nasicornis*<sup>a</sup>

protein family	% of total venom proteins				
	<i>B. g. rhinoceros</i>	<i>B. nasicornis</i>	<i>B. g. gabonica</i>	<i>B. a. arietans</i>	<i>B. caudalis</i>
Bradykinin-potentiating peptides	0.3	–	2.8	–	–
Dimeric disintegrin	8.5	3.5	3.4	–	2.3
Long disintegrin	–	–	–	17.8	–
Kunitz-type inhibitors	7.5	–	3.0	4.2	3.2
Cystatin	5.3	4.2	9.8	1.7	–
DC-fragment	0.6	<0.1	0.5	–	–
svVEGF	–	–	1.0	–	–
PLA <sub>2</sub>	4.8	20.1	11.4	4.3	59.8
Serine proteinase	23.9	21.9	26.4	19.5	15.1
CRISP	1.2	1.3	2.0	–	1.2
C-type lectin	14.1	4.2	14.3	13.2	4.9
L-amino acid oxidase	2.2	3.2	1.3	–	1.7
Zn <sup>2+</sup> -metalloproteinase	30.8	40.9	22.9	38.5	11.5
Unknown peptides	0.8	0.7	1.2	0.9	0.3

<sup>a</sup> For comparison, the compositions of the venoms of *Bitis gabonica gabonica*<sup>21</sup> and *Bitis arietans*<sup>20</sup> are listed.

**Table 5.** Adults Average Length and Venom Features of *Bitis* Species<sup>a</sup>

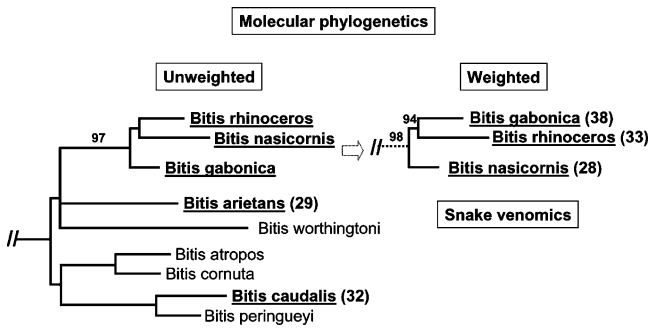
	average	intravenous		
	length (cm)	LD <sup>50</sup> in mice (mg/Kg)	wet venom yield (mg)	deadly for humans (mg)
<i>B. arietans</i>	80	0.4–2.0	100–350	100
<i>B. gabonica</i>	80–130	0.8–5.0	200–600	90–100
<i>B. nasicornis</i>	60–90	1.1	200	80
<i>B. caudalis</i>	30–40	1.2	85	300

<sup>a</sup> Source, <http://en.wikipedia.org/wiki/Bitis>.

*B. nasicornis*, displaying a PSC of 12, share 5 venom components, whereas *B. g. rhinoceros* and *B. nasicornis* share only 2 toxins (PSC = 7). On the other hand, the venoms of *Bitis arietans* and *Bitis caudalis* have diverged to a point where no similarity relationships to the other species of *Bitis* can be established. The distinctness of *Bitis arietans* among its congeneric species is highlighted by the occurrence in its venom of large amounts of the long disintegrin bitistatin,<sup>20</sup> whereas venoms from all the other *Bitis* species investigated contain dimeric disintegrins (Table 4). Moreover,

*Bitis arietans* venom lacks both, the ( $\alpha\beta$ )<sub>3</sub> C-type lectin-like molecule and the dimeric snake venom PIII-metalloproteases, which are conserved in the heavy-bodied *B. g. gabonica*, *B. g. rhinoceros*, *B. nasicornis* and in the small species, *Bitis caudalis*.

Our data support the monophyly of the three West African taxa (*B. g. gabonica*, *B. g. rhinoceros*, and *B. nasicornis*) based on genetic distance reconstructions, the lack of alliances between *B. arietans* and any other *Bitis* species, and are also consistent with the taxonomic association of *Bitis caudalis* within the differentiated group of small *Bitis* species<sup>7</sup> (Figure 5). From the apparently ancient divergence between the conventionally recognized subspecies of *Bitis gabonica*, *B. g. gabonica*, and *B. g. rhinoceros*, some authors<sup>8</sup> consider *B. g. rhinoceros* as a separate species, *Bitis rhinoceros*. The low level of venom toxin composition similarity between the two *B. gabonica* taxa appears to be consistent with this proposal. Moreover, our proteomic data fit better to a weighted phylogram based on overall genetic distances than to an unweighted maximum-parsimony tree (Figure 5).



**Figure 5.** Phylogenetic relationships among *Bitis*. Details of maximum-parsimony trees generated with *Causus* serving as the outgroup.<sup>7</sup> Left, the 75% majority-rule cladogram of the unweighted maximum-parsimony analysis. Right, weighted maximum-parsimony phylogram based on overall genetic distance-adjusted step matrix and the successive approximation approach. Bootstrap values of nodes with confidence values >90% are indicated. *Bitis* species for which the venom proteome has been analyzed are underlined. The number of distinct proteins identified using a venomomics approach are given in parentheses. Compositional data from *Bitis gabonica gabonica* and *Bitis arietans* venoms are from Juárez et al. (2006)<sup>20</sup> and Calvete et al. (2007).<sup>21</sup>

### Concluding Remarks

Proteomic analysis of the venoms of Afrotropical *Bitis* species supports the hypothesis that snake venom proteomes are composed of proteins belonging to only a few toxin families exhibiting large structural divergence and distinct relative abundances in even closely related species. A comprehensive catalog of venom composition may serve as a starting point for studying structure–function correlations of individual toxins for the development of new research tools and drugs of potential clinical use,<sup>50–52</sup> and for structure-based antivenom production strategies. Our study also highlights the relevance of detailed proteomic studies for a thorough characterization of the protein composition of snake venoms and for addressing how venoms evolve. The venom composition appears to keep information on the evolutionary history of congeneric taxa, which may be useful for resolving or supporting cladogenetic events inferred from DNA sequence data.

**Acknowledgment.** This study has been financed by grant BFU2004-01432/BMC from the Ministerio de Educación y Ciencia, Madrid, Spain.

### References

- (1) Scanlon, J. D.; Lee, M. S. Y. The pleistocene serpent *Wonambi* and the early evolution of snakes. *Nature* **2000**, *403*, 416–420.
- (2) Apesteguía, S.; Zaher, H. A cretaceous terrestrial snake with robust hindlimb and a sacrum. *Nature* **2006**, *440*, 1037–1040.
- (3) Alvarez, L. W.; Alvarez, W.; Asaro, F.; Michel, H. V. Extraterrestrial cause for the Cretaceous-Tertiary extinction. *Science* **1980**, *208*, 1095–1108.
- (4) Greene, H. W. *Snakes: The Evolution of Mystery in Nature*; University of California Press: Berkeley, 1997.
- (5) Heise, P. J.; Maxson, L. R.; Dowling, H. G.; Hedges, S. B. Higher-level snake phylogeny inferred from mitochondrial DNA sequences of 12S rRNA and 16S rRNA genes. *Mol. Biol. Evol.* **1995**, *12*, 259–265.
- (6) Vidal, N.; Hedges, S. B. Higher-level relationships of snakes inferred from four nuclear and mitochondrial genes. *C. R. Biol.* **2002**, *325*, 977–985.
- (7) Lenk, P.; Kalyabina, S.; Wink, M.; Joger, U. Evolutionary relationships among true vipers (Reptilia: Viperidae) inferred from mitochondrial DNA sequences. *Mol. Phylogenet. Evol.* **2001**, *19*, 94–104.

- (8) Lenk, P.; Herrmann, H. W.; Joger, U.; Wink, M. Phylogeny and systematics of *Bitis* (Reptilia: Viperidae) based on molecular evidences. *Kaupia* **1999**, *8*, 31–38.
- (9) Groombridge, B. C. Phyletic relationships among viperine snakes. In *Studies in Herpetology*, Proceedings of the 3rd Ord. Gen. Mtg. Societas European Herpetologica; Rocek, Z., Ed.; Charles Univ.: Prague, Czech Rep., 1986; pp 219–222.
- (10) Herrmann, H.-W.; Joger, U. Evolution of viperine snakes. In *Venomous Snakes: Ecology and Snakebite*; Thorpe, R. S., Wüster, W., Malhotra, A., Eds.; Oxford Univ. Press: London, UK, 1997; pp 43–61.
- (11) Vidal, N. Colubroid systematics: evidence for an early appearance of the venom apparatus followed by extensive evolutionary tinkering. *J. Toxicol. Toxin Rev.* **2002**, *21*, 21–41.
- (12) Fry, B. G.; Wüster, W. Assembling an arsenal: origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences. *Mol. Biol. Evol.* **2004**, *21*, 870–883.
- (13) Fry, B. G. From genome to “venome”: molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. *Genome Res.* **2005**, *15*, 403–420.
- (14) Fry, B. G.; Vidal, N.; Norman, J. A.; Vonk, F. J.; Scheib, H.; Ramjan, S. F.; Kuruppu, S.; Fung, K.; Hedges, S. B.; Richardson, M. K.; Hodgson, W. C.; Ignjatovic, V.; Summerhayes, R.; Kochva, E. Early evolution of the venom system in lizards and snakes. *Nature* **2006**, *439*, 584–588.
- (15) Greene, H. W. Dietary correlates of the origin and radiation of snakes. *Am. Zool.* **1983**, *23*, 431–441.
- (16) Ménez, A.; Stöcklin, R.; Mebs, D. “Venomics” or: The venomous systems genome project. *Toxicon* **2006**, *47*, 255–259.
- (17) Juárez, P.; Sanz, L.; Calvete, J. J. Snake venomomics: characterization of protein families in *Sistrurus barbouri* venom by cysteine mapping, N-terminal sequencing, and tandem mass spectrometry analysis. *Proteomics* **2004**, *4*, 327–338.
- (18) Sanz, L.; Gibbs, H. L.; Mackessy, S. P.; Calvete, J. J. Venom proteomes of closely-related *Sistrurus* rattlesnakes with divergent diets. *J. Proteome Res.* **2006**, *5*, 2098–2112.
- (19) Bazaá, A.; Marrakchi, N.; El Ayeb, M.; Sanz, L.; Calvete, J. J. Snake venomomics: comparative analysis of the venom proteomes of the Tunisian snakes *Cerastes cerastes*, *Cerastes vipera* and *Macrovipera lebetina*. *Proteomics* **2005**, *5*, 4223–4235.
- (20) Juárez, P.; Wagstaff, S. C.; Oliver, J.; Sanz, L.; Harrison, R. A.; Calvete, J. J. Molecular cloning of disintegrin-like transcript BA-5A from *Bitis arietans* venom gland cDNA library: a putative intermediate in the evolution of the long chain disintegrin bitistatin. *J. Mol. Evol.* **2006**, *63*, 142–152.
- (21) Calvete, J. J.; Marcinkiewicz, C.; Sanz, L. Snake venomomics of *Bitis gabonica gabonica*. Protein family composition, subunit organization of venom toxins, and characterization of dimeric disintegrins bitisgabinin-1 and bitisgabinin-2. *J. Proteome Res.* **2007**, *6*, 326–336.
- (22) Altschul, S. F.; Madden, T. L.; Schaffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402.
- (23) Le Blanc, J. C.; Hager, J. W.; Ilisiu, A. M.; Hunter, C.; Zhong, F.; Chu, I. Unique scanning capabilities of a new hybrid linear ion trap mass spectrometer (Q TRAP) used for high sensitivity proteomics applications. *Proteomics* **2003**, *3*, 859–869.
- (24) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 690–685.
- (25) Wetton, J. H.; Carter, R. E.; Parkin, D. T.; Walters, D. Demographic study of a wild house sparrow population by DNA fingerprinting. *Nature* **1987**, *327*, 147–149.
- (26) Francischetti, I. M.; My-Pham, V.; Harrison, J.; Garfield, M. K.; Ribeiro, J. M. C. *Bitis gabonica* (Gaboon viper) snake venom gland: towards a catalog of full-length transcripts (cDNA) and proteins. *Gene* **2004**, *337*, 55–69.
- (27) Joubert, F. J.; Townshend, G. S.; Botes, D. P. Snake Venoms. Purification, some properties of two phospholipases A2 (CM-I and CM-II) and the amino-acid sequence of CM-II and *Bitis nasicornis* (horned adder) venom. *Hoppe-Seyler's Z. Physiol. Chem.* **1983**, *364*, 1717–1726.
- (28) Viljoen, C. C.; Botes, D. P.; Kruger, H. Isolation and amino acid sequence of caudoxin, a presynaptic acting toxic phospholipase A2 from the venom of the horned puff adder (*Bitis caudalis*). *Toxicon* **1982**, *20*, 715–737.
- (29) Ménez, A., Ed. *Perspectives in Molecular Toxicology*; John Wiley & Sons, Ltd.: Chichester, UK, 2002.

- (30) Ogawa, T.; Kitajima, M.; Nakashima, K.; Sakaki, Y.; Ohno, M. Molecular evolution of group II phospholipases A<sub>2</sub>. *J. Mol. Evol.* **1995**, *41*, 867–877.
- (31) Ogawa, T.; Chijiwa, T.; Oda-Ueda, N.; Ohno, M. Molecular diversity and accelerated evolution of C-type lectin-like proteins from snake venom. *Toxicon* **2005**, *45*, 1–14.
- (32) Kordis, D.; Gubensek, F. Ammodytoxin C gene helps to elucidate the irregular structure of *Crotalinae* group II phospholipase A<sub>2</sub> genes. *Eur. J. Biochem.* **1996**, *240*, 83–90.
- (33) Deshimaru, M.; Ogawa, T.; Nakashima, K. I.; Nobuhisu, I.; Chijiwa, T.; Shimohigashi, Y.; Fukumaki, Y.; Niwa, M.; Yamashina, I.; Hattori, S.; Ohno, M. Accelerated evolution of crotaline snake venom gland serine proteases. *FEBS Lett.* **1996**, *397*, 83–88.
- (34) Ohno, M.; Menez, R.; Ogawa, T.; Danse, J. M.; Shimohigashi, Y.; Fromen, C.; Ducancel, F.; Zinn-Justin, S.; Le, Du, M. H.; Boulain, J. C.; Tamiya, T.; Menez, A. Molecular evolution of snake toxins: is the functional diversity of snake toxins associated with a mechanism of accelerated evolution? *Prog. Nucleic Acid Res. Mol. Biol.* **1998**, *59*, 307–364.
- (35) Junqueira de Azevedo, I. L.; Ho, P. L. A survey of gene expression and diversity in the venom glands of the pitviper snake *Bothrops insularis* through the generation of expressed sequence tags (ESTs). *Gene* **2002**, *299*, 279–291.
- (36) Kashima, S.; Roberto, P. G.; Soares, A. M.; Astolfi-Filho, S.; Pereira, J. O.; Giuliati, S.; Faria, M., Jr.; Xavier, M. A. S.; Fontes, M. R. M.; Giglio, J. R.; Franca, S. C. Analysis of *Bothrops jararacussu* venomous gland transcriptome focusing on structural and functional aspect: I- gene expression profile of highly expressed phospholipases A<sub>2</sub>. *Biochimie* **2004**, *86*, 211–219.
- (37) Cidade, D. A. P.; Simão, T. A.; Dávila, A. M. R.; Wagner, G.; Junqueira-de-Azevedo, I. L. M.; Ho, P. L.; Bon, C.; Zingali, R.; Albano, R. M. *Bothrops jararaca* venom transcriptome: Analysis of the gene expression pattern. *Toxicon* **2006**, *48*, 437–461.
- (38) Qinghua, L.; Xiaowei, Z.; Wei, Y.; Chenji, L.; Yijun, H.; Pengxin, Q.; Xingwen, S.; Songnian, H.; Guangmei, Y. A catalog for transcripts in the venom gland of the *Agkistrodon acutus*: identification of the toxins potentially involved in coagulopathy. *Biochem. Biophys. Res. Commun.* **2006**, *341*, 522–531.
- (39) Wagstaff, S. C.; Harrison, R. A. Venom gland EST analysis of the saw-scaled viper, *Echis ocellatus*, reveals novel  $\alpha_0\beta_1$  integrin-binding motifs in venom metalloproteinases and a new group of putative toxins, renin-like proteases. *Gene* **2006**, *377*, 21–32.
- (40) Junqueira-de-Azevedo, I. L. M.; Ching, A. T. C.; Carvalho, E.; Faria, F.; Nishiyama, M. Y., Jr.; Ho, P. L.; Diniz, M. R. V. *Lachesis muta* (*Viperidae*) cDNAs reveal diverging pitviper molecules and scaffolds typical of cobra (*Elapidae*) venoms: implications in snake toxin repertoire evolution. *Genetics* **2006**, *173*, 877–889.
- (41) Ching, A. T. C.; Rocha, M. M. T.; Leme, A. F. P.; Pimenta, D. C.; Furtado, M. F. D.; Serrano, S. M. T.; Ho, P. L.; Junqueira-de-Azevedo, I. L. M. Some aspects of the venom proteome of the Colubridae snake *Philodryas olfersii* revealed from a Duvernoy's (venom) gland transcriptome. *FEBS Lett.* **2006**, *580*, 4417–4422.
- (42) Richman, A. Evolution of balanced genetic polymorphism. *Mol. Ecol.* **2000**, *9*, 1953–1963.
- (43) Nei, M.; Gu, X.; Sitnikova, T. Evolution by the birth-and-death process in multigene families of the vertebrate immune system. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 7799–7806.
- (44) Hedrick, P. W.; Kim, T. Genetics of complex polymorphisms: parasites and maintenance of MHC variation. In *Evolutionary Genetics: From Molecules to Morphology*; Singh, R., Krimbas, C., Eds.; Cambridge Univ. Press: Oxford, 2000; pp 204–234.
- (45) Stahl, E. A.; Dwyer, G.; Mauricio, R.; Kreitman, M.; Bergelson, J. Dynamics of disease resistance polymorphism at the Rpm1 locus of *Arabidopsis*. *Nature* **1999**, *400*, 667–671.
- (46) Bergelson, J.; Kreitman, M.; Stahl, E. A.; Tian, D. Evolutionary dynamics of plant R-genes. *Science* **2001**, *292*, 2281–2285.
- (47) Tian, D.; Araki, H.; Stahl, E. A.; Bergelson, J.; Kreitman, M. Signature of balancing selection in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11525–11530.
- (48) Morita, T. Structures and functions of snake venom CLPs (C-type lectin-like proteins) with anticoagulant-, procoagulant-, and platelet-modulating activities. In *Perspectives in Molecular Toxicology*; Ménez, A., Ed.; John Wiley & Sons, Ltd.: Chichester, UK, 2002; pp 1099–1114.
- (49) Morita, T. Structure-function relationships of C-type lectin-related proteins. *Pathophysiol. Haemost. Thromb.* **2005**, *34*, 156–159.
- (50) Menez, A. Functional architectures of animal toxins: a clue to drug design? *Toxicon* **1998**, *36*, 1557–1572.
- (51) Harvey, A. L.; Bradley, K. N.; Cochran, S. A.; Rowan, E. G.; Pratt, J. A.; Quillfeldt, J. A.; Jerusalinsky, D. A. What can toxins tell us for drug discovery? *Toxicon* **1998**, *36*, 1635–1640.
- (52) Koh, D. C. I.; Armugam, A.; Jeyaseelan, K. Snake venom components and their applications in biomedicine. *Cell. Mol. Life Sci.* **2006**, *63*, 3030–3041.

PR0701714