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Analysis of Colubroidea snake venoms by liquid chromatography with mass spectrometry: evolutionary and toxinological implications

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The evolution of the venomous function of snakes and the diversification of the toxins has been of tremendous research interest and considerable debate. It has become recently evident that the evolution of the toxins in the advanced snakes (Colubroidea) predated the evolution of the advanced, front-fanged delivery mechanisms. Historically, the venoms of snakes lacking front-fanged venom-delivery systems (conventionally grouped into the paraphyletic family Colubridae) have been largely neglected. In this study we used liquid chromatography with mass spectrometry (LC/MS) to analyze a large number of venoms from a wide array of species representing the major advanced snake clades Atractaspididae, Colubrinae, Elapidae, Homalopsinae, Natricinae, Psammophiinae, Pseudoxyrhophiinae, Xenodontinae, and Viperidae. We also present the first sequences of toxins from *Azemiops feae* as well as additional toxin sequences from the Colubrinae. The large body of data on molecular masses and retention times thus assembled demonstrates a hitherto unsuspected diversity of toxins in all lineages, having implications ranging from clinical management of envenomings to venom evolution to the use of isolated toxins as leads for drug design and development. Although definitive assignment of a toxin to a protein family can only be done through demonstrated structural studies such as N-terminal sequencing, the molecular mass data complemented by LC retention information, presented here, do permit formulation of reasonable hypotheses concerning snake venom evolution and potential clinical effects to a degree not possible till now, and some hypotheses of this kind are proposed here. The data will also be useful in biodiscovery. Copyright © 2003 John Wiley & Sons, Ltd.

The advanced snakes (superfamily Colubroidea) make up over 80% of the approximately 2900 species of snake currently described, and contain all the known venomous forms.[1,2] The evolution of the venomous function of snakes and the diversification of their toxins has been of tremendous research interest and considerable debate. Only about 20% of the advanced snakes (Atractaspididae, Elapidae and Viperidae) have front-fanged delivery systems, and are typically regarded as of major medical interest. Of the remainder of the advanced snakes, some lack all trace of a venom apparatus, whereas the majority display a venom-delivery system consisting of a venom gland secreting toxins and expressing them near the base of the posterior maxillary teeth, which may display some apparent adaptations to facilitate the injection of these secretions (enlargement, grooving). Conventionally, all the advanced snakes devoid of anterior fangs were placed in the family Colubridae. However, several recent studies[3–6] have demonstrated convincingly that this family, as conventionally understood, is paraphyletic with respect to the front-fanged taxa: some of the distinct 'colubrid' clades (normally accorded subfamily status) are more closely related to viperids, elapids or atractaspidids than to each other (Fig. 1). In this paper, we will use the term 'colubrids' as an informal but convenient term to denote those advanced snakes which do not possess front fangs.

The origin of venom-delivery systems in snakes has been a subject of much research and debate,[2,7,8] with present opinion favoring an early origin of venom at the base of the colubroid radiation, followed by extensive 'evolutionary tinkering' (Vidal,[2] but see Chiszar and Smith[9] for contrasting views). The 'early origin' hypothesis was recently reinforced by us through the isolation of a three-finger toxin (3FTx) from the colubrine *Coelognathus radiatus*, and a phylogenetic analysis

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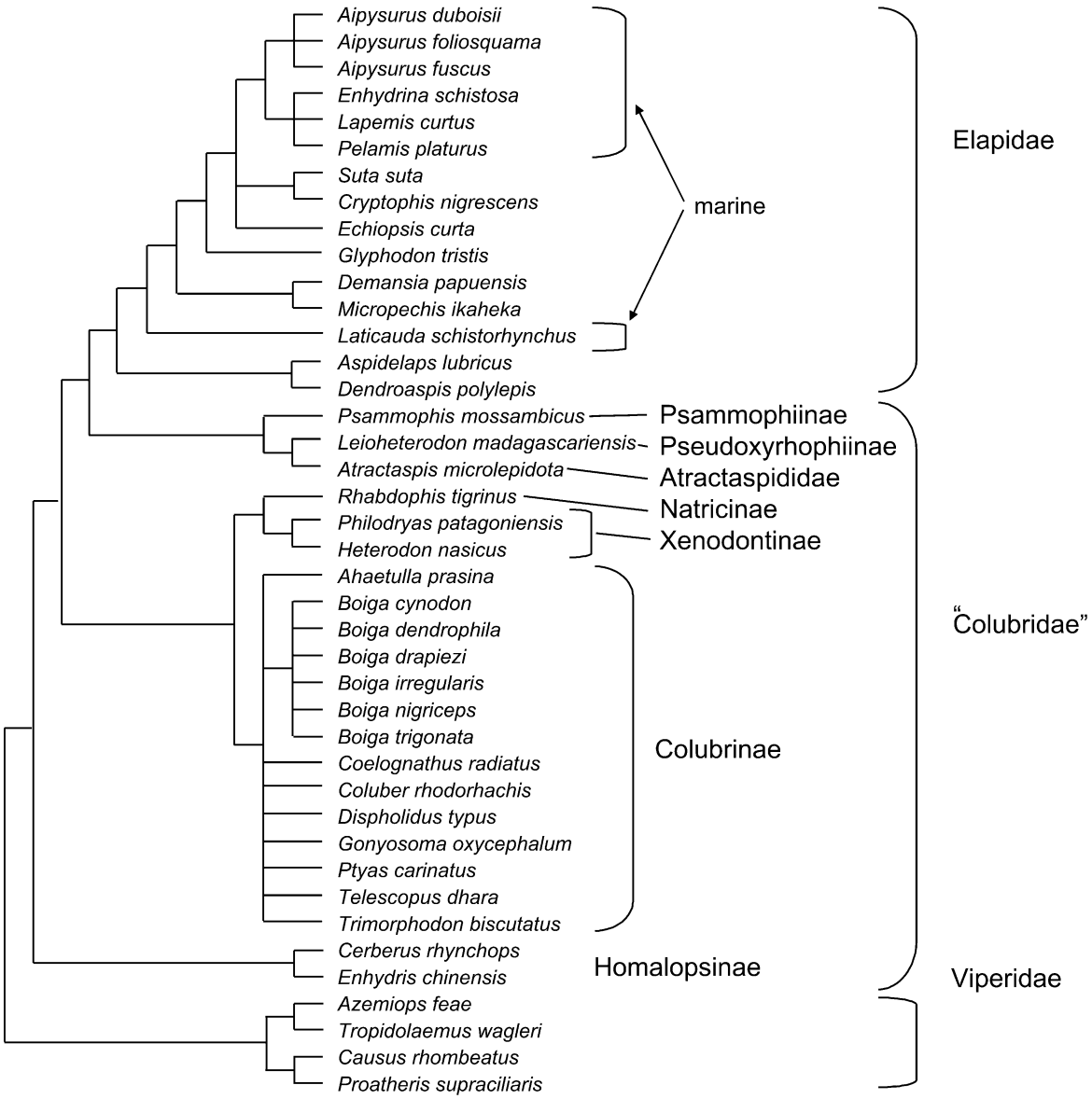

Figure 1. 'Best guess' phylogenetic tree for the species studied. Phylogeny follows principally Vidal and Hedges[6] and Slowinski and Lawson,[5] with additional input from Scanlon and Lee[60] for Australasian elapids.

showing that this gene family diverged before some of the most basal lineage splits within the Colubroidea.[10]

Historically, only the front-fanged families Elapidae and Viperidae, and to a lesser extent the Atractaspididae, have been the focus of intensive toxinological research. Further, even within those families, most of the research has centered on the 'usual suspects', taxa of medical interest or readily accessible for ease of study. In a recent study by us of the diversity of elapid three-finger toxins (3FTx) of the approximately 65 genera in the family Elapidae, the four genera *Bungarus*, *Dendroaspis*, *Laticauda* and *Naj*a accounted for 222 out of 263 (84.4%) sequenced toxins,[11] while, in the Viperidae, of the approximately 35 recognized genera, *Crotalus*, *Trimeresurus* and *Bothrops* account for 602 of 979 (61.5%) listed sequences in the SwissProt database.

The venoms of the remainder of the advanced snakes, the various 'colubrid' clades, have received scant attention. Only two proteins have been fully sequenced, alpha-colubritoxin[10] isolated by us from the venom of the Asian ratsnake *Coelognathus radiatus* (formerly known as *Elaphe radiata*), and tigrin from the Asian keelback snake *Rhabdophis tigrinus*.[12] The former is a 3FTx, with a seven amino acid N-terminal extension relative to the elapid 3FTx,[10] and tigrin is a CRISP toxin that is strongly homologous not only to elapid toxins, but also to viper toxins of the same gene family.[12] Enzymatic and electrophoretic studies of selected rear-fanged colubroid venoms[13] have demonstrated a considerable diversity of components and modes of action, and underscored the potential of the hitherto neglected clades in this radiation as sources of novel proteins.

In this study we use liquid chromatography with mass spectrometry (LC/MS) to analyze a representative selection of Colubroid venoms, including most major 'colubrid' clades, to examine the diversity of toxins, with the specific aim of determining the extent to which individual toxin families are distributed across the major colubroid lineages. In addition to our previous study on snakes,[14] mass spectrometry has been tremendously useful in the investigation of a wide array of

Table 1. Previously characterized snake toxin protein families

Family	~MW (kDa)
Bradykinin-potentiating peptide	1
Waglerin	2.5
Sarafotoxin	2–3.5
Natriuretic peptide	3–4
Myotoxic peptide	4–5
Disintegrin	4–8
BPTI/Kunitz-type protease inhibitor	6–7
Three-finger toxin	6–9
Prokinecitin	8–9
Thaicobrin/Ohanin	11–13
Cystatin	12–15
Pancreatic-type PLA_2	13–14
Synovial-type PLA_2	13–14
C-type lectin	14–19
CRISP	23–26
Peptidase family M12B	20–25
Peptidase family S1	25–28
Prothrombin activator (factor Xa-like)	48–50
L-amino oxidase	55–59
Acetylcholinesterase	60

venoms and toxins, from cone snails[15] to frogs[16] and spiders.[17] Species milked by us for analysis in this study were chosen primarily with a view to sampling the maximum possible phylogenetic breadth across the advanced snakes, and focused on previously unstudied species not closely related to well-studied species, species of potential medical importance, and, for comparative purposes, also included a few species that had been the focus of previous long-term intensive research interest. For the majority of the species studied, this was the first time to our knowledge that the venom had ever been analyzed. Molecular masses and retention times were compared with identified toxin families (Table 1) and previous LC/MS analyses of venoms.[10,14] The results have implications ranging from potential clinical effects to evolution/taxonomy to drug design and development. This collection of molecular masses and retention time information provides a platform upon which to build future evolutionary and biodiscovery studies.

MATERIALS AND METHODS

Species milked

Species and localities for each venom are shown in Table 2. In addition, we included the Henophidian snake *Python reticulatus*, Reticulated Python (Singapore), as a negative control.

Venom collection

All 'colubrid' specimens were milked using a variation of a recently developed colubrid-specific method.[18] Rather than ketamine, snakes were anesthetized with Zoletil 100 (Virbac; Tiletamine 50 mg and Zolazepam 50 mg) at a starting dose of 3 mg/kg. Rather than intramuscular injections on the body, pilocarpine (Sigma) was injected subcutaneously peri-glandular to the venom gland. As an additional step, atropine (Atrosite) was injected intramuscularly, subsequent to milkings, at a concentration of 0.04–0.08 mg/kg in order to counteract the excessive salivation produced by the pilocarpine and thus reduce the likelihood of the snakes asphyxiating while anesthetized. Atractaspididae and small Elapidae were milked by sliding pipette tips over the fang and wiggling to stimulate venom delivery; large Elapidae and all the Viperidae were milked by having the restrained snake bite a latex-covered specimen jar. Pooled samples from at least six unrelated adults were used for all species to minimize the effects of individual variation.[19] All venoms underwent a 20-micron filtration to remove any potential mucosal contaminents. In all cases, polyethylene materials (pipette tips, Eppendorf tubes, specimen bottles) were used to handle and contain the milkings due to the strong affinity some peptides possess for glass and polystyrene.

LC/MS

On-line liquid chromatography/mass spectrometry (LC/MS) analysis of venom samples dissolved in 0.1% trifluoroacetic acid (TFA) to a concentration of ~3 mg/mL was performed on a Phenomenex Jupiter C_{18} column (1 × 150 mm, 5 μ particle size, 300 Å) with solvent A (0.05% TFA) and solvent B (90% acetonitrile in 0.045% TFA) at a flow rate of 50 μL/min. The solvent delivery and gradient formation of a 1% gradient from 0–60% acetonitrile/0.05% TFA over 60 min were achieved using a Shimadzu LC 10AD solvent delivery system. Electrospray mass spectra were acquired using a PE-SCIEX API 300 system with a turbo ionspray atmospheric pressure ionization source. Samples (25 μL) were injected manually into the LC/MS system and analyzed in positive ion mode. Full-scan data were acquired with the electrospray sprayer voltage at 4600 V, orifice 45 V and ring 350 V, over the m/z range 600–3000 with a step size of 0.2 Th. Data processing was performed with the aid of the software package Biomultiview (PE-SCIEX, Canada). Isolation, characterization and Edman degradation sequencing of venom components were performed as previously described.[10] Removal of pyroglutamic acid was accomplished using pyroglutamase (Takara Biomedicals, cat. no. 7334) following the standard protocol.

RESULTS

The venoms of the species studied all yielded a number of components of varying molecular masses and retention times. Results are shown in Figs. 2–8 and in Tables 3–5. Between 5 and 26 different molecular masses were identified for each venom. These numbers are not intended as absolute upper bounds, as ion suppression remains a problem in electrospray mass spectrometry for co-eluting compounds. In the present study this was particularly acute for low molecular weight peptides present in lesser amounts, such as the natriuretic peptides,[14] that co-elute with the abundant proteins such as the PLA_2 toxins. Similarly, large hydrophobic components in some venoms did not behave well chromatographically under the conditions used, and reliable molecular masses could not be obtained for these HPLC peaks such as the large peak in *Dispholidus typus* venom that putatively contains the prothombin-activating enzyme.

As a general rule, terrestrial elapids and pit vipers yielded the highest diversity, whereas most 'colubrid' venoms (with

Table 2. Species and localities of snake milked for this study

Atractaspididae		
Atractaspis microlepidota	Small-scaled Stiletto Snake	Kenya
Colubrinae		
Ahaetulla prasina	Oriental Whip Snake	Singapore
Boiga cynodon	Dog-toothed Catsnake	Singapore
Boiga dendrophila dendrophila	Mangrove Catsnake	Bali
Boiga dendrophila gemmicincta	Black Catsnake	Sulawesi
Boiga drapiezii	White-spotted Catsnake	Bali
Boiga irregularis	Brown Tree Snake	Merauke, West Papua
Boiga nigriceps	Dark-headed Catsnake	West Java
Boiga trigonata	Common Catsnake	Pakistan
Coelognathus radiatus	Radiated Ratsnake	West Java
Coluber rhodorachis	Cliff Racer	Egypt
Dispholidus typus	Boomslang	Uganda
Gonyosoma oxycephalum	Red-tailed Ratsnake	Singapore
Ptyas carinatus	Keeled Ratsnake	Singapore
Telescopus dhara	Egyptian Catsnake	Egypt
Trimorphodon biscutatus	Lyre Snake	Arizona, USA
Elapidae		
Aipysurus duboisii	Dubois's Sea Snake	Ashmore Reef, Australia
Aipysurus foliosquama	Leaf-scaled Sea Snake	Ashmore Reef, Australia
Aipysurus fuscus	Tawny Sea Snake	Ashmore Reef, Australia
Aspidelaps lubricus	Cape Coral Cobra	South Africa
Cryptophis nigrescens	Australian Small-eyed Snake	Queensland, Australia
Demansia papuensis	Northern Whip Snake	Northern Territory, Australia
Dendroaspis polylepis	Black Mamba	Tanzania
Echiopsis curta	Bardick Snake	South Australia, Australia
Enhydrina schistosa	Beaked Sea Snake	Peninsular Malaysia and Albatross Bay Queensland, Australia
Glyphodon tristis	Brown-headed Snake	Queensland, Australia
Lapemis curtus	Spine-bellied Sea Snake	Peninsular Malaysia and Albatross Bay, Queensland, Australia
Laticauda schistorhynchus	Katuali	Niue
Micropechis ikaheka	New Guinea Small-eyed Snake	Jaya Pura, West Papua
Pelamis platurus	Pelagic Sea Snake	New South Wales, Australia
Suta suta	Curl Snake	South Australia, Australia
Homalopsinae		
Cerberus rynchops	Dog-faced Water Snake	Singapore
Enhydris chinensis	Chinese Water Snake	Guangdong, China
Natricinae		
Rhabdophis tigrinus	Tiger Keelback	Hunan, China
Psammophiinae		
Psammophis mossambicus	Olive Sand Snake	Tanzania
Pseudoxyrhophiinae		
Leioheterodon madagascariensis	Madagascar Hognosed Snake	Madagascar
Xenodontinae		
Heterodon nasicus	Western Hognosed Snake	Texas, USA
Philodryas patagoniensis	Chocolate Palm Snake	Brazil
Viperidae		
Azemiops feae	Fea's Viper	Guizhou, China
Causus rhombeatus	Rhombic Night Adder	Tanzania
Proatheris superciliaris	Swamp Viper	Tanzania
Tropidolaemus wagleri	Wagler's Viper	Sulawesi

the notable exception of *Psammophis* and *Heterodon*) yielded fewer components, as did the marine elapids and the viperines. Considerable variation in the diversity of molecular masses was found in most of the major lineages, and the relative importance of different molecular mass categories varied partially with phylogeny. For instance, in the psammophiine venom studied and that of most colubrines, proteins with molecular masses and retention times consistent with 3FTx were important and diverse, whereas they were present in smaller quantities and lower diversity in homalopsines, natricines, pseudoxyrhophiines and xenodontines, and absent altogether in the vipers. On the other hand, larger enzymes were relatively poorly represented in most colubrines, but more heavily so in the homalopsines, natricines, pseudoxyrhophiines and xenodontines. We isolated and partially sequenced components from *Boiga cynodon*, *B. dendrophila* and *Azemiops feae* (Fig. 9). The negative control, saliva from *Python reticulatus*, contained no detectable protein masses.[10]

DISCUSSION

Diversity of venom components

Our survey of colubroid venoms revealed a considerable diversity of proteins in the venoms of practically all Caenophidian lineages (Tables 3–5, Figs. 2–8). This diversity reinforces the potential of the almost entirely unstudied rear-fanged colubroids to yield a plethora of novel toxins of

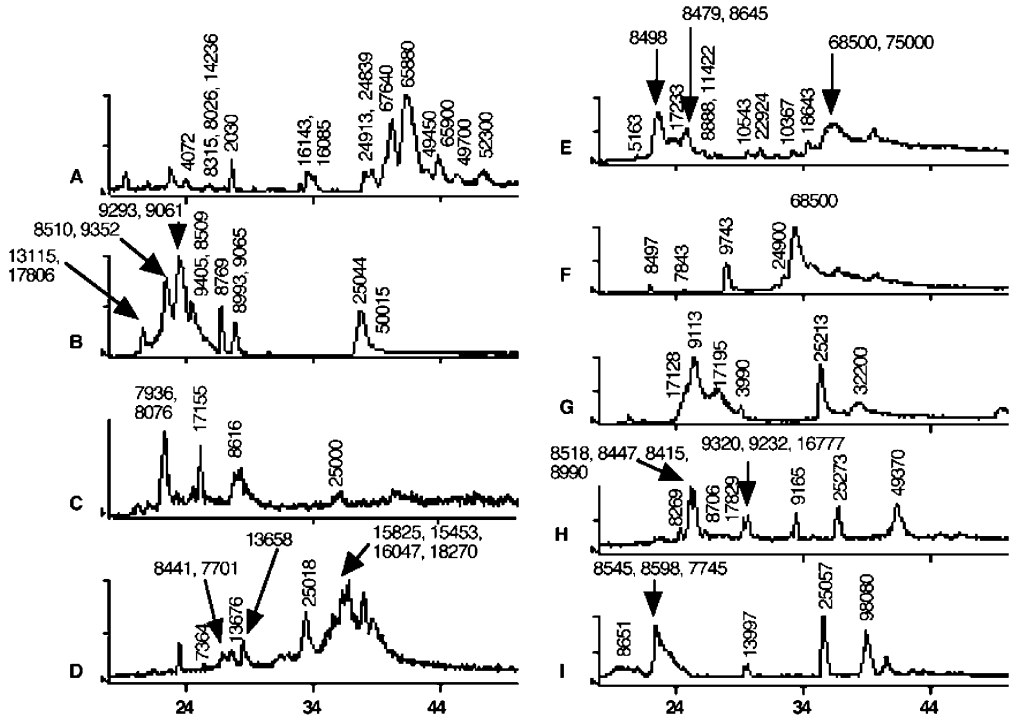

Figure 2. LC/MS analysis of the Colubrinae venoms (A) *Ahaetulla prasina*, (B) *Boiga dendrophila*, (C) *Coluber rhodorhachis*, (D) *Dispholidus typus*, (E) *Coelognathus radiatus* (formerly *Elaphe radiata*), (F) *Gonyosoma oxycephalum*, (G) *Ptyas carinatus*, (H) *Telescopus dhara*, and (I) *Trimorphodon biscutatus*. X-axis is percentage of acetonitrile at time of elution; Y-axis is relative intensity (0–100%). Reconstructed masses in Daltons are shown above each peak.

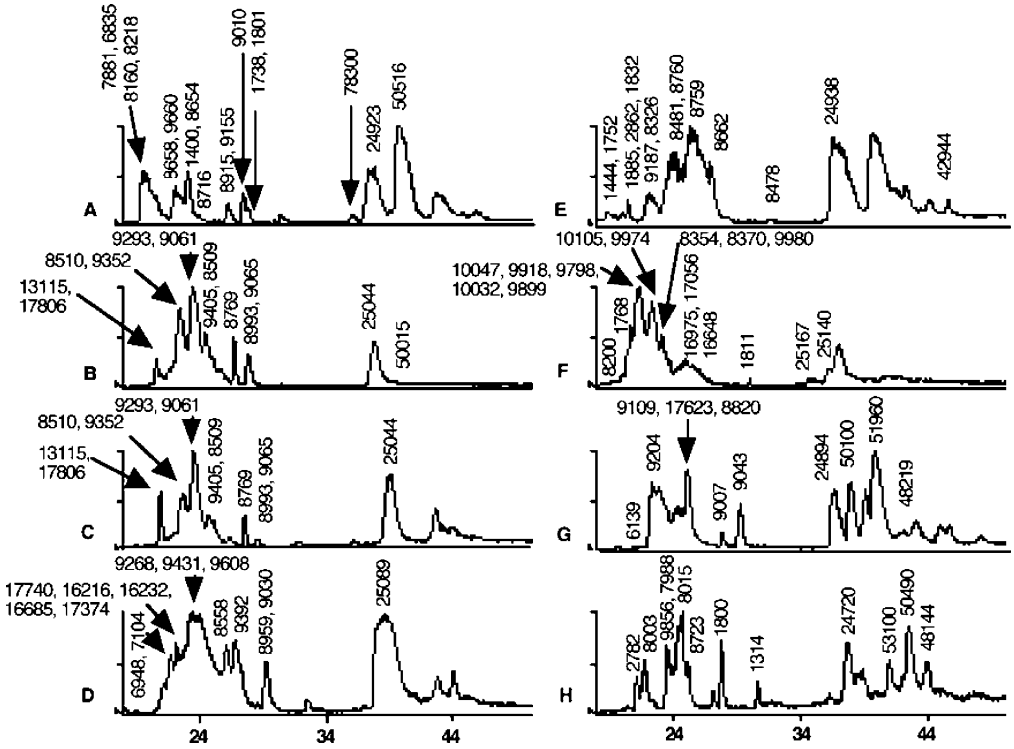

Figure 3. LC/MS analysis of *Boiga* species (A) *B. cynodon*, (B) *B. dendrophila dendrophila* (black and yellow population), (C) *B. d. dendrophila* (black and white population), (D) *B. dendrophila gemicincta*, (E) *B. drapiezii*, (F) *B. irregularis*, (G) *B. nigriceps*, and (H) *B. trigonata*. X-axis is percentage of acetonitrile at time of elution; Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons in shown above each peak.

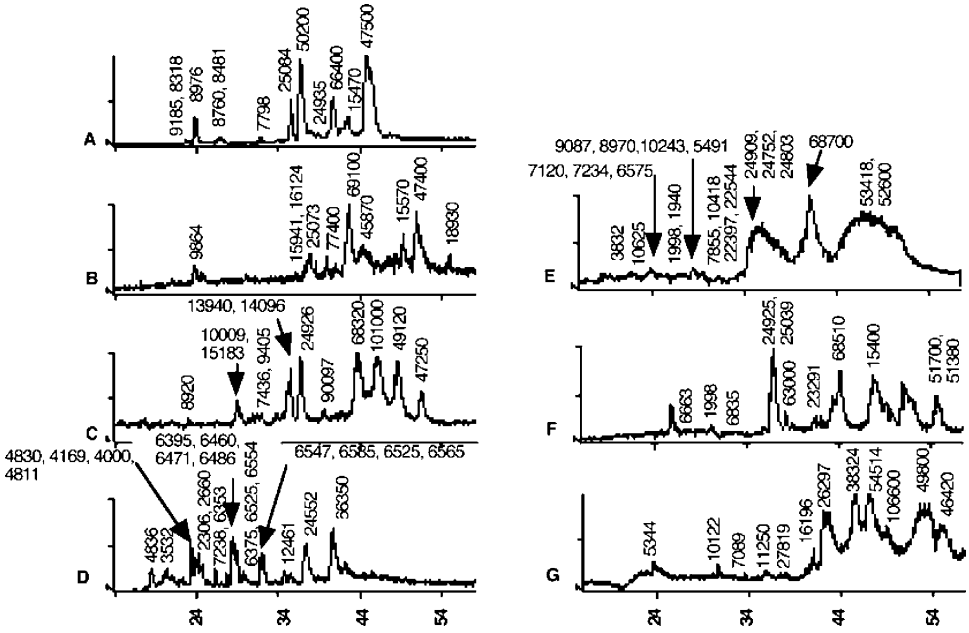

Figure 4. LC/MS analysis of representatives from different 'colubrid' families (A) *Cerberus rynchops* (Homalopsinae), (B) *Enhydris chinensis* (Homalopsinae), (C) *Leioheterodon madagascariensis* (Pseudoxyrhophiinae), (D) *Psammophis mossambicus* (Psammophiinae), (E) *Heterodon nasicus* (Xendontinae), (F) *Philodryas patagoniensis* (Xendontinae), and (G) *Rhabdophis tigrinus* (Natricinae). X-axis is percentage of acetonitrile at time of elution; Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

potential interest for studies ranging from snake evolution to pharmacology, drug discovery and clinical medicine.

Obviously, definitive assignment of a toxin to a protein family can only be done through demonstrated structural studies such as N-terminal sequencing. However, the breadth of coverage of the mass spectrometric data presented here does provide a unique resource upon which reasonable evolutionary and toxinological hypotheses can be based, and later subjected to more detailed examination. (Extensive retention time data are not listed here since, unlike molecular mass data, retention times can vary widely depending on experimental conditions; rather, the chromatographic information obtained in the present experiments is used as subsidiary complementary information used to support assignments of new compounds to toxin families via comparisons with data obtained by us under identical conditions for venoms that had been characterized previously.)

Many of the components detected in this study have molecular masses and elution profiles consistent with well-documented classes of toxins found in elapids or vipers (Table 1). For instance, all lineages with the exception of the viperids contained molecular masses consistent with 3FTx (Figs. 2–7). A partial 3FTx sequence from *Boiga dendrophila* confirmed the presence of this toxin class in the *Boiga* venoms, and thus its widespread distribution in the Colubrinae (Fig. 9). The confirmed presence of 3FTx in both colubrines and elapids leads to the prediction that they should be widespread throughout most of the Colubroidea.

Molecular masses and retention times consistent with PLA_2 (~12–14 kDa) were widespread in the 'colubrid' venoms (Tables 3 and 5, Figs. 2–4). However, reduction and alkylation of the larger 16–17 kDa components in *Boiga irregularis* venoms revealed them to be cysteine-linked heterodimers of 8–9 kDa subunits. It is anticipated that these will ultimately be shown to be 3FTx heterodimers.

Proteins consistent with the masses and retention times of CRISP proteins were widespread in all lineages. However, unlike the 3FTx, these toxins are also present in the Viperidae, and consequently may represent one of the earliest toxin families, predating the basal lineage split between the vipers and the remaining Colubroidea. Sequences have been reported from a variety of advanced snakes,[12,13] including two reported here from *Boiga cynodon* and *B. dendrophila*, and they are highly homologous (Fig. 9). CRISP toxins from *Pseudechis* species block the cyclic nucleotide-gated calcium channel,[12] and CRISP venom proteins isolated from pit vipers (21305550 from *Deinagkistrodon acutus*, 21305552 from *Protobothrops flavoviridis* and 2500712 from *P. mucrosquamatus*) and an elapid (21305554 *Laticauda semifasciata*) relax the peripheral

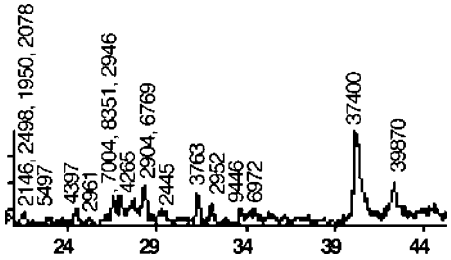

Figure 5. LC/MS analysis of an Atractaspidae venom (*Atractaspis microlepidota*). X-axis is percentage of acetonitrile at time of elution; Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

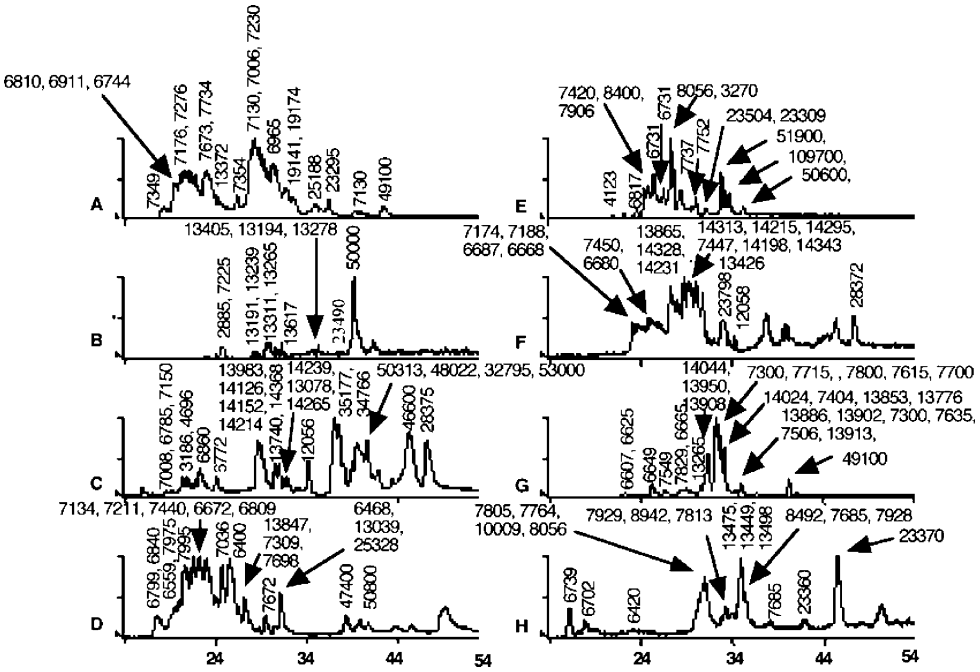

Figure 6. LC/MS analysis of terrestrial Elapidae (A) *Aspidelaps lubricus*, (B) *Cryptophis nigrescens*, (C) *Demansia papuensis*, (D) *Dendroaspis polylepis*, (E) *Echiopsis curta*, (F) *Glyphodon tristis*, (G) *Micropechis ikaheka*, and (H) *Suta suta.* X-axis is percentage of acetonitrile at time of elution; Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

smooth muscle through blockage of K^+-induced contraction, possibly by acting upon voltage-gated Ca^{2+} channels.[12] The component from the sole Natricinae for which a CRISP toxin has been characterized[12] (21326193 from *Rhabdophis tigrinus*) did not relax the smooth muscle and its mode of action remains unclear. However, the CRISP toxins may ultimately prove to have a multiplicity of actions, just as has been the case with other snake venom toxin families such as the 3FTx.[11]

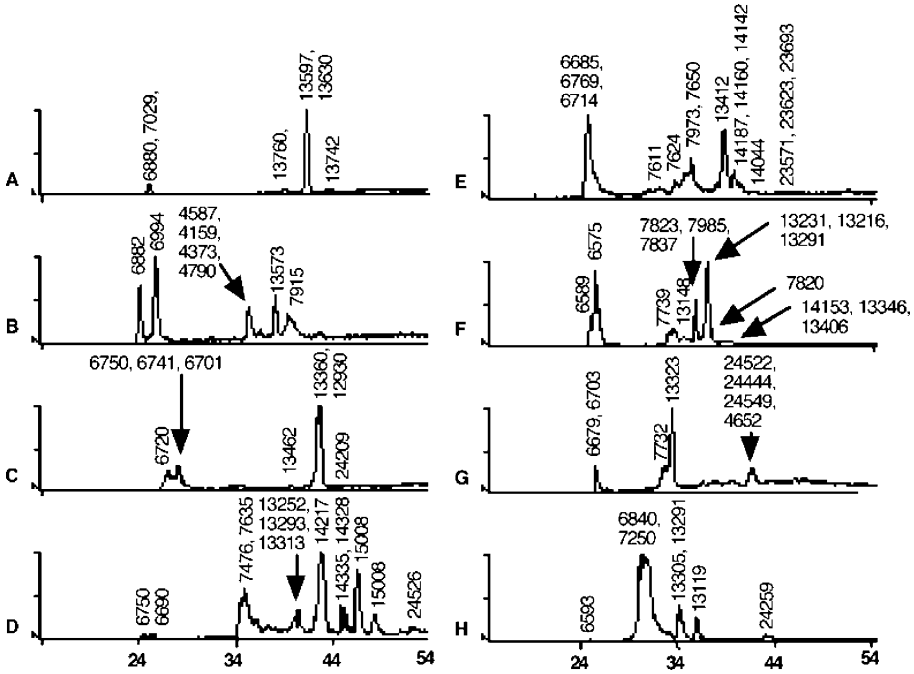

Figure 7. LC/MS analysis of marine Elapidae (A) *Aipysurus duboisii*, (B) *Aipysurus foliosquama*, (C) *Aipysurus fuscus*, (D) *Lapemis curtus*, (E) *Enhydrina schistosa* (Malaysia), (F) *Enhydrina schistosa* (Weipa, Australia), (G) *Pelamis platurus*, and (H) *Laticauda schistorhynchus.* X-axis is percentage of acetonitrile at time of elution; Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

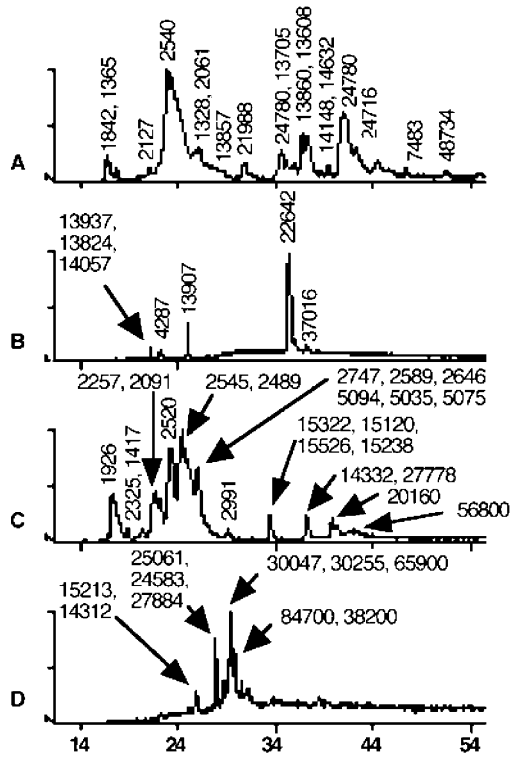

Figure 8. LC/MS analysis of Viperidae (A) *Azemiops feae*, (B) *Proatheris superciliaris*, (C) *Tropidolaeumus wagleri*, and (D) the subfamily Causinae representative *Causus rhombeatus*. X-axis is percentage of acetonitrile at time of elution; Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

The 2–4 kDa components in the *Atractaspis microlepidota* venom examined here can be reasonably hypothesized to be representatives of the well-characterized and abundant sarafotoxins (e.g. NCBI accession numbers 134896 and 289101). Molecular masses consistent with disintegrins and 3FTx were also observed, while the 37400 and 39870 Da components (Fig. 5) are almost certainly similar to metalloproteinase precursor forms sequenced previously (see NCBI accession numbers 6007791, 6007787, 6007785, 6007789). These toxins are highly homologous to *Naja naja* (6006966) and *Naja mossambica* (21435683) sequences, and to viperid toxins (e.g. 2231613 from *Agkistrodon contortrix laticinctus* and 20530121 from *Protobothrops flavoviridis*). This would indicate that the M12B class of metalloproteinase is widespread across the Colubroidea families and most likely represents yet another class of proteins recruited into the venom function at the very base of the colubroid radiation. Further investigations of the gene phylogeny of these proteins would be revealing in this context.

Among the elapids, our study revealed complex venoms with a variety of different molecular mass classes across a diversity of small elapids hitherto largely neglected by toxinological research (Fig. 6). In the African *Aspidelaps lubricus*, 3FTx and PLA_2 toxins,[20–22] with toxicities comparable to those of African species of *Naja* (Cobra), have been purified. Consistent with these findings, the venom is rich in molecular masses characteristic of 3FTx, and also contains 19 kDa components (possibly C-type lectins). The neurotoxic venom of the Australian *Echiopsis curta* has been reported to cross-react with death adder antivenom,[23] reflective of phylogenetic relationships. While not sharing any of the individual components isolated from *Acanthophis* venoms,[14] the venom of *Echiopsis curta* is similarly rich in 3FTx, which may explain the cross-reactivity. The cause of the anticoagulant action of the venom[23] is unclear, as the anti-platelet PLA_2 toxins (similar to those reported from other Australian elapids[24–26]) appear to be lacking in the venom. This action upon the blood may be due to the large hydrophobic components present. The venoms of *Glyphodon tristis* and *Suta suta* were rich in molecular masses characteristic of 3FTx and PLA_2, but, intriguingly, the largest peak in the latter was a component with retention time and molecular mass consistent with those of the smooth muscle paralyzing CRISP toxins.[12] LC/MS analysis of *Cryptophis nigrescens* venom revealed the overwhelmingly dominant component to be a 50 kDa component, and this venom was quite unusual for an elapid in being almost devoid of masses typical of 3FTx.

In addition to the hitherto neglected 'colubrid' lineages and the understudied small elapids, our analysis also revealed the presence of previously undocumented molecular mass classes in well-studied venoms. PLA_2 were not previously documented from the heavily studied mamba (*Dendroaspis*) venoms, but our study revealed two peaks consistent with PLA_2 in both molecular mass and retention times in the venom of *D. polylepis* (Fig. 6). Proteins of unknown affinities were also documented in the venoms of the elapids *Cryptophis nigrescens* and *Demansia papuensis*. Similarly, our analysis of the venom of the obscure viperid *Azemiops feae*, most likely the sister group of pit vipers, revealed a diversity of toxins and a remarkable similarity with the venom of the well-studied pit viper *Tropidolaemus wagleri* (Fig. 8).

The demonstration of the diversity of toxins present (Tables 3–5) is useful for researchers interested in isolating and characterizing novel toxins for use as investigational ligands or as scaffolds in drug design and development. The so-called subfamilies of the artificial 'Colubridae' all represent major independent radiations of advanced snakes, being in many cases no more closely related to each other than they are to the Elapidae (Fig. 1). As such, the toxins contained in the venoms should also differ appreciably. Consequently, these venoms should prove to be a rich source of unique toxins, as exemplified by the novel 3FTx alpha-colubritoxin from *Coelognathus radiatus*.[10]

The potential variation is demonstrated by the N-terminal sequence of the Colubrinae toxin isolated here from *Boiga dendrophila*, that shows similarity to, but nevertheless differs from, alpha-colubritoxin from *Coelognathus radiatus* (Fig. 9). Like the fully sequenced 3FTx from *Coelognathus radiatus*,[10] molecular mass gains during reduction and alkylation revealed that this toxin contains 10 cysteine residues, and it is anticipated that at least some *Boiga* 3FTx will have the same ancestral cysteine pattern as alpha-colubritoxin.[10,11]

The presence of such considerable variation within the Colubrinae gives an indication of the far greater degree of sequence and activity diversity likely to be found across the range of 'rear-fanged' colubroids. LC/MS analysis revealed numerous peaks containing multiple protein isoforms, indicative of a high rate of gene duplication and sequence

Table 3. Molecular masses observed in Colubrinae venoms

Species	<5	6–10	12–15	15–19	20–26	26–30	30–35	36–40	40–55	>55
Ahaetulla prasina	4072, 2030	8315, 8026	14236	16143, 16085	24913, 24839				49450, 49700, 52300	67640, 65880
Boiga cynodon	1400, 1738, 1801	7881, 6835, 8160, 8218, 8654, 8716, 8915, 8658 9155, 9010, 9660			24923				50516	78300
Boiga dendrophila		8510, 9352, 9293, 9061, 9405, 8509, 8769, 8993, 9065	13115	17806	25044				50015	
Boiga dendrophila gemmicincta		6948, 7104, 9268, 9431, 9608, 8558, 9392, 8959, 9030		17740, 16216, 16232, 16685, 17374	25089					
Boiga drapiezii	1444, 1752, 1885, 2862, 1832	9187, 8326, 8481, 8760, 8759, 8662, 8478			24938				42944	
Boiga irregularis	1768, 1811	8200, 10047, 9918, 9798, 10032, 9899, 10105, 9974, 8354, 8370, 9980		16975, 17056, 16648	25167, 25140					
Boiga nigriceps		6139, 9204, 9007, 9043, 9109, 8820		17623	24894				50100, 51960, 48219	
Boiga trigonata	1314, 1800, 2782	8003, 9856, 7988, 8015, 8723			24720				53100, 50490, 48144	
Coelognathus radiatus	5163	8498, 8479, 8645, 8888	11422, 10543, 10367	17233, 18643	22924					68500, 75000
Coluber rhodorachis		7936, 8076, 8616		17155	25000					
Dispholidus typus		8441, 7701, 7364	13676, 13658	15825, 15453, 16047, 18270	25018					
Gonyosoma oxycephalum		8497, 7843, 9743			24900					68500
Ptyas carinatus	3990	9113		17128, 17195	25213		32200			
Telescopus dhara		8518, 8447, 8415, 8990, 8269, 8706, 9320, 9232, 9165		16777, 17829	25273				49370	
Trimorphodon biscutatus		8651, 8545, 8598, 7745	13997		25057					98080

Table 4. Molecular masses observed in Elapidae venoms

Species	<5	6–10	12–15	15–19	20–26	26–30	30–40	>40
Aipysurus duboisii		6880, 7029	13760, 13742, 13597, 13630					
A. foliosquama	4587, 4159, 4373, 4790	6882, 6994, 7915	13573					
A. fuscus		6720, 6750, 6741, 6701	13462, 13360, 12930		24209			
Aspidelaps lubricus		6810, 6911, 6744, 7349, 7176, 7276, 7673, 7734, 7354, 7130, 7006, 7230, 6965, 7130	13372	19141, 19174	23295, 25188			49100
Cryptophis nigrescens	2885	7225	13191, 13239, 13311, 13265, 13617, 13405, 13194, 13278		23490			50000
Demansia papuensis	3186, 4696	7008, 6785, 7150, 6860, 6772	13983, 14126, 14152, 14214, 13740, 14368, 14239, 13078, 14265, 12056			28375	35177, 34766, 32795	46600, 48022, 50313, 53000
Dendroaspis polylepis		7134, 7211, 7440, 6672, 6809, 6799, 6840, 6559, 7975, 7995, 7036, 6400, 7698, 7672, 6468	13847, 13039		25328			47400, 50800
Echiopsis curta	3270, 4123	7420, 8400, 7906, 6817, 6731, 6731, 8056, 7737, 7752			23504, 23309			51900, 50600
Enhydrina shistosa (Australia)		6589, 6675, 7739, 7823, 7985, 7837, 7820	13231, 13216, 13291, 14153, 13346, 13406					
Enhydrina schistosa (Malaysia)		6685, 6769, 6714, 7611, 7624, 7973, 7650	13412, 14187, 14160, 14142		23571, 23623, 23693			
Glyphodon tristis		7174, 7188, 6687, 6668, 7450, 6680, 7447	13865, 14328, 14231, 14313, 14215, 14295, 14198, 14343, 13426, 12058		23798	28372		
Lapemis curtus (Australia)		6750, 6690, 7476, 7635	13252, 13293, 13313, 14217, 14328, 14335, 15008, 15008		24526			
Lapemis curtus (Malaysia)		6750, 6608, 6690, 7734, 7521, 7333, 7925	12037, 13206, 13313, 14216, 15048, 15008		24324			
Laticauda schistorhyncha		6593, 6840, 7250	13305, 13291, 13119		24259			
Micropechis ikaheka		6607, 6625, 6649, 7549, 7829, 6665, 7300, 7715, 7800, 7615, 7700, 7404, 7300, 7635, 7506	13265, 14044, 13950, 13908, 13265, 14024, 13853, 13776, 13886, 13902, 13913					49100
Pelamis platurus	4652	6679, 6703, 7732	13323		24522, 24444, 24549			
Suta suta		6739, 6702, 6420, 7805, 7764, 10009, 8056, 7929, 8942, 7813, 8492, 7685, 7928, 7685	13475, 13449, 13498		23360, 23370			

RCM

Table 5. Molecular masses observed in Atractaspididae, Homalopsinae, Psammophiinae, Pseudoxyrhophiinae, Xenodontinae and Viperidae venoms

Species	<5	6–10	12–15	15–19	20–26	26–30	30–40	40–55	>55
Atractaspididae									
Atractaspis microlepidota	2146, 2498, 1950, 2078, 2961, 2946, 2904, 2952, 4397, 4265, 3763, 5497	7004, 8351, 6769, 6972, 9446					37400, 39870		
Homalopsinae									
Cerberus rynchops		9185, 8318, 8976, 8760, 8481, 7798			25084, 24935			47600, 50200	
Enhydris enhydris		9864		15941, 16124, 15570, 18930	25073			45870, 47400	69100, 77400
Natricinae									
Rhabdophis tigrinus	5344	7089	10122, 11250	16196		27819, 26297	38324	46420, 49800, 54514	106600
Psammophiinae									
Psammophis mossambicus	4830, 4169, 4000, 4811, 4836, 3532, 2306, 2660	6395, 6460, 6471, 6486, 7238, 6353, 6375, 6525, 6554, 6547, 6585, 6525, 6565	12461		24552				66350
Pseudoxyrhophiinae									
Leioheterodon madagascariensis		8920, 7436, 9405 10009	13940, 14096, 15183		24926			49120, 47250	68320, 90007, 101000
Xenodontinae									
Heterodon nasicus	3832, 1998, 1940, 5491	7120, 7234, 6575, 9087, 8970, 7855, 10243, 10625, 10418			22397, 22544, 24909, 24752, 24803			53418, 52600	68700
Philodryas patagoniensis	1998	6663, 6835		15400	24925, 25039, 23291			51700, 51380	63000, 68510
Viperidae									
Azemiops feae	1842, 1365, 1328, 2127, 2061, 2540	7483	13857, 13705, 13860, 13608, 14148, 14632		21988, 24780, 24780, 24716				48734
Causus rhombeatus			14312	15213	25061, 24583, 27884		30047, 30255, 38200		65900, 84700
Proatheris superciliaris	4287		13937, 13824, 14057, 13907		22642		37016		
Tropidolaemus wagleri	1926, 2325, 1417, 2257, 2091, 2520, 2545, 2489, 2291, 2747, 2589, 2646, 2291	5094, 5035, 5075	14332	15322, 15120, 15526, 15238	20160	27778			56800

A.

1. QAIGLPHTACIQCNRKTSSKCLSGQICLPYHMTCYTLYKPDENGELK
2. QAIGPPYGLCFQCNQKTSSDCTEARRCSPFHEKCYTLYQPDENWMKS

B.

1. NVDFNSESPRRKNKQQEIVDMHNSFRRSVNPTARN
2. NVDFNSESPRRKNKQQEIVDMHNSFRRSVNPTARN
3. QDFNSEPPRKPEIQRVSVDTN
4. YDDFNSQSPRRPEIQRSIAN
5. NVDFN
6. NVDFNSESPRNPGKQQEIVNIHNSFRRSVRPTARN
7. TADFASESSNKKNYQKEIVDKHNALRRSVKPTARN
8. NVDFDSESPRKPEIQNEIIDLHNSLRRSVNPTASN

C.

1. SLITFETLILKVAGRSGIWYYGSYGCFCGA
2. HLMQFETLIMKVAGRSGVWYYGSYGCFCGA

D.

1. VAGGDECNI-EHRSL-VLFNSNGFLCGGTL
2. VFGGDECNINEHRSLVVLFNSNGFLCGGTL

Figure 9. N-Terminal sequence comparison of isolated toxins with previously reported sequences. (A) 1. *Boiga dendrophila* 8679 Da peptide with 2. the 3FTX alpha-colubritoxin[10] (N-terminal Q is pyroglutamic acid). (B) 1. Colubrinae (*Boiga dendrophila*) 25044 and 2. Colubrinae (*Boiga cynodon*) 24923 Da proteins and ~26 kDa proteins from 3. Xenodontinae (*Hydrodynastes gigas*[13]), 4. Dipsadinae (*Hypsiglena torquata*[13]) and 5. Colubrinae (*Trimorphodon biscutatus lambda*[13]), compared with the CRISP toxins from 6. Natricinae (*Rhabdophis subminiatus*) (NCBI accession number 21326193), 7. Elapidae (*Pseudechis australis*) (NCBI accession number 23264041) and 8. Viperidae (*Protobothrops mucrosquamatus*) (NCBI accession number 2500712) (C) 1. *Azemiops feae* 13608 Da component with 2. a synovial-type PLA_2 from *Trimeresurus gramineus* (NCBI accession number 3914269). (D) 1. *Azemiops feae* 24780 Da with 2. a plasminogen activator from *Trimeresurus stejnegeri* (NCBI accession number 13959636).

evolution similar to that of the Elapidae.[11] We anticipate that, like various toxins isolated from Elapidae venom, 3FTx from other lineages will be discovered with alternately evolved cysteines and phylogenetic groupings.[10,11] These variations will also greatly facilitate the search for novel toxins for use as investigational ligands or even as scaffolds for drug design and development. The potent yet easily reversible neurotoxicity of alpha-colubritoxin[10] demonstrates the potential of these toxins in biodiscovery.

Evolutionary implications

Our results indicate that the venoms of the 'colubrid' snakes are, in many cases, as complex as those of many front-fanged snakes, both in terms of absolute numbers of resolved molecular masses as well as in terms of molecular mass categories (Tables 3–5). Moreover, as demonstrated previously[10] and in this study (Fig. 9), at least some of the toxins present in the venoms of the 'colubrid' snakes belong to widespread toxin families shared with front-fanged snakes, such as the 3FTx of the Elapidae[11] and the CRISP toxins found in both Elapidae and Viperidae venoms.[12] In addition to our LC/MS analysis of venoms indicating the widespread presence of 3FTx and CRISP toxins, at least one of these toxin classes has been confirmed by us through protein sequencing to be present in the venom of the divergent lineages Colubrinae (3FTx, CRISP), Elapidae (3FTx, CRISP), Natricinae (CRISP), Xendodontinae (CRISP) and Viperidae (CRISP), with the toxin sequences being highly homologous, including sequences presented here (Fig. 9).

Our phylogenetic analysis of full-length 3FTx sequences[10] indicates that this multi-gene family was recruited early into the chemical arsenal of the Colubroidea, and started to diversify before the lineages leading to the present-day Elapidae and Colubrinae diverged, but after the split by Viperidae. The presence of CRISP toxins with high degrees of sequence similarity in colubrines, elapids, natricines, vipers and xenodontines suggests even earlier recruitment and diversification, before the divergence between the Viperidae and the remaining Colubroids. In contrast, the synovial-type PLA_2 toxins found in viper venoms, such as the sequence we present here from *Azemiops feae* (Fig. 9), represent an independent recruitment event that occurred after Viperidae left the main Colubroidea lineage. Consequently, based upon phylogenetic relationships, we anticipate that, should PLA_2 toxins indeed be confirmed as present in the 'colubrid' venoms, they will most likely be homologous to the elapid ('pancreatic-type' PLA_2) rather than viper ('synovial-type' PLA_2) toxins.

Our results provide further evidence of the antiquity of these various toxin groups and their early recruitment into

the chemical arsenal of the Colubroidea snakes. This highlights the shared history of the toxin-secreting gland; the presence of a toxin-secreting supralabial gland is a synapomorphy of the Colubroidea. Toxins pre-dating intricate delivery mechanisms is a logical scenario, since there cannot be any selection for an effective delivery system in the absence of potent venoms. These findings have profound implications for our understanding of the evolution of the toxin-secreting oral glands of the advanced snakes.

The 'colubrid' toxin-secreting gland, usually termed Duvernoy's gland,[27] is homologous with the venom glands of viperids, elapids and atractaspidids,[28] but there are considerable anatomical and functional differences between them.[29] However, at the same time it is important to note that, since the front-fanged venom-delivery apparatus evolved independently in these three families,[2] the 'venom glands' of these front-fanged radiations are not homologous, except through their more general homology with the Duvernoy's gland of 'colubrid' snakes. Duvernoy's glands almost certainly represent the primitive condition of the venom-delivery system within the advanced snakes, from which the venom glands of front-fanged snakes, with their associated high-pressure delivery system (compressor muscles, storage lumen, venom duct), have evolved on three separate occasions. Moreover, the colubrine *Dispholidus typus* displays some convergence with front-fanged snakes, as it has rudimentary compressor muscles associated with its venom delivery system.

In view of the homology of the toxin-secreting glands of all advanced snakes and their toxins, the fact that Duvernoy's glands represent a primitive condition, and that the derived glands of the front-fanged snakes are independently derived from these, we suggest that the distinction between Duvernoy's glands and venom glands is an artificial one that impedes our understanding of the evolution of the venom apparatus of snakes. For this reason, we propose that the term 'Duvernoy's gland' should be abandoned, and that the term 'venom gland' be used for the toxin-secreting oral glands of all snakes, regardless of the degree of anatomical specialization in the venom-delivery apparatus. In so doing, we accept that the precise biological role of 'colubrid' toxins in the foraging biology of these snakes remains poorly understood, and may be diverse and not restricted to rapid prey death,[30] although the neurotoxic activity in at least some 'colubrid' venoms[10] is most consistent with the role of killing or incapacitating prey rather than digestion or other functions. However, in any case, we reject the suggestion that rapid prey death should be the sole determinant of whether a toxic secretion should be termed a 'venom'.[31] Apart from the difficulty in defining rapid prey death,[32] such an overly restrictive and arbitrary definition obscures the evolutionary homology between the toxin-secreting glands of 'colubrid' and front-fanged snakes, and may introduce entirely artificial distinctions between anatomically similar and homologous structures and secretions within the 'colubrids'. For instance, classifying the toxins and glands of *Dispholidus typus* as a venom apparatus, but not the homologous secretions and structures of *Boiga irregularis*,[31] on the basis of observed differences in predatory behavior,[31,33] contributes little to our understanding of the evolution of venom in snakes.

In the same vein, we also agree with the suggestion that the distinction between opisthoglyphous and aglyphous 'colubrids' should be abandoned.[34] Not only does this distinction shoehorn a wide variety of dentitional types into two artificial, non-monophyletic categories, but it similarly ignores the fact that a wide variety of 'colubrids' possess complex venoms, with widely shared toxin gene families that transcend any divisions based on dentition types.

Another startling result of the present study is the extreme lack of diversity found in the venoms of the marine elapids; fewer than 10 components were identified in all except *Enhydrina* and *Lapemis*, compared with 16 or more in all but one terrestrial elapid (Figs. 6 and 7, Table 4). All marine elapid venoms are dominated by a few 6–7 kDa and 12–16 kDa proteins (presumably 3FTx and PLA_2), with small quantities of 4 kDa and ~24 kDa proteins present in some venoms. These latter peaks probably correspond to natriuretic peptides and CRISPs, respectively. Whereas most terrestrial elapids have multiple isoforms in each peak and several peaks for each toxin family, the marine elapid venoms have fewer isoforms present in each peak and fewer peaks overall. Moreover, whereas many terrestrial elapid venoms contain proteins of various high molecular mass classes, none of the marine venoms contained any components larger than ~26 kDa. Low diversity within toxin classes is also the case in terms of gene phylogeny. Extensive previous sequencing of sea snake 3FTx did not reveal any proteins that were not phylogenetically aligned with the type I or type II alpha-neurotoxins,[11] even from the rigorously studied *Laticauda* venoms.[11] Other Elapidae for which extensive sequencing of 3FTx has been undertaken (e.g. *Bungarus*, *Dendroaspis*, *Naja*) have revealed a number of other phylogenetically distinct groups of toxins with divergent or unknown functions.[11]

The streamlined pattern of sea snake venoms is all the more striking due to its parallel occurrence in two independent marine radiations; sea kraits (*Laticauda*) and the true sea snakes (e.g. *Aipysurus*, *Enhydrina*, *Lapemis* and *Pelamis*) evolved their marine, ichthyophagous habits, and their simple venom structures, independently from within the Australasian elapid radiation (Fig. 1). This suggests a strong functional association between the relatively simple venoms of these snakes and their teleost-based diet. It is possible that the venoms of sea snakes are relatively simple because of their specialized diet, consisting of a single class of vertebrates. On the other hand, the documented high levels of resistance to marine elapid venoms in at least some potential prey species[35,36] would normally lead to the prediction that these snakes should have complex venoms to overcome prey resistance to particular toxins or toxin classes. However, recent research suggests that broad resistance to alpha-neurotoxins may exist at the receptor level,[37] as opposed to being antibody-mediated, so that a greater diversity of alpha-neurotoxins would not in fact be helpful in overcoming that resistance. Additional studies on the action of sea snake venoms on their natural prey items may be revealing in this context.

Clinical implications

The clinical implications of this study center around the many previously unstudied types of venom examined, and

consequently the results provide information that may be useful with regard to the potential clinical effects of these bites as well as guiding antivenom choices. This is particularly the case for the poorly documented clinical effects of 'colubrid' envenomings, especially with regard to neurotoxicity. Neurotoxicity rarely features in clinical reports of 'colubrid' envenomings, but has been documented *in vitro* in a number of species belonging to the Colubrinae[10,38–40] and Xenodontinae.[41,42] Only two 'colubrid' species, *Boiga irregularis* (Colubrinae) and *Malpolon monspessulanus* (Psammophiinae), have been recorded in the literature as causing unequivocal, clinically significant neurotoxicity,[43,44] and there are less clear-cut reports for two others, *Coluber viridiflavus*[45] (Colubrinae) and *Hydrodynastes gigas*[46] (Xenodontinae).

The richness of the colubrine and psammophiine venoms in molecular masses consistent with 3FTx (Figs. 2–4) indicates that neurotoxic envenomings may be a more likely occurrence than hitherto appreciated, and in a wider range of 'colubrid' species. One of us (B.G.F.) has in the last 6 months consulted on significantly neurotoxic envenomings to pet store employees by *Coluber rhodorachis* and *Boiga blandingii* (both Colubrinae). In both cases, envenoming resulted in moderate systemic paralysis of the skeletal muscles and breathing difficulty, which resolved spontaneously after 7 h. These incidents underscore the potential for unexpectedly serious neurotoxic envenoming after bites by 'colubrid' snakes, normally regarded as inoffensive.

In most 'colubrids', the potential clinical effects are largely unknown. While the venom delivery in most lineages is typically not as efficient as that of the Atractaspididae, Elapidae or Viperidae (with the notable exception of the advanced fang architecture found in *Dispholidus typus*), dangerous or even lethal bites have been documented in the Colubrinae (*Dispholidus*, *Thelotornis*), Psammophiinae (*Malpolon*), Natricinae (*Rhabdophis*) and Xenodontinae (*Philodryas*, *Phalotris*). In view of the growing popularity of herpetoculture and the general assumption that all 'colubrids', with the exceptions of the above-named examples, are harmless, the diversity of toxins found in many of these lineages suggests that some may be potentially medically significant.

Some 'colubrids' have a long history of interaction with humans due to their popularity in herpetoculture, and one can thus be confident that the full envelope of possible reactions to bites is known. For instance, *Thamnophis* spp. (garter snakes) have been kept safely by tens of thousands of people, with a very low incidence of mild local symptoms,[47–49] so that the possibility of life-threatening garter snake bites can be discounted. However, as different genera that were previously rarely kept become available, it is possible that some of these may eventually turn out to be more dangerous than previously suspected. This has already happened; *Rhabdophis* species were very popular in the pet trade in the 1970s and 1980s, until they caused several life-threatening envenomings.[50,51]

The potential danger of some presently unsuspected 'colubrid' species is underscored by the fact that, despite its demonstrated lethal potential, the *Rhabdophis tigrinus* examined in the course of this study displayed relatively unremarkable fang size and venom yields (5–8 mg dry weight). In contrast, the closely related genus *Macropisthodon* reaches similar lengths yet has much larger fangs and thus should be treated with caution. We flag the psammophiines in general and *Psammophis* in particular as of potential clinical significance, due to complex venoms rich in likely neurotoxins, combined with large venom glands (larger than the glands in some Elapidae), well-developed dentition and significant venom yields (an average of 12+ mg for 1 m specimens of *Psammophis mossambicus*, which reaches in excess of 1.5 m in length). On the other hand, the *Telescopus dhara* studied here had even larger venom glands (larger than many elapids), extremely large venom yields (15 mg from 30–40 cm specimens of a species that reaches in excess of a meter), yet has very small fangs located in the rear of the mouth. Thus, the likelihood of significant envenoming from this species is lower than in species such as *Psammophis mossambicus*, but severe consequences from an exceptional bite cannot be ruled out.

The present analysis of previously understudied venoms from small elapid snakes (Fig. 6) has also revealed the presence of complex venoms, and underscores that these snakes, often regarded as of little medical significance, may be more dangerous than generally realized. *Aspidelaps* species (coral and shield-nose cobras) are very popular pet venomous snakes due to their small size, colorful pattern and reputed inability to cause medically significant envenomings. However, the large number and quantity of 3FTx molecular masses suggest that these species may be dangerous, and lethal bites are in fact on record.[52] Similarly, *Glyphodon tristis*bites were considered to be of little medical consequence until a near-lethal evenomation was suffered by a tourist on Lizard Island,[53] consistent with the 3FTx and PLA_2 molecular masses found in the present study. *Cryptophis nigrescens* is another small but lethal[53] Australian elapid with understudied venom. The lack of 3FTX masses in this venom was consistent with clinical cases reporting severe myotoxicity coupled with virtual lack of neurotoxicity. Inconsistent with the reported lack of coagulopathic action by the venom, the dominant component had a molecular mass and retention time consistent with that of a prothrombin activator (Fig. 6). Studies on the bioactivity of this component would be insightful and may reveal a novel enzyme.

The two other small elapids included, *Echiopsis curta* and *Suta suta*, are not normally considered as being of substantial medical importance, but the abundance of 3FTx and PLA_2 masses in their venoms suggests that dangerously neurotoxic bites may be a real possibility. In particular, the little known *Suta suta*, a denizen of the remote outback in Australia and rarely involved in documented bites,[53] yielded an average of 15 mg of 3FTx-rich venom per 20 cm specimen of a species that grows in excess of 90 cm.

Many field guides and standard works tend to dismiss the smaller elapids as of little concern. However, the complex nature of the venoms of these small snakes, and higher venom yields than anticipated, underscores the fundamental consideration that even lesser-known, small species may still be capable of clinically significant envenomings after exceptional bites in which a larger than usual quantity of venom is injected. Lethal evenomations have already occurred with other small elapids thought to be harmless, such as the tragic

death of Hans Schnurrenberger by a 30 cm *Sinomicrurus macclellandi* (formerly *Calliophis macclellandi*); the initial bite was ignored until neurotoxic symptoms showed up 6 h later, and death was 8 h after envenomation.[54]

In addition to revealing the potentially clinically important diversity of components in 'colubrid' and understudied elapid venoms, LC/MS analysis of some venoms revealed the presence of molecular masses not present in the venom of the nearest antivenom match, thus flagging potential treatment problems. *Demansia papuensis* venom, for example, is rich in molecular mass classes not found in any of the other Australian elapids (Fig. 6, Table 4). This suggests that the standard Australian antivenoms will be unlikely to neutralize these components. In contrast, from a medical point of view, a positive discovery is the lack of diversity in the sea snake venoms (Fig. 7, Table 4). It is likely that this is why the sole sea snake antivenom (made using only *Enhydrina schistosa* venom) has been attributed with a wider cross-reactivity with other sea snake genera[53] than would normally be the case, considering the genetic distance separating them.

It is unknown what antivenom will neutralize *Azemiops feae* venom. Due to its rarity, little is known about the clinical effects, and the only few fleetingly reported bites caused only mild local envenoming.[55] However, as has recently become established in the pet trade, bites are now much more likely. A previous study on the venom examined only the enzymatic activity of the venom, and concluded that it had no blood clotting, hemorrhagic or myolytic activities.[56] However, our LC/MS profiling of the venom (Fig. 8), demonstrating abundant molecules with molecular masses and retention times similar to those of the neurotoxic peptides from *Tropidolaeumus wagleri* venom, as well as a PLA_2 and a plasminogen activator, both with N-terminals typical of the widespread 'typical' forms of both toxin classes (Fig. 9), indicates that envenoming may potentially result in neurotoxicity and coagulation disturbances. Similarly, *Proatheris superciliaris* is another species available in the pet trade for which antivenom is not available. As the venom profile is similar to that of *Atheris squamigera* reported by us previously,[10] this venom may produce similar severe coagulation disturbances.[57] The venom profile of the basal viperine *Causus rhombeatus* does not share any similarity with the other vipers, and is rich in molecular masses corresponding to those of proteases that may affect coagulation or cause localized swelling; the latter is the principal manifestation of night adder envenoming in humans,[58] although potent coagulopathic toxins have been isolated from this venom.[59]

CONCLUSIONS

This study demonstrates the multiple applications of a single data set, mostly comprising molecular masses with complementary LC retention information, generated by comprehensive venom analysis using LC/MS. The results demonstrate the tremendous variation and diversity of snake venoms, with implications ranging from clinical management to drug design and discovery, to evolution and taxonomy. Our LC/MS results, even without definitive information such as N-terminal sequencing, demonstrate that even extremely well-studied venoms, such as that of *Dendroaspis polylepis*, are more diverse than previously documented, and revealed hitherto undocumented diversity among the numerous largely neglected 'colubrid' snake venoms. We have also uncovered trends of diversity in relation to phylogeny, venom apparatus, and terrestrial vs. marine lifestyles. This study thus provides a solid initial platform upon which to build further research on snake venom evolution and potential clinical effects, but also is useful in biodiscovery.

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