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Electrospray liquid chromatography/mass spectrometry fingerprinting of *Acanthophis* (death adder) venoms: taxonomic and toxinological implications

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Death adders (genus *Acanthophis*) are unique among elapid snakes in both morphology and venom composition. Despite this genus being among the most divergent of all elapids, the venom has been historically regarded as relatively quite simple. In this study, liquid chromatography/mass spectrometry (LC/MS) analysis has revealed a much greater diversity in venom composition, including the presence of molecules of novel molecular weights that may represent a new class of venom component. Furthermore, significant variation exists between species and populations, which allow for the LC/MS fingerprinting of each species. Mass profiling of *Acanthophis* venoms clearly demonstrates the effectiveness of this technique which underpins fundamental studies ranging from chemotaxonomy to drug design. Copyright © 2002 John Wiley & Sons, Ltd.

Death adders (genus *Acanthophis*) are unique among elapids in both morphology and venom composition. They are the only elapids that are viper-like in appearance and habit, with all species characterised by a somewhat flattened, triangular head, short stout body and thin rat-like tail ending in a curved spine.[1] The tail is used as a caudal lure to attract birds and reptiles. In addition, death adders are the only elapids with semi-mobile fangs. This, in some respects, makes them the most evolved of all elapids, and morphologically intermediate between the Elapidae and the Viperidae. The species-level taxonomy of these animals is poorly resolved, with a recent amateur revision[2] only serving to further muddy the waters.[3–5]

Death adders are the widest ranging of the Australian elapids, being found not only in continental Australia, but north throughout the Torres Straight Islands, Papua New Guinea, Irian Jaya and through the Indonesian islands such as Seram, Halmahera, Obi and Tanimbar. In addition, due to the cryptozoic nature of these snakes, great differences in morphology can be observed even between geographically adjacent populations. This is particularly true of island forms, since these snakes are far less adept swimmers than other elapids, and gene flow between islands may thus be very restricted.

Death adder venoms have long been considered unique in being made up overwhelmingly by alpha postsynaptic neurotoxins.[6] Only recently have phospholipase A_2 toxins been isolated.[7–9] The peptidic neurotoxins found in Australian elapids all bind with high affinity to skeletal nicotinic acetylcholine receptors. However, two subgroups exist, differing in size, from an average of slightly above 60 amino acids (short chain) versus an average of 73 amino acids (long chain), and having either four or five disulfide bridges, respectively. These structural differences are due to the long-chain toxins having a C-terminal extension.[10] The majority of the Australian elapid PLA_2s are basic, 118 amino acids, have seven disulfide bonds and molecular weights in the range 12–14 kDa.

Death adder envenomations are a rare occurrence in Australia, but remain a significant health problem in Papua New Guinea.[11–13] Clinical symptoms of envenomation by *Acanthophis* spp. include those relating to the paralysis of bulbar and ocular muscles, and death occurs through inhibition of respiration due to paralysis of the voluntary muscles.[14,15] However, these studies also unexpectedly reported rhabdomyolysis in rodent assays and bleeding/coagulopathy in proven *Acanthophis* victims in Port Moresby (Papua New Guinea). These effects indicate that in some populations significant amounts of toxins other than neurotoxins are present. A previous study also showed

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significant differences in the ability of antivenom to reverse the effects of venom *in vitro*.[16]

The current study focused upon the major *Acanthophis* variants from continental Australia and islands in the Indo-Pacific region. Venom was profiled using liquid chromatography/mass spectrometry (LC/MS), a technique previously used to fingerprint a diverse array of venoms ranging from the *Brachypelma* genus of tarantula[17] through cone snails.[18] Further, mass spectrometric analysis was uniquely utilised to examine evolutionary trends in amphibians through the examination of skin toxins.[19] This study used LC/MS to profile the venoms to determine if patterns between species can be distinguished, and whether such profiles can contribute to the elucidation of the taxonomy of these animals. Particular attention was paid to the relationships between the *A. praelongus* populations.

MATERIALS AND METHODS

Species examined

Venoms from a number of populations of death adder were examined. In view of the confusion surrounding the systematics of this group, a discussion of the affinities of the venoms involved is crucial to allow the interpretation of our results in the light of future systematic findings.[4] *Acanthophis antarcticus* (common death adder) is the widest ranging species, with two disjunct geographic ranges, New South Wales/Queensland and South Australia/Western Australia. Venom was obtained from populations in New South Wales (Eden), Queensland (Gold Coast), South Australia (Eyre Penninsula), and Western Australia (Darling Range). The Seram death adder (*Acanthophis* sp.) is an unnamed species from the island of Seram. Similar populations may be found on the islands of Halmahera, Obi and Tanimbar. The Barkly Tableland death adder is an isolated form from the Barkly Tableland region of the Northern Territory. It was described as a new species, *Acanthophis hawkei*.[20] However, this was not recognised by most subsequent authors,[1] and its status remains to be resolved. *Acanthophis praelongus* (northern death adder) is a wide ranging species found in northern Queensland and the Northern Territory, and may in fact be a species complex, but its status at this time is far from resolved (K. Aplin, Western Australian Museum, personal communication). Our venoms are represented by the four main geographical variations, referred to by Hoser[2] as *A. praelongus* (Cairns, Queensland), *A. woolfi* (Mt. Isa, Queensland), *A. lancasteri* (Hayes Creek, Northern Territory) and *A. cummingi* (Humpty Doo, Northern Territory). However, in view of the lack of evidence for this taxonomic arrangement, the *praelongus* populations will be referred to in this text simply by geographical region, i.e., Cairns (pra-cai), Mt. Isa (pra-isa), Hayes Creek (pra-hay) and Humpty Doo (pra-hum). *Acanthophis pyrrhus* (desert death adder) is found in desert regions from western to central Australia. The venoms used in this study originate from specimens from the Alice Springs area. The Irian Jaya death adder is a problematic taxon, which was first described as *Acanthophis antarcticus rugosus*.[21] This taxon is not widely recognised in the literature, and is usually regarded as a synonym of *A. antarcticus* or *A. praelongus*. However, in the absence of clear evidence of conspecificity with any of the Australian mainland *Acanthophis*, we tentatively treat this form as a full species, *A. rugosus*. Our venoms come from the Merauke area of Irian Jaya. *Acanthophis wellsi* (Pilbara death adder, also called Black Head death adder) is found in the Pilbara region of Western Australia. The validity of this species has recently been confirmed.[22]

Venom collection

Pooled venoms for particular populations or geographic ranges were used for all species to minimise the effects of individual variations,[23] with at least six unrelated adults of both sexes making up each pool. Collection of venom from snakes utilised the naturally aggressive nature of these species, where venom is collected from the fang tips of an irritated specimen through the specimen biting down on a latex covered specimen bottle and injecting the venom. Polyethylene materials (polyethylene pipette tips, polyethylene Eppendorf tubes, specimen bottles) were used to handle and contain the venom due to the strong affinity some peptides possess for glass and polystyrene. The venom was dissolved in 0.1% trifluoroacetic acid (TFA)/H_2O solution for sample transfer and then lyophilised for storage. Venoms were collected by the first author with the exception of *A. rugosus* and *A.* sp. Seram, which were purchased from Venom Supplies, Tanunda, South Australia.

Liquid chromatography/mass spectrometry

On-line LC/MS of venom samples dissolved in 0.1% TFA to a concentration of ~1 mg/mL was performed on a Phenomenex C^{18} analytical 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 was a 1% gradient from 0 to 60% acetonitrile/0.05% TFA over 60 min. Electrospray mass spectra were acquired on a PE-SCIEX API 300 LC/MS/MS system with an Ionspray atmospheric pressure ionisation source. Samples (10 μL) were injected manually into the LC/MS system and analysed in positive ion mode. Full scan data was acquired at an orifice potential of 80 V over the ion range 600–3000 *m/z* with a step size of 0.2 u. Data processing was performed with the aid of the software package Biomultiview (PE-SCIEX, Canada).

RP-HPLC

The column used was a Vydac Preparative C18 column (20 × 250 mm, 10 μ, 300 Å) on a Waters 600 HPLC system and UV absorbance was monitored at 214 nm by a Waters 486 tuneable absorbance detector. Samples were dissolved in 2 mL of buffer A (0.1% TFA), manually injected and then the column run at 100% A to wash off the concentrated salts present. The following gradient conditions of buffer B (90% acetonitrile, 0.09% TFA) were then used: 0 to 60% over 60 min (1% gradient) and then 60 to 80% in 5 min (4% gradient).

Edman degradation sequencing

Reduction/alkylation

Pure peptide (0.1 mg) was dissolved in 100 μL of 0.1 M ammonium bicarbonate at pH 7.0. Ten μL of 0.1 M tris(carboxyethyl)phosphine HCl (TCEP; pH 7) was added and incubated at 50 °C for 20–30 min. Thirty μL of 0.1 M

RCM

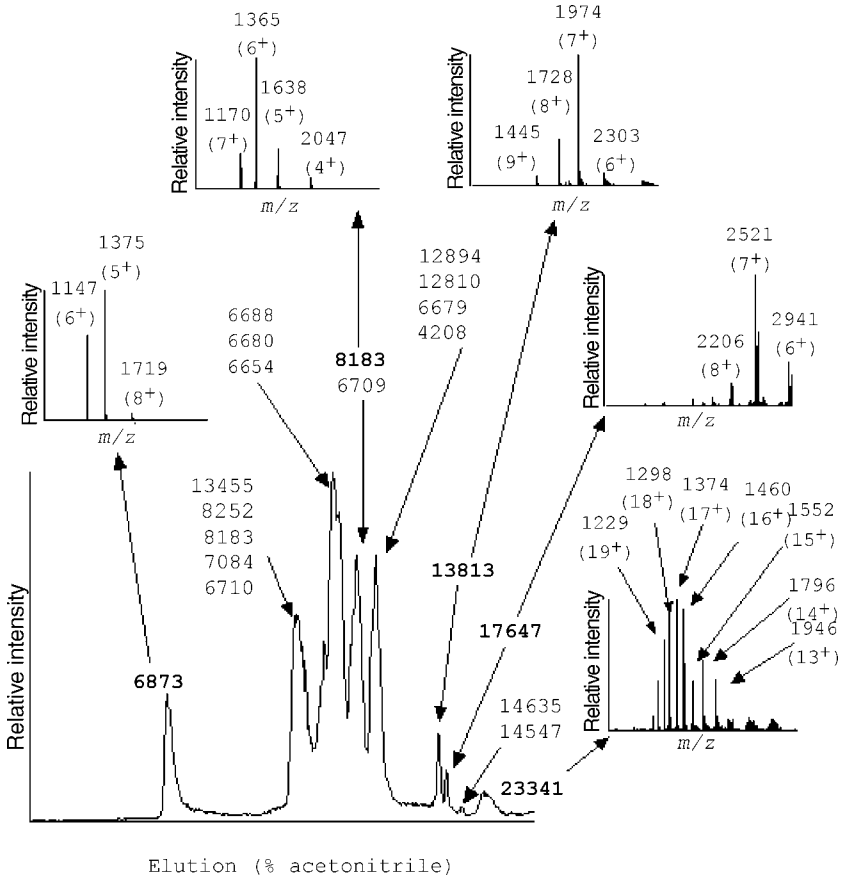

Figure 1. Representative LC/MS profiles of *Acanthophis* venom. Shown is the Western Australian population of *Acanthophis antarticus*. Mass (in Da) is given above each peak for each component present. A 1%/min gradient of acetonitrile was used.

maleimide were then added and incubated at 50 °C for a further 20–30 min. Maleimide added 97 u per cysteine and mass spectroscopy showed an increase of 194 Da per disulfide bond. The reduced/alkylated peptides were N-terminally sequenced using Edman degradation chemistry on an Applied Biosystems 477A protein sequencer.

Data analysis

In order to visualise the pattern of variation in venoms, all components present were scored as present or absent (0 or 1) for each venom. Results were analysed for a tolerance of 1–2 Da for interpretation of the results. Both phenetic and phylogenetic methods of analysis were employed: principal coordinates analysis and UPGMA were implemented using the MVSP (multivariate statistical package) software (Kovach Computing Services, Pentraeth, Anglesey, UK). The phenetic analyses were run on a matrix of Gower general similarity coefficients, which emphasise the shared presence rather than the shared absence of components.

An unrooted parsimony network was constructed using PAUP*4.0b8, using branch-and-bound searching. Due to the somewhat isolated and uncertain phylogenetic position of *Acanthophis* among Australasian elapids,[24,25] choosing an outgroup venom turned out to be impractical. Branch support was assessed by means of bootstrap analysis, using 10000 pseudoreplicates and branch-and-bound searching.

The above analyses were carried out for the combined data of all venom peaks, and separately for the three major classes of toxins, with molecular weights of 6–7, 7–8 and 13 kDa. Other toxin classes were represented by too few peaks to allow a meaningful analysis.

The interpretation of the phylogenetic and phenetic analyses is somewhat complicated by the fact these methods assume independence between characters, i.e., the presence/absence of one peak is assumed to be independent of the presence/absence of another. This is unlikely to apply fully to venom proteins when the homologies of different peaks are unclear: in some cases, different peaks in different venoms are likely to represent alleles of the same homologous locus, so that their presence/absence cannot be independent.

RESULTS

Venoms were profiled using LC/MS to determine differences in venom composition. All venoms had essentially the same generalizsed elution profile, consisting of an early eluting component but with the vast majority of eluting in the mid part of the gradient in Fig. 1). Significant similarities and differences as to the composition of individual molecules were evident in total numbers, molecular weights and retention times of the toxins (Table 1). Representatives of

Table 1. Toxins identified in *Acanthophis* venoms. Toxins are organised by elution and by molecular weight within each time block. Underlined components are the major components present in the venoms

% ACn	A. ant NSW	A. ant Qld	A. ant SA	A. ant WA	A. sp Ser	A. haw	A. pra-Cai	A. pra-Hum	A. pra-Mt. Is a	A. pra-Hay	A. pyr	A. rug	A. wel
8	6873	6873	6873	6873	6912 6816	6785	6853 6802	6854 6799	6854 6786	6952 6853	6913 6866	6913 6884	
11													6747
12										6973			
14	8750	8758	8752	8751			7050	6763	8659 6942	8659	7020 6942		7047
15		8290				8785 7991	8784 7014 7992	8791 7993	7992	8784 8558	7999 6914	7991 6844	6916
16		7291						13498 13456			6763	7656	7769
17	13456 8252 8183 7084	13456 8252 8183 6651	8252 8183 7084	13456 8252 8183 7084	13494 8196 8113	8834 8150	13496	8840	8820 8756 7032	8225 7055	8140	8829	8400 7575
18	6710 6703 5044	6710 5044	6710 5044	6711 5044	6703 5044	5044 7044	6709 5044	7044 5044	14651 8791 8130 6709 5044			6619 5044	6700 5044
19	8125 7073	7070		7070	7091	8148, 7027							7044
20	8635 6687 6651	8375 6686 6652	8376 6677 6652	6688 6680 6654	6535	6653 6623	8784 7269	13511	8784 6758 6654 6744			13151	8737 8509
21	6709	8291 6710	6709	6709	7296	8250 6709 6675	6698 6613	8839 6736 6665	7295 6709 6672	6695 6666 6635		13286	6765 6637
22	8186	6612	8183	8183	8196	6693	6676		7260		6725 6676	7217	6678
23	8635	6676	6676	6679	6704	6737	6709		6622	13510 6737	13485 6753	13048 12983 6657	6733
24	12897 12846	12841	12896	12898	13051	13427 13263 6753		13433 13288 7221	13182	13433 13265 12951 12799 7221	6755	13399	12994
25	12810 4207 4093	12810 4209	12854 12848 6461 4207	12810 4208	4344	8140 4238	13080 8385		12846 8130 4352	13545 6705 4351	12878 8171 4332	4223	13543 8143 7737 7322 4330
26		7280	7219			12879 13111 7219	12957 4353	13111 4351	12798	13037	7573	13084	
27			7272			13251 13139		13081		13126	8171 7507		8173 7505
28	13810	13809	13811	13809		13856 13779	13824	13828		13887	7257	13813	
29	17843 17646 14634 14547	17843 17646 14634 14547	17843 17646 14634 14547	17843 17646 14634 14547	14587 14523	17820 17611 14600 14515	17820 17157 14635 14548	17808 17153 14636 14600 14549 14505 14418	17810 17157	17850 17165 14637	7603	14635 14600 14548	
32	23349	23346	23286 23346	23346	23532	23340	23372 23292	23326	23359 23309	23326	23307	23370	23920

Table 2. N-Terminal sequences and identification of isolated toxins from the different classes in the venom

MW (Da)	Source	N-Terminal sequence	Snake venom toxin similarity[a]
4223	A. rugosus	EKPDSTGNGCFGFPIDRIGS	Natriuretic peptide
6617	*A. rugosus*	KNRPHFCHLPAYPGPCNAFV	Kunitz-type protease inhibitor
6654	*A. antarticus*	KDRPVFCNLPAYTGPCKNVL	Kunitz-type protease inhibitor
6814	*A.* sp Seram	MQCCNQQSSQPKTTTTCPGG	Short-chain alpha-neurotoxin
6854	*A. praelongus* Mt. Isa	MQCCNQQSSQPKTTTTCPGG	Short-chain alpha-neurotoxin
6873	*A. antarticus* WA	MQCCNQQSSQPKTTTTCPGG	Short-chain alpha-neurotoxin
7055	*A. praelongus* Hayes Creek	MQCYNQQPTQPKTTTTCPGG	Short-chain alpha-neurotoxin
7295	*A.* sp Seram	KTCFKTPYNKSEPCPDGQDL	Long-chain alpha-neurotoxin
7991	*A. rugosus*	VICYLGYNYAQPCPPGENVC	Long-chain alpha-neurotoxin
8125	*A. antarticus*	VICYRGYNNPQTCPPGENVC	Long-chain alpha-neurotoxin
8377	*A. antarticus*	VICYRKYTNNKTCPDGENVC	Long-chain alpha-neurotoxin
8751	*A. antarticus* WA	VICYVGYNNPQTCPPGGNVC	Long-chain alpha-neurotoxin
13408	*A. rugosus*	NLAQFGFMIKCANGGSRPVV	Phospholipase A_2
13060	*A. rugosus*	NLAQFGFMIKCAANKGSRPV	Phospholipase A_2
13898	*A.* sp. Seram	NLLQFAFMIECANKMIQPVE	Phospholipase A_2
17843	*A. ant* SA	SIPKPSKNFEQFGNMIQCTI	Taipoxin-gamma chain

[a] Snake venom toxin type as determined by database sequence homology searching.

different toxin classes were sequenced and subsequent database searching revealed the type of toxin (Table 2).

The representative of the 4-kDa class was shown to be a member of the natriuretic peptides. While natriuretic peptides have been previously isolated from venoms ranging from elapids[26] to the platypus,[27] this is the first publication showing the widespread presence within a genus and they also represent the largest natriuretic peptides reported to date. The identity of the peptide of mass 5044 remains to be confirmed but it appears too large for it to be a natriuretic peptide and it does not fall within the range of any previously reported toxins from Australian elapids.[6] As such, it may represent a new class of venom peptide.

The putative peptidic neurotoxins accounted for the vast majority of the venom components present, consistent with the *in vitro* as well as clinical effects of the venoms.[16] As expected, the short-chain neurotoxins displayed strong homology with other members of this class isolated from Australian elapids.[6] The 7.2–8.8 kDa toxins were shown to be long-chain neurotoxins and displayed strong homology with previously isolated toxins. The 6.6–7 kDa class also contained kunitz-type protease inhibitors. Kunitz-type protease inhibitors have been previously isolated from numerous venoms and have been shown to be potent inhibitors of blood chemistry enzymes.[28] Accordingly, these inhibitors may be responsible in part for the bleeding disorders that have been reported from severe *Acanthophis* envenomations or in experimental models.[15,16]

Proteins with molecular weights corresponding to PLA_2 toxins (12–14 kDa) were also present in all the venoms, in greater quantities and molecular weight diversities than expected. N-Terminal sequencing of isolated components confirmed their identity as PLA_2s. As both anti-platelet and presynaptically active neurotoxic PLA_2s have been reported from these venoms,[8,9,29] the large abundance in some of the populations indicates that some envenomations may produce more complex symptoms than others.

In each venom analysed, LC/MS profiling revealed components of unusual size. Interestingly, the 17.6–17.8 kDa components were found only in the continental species *A. antarcticus, A. praelongus* and *A. hawkei*, being absent entirely from the island forms as well as in the continental species *A. pyrrhus* and *A. wellsi*. N-Terminal sequencing revealed them to be highly homologous to the gamma chain of taipoxin, an extremely potent neurotoxin complex from the *Oxyuranus scutellatus* (Coastal Taipan).[6] This raises the interesting question as to whether a similar complex is found in *Acanthophis* venom. The 23-kDa components were ubiquitous in the venoms, at least one isoform being present in each venom. As these components appear to be N-terminally blocked, they were not successfully sequenced for this study. However, these components are consistent in molecular weight and retention time with a group of toxins that share significant homology to the mammalian CRISP (cysteine rich secretory protein) family. These components have been widely reported in reptilian venoms, having been isolated from the gila monster[30] as well as in all the major families of venomous snakes.[31–33] Due to their virtual ubiquity, these toxins almost deserve the name 'fundamental toxin', and their evolution would represent a fascinating study topic from a venom evolution standpoint.

The first eluting component in all species had a mass similar to that of the short-chain peptidic neurotoxin Toxin Aa-c previously isolated from *A. antarcticus* venom.[34] Significantly, the molecular weight of each of the first eluting toxins was unique to each species, which may have uses in 'fingerprinting' the venoms (Table 3). Phenetic and phylogenetic analyses were carried out on a matrix of 192

Table 3. Diagnostic *m/z* fingerprint of each venom based upon the first major eluting peak

Species	A. ant	A. sp Ser	A. haw	A. pra-cai	A. pra-hum	A. pra-isa	A. pra-hay	A. pyr	A. rug	A. wel
m/z	6873	6816	6785	6854	6854	6854	6854	6866	6884	6747

Table 4. Number of components by molecular weight class identified by LC/MS analysis of *Acanthophis* venoms

Species	3–5 kDa	6.6–7.2 kDa	7.3–8.8 kDa	~14 kDa	17 kDa	~23 kDa	Total
A. ant NSW	3	8	7	7	2	1	28
A. ant QLD	2	10	9	6	2	1	30
A. ant SA	2	8	7	9	2	2	31
A. ant WA	2	8	4	6	2	1	23
A. sp. Seram	2	6	4	4	0	1	17
A. hawkei	2	10	5	12	2	1	32
A. pra-cai	2	9	8	6	2	2	29
A. pra-hum	2	6	4	12	2	1	27
A. pra-isa	2	11	10	4	2	2	31
A. pra-hay	2	9	5	9	2	1	28
A. pyrrhus	3	10	8	2	0	1	24
A. rugosus	3	7	4	9	0	1	24
A. wellsi	2	9	12	3	0	1	27

individual peaks and the 13 taxa represented in the study. Sixty-six compounds were shared between two or more venoms, the remainder were unique to one venom. The number of bands for each molecular weight class is shown in Table 4. The *Acanthophis antarcticus* populations share >70% of their components, whereas, in most other species, <60% of components are shared (except in pra-cai and pra-hum).

The parsimony analysis of the combined data of all peaks resulted in 53 equally most parsimonious trees of 245 steps (consistency index 0.7796; c.i. excluding uninformative characters 0.5462) and the parsimony network in Fig. 2(a). The four venoms of *Acanthophis antarcticus* cluster together, as do those of *A. wellsi* and *A. pyrrhus*. Both these nodes are supported by high bootstrap values. The relationships among the remaining taxa are unresolved. Reweighting all characters by the maximum value of their rescaled consistency indices resulted in considerably higher resolution (Fig. 2(b)). However, bootstrap support for the newly supported nodes was low, except for the node linking pra-hay and pra-hum.

The ordination of the individual venoms along the first three axes of the principal coordinates analysis is shown in Fig. 3, and the UPGMA phenogram in Fig. 4. UPGMA and

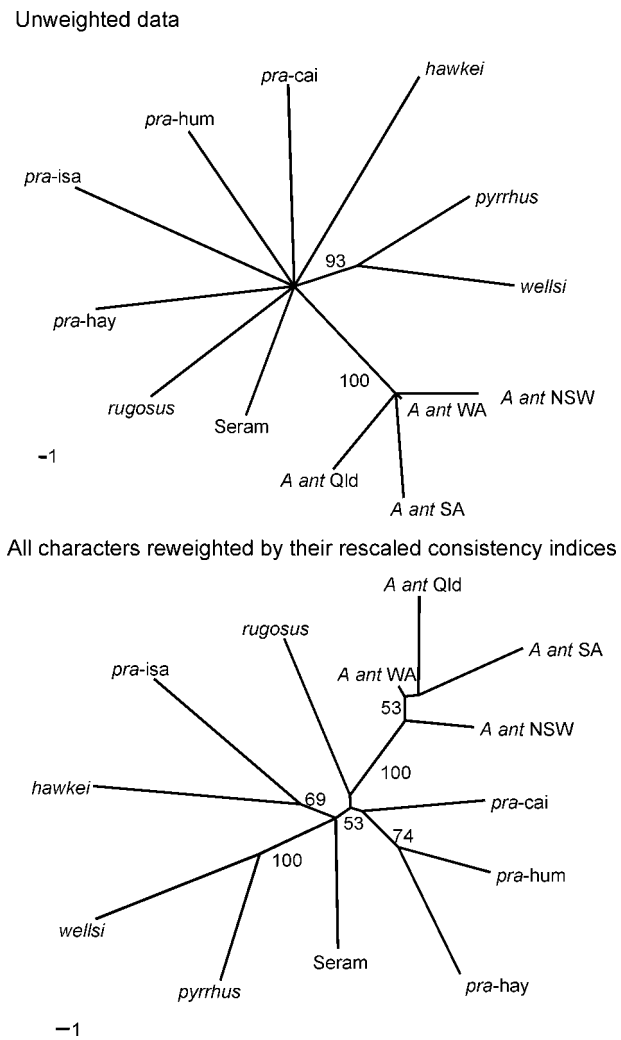

Figure 2. (a) Consensus of 53 equally most parsimonious networks of the 13 *Acanthophis* venoms. Numbers indicate bootstrap support for nodes, branch lengths correspond to the inferred number of evolutionary changes along each branch. (b) Parsimony network of the 13 *Acanthophis* venoms, each character being reweighted by the maximum value of its rescaled consistency index.

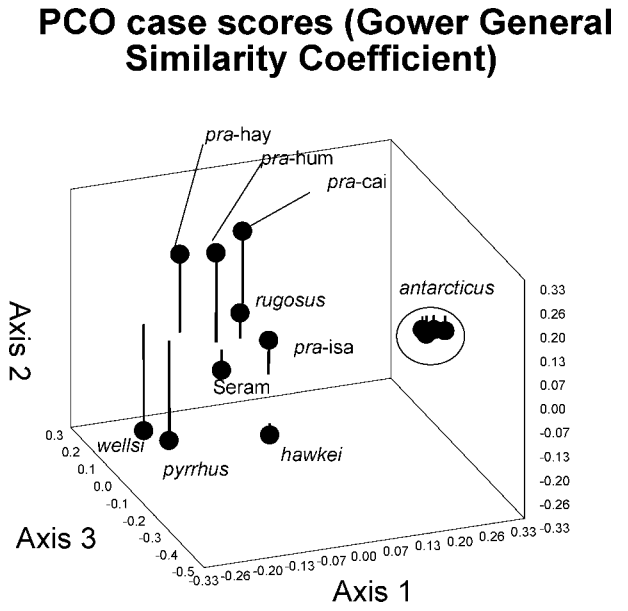

Figure 3. Ordination of *Acanthophis* venoms along the first three axes of the principal coordinate analysis. Axes 1, 2 and 3 summarise 24.9, 17.6 and 14.5% of the total variance, respectively.

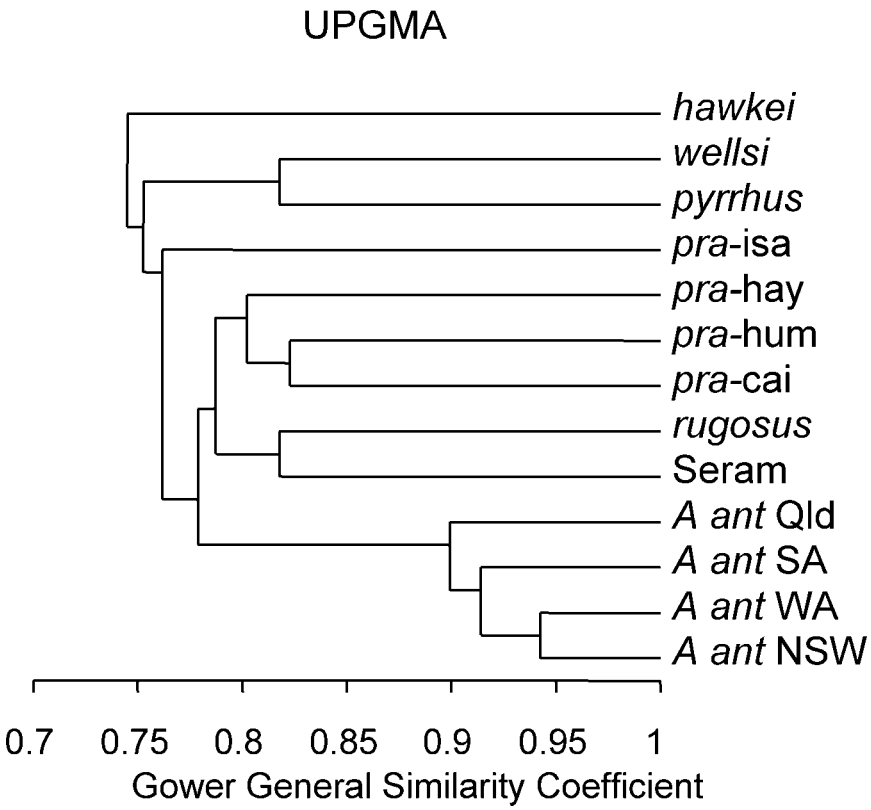

Figure 4. UPGMA phenogram of the 13 *Acanthophis* venoms included in this study.

PCO scatter plots of the three major toxin classes differed considerably from the plots of all peaks combined, and from each other. Both phenetic and phylogenetic analyses of the three major toxin classes revealed considerable incongruence between these toxin classes and the combined database. With the exception of the consistent clustering of the *A. antarcticus* samples, there were no consistent features of either dendrograms or scatter plots between toxin classes (data not shown).

Parsimony networks for three size classes of toxins (6–7 kDa; 7–8 kDa; PLA_2s) agree in joining the four *Acanthophis antarcticus*. However, they differ considerably in terms of the arrangement of the remaining taxa, to the extent where a consensus tree between them would be entirely unresolved except for the grouping of *A. antarcticus* venoms. Reweighting of characters adds little to any of the individual size class networks. The same applies for UPGMA dendrograms and PCO plots for the individual toxin classes: *A. antarcticus* venoms are consistently grouped together, and clearly distinct from venoms of the other taxa (data not shown).

DISCUSSION

The most significant revelation from the LC/MS studies was the diversity of venom molecules not predicted by previous studies.[16] However, the mass spectrometer used in this study was able to scan at a much greater range and sensitivity that that previously used (*m/z* 3000 versus a maximum scan of *m/z* 2300). This allowed for the determination of larger masses, components present in lesser amounts, and also more hydrophobic components. This increased range was particularly important in the revealing of a great diversity of PLA_2s as well as the discovery of new molecular weight classes of components in these venoms. Further, improved chromatography techniques allowed for better separation of the components and thus reduced the potential for ion suppression.

Also significant was the minimal level of conservation of individual molecules. The first eluting components have masses corresponding to isoforms of the short-chain neurotoxin Toxin Aa-c.[6] The characteristic masses for each species of this first peak allow for preliminary *m/z* fingerprinting. This is particularly notable in the case of the *A. praelongus* venoms. Despite these venoms showing far greater variance than the *A. antarticus* venoms, the first eluting peak always contained the 6854 toxin, which may suggest that these snakes are closely related. Another interesting feature revealed by the LC/MS profiling of the venom was components of unusual size. Natriuretic peptides of ~4.2 kDa are present in each venom and a component of mass 5044 is also present in all venoms but does not correspond in molecular weight to other isolated components from elapids and thus may represent a new class of venom molecule.

A. antarcticus crude venom has previously been examined for lethality, neurotoxicity, myotoxicity and its effects on blood coagulation, both experimentally and clinically.[14,35–39] Recently, *A. antarticus, A. praelongus* and *A. pyrrhus* venoms were studied for *in vitro* neurotoxicity, myotoxicity and phospholipase A_2 activity[29,40] and the entire genus studied for neurotoxicity and relative neutralisation by antivenom.[16]

Species variations in chromatographic profiles have been previously observed for *A. antarcticus, A. praelongus* and *A. pyrrhus* venoms.[29] In this study, venoms were profiled using on-line liquid chromatography/mass spectrometry to determine basic biochemical differences. As previously detailed, all venoms had essentially the same generalised elution profile. Given that these venoms are from snakes belonging to the same genus this is not surprising. However, close examination and comparison of each profile showed many differences in peak distribution and complexity between venoms from different species of death adder.

Previous reports suggest that variations in venom composition as a result of geographic location or differences in species are not unique to death adders.[41–44] This suggests that, potentially, LC/MS venom profiles may be of value in illuminating taxonomic relationships among death adder species, as has been previously suggested for some spider venoms.[17] Preliminary work on the LC/MS profiles of *Acanthophis* species showed the venoms of one species (A. *praelongus*) to be much more complex than the other species examined. It was hypothesised that this was due to *A. praelongus* actually being a species complex.[16]

Our analyses tend to agree with other studies on several points of potential taxonomic relevance[22] while differing from others.[2,22,20] The four samples of *Acanthophis antarcticus* invariably cluster together, and their separation from the remaining venoms is supported by high bootstrap values in the parsimony analysis. Moreover, the different *A. antarcticus* venoms have relatively few unique components. Among all *A. antarcticus* venoms, less than 30% of the compounds are unique to any one venom. In none of the analyses did the *A. antarcticus* samples fall into categories corresponding to the two geographically isolated populations (SA and WA vs. Qld and NSW), unlike the allozyme study of Aplin and Donnellan.[22] The venom data presented here thus do not support the recognition of western *A. acanthophis* as a separate subspecies or species, as has been suggested previously.[2,20]

Among the remaining species, each venom is made up to a far greater extent of unique compounds not found in other venoms examined: between 30 and 65% of observed peaks in any one venom are unique to that venom. These other venoms are all highly divergent from each other, and both phenetic and phylogenetic relationships between them are poorly defined (Figs 2–4), except that *A. pyrrhus* and *A. wellsi* cluster together in all analyses of the combined data, with high levels of bootstrap support in the parsimony analysis. Populations currently assigned to *Acanthophis praelongus* differed far more from each other than the even more widespread *A. antarticus* populations did, and did not form a cohesive cluster in any of our analyses. The greater complexity of the pooled *A. praelongus* venom in a previous study[16] was thus the result of the heterogeneity of the included venoms. Further work is needed to confirm whether the variations in venom are indicative of differences in genetic relatedness.

The consistent clustering of the four *A. antarcticus* samples, and that of *A. pyrrhus* with *A. wellsi*, are in agreement with the allozyme analysis presented previously.[22] The remaining venoms are all highly different from each other. Taken at face value, this validates the hypothesis that these different forms may constitute different species. Our results thus provide some evidence for the species status of *A. hawkei* and *A. rugosus*. The position of the undescribed Seram death adder requires further investigation. Its venom shows no particular affinities with any of the venoms included in this study, and differs considerably from that of its nearest neighbor (*A. rugosus*). However, several further *Acanthophis* 'forms' of uncertain status are found in New Guinea,[45] and the relationship between the Seram death adder and these forms requires further investigation. While it is beyond the scope of this paper to propose species status for the Seram death adder, the data presented here clearly marks this population as a potential candidate for being a new species but probably with strong affinities to *A. laevis* from New Guinea.

The congruence between venom profiles and allozyme evidence suggests that the LC/MS profiles may be of systematic usefulness, and that venom composition in these snakes may be associated with their phylogeny. Nevertheless, the taxonomic interpretation of venom data requires caution, as considerable differences in venom can occur between taxonomically undifferentiated populations,[46] perhaps as a result of natural selection for different prey in different regions.[47] There are no published studies of geographic variation in the diet of *Acanthophis*. Consequently, it is not presently possible to separate the effects of phylogeny and natural selection for different prey types on the pattern of variation in venom composition of these snakes.

The present study illustrates the usefulness of LC/MS profiles in the study of variation in snake venoms at low taxonomic levels. In this study, the LC/MS technique has helped develop a fingerprint for the venoms of different populations and species of death adder, as well as revealing the existence of classes of toxin in quantities greater than those reported for this genus in the past or revealing entirely new classes to be found in these venoms. This is in line with previous clinical reports as well as the *in vitro* studies undertaken in which the venoms were shown to have significant differences in activities and relative neutralisation by antivenom.[16] Furthermore, the venom profiles have revealed considerable potential to contribute towards the resolution of taxonomic problems within the *Acanthophis* genus.

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