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Tentacles of Venom: Toxic Protein Convergence in the Kingdom Animalia

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Abstract The origin and evolution of venom in many animal orders remain controversial or almost entirely uninvestigated. Here we use cDNA studies of cephalopod posterior and anterior glands to reveal a single early origin of the associated secreted proteins. Protein types recoverd were CAP (*C*RISP, *A*ntigen 5 [Ag5] and *P*athogenesis-related [PR-1]), chitinase, peptidase S1, PLA_2 (phospholipase A_2), and six novel peptide types. CAP, chitinase, and PLA_2 were each recovered from a single species (*Hapalochlaena maculosa*, *Octopus kaurna*, and *Sepia latimanus*, respectively), while peptidase S1 transcripts were found in large numbers in all three posterior gland libraries. In addition, peptidase S1 transcripts were recovered from the anterior gland of *H. maculata*. We compare their molecular evolution to that of related proteins found in invertebrate and vertebrate venoms, revealing striking similarities in the types of proteins selected for toxic mutation and thus shedding light on what makes a protein amenable for use as a toxin.

Keywords Venom · Protein · Phylogeny · Cephalopod · Convergence

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Introduction

New insights into the evolution of venom systems and the medical importance of the associated toxins cannot be advanced without recognition of the true biochemical, ecological, morphological, and pharmacological diversity of venoms and associated venom systems. A major limitation of the study of venom proteins has been the very narrow taxonomical range examined (Fry et al. 2003). As a consequence, several major animal groups with known or suspected venom systems have remained largely unexplored. The mollusk lineage Cephalopoda (including squids, cuttlefish, and octopuses) is one such major clade. With approximately 800 known species, cephalopods represent an important element in marine trophic systems worldwide, displaying an impressive variation in shape, size (from 2 to >10 m), and habitat (benthic to abyssal, tropical to Antarctic).

Several cephalopod species have been confirmed to use envenomation as a mechanism to neutralize captured prey and/or as a defense against predators (Norman and Reid 2000). The observation of high concentrations of the neurotoxic compound tetrodotoxin (TTX) in the posterior glands of the blue-ringed octopus species (genus *Hapalochlaena*) led to the perception that this molecule represents the major ingredient of the venom (Sutherland and Lane 1969). Consequently, TTx and TTx-like organic compounds (e.g., saxitoxin; Robertson et al. 2004) have become the major point of focus in cephalopod toxinological research. Toxins in these species are thought to be mixed with secretoin in the posterior glands, situated in the abdomen, and connected to the beak by a long secretory duct (Fig. 1). As in many other marine species, TTx in *Hapalochlaena* is produced by endosymbiotic bacteria of the genus *Vibrio* and recent studies have shown that the

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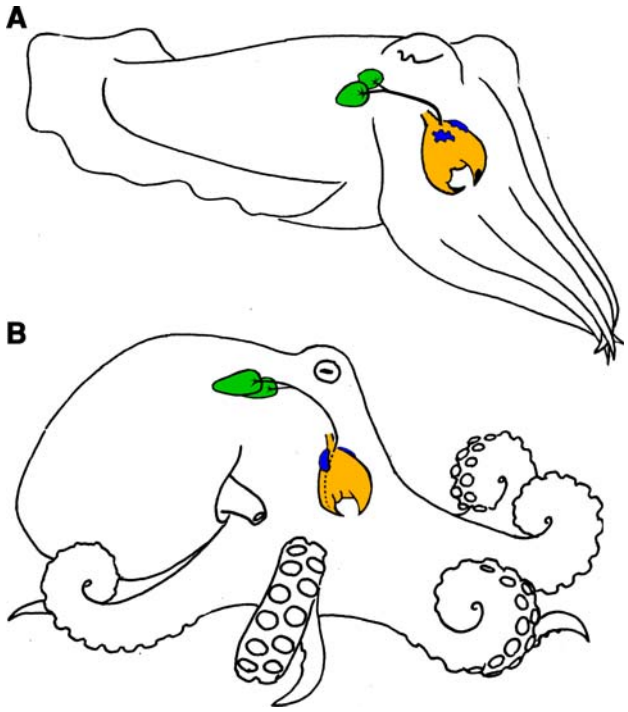


Fig. 1 Relative glandular arrangements of **a** cuttlefish and **b** octopus. Posterior gland is shown in green; anterior, in blue

toxin is present in multiple tissues and body parts of the animals (Yotsu-Yamashita et al. 2007). In contrast, little attention has been paid to the potential synthesis of endogenous proteins in cephalopods for use in predation even though three tachykinin-like peptides have been identified in the posterior secretory gland of octopuses (Eledoisin, OctTK-1, and OctTK-2), with full transcripts isolated for two of those (Kanda et al. 2007). It has been shown that new protein-scaffold classes are added to existing secretory arsenals via a process of recruitment whereby a gene encoding a normal body protein is duplicated and one copy is selectively expressed in the secretory gland (Fry 2005). The molecular diversity of secreted proteins in cephalopod anterior and posterior glands has remained otherwise remarkably uninvestigated. Unlike the posterior glands, the cephalopod anterior glands are tightly associated with the buccal mass, and although it is generally thought that they account for most of mucus secretion in the mouth (Gennaro et al. 1965), their exact function also remains poorly understood.

To obtain an overview of the composition and diversity of cephalopod posterior and anterior gland secreted proteins, we constructed cDNA libraries from the glands of three species, including representatives of the two major lineages of coleoid cephalopods (Strugnell et al. 2005). Comparative analyses identify complex mixtures of proteins, some of which may represent striking cases of protein recruitment convergence compared to other animal phyla, and others of which may represent novel peptide classes with unique structural features.

Materials and Methods

Tissue Sampling and Taxon Selection

Posterior secretory glands were sampled from *Hapalochlaena maculosa*, *Octopus kaurna* (both Octopodiformes), and *Sepia latimanus* (Decapodiformes); divergent lineages of the coleoid cephalopods (Strugnell et al. 2005). We also sampled anterior secretory glands from *H. maculosa*. *S. latimanus* were collected from Osprey Reef, Coral Sea, while *H. maculosa* and *O. kaurna* were collected from the Mornington Peninsula in Victoria, Australia.

cDNA Library Construction and Analysis

Freshly dissected gland tissue was preserved immediately in liquid nitrogen. RNA was isolated using the Qiagen RNeasy Midi Kit with subsequent selection of mRNAs using the Oligotex Midi Kit. cDNA libraries were constructed using the Clonetech Creator SMART cDNA Library Construction Kit and transformed into One Shot Electrocompetent GeneHog *E. coli* cells (Invitrogen Corp., USA) as described previously (4). Isolation and sequencing of inserts were undertaken at the Australian Genome Research Facility, using BDTv3.1 chemistry with electrophoretic separation on an AB330xl. Up to 384 colonies were sequenced per library, inserts screened for vector sequences, and those parts removed prior to analysis and identification. Sequences were identified by homology of the translated DNA sequences with previously characterised toxins using BLAST search of the SWISS-PROT protein database (http://www.expasy.org/tools/blast/).

Molecular Phylogeny

Molecular phylogenetic analyses of cDNA library transcripts were conducted using the translated amino acid sequences. Comparative sequences from venomous taxa and homologous body proteins were obtained through BLAST searches against the UniProt database (http://www.expasy.org/tools/blast/) using representative toxin sequences. To minimize confusion, all sequences obtained in this study are referred to by their GenBank accession numbers (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide) and sequences from previous studies are referred to by their UniProt/Swiss-Prot accession numbers (http://www.expasy.org/cgi-bin/sprot-search-ful). Homologous sequences were aligned using the program CLUSTAL-X 1.83, followed by visual inspection for errors. When

presented as sequence alignments, leader sequence (determined using http://www.cbs.dtu.dk/services/SignalP) is shown in lowercase, prepro region (determined using http://www.cbs.dtu.dk/services/ProP) is underlined, cysteines are highlighted in black, and functional residues are in boldface. Phylogenetic relationships were estimated by Bayesian MCMC analyses using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). For each set of aligned sequences, we implemented a mixed model of amino acid substitution, with gamma-correction for rate heterogeneity among residues and correction for invariable residues. Each MCMC run consisted of four chains (one cold and three heated, temperature parameter = 0.2), with a length of 1 million generations, a sampling frequency of 1 per 100 generations, and a burn-in phase corresponding to the first 100,000 generations. Stationarity of model parameter and likelihood values was confirmed by time series plots. Sequence alignments can be obtained by e-mailing Dr. Bryan Grieg Fry (bgf@unimelb.edu.au).

Results

Analysis of cephalopod posterior gland cDNA libraries revealed transcripts encoding for four protein types with sequence similarity, and conservation of the structurally crucial cysteines, to previously characterized toxins from venomous animals: CAP (*C*RISP [cysteine-rich secretory proteins], *A*ntigen 5 [Ag5], and *P*athogenesis-related [PR-1] proteins) (Figs. 2 and 3), chitinase (Figs. 4 and 5), peptidase S1 (Figs. 6 and 7), and PLA_2 (phospholipase A_2) (Figs. 8 and 9). While CAP-, chitinase-, and PLA_2-encoding transcripts were each recovered from a single species (*Hapalochlaena maculosa*, *Octopus kaurna*, and

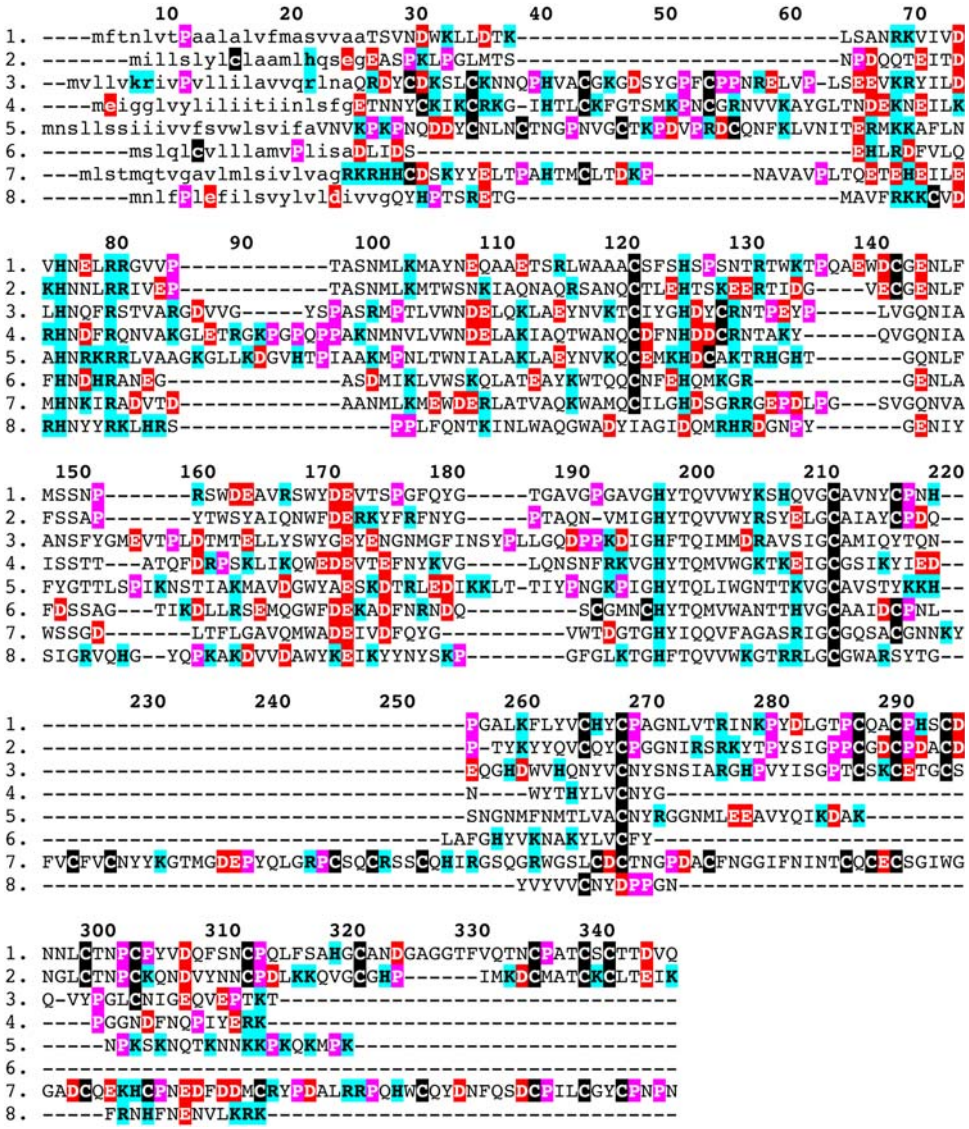


Fig. 2 Sequence alignment of representative cephalopod and venom CAP proteins: 1, A4PIZ5 (*Lampetra japonica*); 2, Q91055 (*Heloderma horridum*); 3, Q16TE8 (*Aedes aegypti*); 4, P10736 (*Dolichovespula maculata*); 5, Q9NH66 (*Ctenocephalides felis*); 6, EU790590 (*Hapalochlaena maculosa*); 7, Q7YT83 (*Conus textile*); 8, Q4PN19 (*Ixodes scapularis*). Highlighted amino acids: negatively charged (red); positive (blue); prolines (magenta); cysteines (black). Signal peptides are in lowercase letters

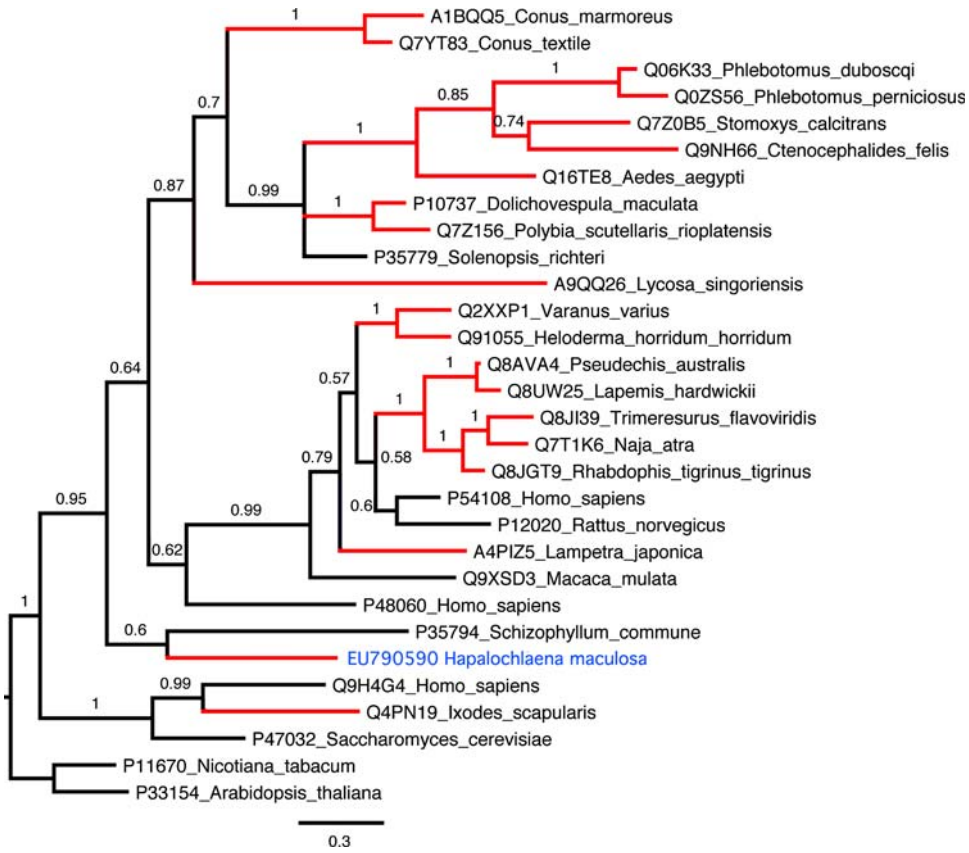


Fig. 3 Bayesian phylogenetic reconstruction of representative CAP proteins. The cephalopod sequence from this study is shown in blue, and venom clades in red

Sepia latimanus, respectively), peptidase S1 transcripts were found in large numbers in all three libraries. One additional peptide-encoding transcript type recovered from *Octopus kaurna* was homologous to the tachykinin sequenced from *Octopus vulgaris* (Kanda et al. 2003) (Fig. 10). We also recovered six peptide-encoding transcript types (Fig. 11) that showed no sequence similarity to any toxin class previously reported in animal venoms or even with any previous reported peptide type from any source. In addition, peptidase S1 transcripts were recovered from the anterior gland of *H. maculata*. Alignment of the translated amino acid sequences revealed extensive variation in the primary structures for all protein types.

The single CAP-encoding transcript recovered from *H. maculosa* differs markedly from CAP toxin sequences reported from the major venomous mollusk clade *Conus* (Figs. 2 and 3). The N-terminal region of the cephalopod translated protein sequence subsequent to the signal peptide is shorter than the *Conus* sequences and lacks two cysteines present in this region. The octopus sequences also lack the long internal extension with its five additional cysteines (alignment positions 200–256) and the cysteine residue at alignment position 267 found in the *Conus* form. Conversely, the *H. maculosa*-encoded protein has two extra cysteines (alignment positions 193 and 197) that are absent in all other CAP toxins. Phylogenetic analysis of the CAP proteins confirms that the cephalopod protein is not closely related to the other mollusk toxins. Instead, the sequence was recovered as the only representative of a distinct CAP lineage within animals.

The translated chitinase transcript recovered from *Octopus kaurna* was similar in form to proteins present in wasp venom (Figs. 4 and 5). Bayesian phylogenetic analyses identify the presence of major chitinase clades in arthropods and vertebrates but provide little resolution among these clades (Fig. 5). The cephalopod sequence is recovered as a distinct lineage in an unresolved polytomy at the base of Metazoa. Compared to other toxin classes, chitinase proteins display a relatively low level of structural variation, due to the functional and structural constraints in order to preserve the globular and enzymatic properties (Fry 2005).

The peptidase S1 transcripts were particularly diverse and multiple transcripts, which varied in their translated protein primary structures, were recovered from each species (Figs. 6 and 7). The signal peptides showed considerable sequence variation, even within a single species, although there were ten invariant cysteines in the processed (signal peptide excised) protein. One of these cysteines (alignment position 171) is missing in the peptidase S1 proteins found in forms sequenced from animal venoms. The distribution and quantity of charged residues were also

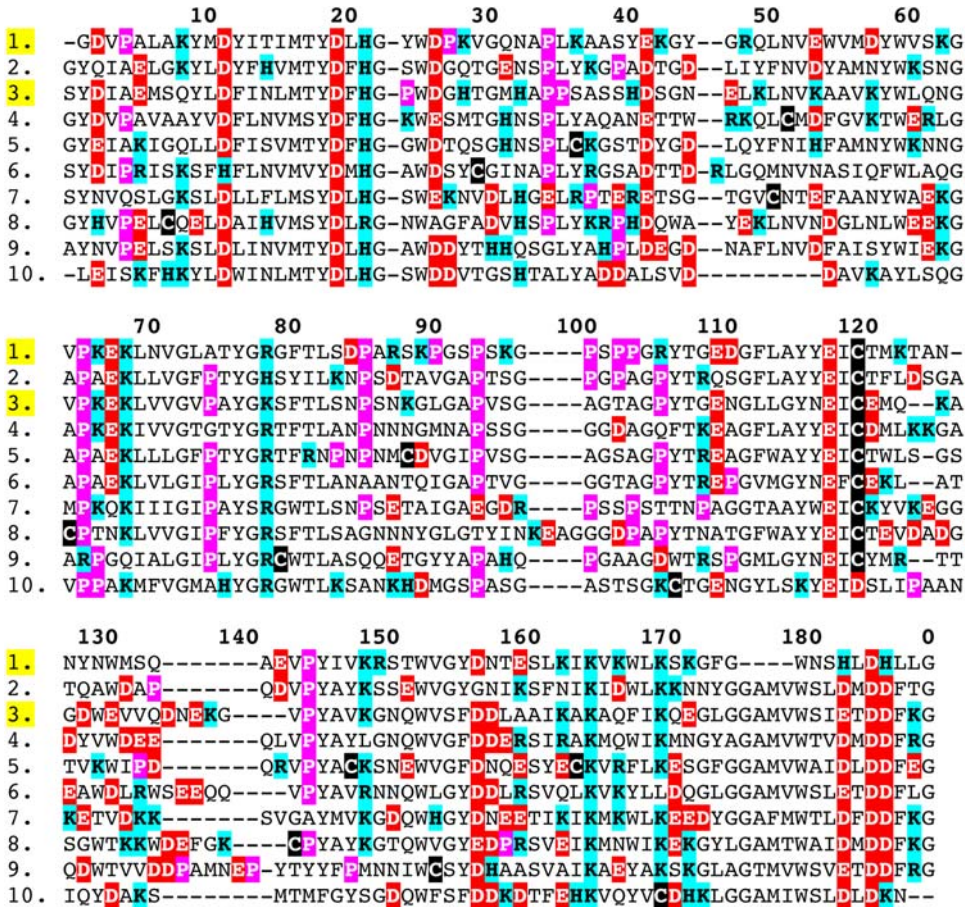


Fig. 4 Sequence alignment of representative chitinase proteins (venom forms are highlighted in yellow): 1, EU790591 (*Octopus kaurna*); 2, Q8AV87 (*Gallus gallus*); 3, Q23737 (*Chelonus* sp,); 4, Q8MY79 (*Haemaphysalis longicornis*); 5, Q90W34 (*Bufo japonicus*); 6, Q7Q5I7 (*Anopheles gambiae*); 7, Q7JQ23 (*Acanthocheilonema viteae*); 8, Q9GV05 (*Bombyx mori*); 9, Q8ITU3 (*Penaeus vannamei*); 10, P90547 (*Entamoeba invadens*). Highlighted amino acids: negatively charged (red); positive (blue); prolines (magenta); cysteines (black)

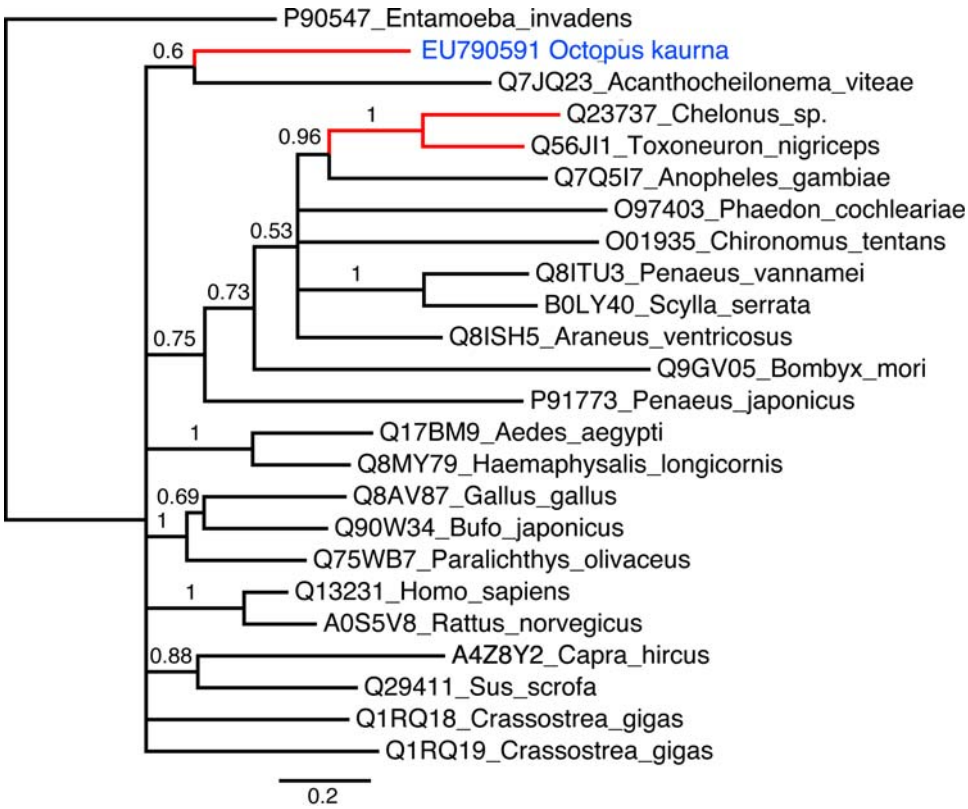


Fig. 5 Bayesian phylogenetic reconstruction of representative chitinase proteins. The cephalopod sequence obtained in this study is shown in blue, and venom clades in red

variable among the cephalopod translated proteins, with calculated isoelectric points ranging from acidic (5.02 for EU790592 from *Sepia latimanus*) to basic (9.18 for EU790601 from *Octopus kaurna*). Molecular weights of processed proteins (signal peptide excised) ranged from 25,247.69 Da for EU790597 from *O. kaurna* to 26,890.93 Da for EU790599 (also from *O. kaurna*). Phylogenetic analyses show that the cephalopod sequences constitute a single major clade among invertebrate peptidase S1s. The peptidase S1 transcript recovered from the anterior glands of *H. maculosa* was recovered as a close relative of a posterior gland transcript from the same species and phylogenetically is placed within the posterior transcripts (Fig. 7).

The PLA_2 transcripts recovered from the *S. latimanus* cDNA library were from type III proteins (Figs. 8 and 9) and, thus, are similar to PLA_2 toxins independently used in the venoms of lizards, *Lonomia* caterpillars, and scorpions. As with these venomous forms, it was highly charged, with the positively charged residue lysine being particularly abundant, resulting in a calculated pI of 9.74. As a consequence of the longer-than-typical N-terminal region, with a contribution from a mid-sequence insertion, this PLA_2 form was larger than average (22,121.59 Da).

The tachykinin peptide-encoding transcript recovered from *Octopus kaurna* differed from that of the previously reported *O. vulgaris* peptides (Kanda et al. 2003) in having a negatively charged aspartic acid instead of a positively charged lysine at alignment position 40 (position 1 of the functional peptide) (Fig. 10). The net charge of the peptide was further affected by an aspartic acid doublet in place of a serine doublet at alignment positions 44–45 (positions 5 and 6 of the functional peptide). Consequently, the *O. kauma* peptide has the calculated low pI of 3.37, compared to pI's of 6.0 for both *O. vulgaris* forms. The methionine C-terminal amide, however, seems preserved in all forms. The *O. kaurna* sequence also had a significantly shorter C-terminal propep region than either of the two *O. vulgaris* forms but was two residues longer in the N-terminal propep region.

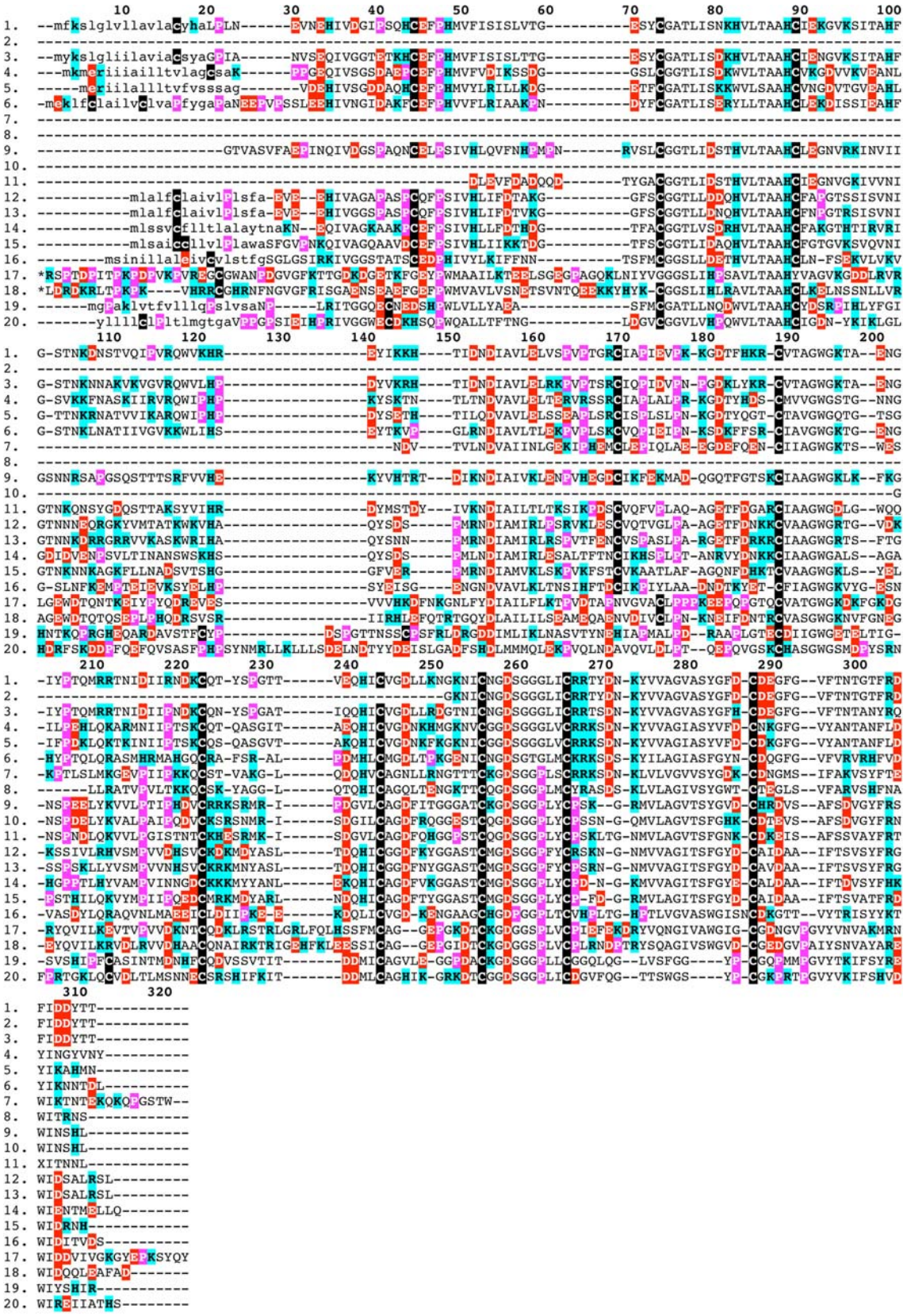
The six novel transcript types (NP1–NP6) recovered from the cephalopod cDNA libraries all possessed, in the translated forms, the N-terminal signal peptide characteristic of secreted proteins (Fig. 11). All contained multiple cysteines (8, 6, 12, 11, 12, and 2, respectively); the free cysteine in NP4 potentially facilitating dimerization. Myriad charged residues were present in each and the calculated pI/MW values were 8.49/8149.14, 9.94/5585.65, 6.46/14657.94, 6.03/9692.92, 5.18/12346.03, and 9.79/5425.36, respectively. These peptides lacked any significant sequences similarity to any known proteins (whether venom or nonvenom), with E values >10 and little similarity of residues, particularly of cysteines.

Fig. 6 Sequence alignment of representative venom S1 peptidase ▶ from *Hapalochlaena maculosa* (1, EU790595; 2, EU790607), *Octopus kaurna* (3, EU790593; 4, EU790594; 5, EU790597; 6, EU790599; 7, EU790604; 9, EU790596; 10, XXXXXX; 11, EU790602; 12, EU790600; 13, EU790601; 14, EU790603), *Sepia latimanus* (8, EU790606; 15, EU790598; 16, EU790592), *Lonomia obliqua* (17, Q5MGE2), *Cotesia rubecula* (18, Q7Z1F0), *Varanus_mitchelli* (19, Q2XXN0), and *Blarina brevicauda* (20, Q5FBW2). Highlighted amino acids: negatively charged (red); positive (blue); prolines (magenta); cysteines (black). Signal peptides are shown in lowercase letters

Discussion

Our analyses demonstrate that these cephalopod transcriptomes represent a mixture of novel proteins, some of which may represent new peptide classes. The peptidase S1 transcripts do not form cephalopod species specific monophyletic clades but, instead, are highly interspersed. The resulting phylogenetic arrangement suggests at least four successive gene duplication events occurred prior to the divergence of octopuses and cuttlefish (Fig. 7). Given that these represent the two major lineages of living coleoid cephalopods (Strugnell et al. 2005), our data provide evidence for a basal radiation of peptidase S1 transcription in the posterior glands of this group. The extensive diversification of peptidase S1 prior to the divergence of octopuses and cuttlefish reveals a single, early origin of these secreted in coleoid cephalopods, a clade that contains >99% of all living species in this class. Moreover, their extensive diversification is shown by at least eight additional gene duplications occurring within the octopus lineage (Fig. 7). The molecular diversity and variation in functional (intraloop) residues of the encoded proteins is consistent with the molecular adaptive pattern of neofunctionalization observed in multigene toxin families in venomous taxa such as cone snails, reptiles, spiders, and scorpions (e.g., Froy et al. 1999; Fry et al. 2003; Rodríguez de la Vega et al. 2003). The nonmonophyly of the posterior gland sequences relative to the anterior sequence also demonstrates a common tissue origin of these two gland structures. The expression of peptidase S1s in the anterior gland of *H. maculate* also may indicate that these organs are involved in cephalopod toxicity and, hence, that the associated secretory system may be anatomically more complex than previously assumed.

Of the cephalopod transcripts types recovered CAP (e.g., Fang et al. 1998; Nobile et al. 1994; Brown et al. 2003; Milne et al. 2003), chitinase (e.g., Krishnan et al. 1994), peptidase S1 (e.g., Amarant et al. 1991; Asgari et al. 2003; Kita et al. 2004), and PLA_2 (e.g., Alape-Girón et al. 1999; Nevalainen et al. 2004) are known independently recruited venom components of other taxa (Table 1). Despite evidence for the convergent recruitment of secreted proteins, our phylogenetic analyses indicate that the cephalopod



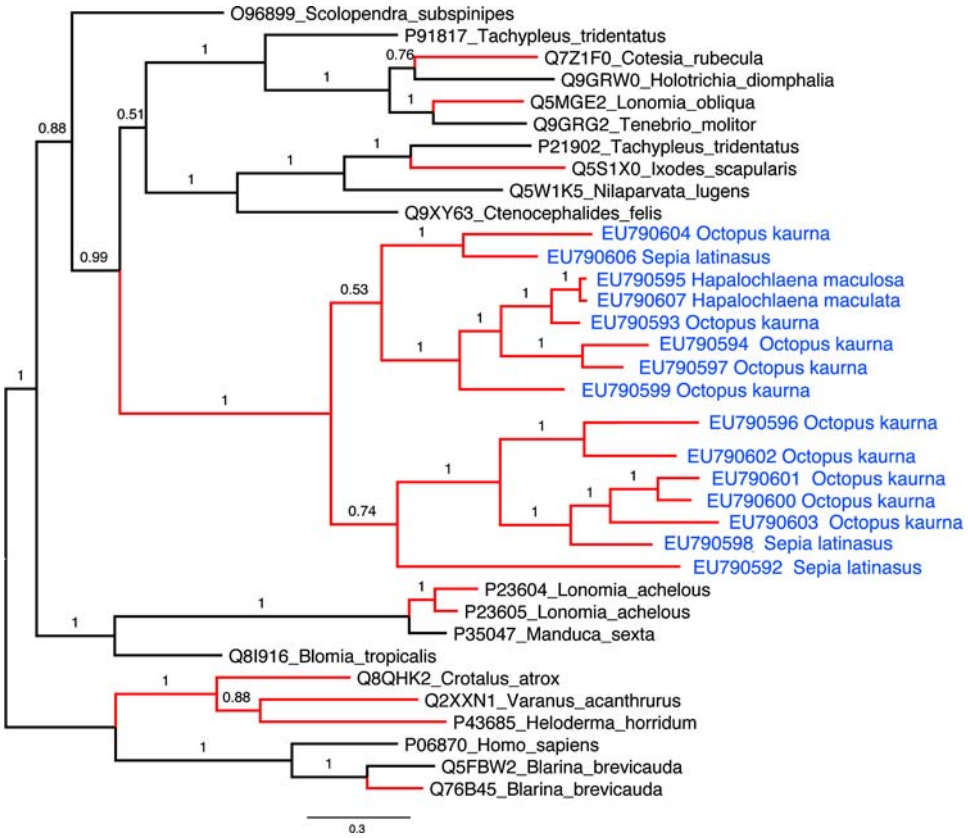


Fig. 7 Bayesian phylogenetic reconstruction of representative S1 peptidase. Cephalopod sequences obtained in this study are shown in blue, and venom forms in red

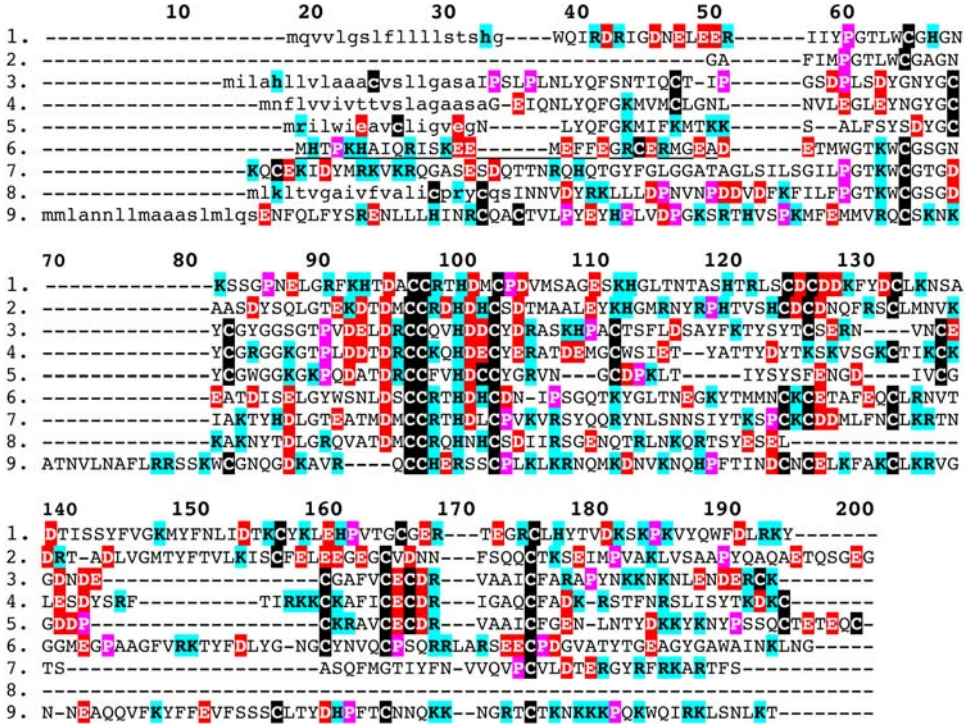


Fig. 8 Sequence alignment of representative toxic-mutant phospholipase A_2: 1, P00630 (*Apis mellifera*); 2, P80003 (*Heloderma suspectum*); 3, A7X418 (*Trimorphodon biscutatus*); 4, Q3C2C2 (*Acanthaster planci*); 5, Q6A3A7 (*Vipera ammodytes meridionalis*); 6, P59888 (*Pandinus imperator*); 7, Q0ZS49 (*Phlebotomus perniciosus*); 8, Q5MGE1 (*Lonomia obliqua*); 9, EU790608 (*Sepia latimanus*). Highlighted amino acids: negatively charged (red); positive (blue); Prolines (magenta); cysteines (black). Signal peptides are shown in lowercase letters

proteins form distinct clades or lineages, consistent with their evolutionary divergence. This is partly due to the large phylogenetic distance between mollusks and most well-studied venomous taxa. The majority of animal toxins within the above-mentioned protein classes have been isolated from vertebrates and arthropods, which are,

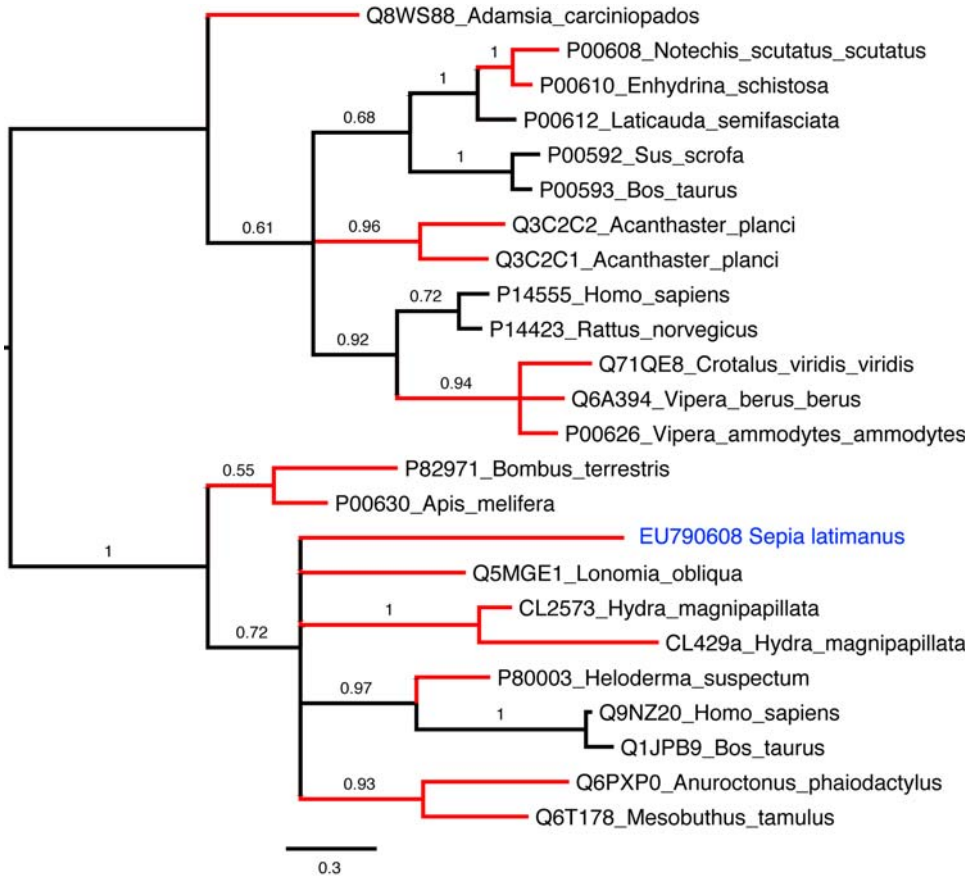


Fig. 9 Bayesian phylogenetic reconstruction of representative phospholipase A_2 proteins. Cephalopod sequences obtained in this study are shown in blue, and venom clades in red

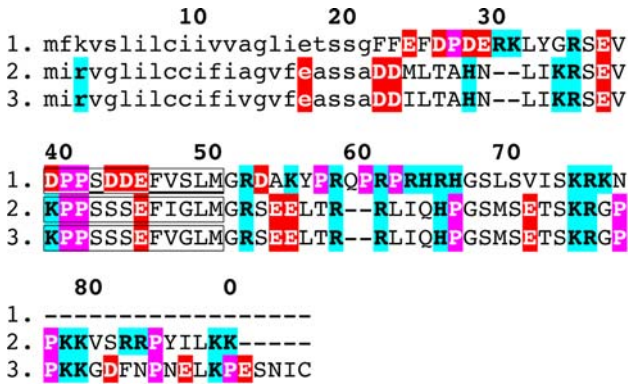


Fig. 10 Sequence alignment of tachykinin peptides: 1, EU790609 (*Octopus kaurna*); 2, Q8I6S3; 3, Q8I6S2 (*Octopus vulgaris*). Highlighted amino acids: negatively charged (red); positive (blue); prolines (magenta); cysteines (black). Posttranslationally processed functional peptide is shown in the box. Signal peptides are shown in lowercase letters

respectively, nested in the bilaterian clades Ecdysozoa and Deuterostomia. The phylum Mollusca (including Cephalopoda) instead belongs to the third major clade of bilaterian metazoa, Lophotrochozoa. Our study hence confirms that convergent protein recruitment is not limited to the well-documented arthropod and reptile venom clades but, instead, spans all major animal phyla. The discovery of structurally distinct forms of well-known toxin classes in cephalopods emphasizes the virtues of screening currently understudied taxa with known or suspected bioactive secretions. A similar strategy may reveal the presence of bioactive proteins in other major unexplored clades. While these data provide an overview of the complexity and composition of the cephalopod anterior and posterior gland transcriptomes, we consider it likely that more detailed exploration of their secretions will reveal additional protein classes, especially those that may be expressed at lower concentrations. The presence of multiple sequences in phylogenetically distant cephalopods indicates that the transcriptomes in this group are diverse and ancient. Functional and ecologically specialized systems may have been preserved in multiple other coleoid lineages as well, including enigmatic taxa inhabiting largely unexplored biomes, such as deepsea squids and giant octopuses.

The remarkably similar biochemical compositions of the cephalopod glandular secretions and the complex venoms across the Animal Kingdom suggests that there are structural and/or functional constraints as to what makes a protein suitable for recruitment. In addition to the cephalopod proteins discussed above, other protein classes that

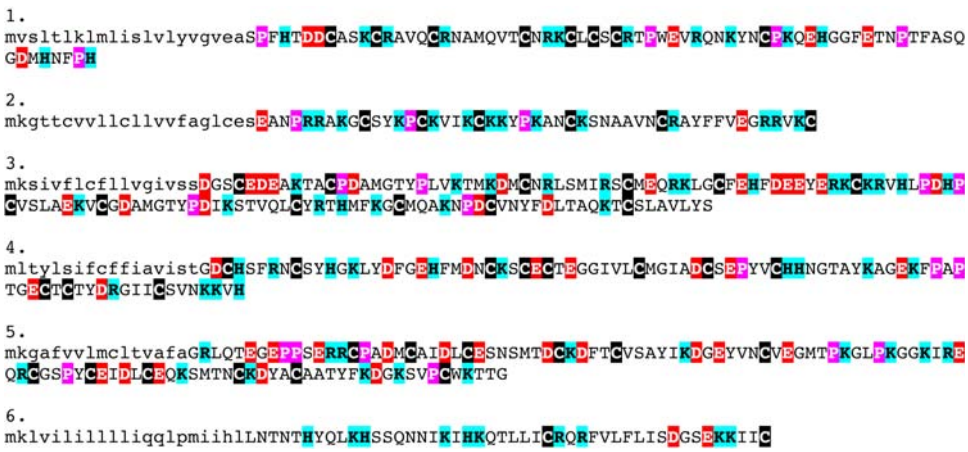


Fig. 11 Novel cephalopod peptides from *Hapalochlaena maculosa* (1, EU790610; 4, EU790613; 6, EU790615) and *Octopus kaurna* (2, EU790611; 3, EU790612; 5, EU790614). Highlighted amino acids: negatively charged (red); positive (blue); prolines (magenta); cysteines (black). Signal peptides are shown in lowercase letters

Table 1 Cephalopod toxic mutant proteins convergently recuited into other venomous lineages

	CAP	Chi	Hya	Kal	PLA_2
Cephalopod	X	X	X	X	X
Cnidarian					X
Cone snail	X				
Fish			X		
Insect Bristle				X	X
Proboscis	X		X	X	X
Stinger	X	X	X	X	X
Hook worm	X				
Scorpion			X		X
Shrew				X	
Spider	X		X		
Reptile	X		X	X	X(3)
Tick	X			X	X

CAP CRISP, Antigen 5 (Ag5) and Pathogenesis-related (PR-1), *Chi* chitinase, *Hya* hyaluronidase, *Kal* kallikrein, *PLA_2* phospholipase A_2. X(3): independent recruitment of Group IB, IIA, and III PLA_2 into reptile venoms

have been recruited into venoms on multiple occasions include AVIT peptides, cystatin, defensin, hyaluronidase, kunitz, lectin, lipocalin, natriuretic, sphingomyelinase, and SPRY (Fry et al. 2009). These protein classes span a broad spectrum of different structures and biochemical activities. However, we notice that the major classes share some general features. Typically the proteins chosen are from widely dispersed multigene secretory protein families with extensive cysteine cross-linking. These proteins are collectively much more numerous than globular enzymes, transmembrane proteins, or intracellular protein. Although the relative abundance of these protein types in animal venoms may reflect stochastic recruitment processes, there has not been a single reported case of a signal peptide added onto a transmembrane or intracellular protein or a hybrid protein expressed in a venom gland. A strong bias is also evident for all of the protein-scaffold types, whether from peptides or enzymes. Although the protein scaffolds present in venoms represent functionally and structurally versatile kinds, they share an underlying biochemistry that would produce toxic effects when delivered as an "overdose" (Fry et al. 2009). Toxic effects include taking advantage of a universally present substrate to cause physical damage or causing changes in physiological chemistry though agonistic or antagonistic targeting (Fry 2005). This allows the new venom gland protein to have an immediate effect based on overexpression of the original bioactivity. Furthermore, the features of widely dispersed body proteins, particularly the presence of a molecular scaffold amenable to functional diversification, are features that make a protein suitable for accelerated gene duplication and diversification in the venom gland.

Further work into the bioactivity of these proteins will be illuminating with regard to their functional diversity and role in predation.

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