Convergent molecular evolution of toxins in the venom of advanced snakes (Colubroidea)

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Chapter 5. Evolution of Novel Structures and Functions in Snake Venom Metalloproteinases

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Abstract

Snake Venom Metalloproteinases (SVMPs) are toxins found in snake venom that may be used to study the development of protein structure and function. SVMP has undergone extensive gene duplication and domain loss over its evolution. However, nothing is known about the development of the P-IIId SVMP and shortened variants of SVMPs. This research looked at the evolutionary mechanism that led to structural and functional diversity within the P-IIId subfamily and the shortened SVMP type. The discovery of a subset of amino acid positions that are targets of positive Darwinian selection, resulting in rapid structural and functional diversity within and within disintegrin subfamilies, is shown here. We can establish genetic and temporal characteristics throughout the evolutionary pathway of distinct P-III SVMP lineages by clustering within the phylogeny and connecting positively selected sites to structural and functional areas. We also discovered that the shortened SVMP propeptide undergoes a rapid evolution.

Keywords: PIII-d SVMP, SVMP propeptide, novel domain, evolution

Introduction

Snake metalloproteinases venoms (SVMPs), are discovered in a variety of sophisticated snake lineages, showing hemorrhagic properties in snake prey. Snake venom glands on the base of the advanced snake (caenophidian) radiation is considered to have recruited SVMPs (Gutiérrez, et al. 2008; Casewell, et al. 2009; Wagstaff, et al. 2009; Jiang, et al. 2011; Petras, et al. 2011; Ching, et al. 2012). They are frequently reported to be the major venom components in viperid snake venom, although they are generally considerably less important in the venom of other snake families (Gutiérrez, et al. 2008; Wagstaff, et al. 2009; Casewell, et al. 2011; Jiang, et al. 2011; Petras, et al. 2011; Ching, et al. 2012).

SVMPs are classified into three classes according to their domain structures in the C-terminal region (Casewell, et al. 2015b): PI SVMP contains (in downstream order) three domains: signal peptide+ propeptide + metalloprotease domains; PII SVMP contains four domains: signal peptide+ propeptide + metalloprotease + disintegrin domains; and ancestral PIII SVMP contains five domains: signal peptide+ propeptide + metalloprotease + disintegrin-like + cysteine-rich domains.

P-III SVMP have been isolated from a wide lineage of snakes, including both three FFS lineages and some of RFS lineages (Fry, et al. 2008; Casewell, et al. 2011). To date, all SVMP sequences recovered from non-viper lineages belong to the P-III SVMP type and form mono clade on the base of the SVMP toxin radiation with undergoing considerable structural and functional alteration over evolutionary time (Fry, et al. 2008; Casewell, et al. 2011). Apotypic P-III SVMP subclasses include those that remain intact (P-IIIa), those that proteolytically process the disintegrin-like and cysteine-rich domains (P-IIIb), those that form intact dimeric structures (P-IIIc), and those that bond covalently with C-type lectin venom components (P-IIId) (Fox and Serrano 2008).

Following the split of the viperid snakes from the remaining caenophidian snakes, gene duplication resulted in considerable diversification of P-III SVMPs within the former, with multiple P-III isoforms typically retained in the venom of any one species (Casewell, et al. 2009; Wagstaff, et al. 2009). In light of the conservation of cysteines in the P-III plesiotypic SVMPs, amino acid alterations following the recruitment of the SVMP scaffold into venom result in the acquisition and removal of additional cysteine residues crucial for enabling structural changes and posttranslational modifications of apotypic SVMPs. Some P-III SVMPs have evolved additional procoagulant functions by activating other components of the clotting cascade, such as Factor X (Kisiel, et al. 1976; Hofmann and Bon 1987; Takeya, et al. 1992; Siigur, et al. 2004). By now, the majority of the P-III SVMP sequences remain structurally uncharacterized also with their evolutionary history.

There are two known examples of SVMP genes that are extensively truncated, resulting in the expression of proteins containing only parts of the propeptide domain (Fry, et al. 2008; Casewell, et al. 2011; Brust, et al. 2013). These atypical SVMPs have been identified from the Lamprophiid genus *Psammophi*s and the viperid snake genus *Echis*, and they lack the metalloprotease domain (and additional C-terminal domains) and zinc-binding motif that characterize the adamalysins. The propeptide-only expression in *Psammophis* and *Echis* are convergent derivations (Brust, et al. 2013). The *Psammophis* monodomain

form had a lot of variance in sequence, but the *Echis* monodomain pre-propeptide form was virtually similar to the pre-propeptide region produced in the multidomain gene.

Although these genes have yet to be identified as translated and secreted in *Echis* venom, the resultant toxins are present in *Psammophis* venom (Fry, Lumsden, et al. 2003b; Brust, et al. 2013), where they exhibit an entirely novel neurotoxic activity: inhibition of postsynaptic α7 nicotinic acetylcholine receptors (Brust, et al. 2013). Notably, domain loss in *Psammophis* has resulted in increased selection pressure, which has driven a rapid rate of mutations in the propeptide domain and resulted in protein neofunctionalization, analogous to the processes observed in the evolution of P-I and P-II SVMPs (Casewell, et al. 2011; Brust, et al. 2013). Bioassays on two post-translationally cleaved new proline-rich peptides from the *P. mossambicus* propeptide domain revealed that they had been neofunctionalizationed for selective inhibition of human a7 neuronal nicotinic acetylcholine receptors, according to Fry (Brust, et al. 2013).

The goal of this study was to see if (1) the multi domains within P-III SVMP have had the same evolutionary history, and if so, what impact changes in molecular structure have had on the rate of evolution and neofunctionalization; and (2) whether the truncated SVMP type is widespread within the genus *Psammophis*, and if so, what phylogenetic history it has. As a result, we use Bayesian inference analyses on multiple domain partitions of an extensive P-III SVMP data set to trace the evolutionary history of the ancestral P-III SVMPs and truncated SVMPs, as well as their constituent domains, before using adaptive molecular evolution tests on points of the tree where domain alterations were inferred to have occurred. The results of this study's studies demonstrate the uniqueness of SVMP subtype development with neofunctionalization.

Materials and Methods

Sequence Alignments and Phylogenetic Reconstruction

Protein sequences for all toxin sequences were retrieved from the UniProt database (https://www.uniprot.org) and NCBI database (http://www.ncbi.nlm.nih.gov), then combined with the toxin transcripts from our assembly and annotation (Chapter 2). Partial sequences, sequences with suspect assembling errors were excluded. For the blocks of sequence in between these sites, the sequences were aligned using a mix of manual alignment of the conserved cysteine locations and alignment using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) method (Edgar 2004) implemented in AliView (Larsson 2014). Manual refinement of the alignment was also involved because there are structural differences within different toxin families. The phylogenetic trees for different toxin families were reconstructed with MrBayes 3.2 (Ronquist, et al. 2012) based on the amino acid sequence alignment. The settings for MrBayes can be found in the Supplementary File 2. The output trees from MrBayes were midpoint rooting, then further edited and annotated with iTol (Letunic and Bork 2007).

Tests for Selection

Coding DNA sequences, which are corresponding to the toxin sequences used for phylogenetic analysis, were retrieved from GenBank (Benson, et al. 2012) and our assembly and annotation (Chapter 2). Using

AliView and the MUSCLE method, the sequences were trimmed to only contain codons that translate to the mature protein, then translated, aligned, and reverse translated. Clades were created based on taxonomy and structural differences (functional domains/motifs, for example). The resultant codon alignments were used to create phylogenetic trees for each clade using the same methods outlined in the above 'Phylogenetic Reconstruction' section. All following studies were conducted using these tree topologies.

Calculating the ratio of nonsynonymous nucleotide substitutions per nonsynonymous site (dN) to synonymous substitutions per synonymous sites (dS) (ω=dN/dS) for each codon in the alignment might reveal if a gene is undergoing rapid evolution or stays functionally restricted. Codons developing with ω >1 are thought to have evolved under positive selection (functional diversity), whereas codons evolving with ω<1 are thought to have evolved under purifying selection. Sites with a value of ω=1 are believed to evolve in a neutral manner. In order to find the most likely groups on which positive selection has been working, we conducted a series of experiments integrating data from site-based and lineage-specific studies.

Due to their various emphases, we employed many of the selection tests developed in HyPhy v 2.220150316 beta (Pond and Muse 2005) to study the patterns of selection acting on distinct toxin families. The Analyze Codon Data analysis in HyPhy produces overall alignment values, whereas the FUBAR technique assesses the intensity of persistent positive or negative selection on individual amino acids (Murrell, et al. 2013). The Mixed Effects Model of Evolution (MEME) approach, on the other hand, finds particular locations that have been exposed to positive selection in the past (Murrell, et al. 2012).

Protein Modelling

To map residues evolving under positive selection in three-dimensional (3D) structures, sample sequences from the RCSB PDB database (Rose, et al. 2010) were used to create bespoke models for each clade belonging to various toxin families (Table 1). Alignments of each clade were trimmed to match these PDB structures. To render and colour the 3D structure of the proteins, we utilized the UCSF Chimera program v 1.10.2 with attribute files generated from FUBAR and MEME results. For FUBAR, we used the value from the beta-alpha column which is a measure of the difference between the rates of non-synonymous (beta) and synonymous (alpha) mutations. For MEME, since MEME estimates two rates of positive selection and gives each a probability, we take the weighted average of those two and then subtract alpha to arrive at a similar value to the one we used for FUBAR.

Results and Discussion

Snake Venom Metalloproteinases (SVMPs)

While SVMPs have long been known as one of the dominant venom types in viperid venoms, increasing evidence is emerging of their importance in the venoms of other families (Kamiguti, et al. 2000; Peichoto, et al. 2007; Fry, et al. 2008; Casewell, et al. 2015a; Debono, et al. 2017; Modahl, et al. 2018; Debono, et al. 2020). The basal SVMP structural form (P-III) is a final processed protein consisting of three domains: protease + disintegrin + cysteine-rich. Domain-deletion forms are largely known only from viperid

venoms including the P-II (protease + disintegrin, with the cysteine-rich domain deleted), and P-I (protease only, with both the disintegrin and cysteine-rich domains deleted) (Casewell, et al. 2015a). Intriguingly the P-I derived condition appears to have evolved convergently in the dipsadinae lineage within the Colubridae rear-fanged snake family (Campos, et al. 2016).The P-III form, however, remains a major constituent of viperid venoms and other than select dipsadinae lineages, it is the only form present in non-viperid snakes. Consistent with the structural and functional diversification of SVMP within the viperids, phylogenetic analysis in this study revealed evidence of extensive gene duplication in the last common ancestor of the viperid snakes (Figure 1). In contrast, for the colubrid and elapid snakes, the sequences broadly follow organismal relationships, with diversification events largely confined to within a particular lineage.

Table 1: Custom models for protein modelling.

Clade	Sequence for 3D modeling	PDB ID
Elapidae	Cerberus_rynchops_D8VNS0	2dw2
Colubriae	Naja_atra_D5LMJ3	3k7l
Viperidae	Trimeresurus_stejnegeri_Q2LD49	3k7l

Within the P-III SVMP enzymatic toxin class, there have been convergent structural derivations characterized by the evolution of a new cysteine that allows these toxins to form covalently linked multimers with lectin dimers. SVMP with this novel cysteine are termed P-IIId. Phylogenetic analysis suggests that the P-IIId type have evolved on at least three occasions: *Bothrops*; the last common ancestor of the genera *Daboia, Macrovipera, Montivipera* and *Vipera*; and in the genus *Echis* (Figure 1). *Echis* venoms contain two distinct forms of P-IIId which confirms previous hypotheses that were based on sequence similarity but lacked phylogenetic analyses (Casewell, et al. 2009). Sequence analysis in our study shows that while the cysteines have evolved in homologous regions of the SVMP scaffold, suggesting structural constraints in the formation of a multimeric complex, they differ slightly in position and, consistent with independent evolutions, differ in flanking residues (Figure 2).

In addition to structural diversifications, SVMPs have acquired a number of novel functions, the most common of which is procoagulant activity (Casewell, et al. 2015a). Identifying sequences in our phylogeny that have demonstrated procoagulant effects suggest that, within the viperids, the procoagulant trait has independently evolved within the viperine subfamily (such as *Echis, Daboia, Macrovipera, Pseudocerastes,* and *Vipera*) and the crotaline subfamily (*Bothrops*) (Figure 1). Clotting factor activation has been documented in the additional crotaline genera *Calloselasma* and *Crotalus* (Debono, et al. 2019; Seneci, et al. 2021), but the toxins responsible have not been sequenced. Consequently, their phylogenetic affinity to the *Bothrops*-type procoagulant P-III SVMP are unknown, and it cannot be determined whether procoagulant SVMPs have evolved once or several times in the pit vipers. Once the sequences become available, this will be resolved by whether the toxins form a

monophyletic group with the *Bothrops* toxins or if they form distinct clades. In addition to evolving at least twice within the viperids, the procoagulant SVMP trait evolved independently again in the last common ancestor of the colubrid genera *Dispholidus* and *Thelatornis* (Debono, et al. 2017) and also in the elapid genus *Micropechis* (Gao, et al. 2002). If P-III SVMP are responsible for the procoagulant activity shown for *Atractaspis* venoms (Oulion, et al. 2018), then this would represent another convergent evolution of this trait. Similarly, the toxins responsible for the procoagulant toxicity of the *Rhabdophis* genus have not been identified (Iddon and Theakston 1986; Komori, et al. 2017), but if the *Rhabdophis* procoagulant effect is due to a SVMP, this would almost certainly represent another instance of functional convergence considering the tens of millions of years of separation between this genus and the other procoagulant lineages.

The overall ω value for all lineages was consistently higher for the cysteine-rich domains than for the disintegrin or protease domain. This suggests that the cysteine-rich domain is crucial for target binding prior to the interaction of the catalytic site located on the protease domain, and therefore this is a critical domain for the evolution of neofunctionalization. Analysis of selection (Table 2) and 3D modelling (Figure 3) showed that more than half of the positively selected sites detected were confined to the protease domain; of the remaining variations, more were found in the cysteine-rich domains than in the disintegrin-like domains. Again, this pattern suggests a bias in positive selection toward the protease domain, consistent with this domain being the subunit responsible for the enzymatic activity.

Table 2: Molecular evolutionary rates of SVMP (See Figure 3 for modelling).

Clade	Domains	ω	FUBAR (-)[a]	FUBAR (+)[b]	MEME[c]	FUBAR & MEME[d]
Colubridae	Full length secreted form	1.19	74	103	46	33
	Peptidase domain	1.54	19	53	50	39
	Disintegrin domain	0.72	14	12	12	10
	Cys-rich domain	1.64	18	30	36	24
Elapidae	Full length secreted form	1.23	36	75	86	56
	Peptidase domain	1.47	13	25	35	21
	Disintegrin domain	0.86	5	8	9	7
	Cys-rich domain	1.76	7	29	36	21
Viperidae	Full length secreted form	1.37	79	132	159	124
	Peptidase domain	1.44	22	57	78	55
	Disintegrin domain	0.80	23	21	21	18
	Cys-rich domain	1.63	27	41	50	38

[a] Number of codons under negative selection according to FUBAR
[b] Number of codons under positive selection according to FUBAR
[c] Number of codons under episodic diversifying selection according to MEME
[d] Number of codons that fit criteria [b] and [c]

Figure 1: Molecular phylogenetic reconstruction of SVMP toxins with the lineage specific amplification of particular forms shaded in pink (Colubridae), blue (Elapidae), or yellow (Viperidae). Gray shading shows the convergent evolutions of procoagulant functionally derived forms. Colours on the outside of the ring designate the convergent evolutions of the P-IIId structurally derived forms. Sequence alignment for constructing phylogenetic tree can be viewed in Supplementary File 7. For tree output file for SVMP toxins, see Supplementary File 8.

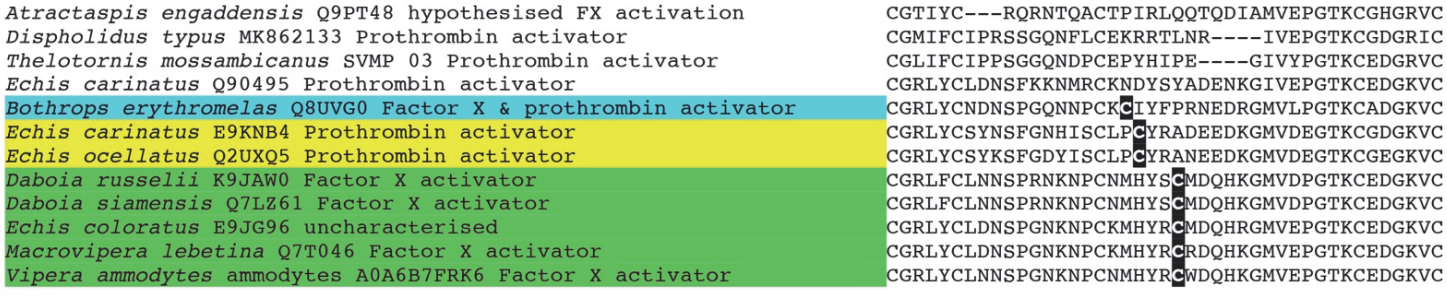

Figure 2: Partial amino acid sequence alignment of representative SVMP with coloured shading of species names indicating the three convergent evolutions of interchain cysteines, diagnostic of the P-IIId structurally derived forms. Other procoagulant functionally derived forms (species names not shaded) are included for comparison. Cysteines forming links to lectin dimers are highlighted in black background.

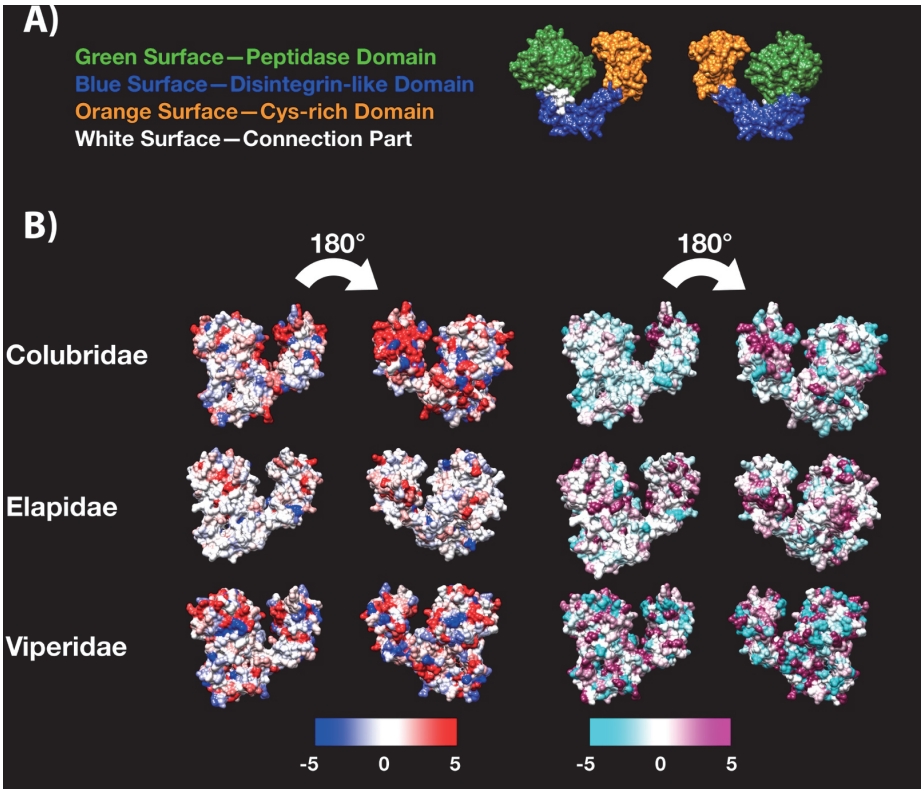

Figure3: 3D models of PIII SVMP. A) location of the three domains which make up the SVMP toxin type. B) Molecular modelling of SVMP showing sites under selection by FUBAR (left) and MEME (right) colour coded to show sites that are negatively, neutrally, or positively selected. See Table 1 for values. Protein models show front and back views colored according to FUBAR's estimated strength of selection (β-α, left) and MEME's significance levels (right). Table 2 contains the information regarding template choice for each toxin subclass.

SVMP propeptide domain novel toxins

In addition to the structural variations noted in the above section, on at least two independent occasions the propeptide domain of SVMP genes have been recruited as toxins in their own right, without accompanying expression of any of the three domains making up the P-III enzyme. This was first noted in *Echis* venoms, where the truncation is formed by stop codons terminating otherwise unremarkable sequences (Casewell, et al. 2011). More intriguing toxins are found in the venoms psammophiine snakes which were first noted in the species *Psammophis mossambica* (Fry, et al. 2008), where the propeptide domain was selectively expressed. Unlike the *Echis* forms, there was explosive diversification of these novel toxins: 26 variants were discovered in this species alone, including forms with novel cysteines which could potentially form disulphide bonds. Subsequent testing of two of these toxins revealed them

to be novel neurotoxins (Brust, et al. 2013). The activity of the other variants is unknown. In this study, this novel toxin class was shown to be present with staggering sequence diversity across the psammophiine snakes, including not only the additional *Psammophis* species we sequenced but also the *Malpolon* and *Rhamphiophis* species (Figures 4 and 5). Sequence analysis revealed that the first half of the toxins are homologous to the propeptide region of typical SVMP P-III genes. However, there is then an abrupt shift in sequence patterns, which is consistent with a frame-shift mutation providing the starting substrate for the evolution of this novel toxin class. The subsequent evolution resulted in such sequence diversity that calculating rates of was evolution impossible due to the unalignable diversity in the second half of the peptides. Such incredible diversity suggests there may be extensive neofunctionalization beyond the previously characterized neurotoxicity. Therefore, this toxin class represents a particularly rich area for future research, especially as most of these toxins are either short linear or with a single disulphide-bond, which would allow for efficient synthesis.

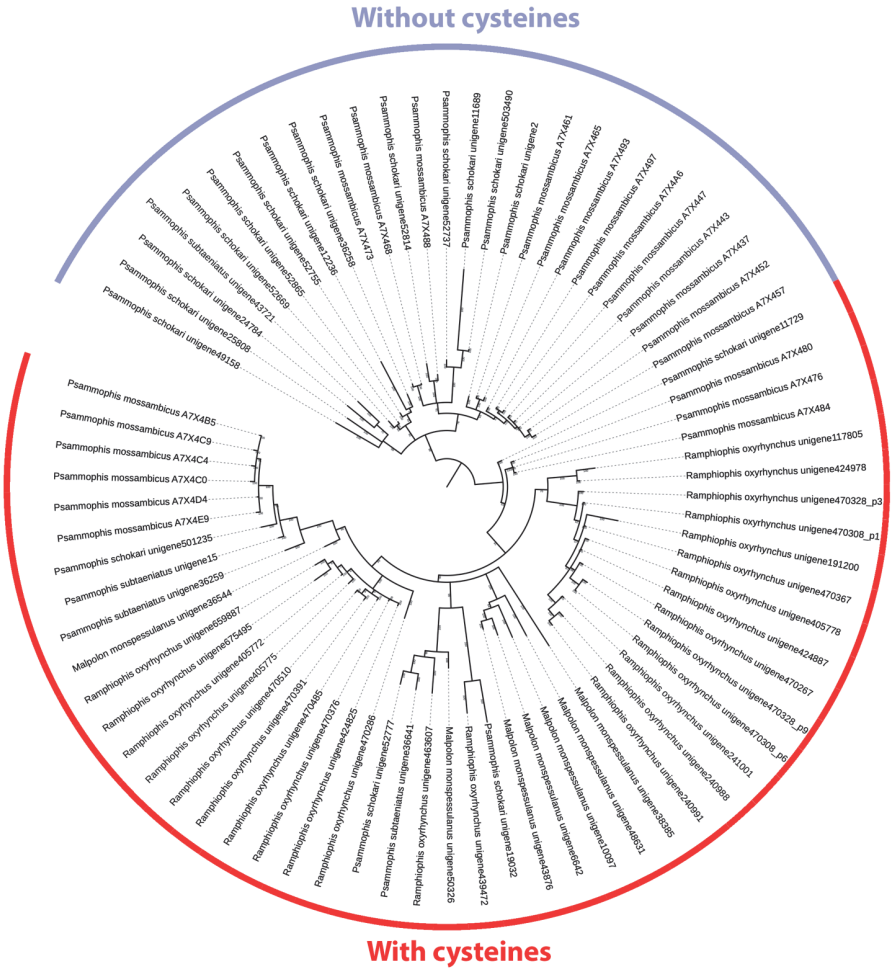

Figure 4: Molecular phylogenetic reconstruction of the psammophiine, lineage-specific derivation of the SVMP propeptide domain into a novel toxin family, with the subsequent explosive diversification of the cysteine-linked forms. Sequence alignment for constructing phylogenetic tree can be viewed in Supplementary File 9. For tree output file for SVMP propeptide domain toxins, see Supplementary File 10.

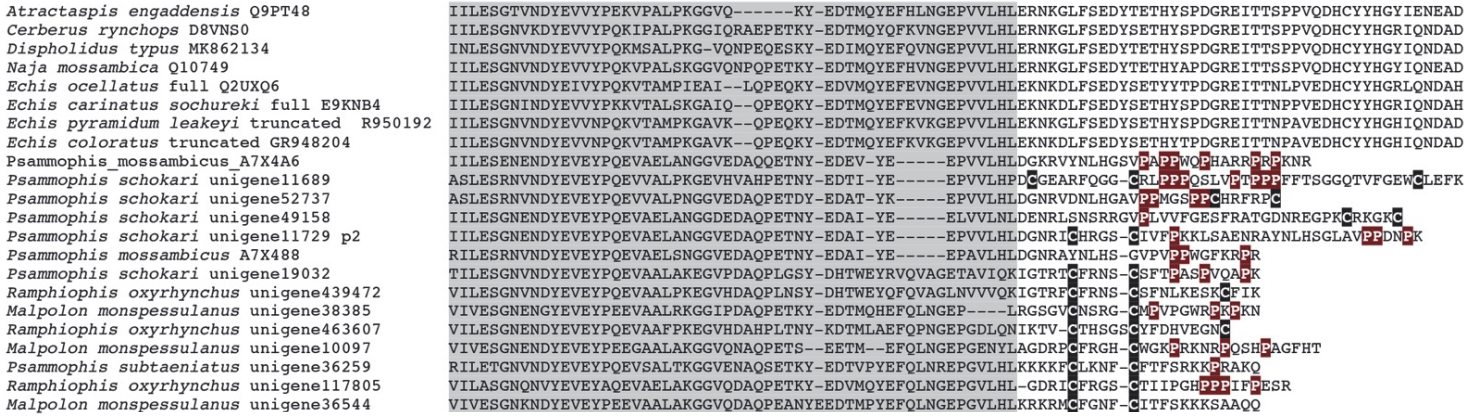

Figure 5: Alignment of representative SVMP propeptide domains. Region shaded in gray is that which is homologous across all representatives, with the unshaded region indicating the location of the putative frameshift mutation that led to the evolution of the new toxin family in psammophiine snakes. Cysteines and Prolines are indicated in black and red boxes.

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