Review

Evolution and diversification of the Toxicofera reptile venom system

Bryan G. Fry[a],*, Nicolas Vidal[b], Louise van der Weerd[c,d], Elazar Kochva[e], Camila Renjifo[f]

[a]Department of Biochemistry & Molecular Biology, Bio21 Institute, University of Melbourne, Melbourne, 3010, Australia
[b]UMR 7138, Département Systématique et Evolution, Muséum National d'Histoire Naturelle, CP 26, 57 rue Cuvier, 75005 Paris, France
[c]Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands
[d]Department of Radiology, Leiden University Medical Center, Leiden, The Netherlands
[e]Department of Zoology, Tel Aviv University, Tel Aviv 69978
[f]Department of Physiological sciences, Faculty of Medicine, Pontificia Universidad Javeriana, Bogotá, Colombia

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ABSTRACT

The diversification of the reptile venom system has been an area of major research but of great controversy. In this review we examine the historical and modern-day efforts of all aspects of the venom system including dentition, glands and secreted toxins and highlight areas of future research opportunities. We use multidisciplinary techniques, including magnetic resonance imaging of venom glands through to molecular phylogenetic reconstruction of toxin evolutionary history, to illustrate the diversity within this integrated weapons system and map the timing of toxin recruitment events over the toxicoferan organismal evolutionary tree.

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* Corresponding author.
  E-mail address: bgf@unimelb.edu.au (B.G. Fry).

1. Background

The evolution of the reptile venom system has been of long-standing interest and considerable controversy. With the scarcity of the fossil record, particularly the inherently poor preservation of soft-tissue, the origin and subsequent diversification of venomous reptiles must be mostly inferred from the knowledge of the modern representatives. Early evidence was based mainly on morphological characters such as skull, dentition, glands, and the compressor muscles associated with the venom delivery system (e.g. [1–3]). Of the approximately 2,650 species of advanced snakes (Caenophidia), only the ~650 front-fanged species have traditionally been considered venomous by the conventional anthropocentric definition. The relative venomous nature of the rest of the advanced snakes remained almost entirely uninvestigated until recently [4–6]. Similarly, the helodermatid lizard venom system is poorly known despite extensive accumulated literature [7] and, until very recently, the potential for other lizards to be venomous had been neglected [8].

2. Origin and evolution of the reptilian venom system

Among snakes, species within the Elapidae and Viperidae families and the sister-genera *Atractaspis* and *Homoroselaps* within the Atractaspidine subfamily of the Lamprophiidae family have elaborate, morphologically-specialized high-pressure front-fang venom systems used to conduct venom into their prey [4]. These systems, including skeletal, muscle and gland components, show a characteristic, but different, pattern for each group [4]. In the front-fanged delivery-system the fang canal lies in close approximation with the venom duct via the fang sheath and the closed channel runs through
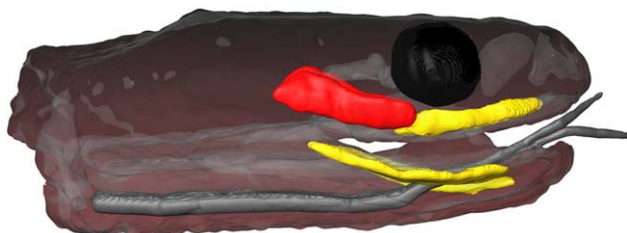

Fig. 1 – Magnetic resonance imaging of a formalin–ethanol preserved *Crotaphopeltis hippocrepis* specimen submersed in Fomblin (Solvay Solexis) performed on a 9.4T vertical 89 mm bore system (Bruker BioSpin, Rheinstetten, Germany) with a Bruker Micro2.5 gradient system of 1T/m and transmit/receive birdcage radiofrequency coil with diameter of 10 mm. Bruker ParaVision 3.0 software was used for image acquisition. Anatomical images were acquired using a 3D gradient echo sequence. Voxel size was (40)³ mm³. Imaging parameters were: TE = 8 ms, TR = 40 ms, flip angle 20°, 8 averages, total scan time of 3 h. Image segmentation of the glands was performed manually in Amira 4.1 (Mercury Computer Systems Inc.) and 3D surface renderings generated. Maxillary venom gland is shown in red and labial mucous glands are shown in yellow.
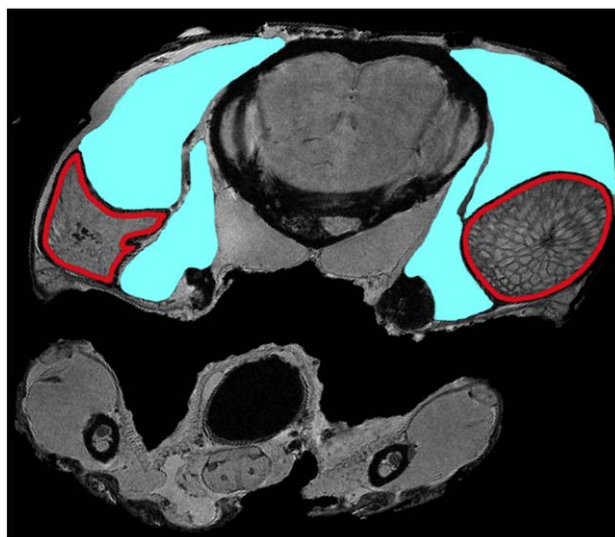

Fig. 2 – Magnetic resonance imaging transverse slice of a formalin–ethanol preserved *Dendroaspis jamesoni* specimen submersed in Fomblin (Solvay Solexis) performed on a 9.4T vertical 89 mm bore system (Bruker BioSpin, Rheinstetten, Germany) with a Bruker Micro2.5 gradient system of 1T/m and transmit/receive birdcage radiofrequency coil with diameter of 10 mm. Bruker ParaVision 3.0 software was used for image acquisition. Anatomical images were acquired using a 3D gradient echo sequence. Voxel size was (40)³ mm³. Imaging parameters were: TE = 8 ms, TR = 40 ms, flip angle 20°, 8 averages, total scan time of 3 hours. Venom gland is outlined in red, compressor muscles filled in blue; right side shows muscles in normal position, left side shows venom gland being compressed to propel venom through the duct and into the fang.

the shaft of the fang for the conduction of venom into a bite wound [4,9,10]. In elapids (proteroglyph) the fangs are positioned at the anterior end of the maxilla while in viperids (solenoglyph) and atractaspidines the maxilla is reduced and there are no maxillary teeth other than the fangs.

Among the various non-front-fanged families, an impressive array of relative dentition is evident that is independent of taxonomical groupings ranging from: aglyph maxillary dentition, in which a posterior fang is unspecialized or nonexistent; to opistoglyph, which consists of a posterior fang which may be variably enlarged in relation to the anterior teeth and may or may not be grooved [4]. Grooved fangs possess a variable-length, open channel along the lateral or anterolateral surface to facilitate the introduction of venom into a bite wound [4,11]. In cases where the posterior teeth are not grooved or enlarged in relation to other teeth, as occurs in the aglyph type of dentition, the posterior teeth are distinguishable from the anterior because of the presence of ridges on the anterior and posterior surfaces [4,11].

Snake venom glands are located posterior to the eye and extend in a line along the upper jaw and ventrally the supralabial mucous glands extend along the entire length of the upper jaw (Fig. 1). Viperid venom glands are long and triangular in shape with the more elongated part extending anteriorly. The gland has a complex tubular structure and may be divided into several

lobes, the lumen is wide and allows the storage of a large amount of venom, it becomes a primary duct anteriorly that expends into an accessory mucous gland and becomes a secondary duct that connects with the sheath of the fangs [12]. Elapid venom glands have usually a smaller lumen and the secretion is stored inside the cells in addition to the lumen of the gland; the accessory mucous gland follows anteriorly surrounding the entire duct [13]. The venom glands of *Atractaspis* show a tubular structure with a lumen running the entire length of the gland. There are no separate accessory glands, but the secretion tubules are covered by mucous cells at their openings into the central lumen. In all three groups there are species with elongated venom glands that reach far beyond the neck region.

In all front-fanged snakes, venom is delivered through muscular compression of the glands (Fig. 2) help propel it along the duct towards the hollow fangs. In contrast, the venom glands of non-front-fanged snakes have a single, short duct extending anteromedially from the lumen of the gland to the base of the posterior teeth or fangs [4,14].

Because of the diversity in dentition and glands among caenophidians, these morphological characters have been studied extensively and indeed guided much of the early phylogenetic groupings of the advanced snakes into the Elapidae and Viperidae families, with the 'Colubridae' family being a taxonomical dumping ground for the non-front-fanged remain-

der of the caenophidians. Since the end of the nineteenth century, many authors have tried to establish transformation series between the four dentition types, the most commonly cited trend being a progressive evolution of the dentition from the aglyph and opisthoglyph types through the proteroglyph and the solenoglyph types. Although the solenoglyph type displayed by viperids was commonly believed to be the most sophisticated and therefore the most derived, both a common origin [15–17] and an independent evolution of the proteroglyph and solenoglyph systems have been inferred [3,18–20].

The development of molecular systematics provided the vital phylogenetic framework necessary for a reconstruction of the evolutionary history of the glands and fangs and thus a resolution of fundamental aspects. Recent molecular phylogenetic studies [21] showed that the non-front-fanged 'Colubridae' is not a single group as previously supposed, but represents many familial-level lineages. Further, the front-fanged snakes (atractaspidines, elapids and viperids) do not form a clade, but are three independent lineages among Caenophidia, with viperids in a basal position. Thus, their glands were not homologous other than through a more inclusive homology with the Duvernoy's gland displayed by non-front-fanged caenophidians. For this reason, the term Duvernoy's gland has been abandoned and the term venom gland is now used for all caenophidians regardless of the
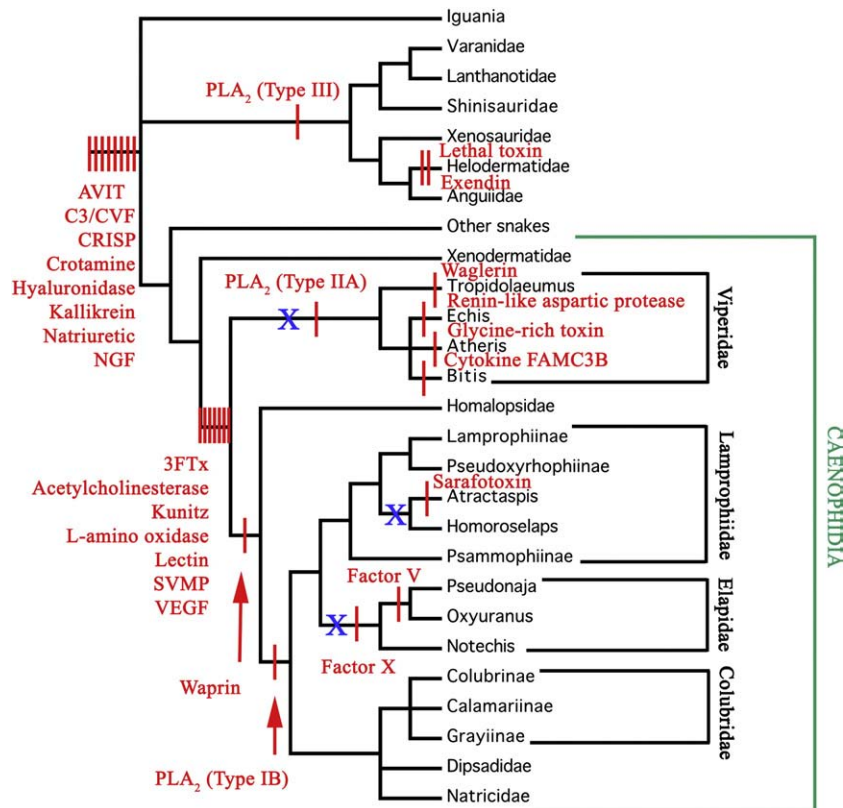

Fig. 3 – Cladogram of evolutionary relationships of Toxicofera reptiles showing the recruitment timing of different protein-scaffold types for use as toxins. Blue X shows independent evolution of hollow front-fanged, high-pressure venom delivery systems. 3FTX = three finger toxin,, C3/CVF = Complement C3/Cobra Venom Factor, CRISP = Cysteine-rich secretory protein, NGF = Nerve Growth Factor, SVMP = snake venom metalloprotease, VEGF = vascular endothelial growth factor. Hyaluronidase and natriuretic peptide presence in *Heloderma* venom based upon Fry et al. unpublished results; Genbank accession numbers EU790961 and EU790965, respectively. Organismal phylogeny based upon [5,6,21,85–88].
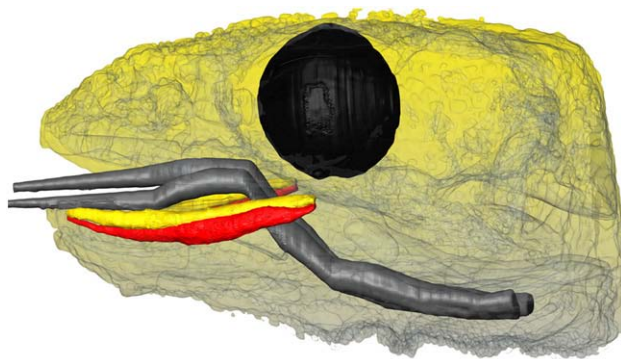
Fig. 4 – Magnetic resonance imaging of a formalin–ethanol preserved *Varanus exanthematicus* specimen submersed in Fomblin (Solvay Solexis) performed on a 9.4T vertical 89 mm bore system (Bruker BioSpin, Rheinstetten, Germany) with a Bruker Micro2.5 gradient system of 1T/m and transmit/receive birdcage radiofrequency coil with diameter of 10 mm. Bruker ParaVision 3.0 software was used for image acquisition. Anatomical images were acquired using a 3D gradient echo sequence. Voxel size was $(40)^3$ mm^3. Imaging parameters were: TE = 8 ms, TR = 40 ms, flip angle 20°, 8 averages, total scan time of 3 h. Image segmentation of the glands was performed manually in Amira 4.1 (Mercury Computer Systems Inc.) and 3D surface renderings generated. Mandibular venom gland is shown in red and infralabial mucous gland is shown in yellow.

degree of anatomical deviation or relative medical importance of human envenomations [4,22]. Supporting the homology, and thus consistent terminology, accumulated developmental evidence supported that all gland types and associated dentition were derivates of the dental glands, developing from a common primordium at the posterior end of the dental lamina, with 'dental uncoupling' being responsible for the fang diversity in both structure and geographical location [1,12,23–26].

In contrast to the venom delivery system of caenophidian snakes in which the single compartment venom glands and delivery teeth are housed in the upper jaw, the venom of the venomous lizards in the genus *Heloderma* is produced by multi-compartmentalised glands on the lower jaw. Due to significant differences in anatomy of the venom delivery system and distant phylogenetic relatedness, it has been long assumed that the venom system of the helodermatid lizards represented the sole lizard venom system and that this represented a second, independent evolution of venom within the squamate reptiles. However, both lineages were revealed to be members of a clade (Toxicofera) that also includes several lineages of other lizards recently shown to be venomous (Fig. 3) [8,27]. These studies demonstrate that a core set of venom genes evolved in the common ancestor of the Toxicofera [8] and subsequently evolved into the more complex venoms observed in snakes and lizards following further toxin recruitment events [28]. In contrast to the hypothesis of independent origins, this new perspective revealed that *Heloderma* and snake venom systems are homologous but highly differentiated descendants of an early-evolved venom system in squamates which possessed

incipient venom glands in both the mandibular and maxillary regions, with snakes favouring the development of the maxillary venom gland and secondarily reducing the mandibular components, while the anguimorph lizards did the reverse, resulting in the modern condition seen today (Fig. 4). In contrast, within the Iguania the mandibular and maxillary glands are not developed past incipient stage and appear to have little ecological/evolutionary relevance. Within the advanced snakes, the maxillary venom glands have become atrophied in egg or slug/snail eating species as well as in the ratsnake clade which has a secondarily evolved form of prey capture (constriction). It is anticipated that the same sort of reduction may have occurred within non-caenophidian snake lineages such as boas and pythons.

2.1.   Protein types recruited

Reptile venom proteins are the result of the duplication of an ordinary body protein, often one involved in a key physiological process, with the subsequent tissue specific expression of the new gene [28]. While toxins have been sourced at different times during toxicoferan evolutionary history (Fig. 3) from a wide range of tissues, are of disparate molecular-scaffolds and have diverse ancestral bioactivities, certain trends are evident. The restriction to secretory proteins on one hand limits the available pool but also pre-selects proteins with certain desirable features: signal peptide, typically useful bioactivity and stable scaffold. The already present signal sequence eliminates the addition of one through interlocus gene conversion (non-reciprocal recombination) and retrotransposition or exon-shuffling of a signal peptide proto-module [29]. Many have pre-existing bioactivities that are highly conserved in a wide suite of potential prey items. This allows for immediately beneficial basal toxic activities: hydrolysis of a universally present substrate (such as hyaluronidase enzymes hydrolysing the 1–4 linkages between N-acetyl-β-D-glucosamine and D-glucuronate residues of hyaluronate); 'mimicking' indigenous body proteins as if they were overexpressed to cause a physiological imbalance (such as the hyper-algesia and intestinal cramping caused by AVIT toxins or the kallikrein toxins over-releasing kinins from kininogen); or acting as a competitive inhibitor to cause an opposite physiological imbalance or disruption of a physiological response (such as the alpha-neurotoxic 3FTx). Most secretory proteins are also extensively covalently-linked as this provides molecular stability and resistance to proteolysis. While extensive neofunctionalisation may occur, such scaffolds are highly conserved in the emergent toxin multigene families [28,30–32].

Functionally important toxin types are reinforced through adaptive evolution involving explosive duplication and diversification, creating a venom gland specific multigene family. The likelihood for neofunctionalisation is increased through random mutation, gene conversion and unequal crossing-over [33]. While the molecular scaffold of the ancestral protein is conserved, derived activities emerge through mutations of the surface chemistry [28,33–35].

This confers a tremendous array of new activities as exemplified in the three finger toxins (Fig. 5). This important toxin class is basally alpha-neurotoxic [28,33], reflective of its neuromodulatory non-toxin molecular ancestry [36–38].
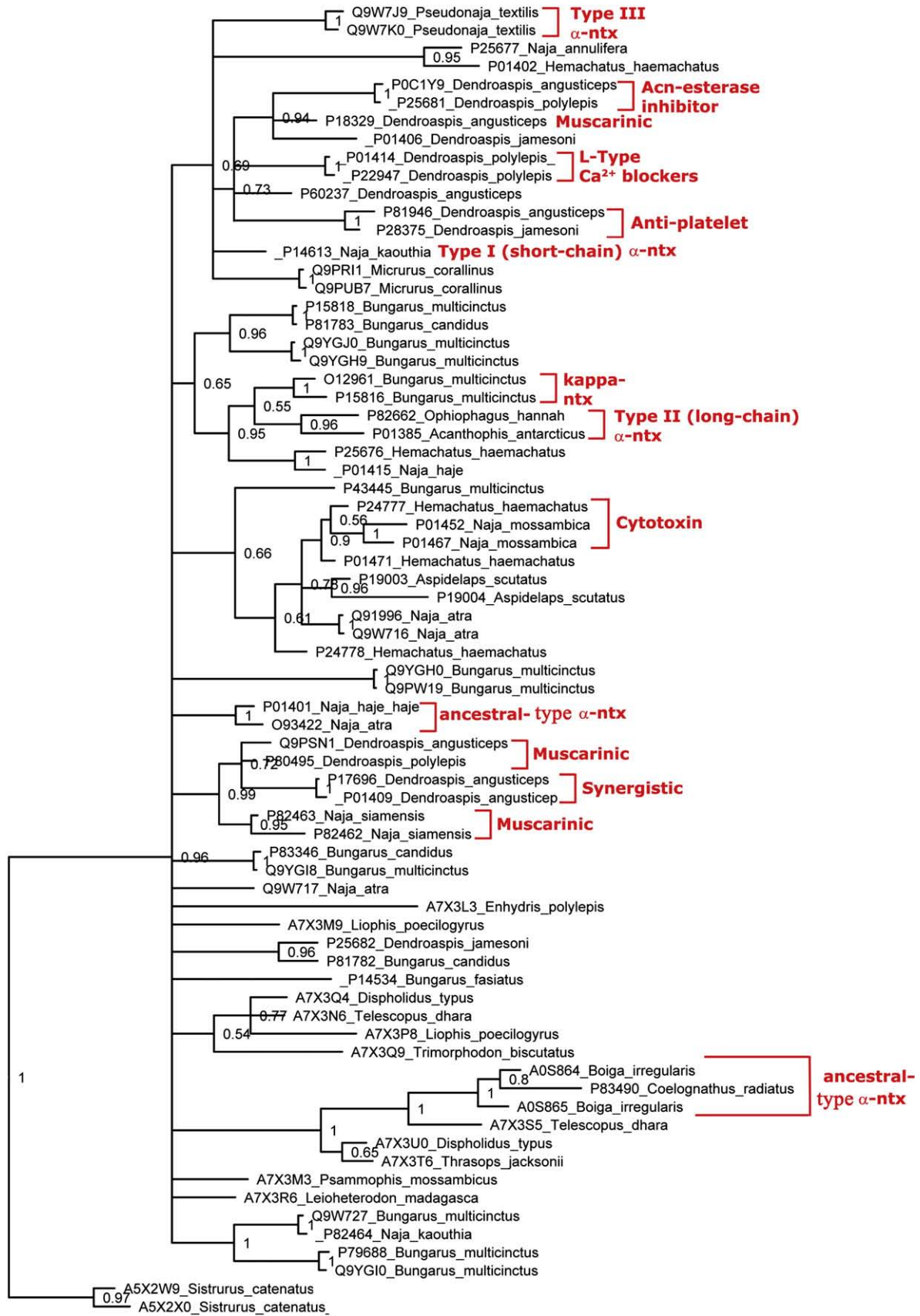
Fig. 5 – Bayesian molecular phylogeny of 3FTx; methodology as per [4]. Outgroup is the non-toxic brain alpha-neuropeptide Q9WVC2 from *Mus musculus* (not shown). Clades with determined bioactivities are indicated in red. ntx = neurotoxin; acn = aceylcholinesterase. All sequences are referred to by their Swiss-Prot accession numbers.

Deletion of the ancestral C^2 and C^3 cysteines greatly potentiated this activity [33] However, the three potent alpha-neurotoxic clades containing only eight of the ancestral ten cysteines (type I [aka: short-chain], type II [aka: long-chain] and type III) are not monophyletic (Fig. 5). The type-II contain two additional newly evolved cysteines, shared with the sister-group kappa-neurotoxins [33]. Neofunctionalisation has resulted in a myriad of novel activities [33]. Additional neurological targets include postsynaptic (muscarinic acetylcholine receptor [39,40]), synaptic (acetylcholinesterase inhibition [41]), pre-synaptic (L-type Ca^{2+} channels [42]) and neuronal (kappa-bungarotoxins [43]). Intriguingly, the muscarinic toxicity has been derived on at least two-separate occasions (Fig. 5). Derived non-neurotoxic activities include cytotoxicity [44] and platelet inhibition [45]. As the name implies, the synergistic toxins, which arose from within one of the muscarinic clades (Fig. 5), are not active by themselves but rather potentiate the alpha-neurotoxins [46]. However, despite the huge amount of effort put into the research of the 3FTx toxin type, our understanding of the full biodiversity is remarkably poor, there being multiple clades lacking defined activities (Fig. 5) [33].

2.2. Domain utilisation

Toxin diversity is also obtained through unique gene mutations ranging from selective expression of a domain, tandem domain repeats or newly evolved, post-translationally liberated multi-product encoding genes.

Three cases of selective domain expression have been documented in toxicoferan venoms: SVMP pre-pro domain; SVMP distintegrin domain; and exendin peptides. Expression of a single domain to the exclusion of the remainder of the multi-domain SVMP (snake venom metalloprotease) encoding
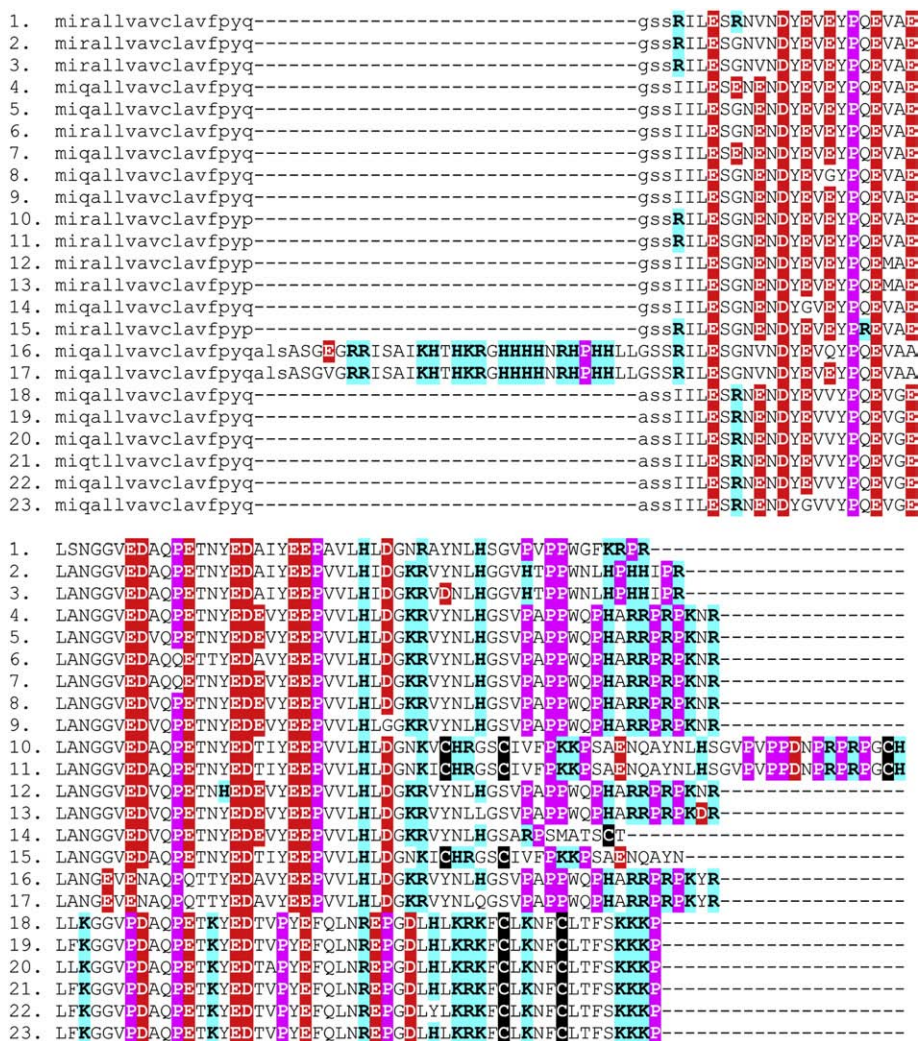

Fig. 6 – Sequence alignment of the selectively expressed SVMP pre-pro domain peptides from *Psammophis mossambicus*. Swiss-Prot accession numbers are: 1. A7X488, 2. A7X468, 3. A7X473, 4. A7X497, 5. A7X437, 6. A7X493, 7. A7X4A, 8. A7X447, 9. A7X443, 10. A7X484, 11. A7X476, 12. A7X452, 13. A7X457, 14. A7X4B0, 15. A7X480, 16. A7X461, 17. A7X465, 18. A7X4D, 19. A7X4B5, 20. A7X4E9, 21. A7X4C0, 22. A7X4C4, and 23. A7X4C9. Highlighted amino acids: negatively charged (red); positive (blue); prolines (magenta); cysteines (black). Signal peptides are shown in lowercase.

gene has occurred twice. The SVMP toxin gene encodes not only a multi-domain enzyme but also includes the N- and C-terminal prepro and disintegrin domains respectively that are post-translationally proteolytically liberated [47,48]. Unlike the disintegrin domain, the N-terminal pro-peptide region has not been attributed as having a role in envenomation. However, multiple transcripts were recently recovered from a *Psammophis mossambicus* cDNA library that encoded only for isoforms of this domain (Fig. 6) [4]. High sequence diversity was evident amongst the isoforms, with significant variations in the distribution of charged residues. Structural changes included not only variations in prolines but four variants had novel cysteines, including two forms with free cysteines and thus able to form dimers. The C-terminal disintegrin domain is post-translationally cleaved and inhibits platelet aggregation by competitively binding to the GIIb/IIIa receptor [47,48]. Novel disintegrin forms have been characterized that are selectively encoded to the exclusion of the rest of the SVMP gene [49]. A different form of selective domain expression has occurred in the helodermatid exendin peptides, where an ancestral tri-domain gene was split into two new separately evolving mono-domain genes (Fry *et al.* unpublished results; Genbank accession numbers EU790959 and EU790960) and these new mono-domain genes each further duplicated, to form exendin-1 and -2 and exendin-3 and -4 respectively.

Tandem repeats of an ancestral domain have been utilized in two very different manners. In the LT1 (lethal toxin 1) multigene family found within helodermatid lizard venoms, the gene encodes for a single product made up of four tandem repeats of an ancestral beta-defensin domain, with the consequent emergence of a novel protein fold (Fry et al. unpublished results; Genbank accession number EU790964). In contrast are the multiple proteolytically liberated sarafotoxin peptides encoded by single-gene tandem repeats of an ancestral domain [50,51]. Intriguingly, the 'short' sarafotoxins are encoded for by almost twice the number of repeats of the 'long' sarafotoxins Fig. 7. Investigation of gene intron/exon boundaries would be revealing in regards to the molecular diversification histories.

The most extreme case of domain mutation is the conversion of the single-product encoding ancestral natriuretic gene to encode for additional, novel, post-translationally liberated peptides. This region has been extensively convergently utilized in such a manner, ranging from the helokinestatin peptides in helodermatid lizards (Fry et al. unpublished results; Genbank EU790965), the antiplatelet peptides from *Macrovipera lebetina* venom [52], the BPP-peptides in viper venoms [53] and the tripeptide metalloprotease inhibitors in *Echis* venoms [54]. All represent striking convergence in use of the upstream region to encode for proline-rich, post-translationally cleaved peptides.

2.3. Subunit utilisation

Another fundamental neofunctionalisation technique is the utilisation of multi-unit toxins. Homomeric toxin complexes include crotamine, disintegrin, lectins and 3FTx. Crotamine toxins exist as covalently-linked homodimers but additional subunits may be non-covalently associated [55]. L-amino oxidase toxins also form dimers, which appear to be covalently-linked [56]. The Viperidae venom specific C-type lectin-like toxins are made up of covalently-linked heterodimers or
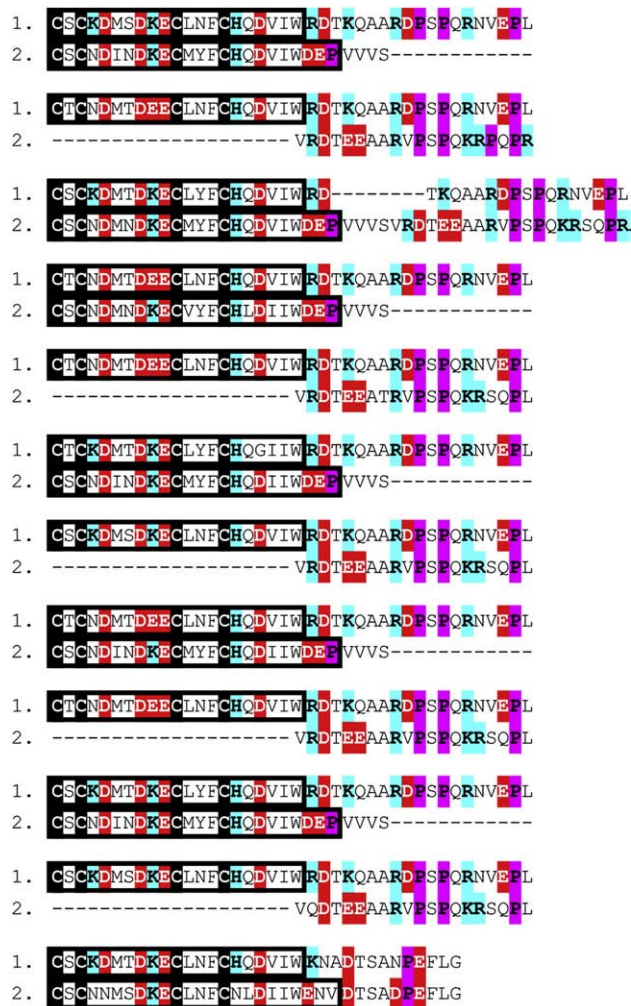

Fig. 7 – Sequence alignment of representative *Atractaspis* sarafotoxin short and long forms. Species/Swiss-Prot accession numbers are 1. *A. engaddensis*/P13208 and 2. *A. microlepidota*/Q6RY98 respectively. Post-translationally cleaved, functional peptides shown in black boxes. Highlighted amino acids: negatively charged (red); positive (blue); prolines (magenta); cysteines (black). Signal peptides are not shown.

non-covalently-linked oligomers of the covalently-linked heterodimers, while snake venom C-type lectin toxins from which they arose [4] are composed exclusively of non-covalently-linked homodimers or homooligomers [57]. As mentioned above, disintegrin toxins can result from cleavage of SVMP or may be selectively expressed. Disintegrins may also be monomeric or covalently-linked dimers. Monomeric forms are the result of cleavage of the full SVMP enzyme or just the selective expression of the disintegrin domain while the dimeric forms are exclusively distintegrin-domain expressed forms [58]. As the cysteines are conserved and even numbered, this means that dimers are the result of the formation of two new disulphide-bonds [59].

3FTx dimers take various forms. Cytotoxins also exist as non-covalently-linked dimers but may also be non-covalently-linked trimers [60]. κ-bungarotoxins also exist as non-covalently-linked homodimers [61]. In contrast, the synergistic 3FTx are covalently-linked dimers [62]. Elapid venom covalently-bound dimers

containing type II (long-chain) alpha-neurotoxins may be homomers or alternatively may be heteromers with various cytotoxins [60]. In both cases the binding to both the muscle-type and $\alpha7$ nicotinic receptors is preserved while the cytotoxin-containing heterodimer lacks the cytotoxic activity. The homodimer has also been conferred a new activity: κ-bungarotoxin-like blockage of the $\alpha3\beta2$ nicotinic acetylcholine receptor subtype. The heterodimeric 3FTx isolated from *Boiga irregularis* venom is specific for the avian neuromuscular junction, accounting for the taxon-specific toxicity [63]. In contrast to the elapid venom covalently-linked dimers, this toxin type uses a newly-evolved free cysteine present on each subunit while the elapid forms use two of the ancestral cysteines. Non-neurotoxic 3FTx multimers have also been isolated, such as the anticoagulant (inhibiting clot initiation and factor VIIa activity) non-covalently-linked hemextin-AB hetero-tetramers from *Hemachatus hemachatus* venom, which is a noncovalently-linked heterotetramer of two different 3FTX subunits [64].

The non-covalently-linked heterodimeric PLA$_2$ complexes from true-viper species (e.g. *Daboia* and *Vipera*) are composed of an acidic and basic subunit and are presynaptic in action [65,66]. Non-covalently-linked dimeric PLA$_2$ have also been characterised from pit-vipers such as *Bothrops* species [67]. Similarly, the potent neurotoxins from *Crotalus durissus terrificus* venom are also heterodimeric PLA$_2$ [68]. Elapid venom PLA$_2$s may be dimers or trimers [69–71]. Taipoxin and paradoxin from the Australian elapid snakes *Oxyuranus scutellatus* and *Oxyuranus microlepidotus* are constructed by three PLA$_2$-related peptide chains: alpha, beta (the identical beta-1 and beta-2) and gamma. However, despite the high degree of homology between the alpha and beta subunits, only the alpha-chain is an extremely potent blocker of the pre-synaptic release of acetylcholine [72–76]. Intriguingly, toxins homologous to the unique gamma subunit have been isolated from *Acanthophis* venom, perhaps indicative of a wider taxonomical distribution of this complex-type [77]. Textilotoxin, isolated from *Pseudonaja textilis*, is a potent presynaptic neuro-myotoxin with phospholipase A$_2$ activity and causes a presynaptic blockade of neuromuscular transmission involving disruption of the regulatory mechanism that controls acetylcholine release [78,79]. This toxin has the most complex structure and highest lethality of any identified snake neurotoxin [80]. The structure was initially reported as being composed of five non-covalently-linked subunits (A,B,C and D, with D existing as a covalently-linked dimer) but more recent evidence points instead to two alternate hexameric structures of $(A/B)_2C_2D_{2a}$ or $(A/B)CD_{2a}D_{2b}$, where D_{2a}, D_{2b} refer to differentially glycosylated dimers of the D dimeric subunit [81].

In addition to multimeric toxins composed of the same protein type, forms exist where different protein types are utilised in the construction of the complex. Multimers taking the form of heterologous heteromers, in that not only are the subunits not homologous but of different protein classes entirely, include β-bungarotoxin and taicatoxin. β-bungarotoxins are covalently-linked heterodimers of kunitz peptides and phospholipase A$_2$ from *Bungarus* venoms [82]. Taicatoxin, isolated from *Oxyuranus scutellatus*, is comprised by three non-covalently-linked subunits: alpha (an alpha-neurotoxic 3FTx), beta (PLA$_2$), and gamma (Kunitz peptide); in ratios of 1:1:4 [83]. This toxin does not affect the low threshold calcium channel currents or has any effect on potassium or sodium channels but is a potent voltage dependent, reversible blocker of high threshold calcium channel currents by binding to the extracellular face of the channel [84].

Such multimeric toxins, whether homomers or heteromers, raise intriguing co-evolutionary questions not only regarding fundamental geometric fits but also neofunctionalisation; such questions remaining largely unexplored and thus represent an exciting, virtually untapped area of toxin molecular evolutionary research.

3. Conclusion

Although molecular techniques have cast a whole new light on our understanding of the history of the venom system among reptiles, this review shows how little we still know about the fundamental evolution of these unique natural bioweapon systems or the molecular evolution of the associated toxins. We hope this review highlights areas of future research and stimulates further interest into this dynamic field.

Acknowledgments

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