Toxicon 59 (2012) 696–708

Contents lists available at SciVerse ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Novel transcripts in the maxillary venom glands of advanced snakes

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ARTICLE INFO

Article history:
Received 21 October 2011
Received in revised form 2 March 2012
Accepted 6 March 2012
Available online 20 March 2012

Keywords:
Protein
Venom
Evolution
Toxin
Phylogeny

ABSTRACT

Venom proteins are added to reptile venoms through duplication of a body protein gene, with the duplicate tissue-specifically expressed in the venom gland. Molecular scaffolds are recruited from a wide range of tissues and with a similar level of diversity of ancestral activity. Transcriptome studies have proven an effective and efficient tool for the discovery of novel toxin scaffolds. In this study, we applied venom gland transcriptomics to a wide taxonomical diversity of advanced snakes and recovered transcripts encoding three novel protein scaffold types lacking sequence homology to any previously characterised snake toxin type: lipocalin, phospholipase A_2 (type IIE) and vitelline membrane outer layer protein. In addition, the first snake maxillary venom gland isoforms were sequenced of ribonuclease, which was only recently sequenced from lizard mandibular venom glands. Further, novel isoforms were also recovered for the only recently characterised veficolin toxin class also shared between lizard and snake venoms. The additional complexity of snake venoms has important implications not only for understanding their molecular evolution, but also reinforces the tremendous importance of venoms as a diverse bio-resource.

1. Introduction

Venom has been a key evolutionary innovation underlying the diversification of the 90 million year old advanced snake clade. However, our understanding of the true diversity of frameworks utilized as toxin scaffolds has been hampered by a focus on characterizing new isoforms of known scaffolds, rather than searching for new scaffolds potentially representing new toxin classes.

Venoms evolve via a process by which a gene encoding for a normal body protein, typically one involved in key regulatory processes or bioactivity, is duplicated and the copy selectively expressed in the venom gland (Fry, 2005; Fry et al., 2009a). A consistent characteristic in toxin recruitment events is a bias towards cysteine-rich proteins, which confers stability to the molecular-scaffold. Proteins are sourced from multi-gene classes expressed in a wide-range of tissues and possessing diverse ancestral normal body functions (Fry, 2005). While some isoforms exhibit useful basal toxic activities, many of the duplicate genes have been mutated, resulting in protein neo-functionalisation and to confer new toxic bioactivities. 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 (Fry et al., 2003). While the molecular scaffold of the ancestral protein is conserved, derived activities emerge through mutations of the surface chemistry (de la Vega et al., 2003; Fry, 2005; Fry et al., 2003; Mouhat et al., 2004; Lynch, 2007; Casewell et al., 2011).

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doi:10.1016/j.toxicon.2012.03.005

As the primary role of snake venom is to aid prey capture it has been suggested that natural selection for the optimisation of venom to differing prey items (e.g. Pawlak et al., 2006) is the most likely evolutionary driving force responsible for generating venom variation (Barlow et al., 2009; Daltry et al., 1996; Gibbs and Mackessy, 2009; Gibbs and Rossiter, 2008). Well documented cases of resistance to envenoming in prey species (Biardi et al., 2006; Poran et al., 1987; Weissenberg et al., 1997) highlight the potential for evolutionary 'arms races' between venom toxicity and prey, resulting in selective pressures to overcome prey resistance. Additionally, the high metabolic cost associated with venom synthesis, evident from snake physiology (McCue, 2006), venom metering (Hayes et al., 1995) and loss of venomous function in snakes feeding on undefended prey (Fry et al., 2008; Li et al., 2005), likely produces a trade-off between synthesis and foraging efficiency.

Twenty two protein-scaffolds have long been known as having been recruited for use as toxin frameworks in snake venoms (Fry, 2005): three finger peptides, acetylcholinesterase, ADAM (a disintegrin and metalloprotease), AVIT, complement 3 (C3) (aka: cobra venom factor (CVF)), C-type natriuretic peptide, CRiSP (cysteine rich secretory protein), the crotasin-type of beta-defensin peptides, cystatin, endothelin, factor V, factor X, kallikrein, kunitz, L-amino oxidase, lectin, prokineticin, nerve growth factor, type IB PLA_2, type IIA PLA_2 , vascular endothelial growth factor, and a unique toxin type (waglerin) for which the corresponding endogenous peptide remains to be elucidated. Most of these are very well characterized although some such as acetylcholinesterase remain enigmatic. Hyaluronidase activity has been known for reptile venoms but the corresponding sequences have only recently been determined (Harrison et al., 2007; Casewell et al., 2009; Fry et al., 2008, 2010a,b). Additional frameworks have recently been shown

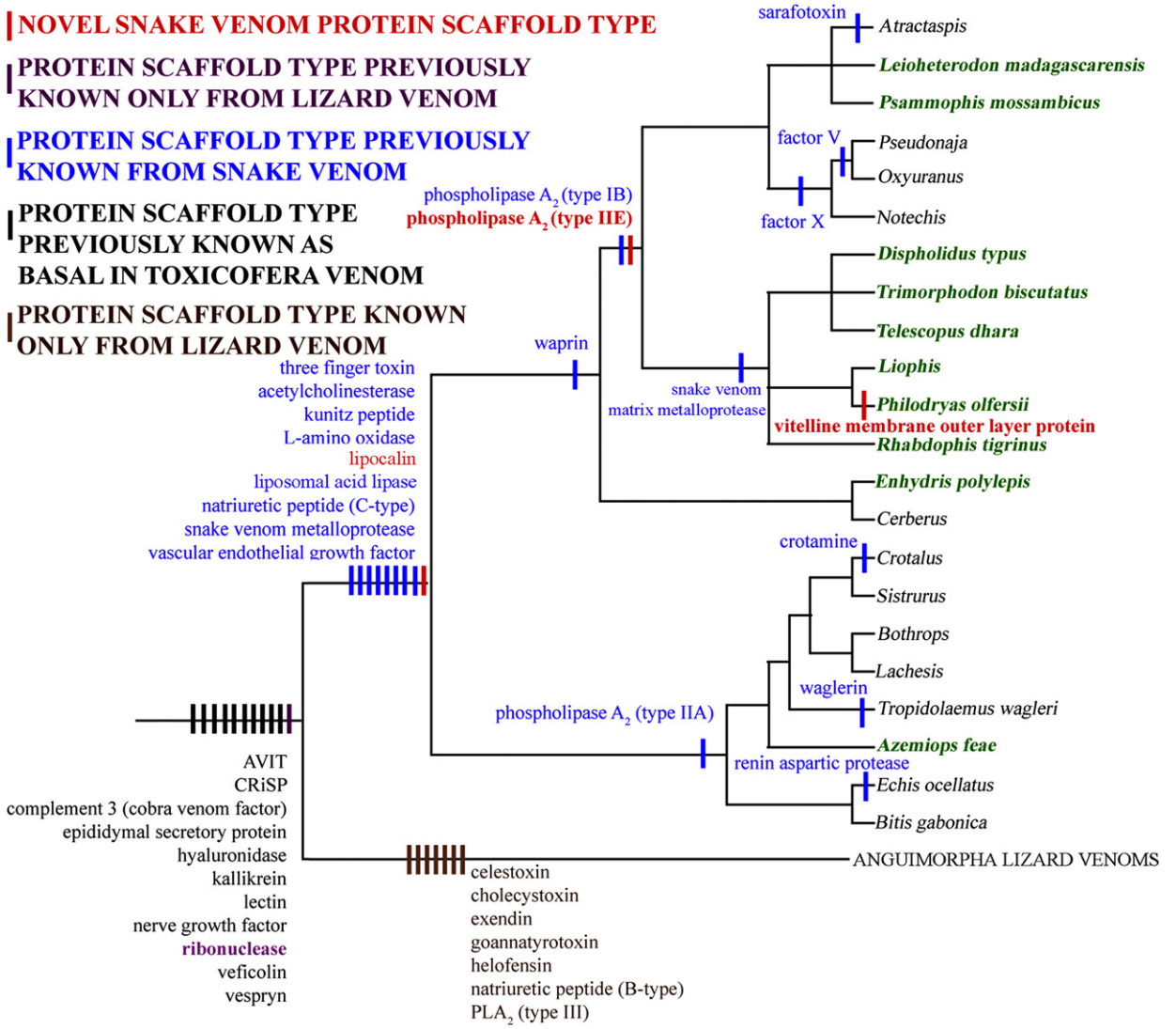


Fig. 1. Cladogram of evolutionary relationships of representative advanced snakes (Vidal ref) showing relative timing of toxin recruitment events. Species from this study are shown in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to be utilised in snake venoms but most of which with relative toxicities remaining to be elucidated: matrix metalloprotease (Ching et al., 2012), phosphodiesterase (Pahari et al., 2007; Santoro et al., 2009), veficolin (Ching et al., 2006; OmPraba et al., 2010; Fry et al., 2010a,b), vespryn (Pung et al., 2005; Fry et al., 2006) and waprin (Torres et al., 2003; Nair et al., 2007; Fry et al., 2008). Several of these novel scaffolds are shared with the venomous lizards as part of the core chemical composition of Toxicoferan reptiles (Fry et al., 2006, 2008, 2009a,b, 2010a,b): AVIT, CRiSP, C3/CVF, hyaluronidase, kallikrein, lectin, NGF, veficolin and vespryn (Fig. 1). These proteins were recruited from disparate tissues (Table 1) with diverse ancestral activities (Table 2).

As transcriptome analysis by random sequencing of venom gland cDNA libraries has proven an effective way of discovering toxins of new protein classes (*c.f.* Ching et al., 2012; Fry et al., 2006, 2008, 2009b, 2010a,b; Junqueira-de-Azevedo and Ho, 2002; Wagstaff and Harrison, 2006), we investigated a wide taxonomical diversity of advanced snakes utilising this method. Our discovery of novel over-expressed protein classes within the transcriptomes, thus representing putative new toxin types, provides additional insights into the molecular evolutionary origins of snake venoms.

2. Materials and methods

2.1. Species

Tissues were obtained from captive specimens with at least two used from each specimen, with tissues extracted four days after milking (AEC approval UM03216): Colubridae – *Dispholidus typus* (Tanzania founder stock), *Telescopus dhara* (Egypt founder stock), *Trimorphodon biscutatus* (Arizona founder stock); Dipsadidae – *Liophis miliaris* (Brazil founder stock), *Liophis poecilogyrus* (Brasil founder stock), *Philodryas olfersii* (Argentina founder stock); Homalopsidae – *Enhydris polylepis* (Darwin, Australia founder stock); Lamprophiidae – *Leioheterodon madagascarensis* (Madagascar founder stock), *Psammophis mossambicus* (Tanzania founder stock); Natricidae – *Rhabdophis tigrinus* (Hunan, China founder stock); Viperidae – *Azemiops feae* (Guizhou, China founder stock).

2.2. cDNA library construction and analysis

In order to investigate the relationship between toxins and the encoding transcripts, and also facilitate molecular evolutionary analyses, venom gland cDNA libraries were

Table 1
Secretion locations of nearest relations of previously characterized snake venom proteins.

Protein type [toxin class if known by specific name]	Tissue type for normal secretion
3FP [3FTx]	Brain
ACN	Muscle
ADAM [SVMP]	Variety of tissues including epididymis, colon, lung, lymph node and thymus.
C3 [CVF]	Liver
CNP	Heart
CRiSP	Myriad of exocrine tissues including salivary.
Crotasin-type beta-defensin [crotamine]	Brain
Cystatin	Restricted to the stratum granulosum of normal skin, the stratum granulosum/spinosum of psoriatic skin, the secretory coils of exocrine sweat glands with low expression levels also found in the nasal cavity
Endothelin [sarafotoxin]	Endothelium
Factor V	Liver
Factor X	Liver
Ficolin [veficolin]	Peripheral blood leukocytes. Also detected in spleen, lung, and thymus, may be due to the presence of tissue macrophages or trapped blood in these tissues. Not detected on lymphocytes and granulocytes.
Hyaluronidase	Widely expressed
Kallikrein	Variety of exocrine tissues including pancreas as well as the salivary glands
Kunitz	Wide variety of tissues, including brain, conceptus membrane, lung, ovary, placenta, and uterus
LAO	Variety of exocrine and immune tissues
Lectin	Wide-spread
NGF	Wide variety of tissues including the brain, eye, prostate and salivary glands
Phosphodiesterase	Kidney
PLA_2 – type IB	Pancreas.
PLA_2 – type IIA	Synovial fluid
Prokineticin [AVIT]	Expressed at high levels in testis and at lower levels in brain, lung, ovary, spleen, thymus, and uterus
Renin-like aspartic protease	Kidney
Snake venom matrix metalloprotease	Macrophages and granulocytes
SPRY [vespryn]	Hemopoietic lineages.
VEGF	Various tissues ranging from the brain to the heart
Veficolin	Peripheral blood leukocytes. Also detected in spleen, lung, and thymus, may be due to the presence of tissue macrophages or trapped blood in these tissues. Not detected on lymphocytes and granulocytes
WAP [waprin]	Variety of tissues including lactating mammary gland, lung, ovary, and testis
[waglerin]	Unknown; no homology to any known peptide

3FP = three finger peptide; 3FTx = three finger toxin; ACN = acetylcholinesterase; CVF = cobra venom factor; LAO = l-amino oxidase; NGF = nerve growth factor; vascular endothelin growth factor; WAP = whey acidic peptide.

Table 2
Bioactivity of ancestors of previously characterized snake venom proteins.

Protein type [related toxin class if known by specific name]	Normal body function
3FP [3FTx]	Bind to the α7 nicotinic acetylcholine receptor
ACN	Rapidly hydrolyses choline released into the synapse, resulting in less neurotransmitter available for neuromuscular control.
ADAM [SVMP]	Enzymatic cleavage of the extracellular matrix
C3 [CVF]	Central to both classical and alternative complement pathways.
CNP	Lowers blood pressure mediated by the relaxation of vascular smooth muscle by binding the GC-B receptor and stimulating the intracellular production of cGMP
CRiSP	Specific actions largely uncharacterized
crotasin-type beta-defensin [crotamine]	Unknown
Cystatin	Inhibit cysteine proteases such as the cathepsins B, L, and S
Endothelin [sarafotoxin]	Potently vasoconstrictive, modulating the contraction of cardiac and smooth muscle
Factor V	Blood cofactor that participate with factor Xa to activate prothrombin to thrombin
Factor X	Vitamin K-dependent glycoproteins that convert prothrombin to thrombin in the presence of factor Va, calcium, and phospholipid during blood clotting
Ficolin [veficolin]	Involved in serum exerting lectin activity. Binds GlcNAc.
Hyaluronidase	Random hydrolysis of (1 → 4)-linkages between *N*-acetyl-beta-D-glucosamine and D-glucuronate residues in hyaluronate
Kallikrein	Release kinins from circulatory kininogen
Kunitz	Inhibit a diverse array of serine proteinases
LAO	Induce apoptosis in cells by two distinct mechanisms; one rapid and mediated by H2O2, the other delayed and mediated by deprivation of L-lysine
Lectin	Hemagglutination activity
NGF	Stimulate division and differentiation of sympathetic and embryonic sensory neurons
Phosphodiesterase	Cleaves a variety of phosphodiester and phosphosulfate bonds including deoxynucleotides, nucleotide sugars, and NAD
PLA_2 – type IB	Release of arachidonic acid from the *sn*−2positionoftheplasmamembranephospholipids
PLA_2 - type IIA	Release arachidonic acid from the *sn*−2positionoftheplasmamembranephospholipids, involvedininflammatory processesanddiseases,suchasrheumatoidarthritisandasthma
Prokineticin [AVIT]	Constriction of intestinal smooth muscle
Renin-like aspartic protease	Renin is a highly specific endopeptidase, whose only known function is to generate angiotensin I from angiotensinogen in the plasma, initiating a cascade of reactions that produce an elevation of blood pressure and increased sodium retention by the kidney. Cleavage of Leu-|-Xaa bond in angiotensinogen to generate angiotensin I.
Snake venom matrix metalloprotease	Proteolysis of the extracellular matrix
SPRY [vespryn]	Largely uncharacterized
VEGF	Increase the permeability of the vascular bed
Veficolin	Involved in serum exerting lectin activity. Binds GlcNAc
WAP [waprin]	Inhibit leukoproteinases
[waglerin]	Unknown; no homology to any known peptide

3FP = three finger peptide; 3FTx = three finger toxin; ACN = acetylcholinesterase; LAO = L-amino oxidase; NGF = nerve growth factor; vascular endothelin growth factor; WAP = whey acidic peptide.

constructed to obtain full-length toxin-encoding transcripts 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 GeneHogs (Invitrogen Corporation, USA). Isolation and sequencing of inserts was conducted at the Australian Genome Research Facility, using BDTv3.1 chemistry with electrophoretic separation on an AB330xl. 384 colonies were sequenced for each library, inserts screened for vector sequences and those parts removed prior to analysis and identification.

Toxin sequences were identified by comparison of the translated sequences with previously characterised toxins using BLAST search of the Swiss-Prot/Uni-Prot protein database (http://www.expasy.org/tools/blast/). Molecular phylogenetic analyses of toxin transcripts were conducted using the translated amino acid sequences. Comparative sequences from other venomous reptiles and outgroups were obtained through BLAST searching (http://www.expasy.org/tools/blast/) using representative toxin sequences. Resultant sequence sets were aligned using the program CLUSTAL-X, followed by visual inspection for errors. When presented as sequence alignments, leader sequence is shown in lowercase, prepro region underlined, cysteines highlighted in black and functional residues in bold. Datasets were analysed using Bayesian inference implemented on MrBayes, version 3.0b4. The analysis was performed by running a minimum of 1×10^6 generations in four chains, and saving every 100th tree. The log-likelihood score of each saved tree was plotted against the number of generations to establish the point at which the log likelihood scores of the analysis reached their asymptote, and the posterior probabilities for clades established by constructing a majority rule consensus tree for all trees generated after the completion of the burn-in phase. All previously known sequences are referred to by their uniprot accession numbers (http://www.uniprot.org/) while sequences obtained in this study are referred to by their Genbank accession numbers (http://www.ncbi.nlm.nih.gov/genbank/).

Table 3
Novel snake venom gland transcripts recovered in this study.

Molecular scaffold type	Snake species recovered from	Tissue type of ancestral protein	Bioactivity of ancestral protein	AA:cysteine ratio
Lipocalin	*Azemiops feae* (JQ340878), *Disphoids typus* (JQ340879), *Liophis miliaris* (*JQ354966*) *Rhabdophis tigrinus* (JQ340877), *Trimorphodon biscutatus* (JQ340880)	Preferentially synthesized in nonproliferating cells.	Preferentially binds long-chain unsaturated fatty; known allergen	25:1
Phospholipase A_2Type IIE	*Dispholidus typus* (JQ340882, JQ340883), *Leioheterodon madagascarensis* (JQ340884, JQ340885, JQ340886)	Restricted to the brain, heart, lung, and placenta.	Progression of inflammatory processes.	9:1
Ribonuclease	*Liophis poecilogyrus* (JQ340889, JQ340890, JQ340891, JQ340892, JQ340893), *Psammophis mossambicus* (JQ340894) previously known from the venomous lizards *Gerrhonotus infernalis* (E2E4J5, E2E4J7, E2E4J8, E2E4J9) and *Celestus warreni* (E2E4K1)	Expressed predominantly in the pancreas	Pyrimidine-specific C-preferring nuclease	27:1
Veficolin	*Enhydris polylepis* (JQ340895, JQ340896, JQ340897, JQ340898, JQ340899, JQ340900, JQ340902, JQ340903, JQ340904, JQ340905, JQ340906), *Philodryas olfersii* (JQ340901) (previously in the snakes *Cerberus rynchops* (D8VNS7, D8VNS8, D8VNS9, D8VNT0), *Micrurus coralinus* (MCOR0279S) and *Philodryas olfersii* (POLF0700S) plus the venomous lizard *Varanus komodoensis* (E2IYB3))	Peripheral blood leukocytes. in these tissues. Not detected on lymphocytes and granulocytes	Involved in serum exerting lectin activity. Binds GlcNAc.	41:1
Vitelline membrane outer layer protein	*Enhydris polylepis*	Oviduct; component of the outer membrane of the vitelline layer of the egg.	Function unknown	21:1

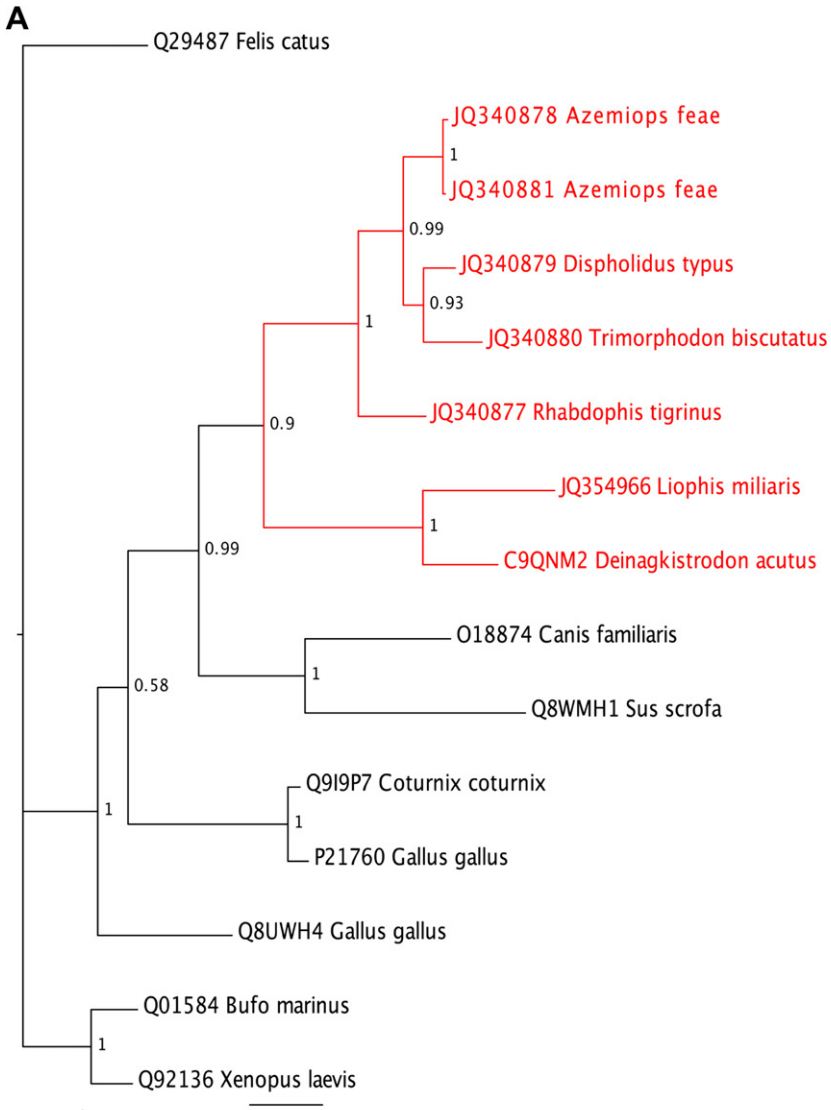
2.3. Molecular modeling of toxins

Phospholipase models were generated based on the assumption that homologous proteins share similar 3D-structures (Chothia and Lesk, 1986). In other words, the three-dimensional structure of a target protein can be modelled if its sequence is homologous to at least one template protein whose 3D-structure has been determined experimentally by applying either X-ray or NMR techniques (Greer, 1981). In this work aligning the protein sequences of target and template(s) was carried out in SPDBV (Guex and Peitsch, 1997) and the initial alignments were refined manually. From these alignments 3D-models were built directly in SPDBV applying the "Build Preliminary Model" option, which is disabled in the currently distributed public version of the software. Loops were built by scanning a database of known loop structures using the same software and suitable specimens were selected after visual inspection. The enthalpy of the resulting models was minimised applying two times 200 steps of Steepest Descent minimisation. Finally, the quality of each model structure was assessed in iMolTalk (Diemand and Scheib, 2004) and a Van-der-Waals surface was calculated in MolMol (Koradi et al., 1996). In MolMol electrostatic potentials were calculated applying the "simplecharge" command and mapped on the model structure surface. The families of venom proteins were analysed by superimposing the structures in SPDBV (Guex and Peitsch, 1997) and conserved and variable structural regions were identified. In the molecular modelling of representative proteins, blue surface areas indicate positive charges, red negative charges and model pairs show to sides of the protein rotated by 180°.

3. Results and discussion

Translated BLAST analyses of transcripts recovered from the venom gland cDNA libraries identified three novel protein scaffold types (Table 3) lacking sequence homology to any previously characterised snake toxin type (Table 1): lipocalin (Fig. 2), phospholipase A_2 (type IIE) (Fig. 3) and vitelline membrane outer layer protein (Fig. 4). Lipocalin had been previously deposited in the Uni-Prot database (accession C9QNM2 from *Deinagkistrodon acutus*) or in the GenBank (accession JQ354966 from *Liophis miliaris*) but not published. In addition, multiple isoforms were sequenced of the only recently discovered veficolin toxin class shared between lizard and snake venoms (Ching et al., 2006; OmPraba et al., 2010; Fry et al., 2010a) and the potential new toxin class ribonuclease previously only sequenced from lizard venoms (Fry et al., 2010a) (Fig. 5) (Figs. 6 and 7). Notably, phylogenetic analyses of these novel/recently-discovered protein scaffolds revealed monophyletic relationships of sequences sampled from the venom glands of multiple advanced snake species and, in some cases, lizards venom glands as well. The use of a well-defined species relationship (Fig. 1) facilitates mapping the likely timing of the recruitment of these novel protein scaffolds into the venom gland.

As with previously characterized known toxins types (Fry, 2005), all the novel protein scaffolds shared the characteristic of extensive cysteine cross-linking and were from multi-gene families (Table 3). The PLA_2 (Fig. 3) and vitelline (Fig. 4) scaffolds in particular showed extensive secondary structural modifications (alpha-helices and beta-sheets respectively) that would confer stability to the molecules. The nearest known normal body homologues



B

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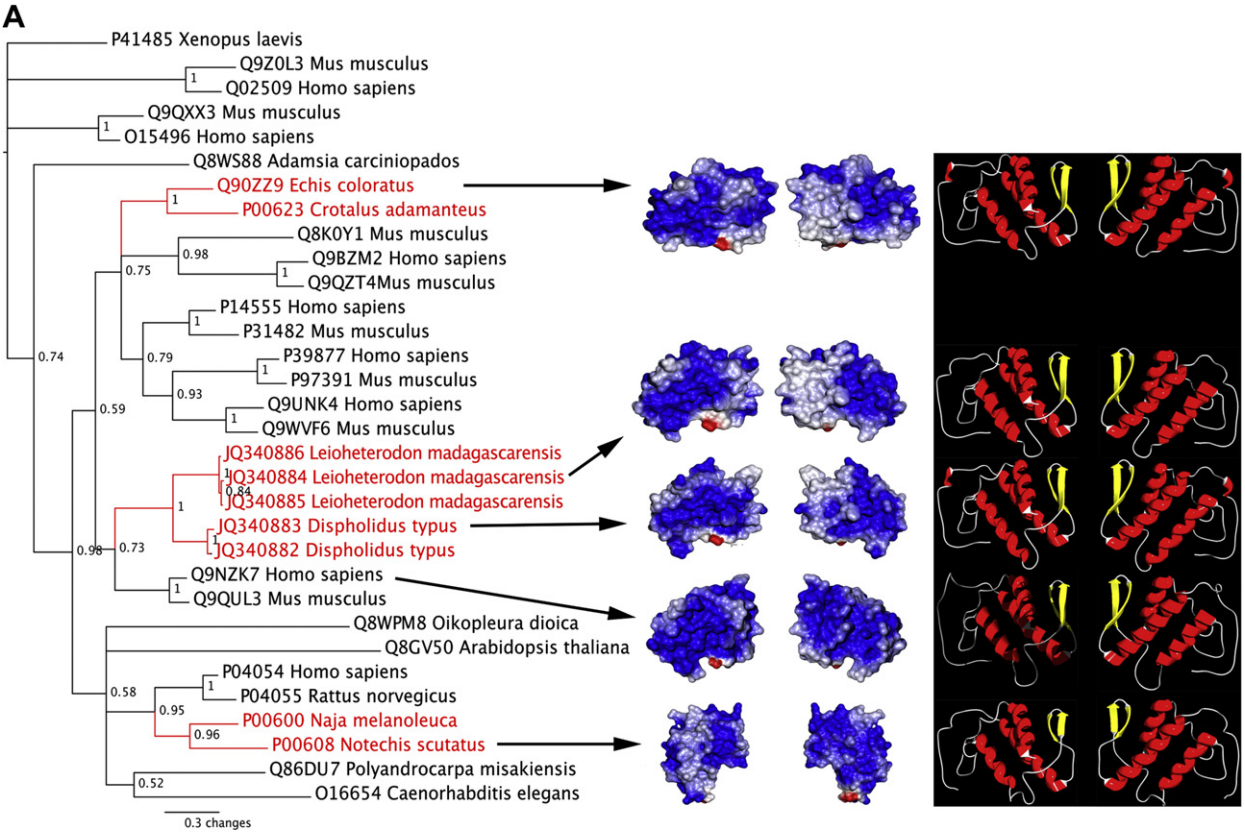
          10        20        30        40        50        60        70        80        90
1. mkswlfalglalccvvqaewKEVTI----TSNKEMGRWITFAIACNSISCLDQIRSLSAFIAEVSQPEEDVFSILVLIHMPDG-CKNVTYQLKK
2.                            >SNKEMGRWITFAIACNSISCLEQIRSLSAFIAEVSQPEEDVFSILVLIHMPDG-CKNVTYQLKK
3. mkslllalglalcyvaqaewKVVIT----TNQENLGRWMTTAVACNSRDTLEQIRGMPAFIADLSRPQEDVFSILAYIPMPDG-CKNVTYQFRK
4. mksllfalglalccfvqaewKEVNT----TSIEDLGRWVIFAVACNSSDTMKVIRKMPAFIAELSRPQEDVLNVVVFLPVPEG-CKNVTGQIRK
5. mksvlftlglafccflqaegEEVTP----NKAQCLGVWVSHAVACSSENHMEQLRGMSMFVTDLSEPRKDVFRISVLVPMPDG-CRNVTHEFRK
6. mrtlalslalallcllhteaaaTVP----DRSEVAGKWYIVALASNTDFFLREKGKMKMVMARISFLGEDELEVSYAAPSPKG-CRKWETTFKK
7. mmrillalslgvaccslwvgaEVQVQPDFQKEKVLGKWYGIGLASNSNWFKDRKSHMKMCTTIITPTADGNVEVTATYPKMDR-CETKSMTYFK
8. mkslllslvlglvca-------QEPQPEQDPFELSGKWITSYIGSSDLEKIGENAPFQVFMRSIEFDDKESKVYLNFFSKENGICEE-FSLIGT

          100       110       120       130       140       150       160       170       180
1. GED-GKYRFLFD----ETVVTVESMKVQDEYVMSTINV---NNEFKTTTPHGRKVTQDPEILQKFKDECKKLGYKEDEIVVLNPTVKCQ
2. GED-GKYRFLFD----ETVVTVESMKVQDEYVMSTINV---NNEFKTTTLHGRKVTQDPEMLQKFKDECKKLGYKEDEIVVLNPTVKCQ
3. GED-GKYHGASD----DTKVTVESVKAYGEFIMTVINV---EDHFRTSSLHGRKVTQDPEILQKFKEECKKLGYSEDEIVILNPTVKCQ
4. GED-GKYHSLSD----NTTVTVKSVKSLNNFIMTTLE----SDNFRSTTLHSKKLTQDPEIIQKFKEECKKLGYSEDQIAVLNPTVKCQ
5. EEN-GIYCFNSG----DTKVTVESVKVEESFVISTLSVKDSANEFITTILHSRQETQDPEMIQKFKDECKKKSYPDDQVVVLSPPVKCR
6. TSDDGELYYSEE---AEKTVEVLDTDYKSYAVIFATRVKDG-RTLHMMRLYSRSREVSPTAMAIFRKLARERNYTDEMVAVLPSQEECSVDEV
7. TEQLGRFRAKSPRYGSEHDMRVVETNYDEYILMYTVKTKGS-ETNQIVSLFGRDKDLRPELLDKFQNFAKSQGLADDNIIILPHTDQCMTEA
8. KQEGNTYDVNYAG----NNKFVVSYASETALIISNINVDEEGDKTIMTGLLGKGTDIEDQDLEKFKEVTRENGIPEENIVNIIERDDCPAK
```

Fig. 2. Lipocalin A) bayesian phylogenetic reconstruction and B) alignment of maxillary venom gland sequences 1. JQ340878 and 2. JQ340881 from *Azemiops feae*, 3. JQ340879 from *Dispholidus typus*, 4. JQ340880 from *Trimorphodon biscutatus*, 5. JQ340877 from *Rhabdophis tigrinus*, 6 JQ354966 from *Liophis miliaris* and 7. C9QNM2 from *Deinagkistrodon acutus* and the representative normal body molecules 7. P21760 from *Gallus gallus*, 8. Q92136 from *Xenopus laevis*, 9. Q8WMH1 from *Sus scrofa*. gt; designates incomplete N-terminal.



B

```
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2.  marplsgsslllllcvlafasaNLIQFGVMIRGLT-GRNG-LDYNGYGCHCGWGGSGQPVDDTDWCCHVHDCGYEAVSLRK-CHP
3.  marplcgislvl-llsvlvfasaNLIQFGWIIKHVT-GRQ-ALIYNGYGCYCGWGGSRQPVDDTDWCCQVHDCGYGALSLRH-CKP
4.  marplcgislvl-llsvlvfasaNLIQFGWIIKHVT-GRQ-ALIYNGYGCYCGWGGSRQPVDDTDWCCQVHDCGYGALSLRQ-CKP
5.  marplcgislvl-llsvlvfasaNLIQFGWIIKHVT-GRQ-ALIYNGYGCYCGWGGSRQPVDDTDWCCQVHDCGYGALSLRQ-CKP
6.  mksphvlvflcl----lvalvtgNLVQFGVMIEKMT-GKS-ALQYNDYGCYCGIGGSHWPVDQTDWCCHAHDCGY-GRLEKLGCEP
7.  mkppialaclcl----lvplaggNLVQFGVMIERMT-GKP-ALQYNDYGCYCGVGGSHWPVDETDWCCHAHDCGY-GRLEKLGCDP
8.                      ~ NLYQFKNMIQCTVPNRS-WWHFANYGCYCGRGGSGTPVDDLDRCCQIHDNCYGEAEKISGC--
9.                      ~ NLVQFGKMIECAIRNRRPALDFMNYGCYCGKGGSGTPVDDLDRCCQVHDECYAEAEK-HGCYP
10. mkllvlavlltvaaa-DSGISPRAVWQFRKMIKCVIPGSDPFLEYNNYGCYCGLGGSGTPVDELDKCCQTHDNCYDQAKKLDSCKF
11. mrtlwivav-------wlmsvegNLYQFGKMIKNKT-GKPAMFSYSAYGCYCGWGGQGKPQDASDRCCFVHDCCY-TRVN--DCSP
12.                     ~SLVQFETLIMKVA-KRSGLLWYSAYGCYCGWGGHGRPQDATDRCCFVHDCCY-GKAT--NCNP
13. mktllllav-imif--gllqahgNLVNFHRMIKLTT-GKEAALSYGFYGCHCGVGGRGSPKDATDRCCVTHDCCY-KRLEKRGCGT

          90        100       110       120       130       140       150       160       170
1.  --KLV---TYFYSGGKD-TITCLGGNE-CQRQTCECDKAAALCFRR--STYRDQYYGYAANK-CQGASPPCANTKRG
2.  --KLV---TYFYSSGKN-TITCLGGNE-CQRQTCECDKAAAICFRR--STYRDQYYGYAANK-CQGASPPCANTKRG
3.  --KIE---KYSYSTEED-TVTCGGETE-CQRETCECDKAAALCFRR--SAFQNRYIAYPDFL-CLGTAPPCEGVRRRSAPTKWGSTP
4.  --KIE---KYSYSTEED-TVTCGGETE-CQRETCECDKAAALCFRR--SAFQNRYIAYPDFL-CLGTAPPCEGVRRRSAPTKWG
5.  --KIE---KYSYSTEED-TVTCGGETE-CQRETCECDKAAAICFRR--SAFRNRYIAYPDFL-CLGTAPPCEGVRRRSAPTKWG
6.  --KLE---KYLFSVSE-RGIFCA-GRTTCQRLTCECDKRAALCFRRNLGTYNRKYAHYPNKL-CTGPTPPC
7.  --KLE---KYLFSITR-DNIFCA-GRTACQRHTCECDKRAALCFRHNLNTYNRKYAHYPNKL-CTGPTPPC
8.  ---WPYIKTYTYESCQGTLTSCGANNK-CAASVCDCDRVAANCFARA--TYNDKNYNIDFNARCQ
9.  ------SLTTYTWECRQVGPYCNSKTQ-CEVFVCACDFAAAKCFAQE--DYNPAHSNINTGERCK
10. LLDNPYTHTYSYS-CSGSAITCSSKNKECEAFICNCDRNAAICFSKA--PYNKAHKNLDTKKYCQS
11. --KMT---SYSYSFEN-RDIICG-DDDSCRKAVCECDREAAICLGENVNTYDEKYRFYSSSH-CVEETEQC
12. --KTV---SYTYSEEN-GEIVCG-GDDPCGTQICECDKAAAICFRDNIPSYDNKYWLFPPKD-CRQEPEPC
13. --KFL---SYKFSNSG-SRITCAK-QDSCRSQLCECDKAAATCFARNKTTYNKKYQYYSNKH-CRGSTPRC
```

Fig. 3. Phospholipase A_2 A) bayesian phylogenetic reconstruction and B) alignment of representative sequences 1. JQ340882 and 2. JQ340883 type IIE from *Dispholidus typus* maxillary venom gland cDNA library, 3. JQ340884, 4. JQ340885 and 5. JQ340886 type IIE from *Leoiheterodon madagascarensis* maxillary venom gland cDNA library, 6. *Homo sapiens* type IIE (Q9NZK7) from brain, heart, lung, and placenta, 7. *Mus musculus* type IIE (Q9QUL3) highly expressed in uterus, and at lower levels in various other tissues, 8. the type IB (P00600) from *Naja melanoleuca* maxillary venom gland, 9. the type IB (P00608) from *Notechis scutatus* maxillary venom gland, 10. *Homo sapiens* type IB (P04054) from the pancreas, 11. type IIA (Q90ZZ9) from *Echis coloratus* maxillary venom gland , 12. type IIA (P00623) from *Crotalus adamanteus* maxillary venom gland, 13. type IIA (P14555) from *Homo sapiens* synovial fluid. In the sequence alignments "~" indicates N-terminal known only from protein sequencings. Clades with red lines are venom molecules. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

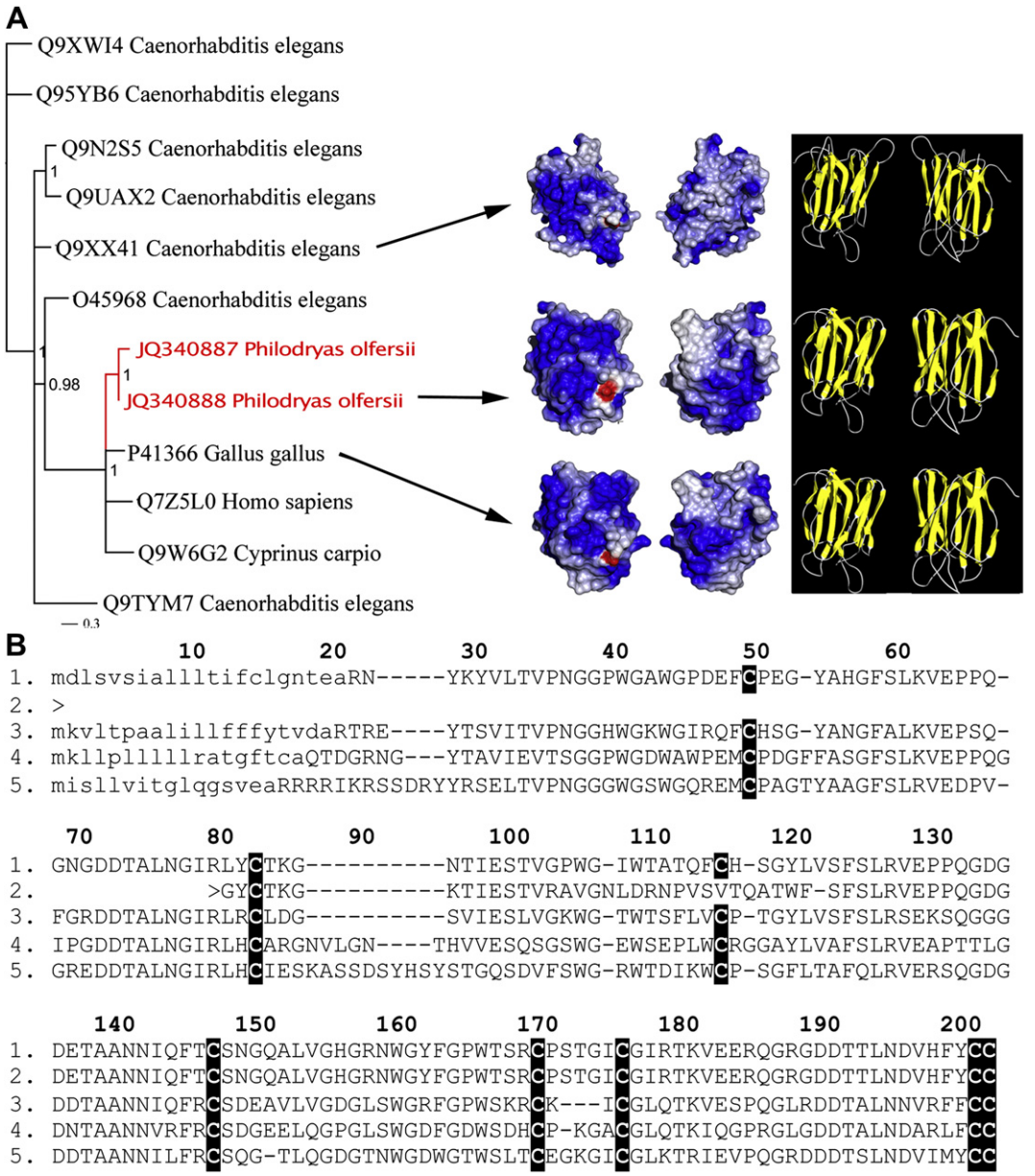
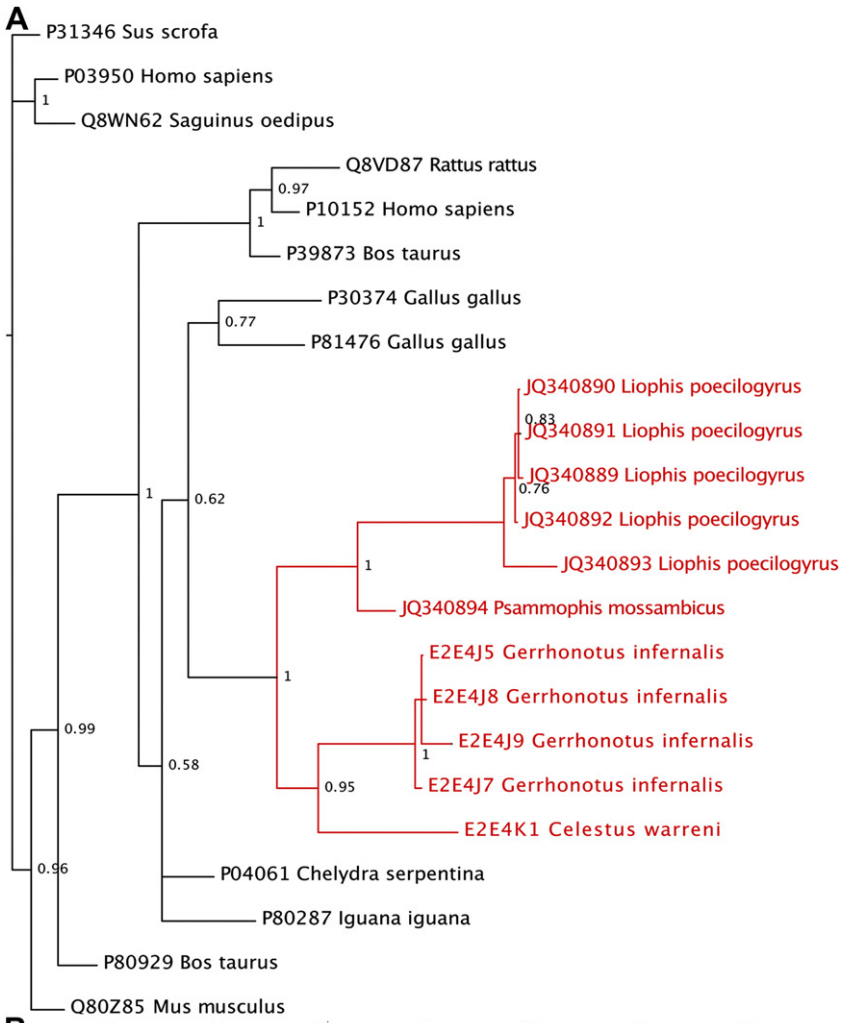


Fig. 4. Vitellin A) bayesian phylogenetic reconstruction and B) alignment of representative sequences 1. JQ340888 and 2. JQ340887 from *Philodryas olfersii* maxillary venom gland cDNA library, and the normal body molecules 3. P41366 from *Gallus gallus*, 4. Q7Z5L0 from *Homo sapiens*, 5. Q9W6G2 from *Cyprinus carpio*. > designates incomplete N-terminal of precursor.

for each of the novel protein types were from a wide range of tissues, ranging from the epididymus to the brain. The nearest normal body proteins also displayed a wide range of endogenous actions. Notably, lipocalin is a known allergen (*c.f.* Saarelainen et al., 2008) while Type IIE PLA_2 is known to be involved in the progression of inflammatory processes (*c.f.* Zhang et al., 2011). However, for vitelline membrane outer later protein the normal, functions have not been elucidated.

As well as changes in functional residues, neofunctionalisation may also be facilitated through changes in tertiary structure as a result of variations in the pattern and number of cysteine residues. Such cysteine changes were evident in lipocalin (Fig. 2), ribonuclease (Fig. 5), and veficolin scaffolds (Fig. 7). Within the lipocalin scaffolds, all venom gland isoforms contain four conserved cysteine residues, however the isoforms from *A. feae* have an additional, novel odd-number of cysteine residues, (five) while the *R. tigrinus* isoform has two novel-evolved cysteines. As with the form recently sequenced from lizard mandibular venom glands (Fry et al., 2010a), the ribonuclease sequences recovered from *L. poecilogyrus* and *P. mossambicus* have deletions of two ancestral cysteines present in the normal body form. The *E. polylepis* veficolin sequences



B

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      10        20        30        40        50        60        70
1.  mtakgihvlcmlfsfawivleseaTT---FDYNKNIDFPRTTTSRAVN-YCDNIIRAKNITNQKY--NVFIHEPE
2.  mtakgihvlcmlfsfawivleseaTT---FDYNKNIDFPRTTTSRAVN-YCDNIIRAKNITNQKY--NVFIHEPE
3.  mtakgihvlcmlfsfawivleseaTT---FDYNRNVDFPRTTTSRAVN-YCDNIIRAKNITNQKY--NVFIHEPE
4.  mtakgihvlcmlfsfawivleseaTT---FDYNKNIDFPMTKTSRAVN-YCDNIIRAKNITNQKY--NVFIHEPE
5.  mtakgihvlrmlfsfawivleseaVFYKPFDYIKNVDYPRPNGSAVVRDYCAYLIKAKNITDKKA--NVFINEPE
6.  maskgihllcllfslawivleteaDNYG-TLKKKHWDYPRTTPPKGVS-YCNYMMKSRRINYLRF--NIFIHASE
7.  maakgscvfwfllasawivmksdaADTFESFKELHVDYPKTEAPNDNE-YCKKVMGGRGQTKLKA--NTYIHAPD
8.  maakgscvfwfllasawivmksdaADTFESFKELHVDYPKTGAPNDDE-YCKKVMGGRGRTKLKA--NTYIHAPD
9.  maakgscvfwfllasacivmksdaADTFESFKELRVDYPKTEAPNDNE-YCKKVMRGRGQTKLKA--NTYIHAPD
10. maakgscvfwfllasawivmksdaADTFESFKELHVDYPKTEAPNDNE-YCKKVMGGRGQTKLKA--NTYIHAPD
11. maakgpcllwlfllftcmtvrteaDHDFEYFQKHHVDFPKPGTWNDDV-YCRMILYKRCIGFGLE--WTYIHATE
12. mvmglgvlllvfvlglgltpptlaqDNSRYTHFLTQHYDAKPQGRDDR-YCESIMRRRGLTSPCKDINTFIHGNK

      80        90        100       110       120       130       130
1.  NVIKNLCPDG---YTGPVRTP-ELHVTL--CR-YLYG--YLVITGRYPLA----IYCENGLITEYSLTLRLGGKL
2.  NDIKNLCPDG---YTGPVRTP-ELHVTL--CR-YLYG--YLVITGRYPLA----IYCENGLITEYSLTLRLGGKL
3.  NVIKNLCPDG---YTGPVRTP-VLHVTL--CR-YLYG--YLVITGRYPLA----IYCENGLITEYSLTLRLGGKL
4.  NVIKNLCPDG---YTGPVRTP-ELHVTL--CR-YLYG--YLVITGRYPLT----IYCENGLITEYSLTLRLGGKL
5.  NVIKSLCPDR---YTGPVETP-VLKLTA--CR-YFGN--YLVILGTYGLS----IYCENGRITGYYLKLRIGGKL
6.  HQLKNICPKY---DDGEWTTDNPFSITT--CTGHVDD--YSTTRSTIMIV----VFCRKGLPIEFRALAP
7.  SELLAACNRKKYKLNHEYGRTSRLPTTL--CT-YGDRV-FLGSSLPGTIK----VLCVNGKPVAFRGFNA
8.  SELLAACNRKKYKLNHEYGRTSRLPTTL--CT-YGDRV-FLGS<
9.  SELLAACNRKKYKLNHEYGRTSRLPTTL--CT-HDDGR-FFGSSLPGTIK----VLCVNGKPVAFRGFTA
10. SELLAACNRKKYKLNHEYGRTSRLPTTLYGCKGILCVLVPFGIRLHSDEE<
11. EQLKAICTVDGKRLTGAYRNLTPLPLAS--CYDTNNDYAFHGESLTGIFE----VQCKDGLPVTFLKFIK
12. RSIKAICENKNGNPHRENLRISKSSFQVTTCKLHGGSPWPPCQYRATAGFRNVVVACENGLPVHLDQSIFRRP
```

Fig. 5. Ribonuclease A) bayesian phylogenetic reconstruction and B) alignment of the venom gland sequences 1. JQ340890, 2. JQ340891, 3. JQ340892, 4. JQ340889, and 5. JQ340893from *Liophis poecilogyrus* maxillary venom gland, 6. JQ340894 from *Psammophis mossambicus* maxillary venom gland cDNA library, 7. E2E4J5, 8. E2E4J8, 9. E2E4J7 and 10. E2E4J9 from *Gerrhonotus infernalis* mandibular venom gland cDNA library, and 11. E2E4K1 from *Celestus warreni* mandibular venom gland cDNA library. Also shown is the normal body form P03950 from *Homo sapiens*. < designates incomplete C-terminal of precursor.

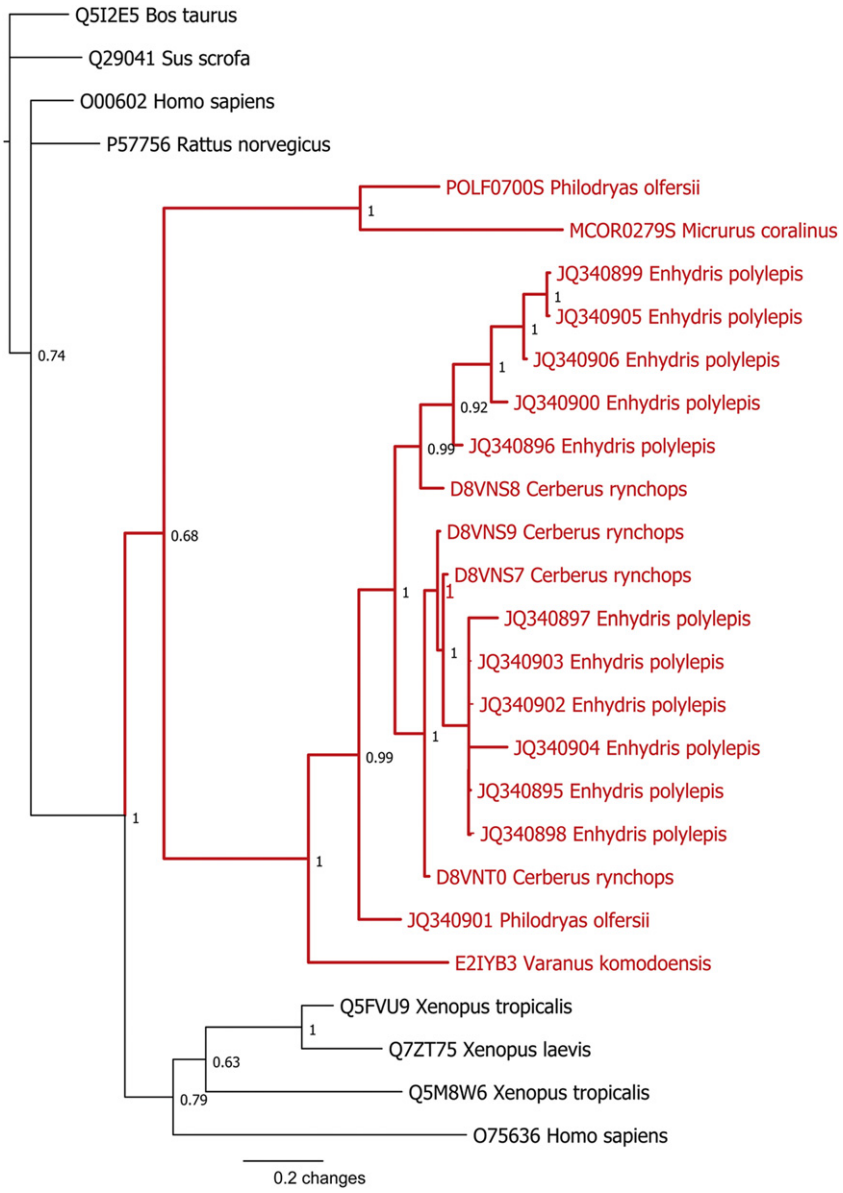

Fig. 6. Veficolin bayesian phylogenetic reconstruction.

also display significant changes to the cysteine patterns, including novel doublets in the N-terminal region. ENH005A01 also has an internal deletion near the C-terminal removing 48 residues (Fig. 7).

Previous work demonstrated that a core set of venom genes evolved in the common Toxicofera ancestor (Fry et al., 2006) and subsequent toxin recruitment events facilitated diversification into complex and varied venoms (Fry et al., 2008, 2009a,b, 2010a,b) (Fig. 1). Of the putative novel toxin types recovered in this study, ribonuclease had previously only been known from lizard mandibular venom gland transcriptome sequencing (Fry et al., 2010a). Thus this study potentially identified another further additional toxin types that share an ancient homology with toxins from lizard venoms and form part of the basal Toxciofera arsenal (Fig. 1, Table 3).

Two of the novel gene types recovered in this study (lipocalin and PLA_2) represent additional convergent recruitment events of the same gene type by independent lineages for use as a toxin-scaffold (Fry et al., 2009a). Lipocalin has been recruited into the venoms of bristle–insects, proboscis–insects, and ticks while PLA_2 toxins have been characterised from the venoms of cephalopods, cnidarians, bristle-insects, proboscis-insects, stinging-insects, scorpions, three times previously in reptiles (two of which were in snakes), and ticks (Fry et al., 2009a). Evidence of such convergent events reinforces the theory that certain structural or functional limitations select as to which protein-scaffolds are amenable for mutation as toxins.

Reflective of their convergent recruitment from a large multi-gene family with generic, conserved features, the two previously known PLA_2-type molecular scaffolds

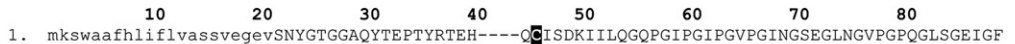

Fig. 7. Veficolin alignment of representative sequences: 1. JQ340897, 2. JQ340903, 3. JQ340902, 4. JQ340895, 5. JQ340898, 6. JQ340904 from *Enhydris polylepis* maxillary venom gland cDNA library, 7. D8VNS9, 8. D8VNT0, 9. D8VNS7 from *Cerberus rynchops* maxillary venom gland cDNA library, 10. JQ340899, 11. JQ340905, 12. JQ340906, 13. JQ340900, 14. JQ340896 from *Enhydris polylepis* maxillary venom gland cDNA library, 15. D8VNS8 from *Cerberus rynchops* maxillary venom gland cDNA library, 16. JQ340901 from *Philodryas olfersii* maxillary venom gland cDNA library, 17. E2IYB3 from *Varanus komodoensis* mandibular venom gland cDNA library, and 18. the normal body O00602 from *Homo sapiens*. In sequence alignments < designates incomplete C-terminal of precursor.

independently recruited in snake venoms (type IB and IIA) are known to display structurally and functionally important features. Most conserved is a binding site for calcium-ions, which is located in a conserved sequence patch around the first CXC-motif (see alignment Fig. 3). The residues interacting with the Ca^{2+}-ion are located in position 51, 53, 54, and 55 (numbers according to the multiple sequence alignment in Fig. 3), respectively. The region is structurally conserved, but sequences are not identical. We consider this not as important, since coordination with calcium happens with the backbone carbonyl-group. Hence, since the overall structure of the protein backbone around the Ca^{2+}-binding site is conserved, it should be irrelevant which amino acids sit in the respective positions.

The recovery of novel protein-scaffolds from the snake venom glands studied here reinforces how little is know about snake venom protein composition. This is underscored by the number and diversity of novel scaffolds recovered despite the relatively limited sampling employed. More extensive sampling will no doubt recover novel isoforms of toxin types identified to-date, potentially placing the toxin recruitment event as earlier in the organismal history, as well as entirely new toxin classes. Further work is also needed to examine the relative relationship between transcription and translation, and thus the presence and representation of the proteins themselves in the venom mixture. These results highlight the relatively untapped potential of such complex mixtures as sources of novel investigation ligands or lead compounds for use in drug design and development.

Acknowledgements

BGF was funded by the Australia & Pacific Science Foundation, the Australian Research Council and the University of Queensland.

Conflict of interest statement

There are no competing interests to declare.

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