

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

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# Chapter 3. Evolutionary Novelties in the Kunitz-type Toxins

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## Abstract

Kunitz-type toxins from snake venoms have undergone extraordinary structural and functional molecular adaptation. These toxins may have functions ranging from enzyme inhibitors to channel-blocking neurotoxins. However, their detailed evolutionary history is not known. We therefore conducted a large-scale phylogenetic and selection analysis to illustrate this issue. Phylogenetic analysis and analysis of selection revealed that the kunitz-type toxins evolved by gene duplication and rapid diversification. We show that the main ancestral function of kunitz-type toxins in Viperidae (plasmin inhibitors) is under neutral selection, while in non-vipers it is under purifying selection. We also show that neurotoxic kunitz-type toxins are found only in non-vipers; the various neurotoxic types clustered in distinct clades under positive selection. These results provide a detailed roadmap for future work to elucidate predator-prey evolutionary arms races, as well as documenting rich biodiscovery resources for lead compounds in the drug design and discovery pipeline.

Keywords: Kunitz, protease inhibitor, neurotoxin, evolution

#### Introduction

Toxins of the Kunitz type are interesting. According to their functions, they may be divided into two groups. Then there are the inhibitors of serine proteases and neurotoxins that block channels, such as dendrotoxins (DTX) and the B chain of bungarotoxin, constituting the second kind (BTX-b). The neurotoxins have a kunitz-like domain, although their protease inhibitory action is minimal. They are an example of dual convergence: not only have they been separately recruited into the venom arsenal of different species, but they are also convergently apotypic in multiple lineages for the same neurotoxic and coagulopathic properties (Beress 1982; Antuch, et al. 1993; Minagawa, et al. 1997; Harvey and Robertson 2004; Bayrhuber, et al. 2005; Honma and Shiomi 2006; Fry, Scheib, van der Weerd, Young, McNaughtan, Ramjan, Vidal, Poelmann and Norman 2008; Koludarov, et al. 2012).

Kunitz-type protease inhibitors attach to the active site of serine proteases predominantly through an exposed binding loop in a canonical configuration. P3, P2, P1, P1, P1, P2, and P3 (nomenclature according to Schechter and Berger) are the six residues that potentially interact with the enzyme in this area. A catalytic trio of residues on the enzyme's pocket (typically Ser, His, and Asp) is responsible for amide bond hydrolysis. In textilinin-1, this hexapeptide is Pro-Cys-Arg-Val-Arg-Phe (PCRVRF) (Flight, et al. 2009), whereas in aprotinin it is Pro-Cys-Lys-Ala-Arg-Ile (PCKARI) (Wells and Strickland 1994). The P1 residue, whose side chain protrudes into the specificity pocket of the protease, is Arg in textilinin-1 and Lys in aprotinin.

Despite the large size of the kunitz-type protease inhibitors, they can be divided into three categories based on their target: trypsin inhibitors (positively charged residues Lys/Arg preferred at P1), elastase inhibitors (small hydrophobic residues Ala/Val at P1), and chymotrypsin inhibitors (large hydrophobic residues Phe/Trp/Tyr/Leu/Val at P1) (Laskowski Jr and Kato 1980; Hedstrom 2002; Wang, et al. 2012). Met, Asn and His at the P1 site residues were also reported for chymotrypsin inhibiting (Laskowski Jr and Kato 1980; Scheidig, et al. 1997; Chang, et al. 2001; Chen, et al. 2001; Guo, et al. 2013). The interaction inhibitor: trypsin is essentially independent of the nature of the basic residue at P1 position, with no substantial changes in the association energies with trypsin following the mutation K15R in BPTI, according to analysis of different mutants of BPTI and other trypsin inhibitors (Navaneetham, et al. 2010). Inhibition of kallikrein prefers Arg over Lys (Fieldler 1987; Grzesiak, et al. 2000), but plasmin inhibition is increased by Lys at the P1 site (Van Nostrand, et al. 1995). A hydrophobic amino acid residue (Ala, Gly, or Phe) binds to the P1' site of venom trypsin/chymotrypsin inhibitors, with Ala being the most prevalent. AvKTI (Araneus ventricosus Kunitz-type serine protease inhibitor), which has the basic amino acid Lys (K) within the P1 site, has dual antitrypsin and antichymotrypsin action (Wan, et al. 2013). A snake venom serine protease inhibitor with Lys (K) at the P1 site and dual inhibitory action against trypsin and chymotrypsin was shown to have a similar effect (Guo, et al. 2013). Furthermore, the P1-P1 residues in AvKTI are Lys (K) and Ala (A), as shown for bovine pancreatic trypsin inhibitor (BPTI), which not only inhibits trypsin but also chymotrypsin, plasmin, and kallikrein. Kunitz peptides that inhibit plasmin are invariably dual-functional, with a P1 site of Arg (R) inhibiting both trypsin and plasmin. P1 and P1 are important residues. P1 fits into the pocket of human plasmin and interacts with six plasmin residues.

In this study, we applied the Bayesian method to reconstruct a large-scale phylogeny so as to reveal the evolution history of the snake venom kunitz-type toxins, particularly the evolutionary traits of the neurotoxic types. Analysis of selection was also utilized to evaluate the selection pressure on different toxin types.

#### **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. Using AliView and the MUSCLE method, the sequences were trimmed to only containing 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 '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) ( $\omega$ =dN/dS) for each codon in the alignment might reveal if a gene is undergoing rapid evolution or stays functionally restricted. Codons developing with  $\omega$  >1 are thought to have evolved under positive selection (functional diversity), whereas codons evolving with  $\omega$ <1 are thought to have evolved under purifying selection. Sites with a value of  $\omega$ =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.

Clade	Sequence for 3D modeling	PDB ID
Viperidae	Vipera_ammodytes_ammodytes_P00992	6a5i
DTX	Dendroaspis_polylepis_P00981	1dtk
BTX	Bungaru_multicinctus_Q1RPT0	1bun
non vipers	Bungarus_multicinctus_Q1RPT0	1bun
Viper type plasmin inhibitor	Vipera_ammodytes_ammodytes_P00992	6a5i
non-viper type plasmin inhibitor	Pseudonaja_textilisQ90WA1	5zj3

#### Table 1: Custom models for protein modelling.

#### **Results and Discussion**

Diverse kunitz peptides have been characterized as neurotoxins (Harvey and Karlsson 1980; Bohlen, et al. 2011; Baconguis, et al. 2014; Possani, et al. 1992), and our phylogenetic analysis combined with differences in sequence, structure, and function suggest that the evolution of this derived activity has occurred on four independent occasions (Figure 1). The new toxins include monomeric toxins and members of toxin complexes. Dendrotoxins are monomeric toxins from *Dendroaspis* venoms that selectively block Kv1.1 voltage-gated potassium channels (Harvey and Karlsson 1980). Kunitz peptides that are subunits of complex neurotoxins may be associated through non-covalent interactions (MitTx and taicatoxin) or covalently linked ( $\beta$ -bungarotoxin). Intriguingly, all such multimers are heteromeric and include PLA<sub>2</sub> toxins. MitTx is a complex of one kunitz subunit and two PLA<sub>2</sub> subunits that activates acid-sensing ion channel ASIC1 to cause intense pain as part of the defensive arsenal of *Micrurus tener* 

(Bohlen, et al. 2011; Baconguis, et al. 2014). Taicatoxin was discovered in the venom of *Oxyuranus scutellatus* and is a complex toxin consisting of one 3FTx subunit, one PLA<sub>2</sub> subunit, and 4 kunitz subunits that blocks cardiac voltage-dependent L-type calcium channels (Cav) (Possani, et al. 1992).  $\beta$ -bungarotoxins are voltage-gated potassium channels (Kv) blocking heterodimers consisting of a kunitz peptide disulphide-linked to a PLA<sub>2</sub> subunit via a newly evolved cysteine not found in other kunitz peptides, linked to a matching novel cysteine in the PLA<sub>2</sub> subunit that is also not found in other PLA<sub>2</sub> toxins. Our phylogenetic analysis indicates that the characteristic cysteine in  $\beta$ -bungarotoxin kunitz peptides evolved independently on two different occasions (Figure 1). In each case, the cysteine is in the same position, suggesting strong structural selection due to inter-chain structural constraints. However, consistent with the phylogenetic placement into two distinct clades, each type differs in the flanking amino acids. Intriguingly, there appears to have been a secondary loss of this trait occurring in the one of the kunitz peptide clades  $\beta$ -bungarotoxins, with the sequences C5H0E4 (Uniprot ID) and B4ESA2 (Uniprot ID) lacking the diagnostic and structurally necessary cysteine (Figure 1).

Other derived kunitz peptides have the pathophysiological action of inhibiting the clotting regulatory enzyme plasmin, which breaks down blood clots in the body. Unsurprisingly, plasmin inhibitors have been isolated and characterized from venoms which are powerfully procoagulant. The venoms allow the snakes to subjugate their prey by triggering the rampant production of endogenous thrombin, leading to the formation of enough blood clots to induce debilitating and lethal strokes. Such toxins have been welldescribed for the Daboia genus within the Viperidae family, and the Oxyuranus/Pseudonaja clade within the Elapidae family (Figures 2 and 3). While Daboia venoms produce procoagulant toxicity through the activation of Factor X and Oxyuranus and Pseudonaja through the activation of prothrombin, both converge on the same functional outcome: the production of high levels of endogenous thrombin which convert fibrinogen to fibrin. Phylogenetic analysis (Figure 1) reveals that they show convergent neofunctionalization of the kunitz peptide such that they inhibit plasmin, thereby prolonging the half-life of the blood clots formed by the venom. Both species also show convergent modification of the same key residue into an arginine, which has been shown to be critical for activity. Both mutants of plasmin inhibitors with only this amino acid changed and native isoforms lacking this arginine from Pseudonaja venom were found to not affect plasmin (Flight, et al. 2005; Flight, et al. 2009). Intriguingly, other phylogenetically distinct sequences contain this mutation (Figure 3), the majority of which are in species with procoagulant venoms, including Oxyuranus variants that are phylogenetically distinct from the functionally characterized plasmin inhibitors, suggesting that this genus may have evolved plasmininhibiting kunitz peptides on multiple occasions. However, functional studies are needed to confirm that these other arginine-containing peptides are indeed plasmin inhibitors.

Selection analyses revealed very different rates of molecular evolution for this toxin type within the snake families (Figure 4 and Table 2), strongly suggesting that there is a multiplicity of undocumented novel activities yet to be discovered across the full range of this toxin class. This is consistent of the documentation of three evolutions of neurotoxin function within just the elapid snakes. The differential rates between the monomeric dendrotoxins ( $\omega$ =2.10) and the disulphide-linked  $\beta$ -bungarotoxin subunits ( $\omega$ =1.23) is consistent with the structural constraints imposed upon the  $\beta$ -bungarotoxin subunits by being

not only part of a multi-subunit complex, but a disulphide-linked one at that. However, despite these structural constraints, the  $\beta$ -bungarotoxin subunits display evidence of individual sites under positive selection. In contrast to the neurotoxins, but consistent with the high structural conservation of the enzymatic pathophysiological target, both independent lineages of plasmin inhibitors are under negative purifying selection pressures (Figure 4 and Table 2).

Toxin group	ω	FUBAR(-)ª	FUBAR(+)⁵	MEMEc	FUBAR & MEME <sup>d</sup>
Viperidae clade	0.96	1	8	8	4
Non-viperid clade	1.18	5	10	13	6
NeurotoxinsDendrotoxins	2.10	0	6	7	3
NeurotoxinsBungarotoxins	1.23	15	25	30	22
Plasmin inhibitors Daboia	0.80	1	0	2	0
Plasmin inhibitors Oxyuranus/ Pseudonaja	0.62	2	0	0	0

 Table 2: Molecular evolutionary rates of kunitz peptides (See Figure 13 for structural models).

<sup>a</sup> Number of codons under negative selection according to FUBAR

<sup>b</sup> Number of codons under positive selection according to FUBAR

<sup>c</sup> Number of codons under episodic diversifying selection according to MEME

 $^{\rm d}$  Number of codons that fit criteria  $^{\rm b}$  and  $^{\rm c}$ 



**Figure 1:** Molecular phylogenetic reconstruction of kunitz peptide toxins. The convergent evolution of coagulotoxins are shaded in red, while the convergent evolution of neurotoxins is highlighted in blue. TCX= taicatoxin. DTX=Dendrotoxin. BTX-b=  $\beta$ -bungarotoxins (characterised by the presence of a novel cysteine). Sequence alignment used for constructing phylogenetic tree can be viewed in Supplementary File 3. For tree output file for kunitz toxins, see Supplementary File 4.

Dendroaspis jamesoni jamesoni GHPA01002366 polylepis GHPD01000414 Dendroaspis polylepis GHPD01001921 Dendroaspis polylepis GHPD01005441 Dendroaspis polylepis GHPD01002964 Bungarus multicinctus Q9W728 Bungarus multicinctus Q1RPS9 Bungarus multicinctus Q1RPS8 Oxyuranus scutellatus B7S4N9 Dendroaspis polylepis P00981 flaviceps D5J9R3 Bungarus flaviceps Q7T2Q6 flaviceps D5J9R2 Bungarus flaviceps D5J9R1 candidus Q8AY46 Micrurus tener G91929 Dendroaspis Bungarus Bungarus Bungarus

CLGVSL LEYS LVYP CSEYF RRT**C**VG NRVCHG CHE CHE HE HE NG GW RRA RRT TYN RRT RT **GYTKIPAFYYNQKKKQCEGFIWSGCGGNSNRFKTIEE** CKGNANRFSTIEK CRGNANRFKTIEE NGFVSAFYYSQKTNKCHSFTYGGCKGNANRFSTIEE GGNANRFKTIEE DVNQNHFTTMSE HGNANSFKTPDE KGNGNHFKTPQL GGNANRFKTIEE KDPYCNLPPDPGP-CHDNKFAFYHHPASNKCKEFVYGGCGGNDNRFKTRNK KGNGNHFKTPQL KGNGNHFKTPQL TGHIPAFYYNPQRKTCERFSYGGCKGNGNHFKTPQI INGNGNHFKSDHI NGNGNHFKTETI GGNDNRFKTRNF KASIPAFYYNWAAKKCOLFHYGG MFFISAFYYSKGSNKCYPFTYSG VHFFYGO CERFSYGG ERFSYGG KAFQYRG CERFSYGG KAFPYRG KRKIPSFYYKWKAKQCLPFDYSG EKFIYGG HDNKFAFYHHPASNKCKEFVYGG LPFDYSG RAAIPRFYYNPHSKO GPVRRAFYYDTRLKT KRKIPSFYYKWKAKQ GAFVDSYYFNRSRIT TGHIPAFYYNPQRKT TGHVPAFYYNPQRKT TGHIPAFYYNPQRKT GSVRRAFYYDTRLKT RORHRDCDKPPDKGN-KPRRKL**G**ILHRDPGR-AAKYGKLPLRIGP-AAKY<mark>C</mark>KLPLRIGL-LQHRTF<mark>C</mark>KLPAEPGP-RPSFCNLPVKPGP-QIRPAF**G**YEDPPFFQK RKRHH-GDKPPNKKR-RKRHPDCDKPPNKKR-RKRHPDCDKPPNKKR-RKRHPDGDKPPNKKR-RKRHPYGNLPPDPGP-RPYACELIVAAGP-KDRPKF<mark>C</mark>HLPPKPGP-RORHRDCDKPPDKGN-

potassium channel blocking dendrotoxins; Micrurus acid-sensing ion channel ASIC1 activating MitTx; Oxyuranus cardiac voltage-dependent Lype calcium channels (Cav) blocking taicatoxin; and Bungarus voltage-gated potassium channels (Kv) blocking bungarotoxins. The convergent Figure 2: Sequence alignment of representative derived neurotoxic forms of kunitz peptides. Shown are: Dendroaspis Ky1.1 voltage-gated evolution of interchain cysteines in the *Bungarus* sequences are indicated by different highlight colours.

Bungarus fasciatus B2KTG2	RERHPDCDKPPDTGRCEKNVRAFYYKPSAKRCVOFTYGGCNANGNHFKSDHLCRCECLEYP
Demansia vestigiata A6MGV1	KDRDEICKLEKERGEDESVLLVFVVNSVEHKCOTFHVGGCEGNENDEHTIFECKSTCAA
Demansia vestigiata A6MEL3	KDI DE LOKI DE DO CONTRA VENERO VENERO VENERO DE MARIA EL TERCESTO A F
Pseudechis australis OGTTB9	KDRDFCFL, DADTGPC, VGFDSFVYNDDEKKCLEFTYGGCGGNANNFTTKEECESTCAA
Ovvuranus microlenidotus OGITB5	
Oxyuranus microlepidotus OfITBA	KDEDECELDIGETCECEVINDEKKCLEFTVCCCECNINNFTWEECESTCA
Oxydranus microreproceds gorrby	
Oxydrands Scatellatus OfTER	NDEDFOIL CHARTER CAN AND EVALUATE FILL CONTRACT AND THE CONTRACT AND
Decudencia textilia 000WD0	
Pseudonaja textilis 000WN1	NDRFELCELFFDIGFCNVRFSFIINFDEVROLEFIIGGCEGNANNFIINFEGCESICAA
Pseudonaja textilis 090WAI	NDRFDFCELFADIGFCNVRFSFINSDEALLEFINGGEGNANNFINEEGESTGAA
Pseudonaja textilis Q90W96	NDRPKY CELPADIGCODT TGAY HISPREHECTEF I IGGCGONANNY NTOECCESTCAA
Pseudonaja textilis Q90W97	KDRPKFCELLPDTGSCEDFTGAFHISTRDRECIEFIIGGGGGNANNFITRECESTCAA
Pseudonaja textilis 090W98	KDHPKFCELPADTGSCKGNVPRFYINADHHQCLKFIIGGCGGNANNFKTIECKSTCAA
Pseudonaja textilis Q90W99	KDRPNFCKLPAETGRCNAKIPRFYYNPRQHQCIEFLYGGCGGNANNFKTIRECESTCAA
Protobothrops mucrosquamatus XP 015686853	KGRPEHCYLPADPGPCRANIRRFYYHSASNTCRPFTYGGCKGNANNFRTRDKCCKACI
Pseudechis porphyriacus B5L5R0	KDRPDFCELPDDRGPCEGIFHAFYYNPDQRQCLEFIYGGCYGNANNFKTIDECKRTCAA
Pseudechis porphyriacus B5G6G6	KDRPDFCELPDDRGPCEGIFHAFYYNPDQRQCLEFIYGGCYGNANNFKTIDECKRTCAA
Tropidechis carinatus Q6ITB0	KDRPDFCELPDDRGPC <mark>E</mark> GIFHAFYYNPDQRQCLEFIYGGCYGNANNFKTIDECERTCAA
Notechis scutatus B5L5R4	KDRPKFCELPADSGPCEGILRAFYYHPVHRTCQMFIYGGCYGNANNFKTIDECKRTCAA
Notechis scutatus Q6ITB2	KDHPEFCELPADSGPC <mark>R</mark> GILHAFYYHPVHRTCLEFIYGGCYGNANNFKTIDECEPPCAA
Oxyuranus scutellatus B5KL30	KDHPEFCELPADSGPC <mark>R</mark> GILHAFYYHPVHRTCLGFIYGGCYGNANNFKTIDECKRTCAA
Tropidechis carinatus B5KL33	KDHPEFCELPADSGPC <mark>R</mark> GILHAFYYHPVHRTCLEFIYGGCYGNANNFKTIDECKRTCAA
Notechis scutatus Q6ITB3	KDHPEFCELPADSGPC <mark>R</mark> GILHAFYYHPVHRTCLEFIYGGCYGNANNFKTIDECKRTCAA
Boiga trigonata Kunitz 00	QDRPKYCDLPSDHGPC <mark>R</mark> ARKTRFYYNSNSKQCVEFNYGGCRGNGNNFMSKDKCHYTCVGPPIRRLTRTGSSVST
Boiga trigonata Kunitz 01	QDRPKYCDLPSDHGPC <mark>R</mark> ARKTRFYYNSNSKQCVEFNYGGCRGNGNNFMSKDKCHYTCVGPPIRRLTRTGSSVST
Boiga dendrophila dendrophila Kunitz 01	QGRPKYCDLPADSGPC <mark>R</mark> AKKTHFYYNSDSKQCEKFTYGGCRGNGNNFKTKDKCHYTCVGSPIRRLTRIGSSVPA
Boiga dendrophila gemmicincta Kunitz 01	QGRPKYCDLPADSGPC <mark>R</mark> AKKTHFYYNSDSKQCEKFTYGGCRGNGNNFKTKDKCHYTCVGSPIRRLTRIGSSVPA
Boiga nigriceps Kunitz 02	RHRPKYCDLPADPGPCRAIKTHFYYNSDSKQCEKFTYGGCRGNENNFETKDKCHYSCVGPPIRRLTRIGSSVPA
Daboia siamensis A8Y7N4	QDRPKFCNLAPESGRC <mark>R</mark> GHLRRIYYNPDSNKCEVFFYGGCGGNDNNFETRKKCRQTCGAPRKGRPT
Daboia siamensis A8Y7N8	HDRPTFCNLAPESGRC <mark>R</mark> GHLRRIYYNPDSNKCEVFFYGGCGGNDNNFETRKKCRQTCGAPRKGRPT
Daboia siamensis A8Y7P3	HDRPTFCNLAPESGRCECHLRRIYYNLESNKCEVFFYGGCGGNDNNFSTRDECRHTCVGK
Daboia siamensis A8Y7P4	HDRPTFCNLAPESGRC <mark>R</mark> GHLRRIYYNLESNKCEVFFYGGCGGNDNNFSTWDECRHTCVGK
Daboia siamensis A8Y7N5	HDRPTFCNLAPESGRC <mark>R</mark> AHLRRIYYNLESNKCEVFFYGGCGGNDNNFSSWDECRHTCVGK
Daboia russelii H6VC06	HDRPTFCNLAPESGRCEAHLRRIYYNLESNKCEVFFYGGCGGNDNNFSTWDECRHTCVGK
Daboia siamensis A8Y7P6	HDRPKFCNLAPESGRCEGHLRRIYYNLESNKCKVFFYGGCGGNANNFETRDECRQTCGGK
Daboia siamensis A8Y7P0	HDRPTFCNLAPESGRCEGHLRRIYYNLESNKCKVFFYGGCGGNANNFETRDECROTCGGK
Daboia siamensis A8Y7P2	HDRPTFCNLAPESGRCEGHLRRIYYNLESNKCNVFFYGGCGGNDNNFETRDECROTCGGK
Daboia russelii H9CCK4	HDRPTFCNLAPESGRCEGHLRRIYYNLESNKCKVFFYGGCGGNDNNFETRDECROTCGGK
Daboia russelii 02ES47	ODRPKFCHLPVDSGIC AHIPRFYYNPASNOCOGFIYGGCGGNANNFETRDOCRHTCGGK
Daboia russelii O2ES46	ODRPKFCHLPVDSGICEAHIPRFYYNPASNOCOGFIYGGCEGNANNFETRDOCRHTCGASGKEGPRPRIASN
Oxvuranus scutellatus B7S4N9	ODRPKFCHLPPNPGPCRAAIPRFYYNPHSKOCEKFIYGGCHGNANSFKTPDECNYTCLGVSLPK
Notechis scutatus XP 026549839	ODRPKFCHLPANPGPCRATITRFYYNSDSKOCEKFTYGGCHGNDNNFETKDKCHYTCVGK
Austrelaps superbus B5L5M7	ODRPKFCHLPANPGPCRATITRFYYNSDSKOCEKFTYGGCHGNENNFETKDKCHYTCVGK
Telescopus dhara A7X3V4	ODRPKYCDLPADSGPCTARKRHFYYNSRSNOCEEFTYGGCRGNENNFETKDKCHYTCVAPPRRRPL
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**Figure 3:** Sequence alignment of representative plasmin-inhibiting derived forms of kunitz peptide toxins, with the functionally important arginine residue shaded in red. Procoagulant species with confirmed plasmin inhibiting activating are shaded in green. Procoagulant species which contain derived kunitz forms with the functionally important arginine but with phylogenetically distinct sequences that have not been functionally confirmed as plasmin-inhibiting are highlighted in yellow. *Pseudonaja* variants which lack the diagnostic arginine and have been bioactivity tested confirm the lack of plasmin inhibition activity are highlighted in gray.



**Figure 4:** Molecular modelling of kunitz toxins showing sites under selection by FUBAR (left) and MEME (right) colour coded to show sites that are negatively, neutrally, or positively selected. See Table 2 for values. Protein models show front and back views colored according to FUBAR's estimated strength of selection ( $\beta$ - $\alpha$ , left) and MEME's significance levels (right). Table 1 contains the information regarding template choice for each toxin subclass.

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