Evolution of molecular resistance to snake venom α-neurotoxins in vertebrates

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Chapter 5: Evolution of Molecular Resistance to Snake Venom α-Neurotoxins in Snakes, and the Broader Implications of this Thesis Research

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Abstract

Venomous snakes are important subjects of study in evolution, ecology, and biomedicine. Many venomous snakes have alpha-neurotoxins (α-neurotoxins) in their venom. These toxins bind the alpha-1 nicotinic acetylcholine receptor (nAChR) at the neuromuscular junction, causing paralysis and asphyxia. Several venomous snakes and their predators have evolved resistance to α-neurotoxins. The resistance is conferred by steric hindrance from N-glycosylated asparagines at amino acids 187 or 189, by an arginine at position 187 that has been hypothesized to either electrostatically repulse positively charged neurotoxins or sterically interfere with α-neurotoxin binding, or proline replacements at positions 194 or 197 of the nAChR ligand-binding domain to inhibit α-neurotoxin binding through structural changes in the receptor. In this chapter, we analyzed this domain in 76 snakes species, and assessed its amino acid sequences for resistance-associated mutations. We also looked for signals of selection in the sequences. Of the snake sequences analysed, 66 were sequenced *de novo*. This represents a major new data set studying toxin resistance in reptiles. We find widespread convergent evolution of the N-glycosylation form of resistance in several snake subfamilies, namely: Viperinae (5/6 species), Natricinae (3/3 species), Colubrinae (4/13 species), and Dipsadinae (1/5 species). We discuss our data in the context of the arms race model of co-ecvolution. We also make a sysntesis of the data in the context of the previous two experimental chapters. Looking at the broader implications of this thesis research, we show important venom resistance – or the lack of it - can be in the context of invasive snakes species. The work in this thesis underscores the inter-connectedness of the biosphere and the ripple effects that one adaption can have across global ecosystems.

Introduction

Venoms have evolved independently in multiple animal lineages (Casewell, Wüster, Vonk *et al.*, 2013; Fry, Roelants, Champagne *et al.*, 2009). When a venomous animal injects venom into a target animal (an event called 'envenomation'), venom toxins disrupt physiological processes, causing pain, incapacitation or death. The fitness costs associated with envenomation can spur a co-evolutionary "arms race" between predator and prey (Brodie & Brodie, 1999; Cott, 1940; Dawkins & Krebs, 1979; Thompson, 1999). The snake α-neurotoxins are members of the three-finger toxin (3FTx) family (Barber, Isbister & Hodgson, 2013; Chang, 1999; Dutertre, Nicke & Tsetlin, 2017; Utkin, Sunagar, Jackson *et al.*, 2015; Vonk, Casewell, Henkel *et al.*, 2013) and are major components of venoms from the families Elapidae and Colubridae (Bourne, Talley, Hansen *et al.*, 2005; Chang & Lee, 1963; Fry, Lumsden, Wuster *et al.*, 2003; Fry, Wuster, Ryan Ramjan *et al.*, 2003; Pawlak, Mackessy, Sixberry *et al.*, 2009; Suryamohan, Krishnankutty, Guillory *et al.*, 2020). Venomous snakes in these families are of considerable scientific interest, not least because they are responsible for numerous human fatalities (Chauhan & Thakur, 2016), and because they can cause ecological destruction as invasive species (Savidge, 1987).

In species susceptible to α-neurotoxins, the toxins bind to the nicotinic acetylcholine receptor (nAChR) α1-subunit (Figure 14A). A number of species that are frequently envenomated by elapids, including predators and prey, or the snakes when they accidently bite themselves, have evolved resistance to these toxins (Arbuckle, Rodriguez de la Vega & Casewell, 2017; Edmunds, 1974; Geffeney, Fujimoto, Brodie *et al.*, 2005; Takacs, Wilhelmsen & Sorota, 2001;

Toledo, Hanifin, Geffeney *et al.*, 2016; Ujvari, Casewell, Sunagar *et al.*, 2015; Venkatesh, Lu, Dandona *et al.*, 2005).

The mechanism of resistance in these cases is modification of the ligand-binding domain of the nAChR. For example, several studies demonstrate that the binding of α-neurotoxins is disrupted by glycosylation of asparagine residues. The NXS/T motif (where X = any amino acid except proline), is an indicator of N-glycosylation (Gavel & Heijne, 1990; Mellquist, Kasturi, Spitalnik *et al.*, 1998; Ohtsubo & Marth, 2006). Previous research has shown this motif to have evolved convergently in the Egyptian cobra (*Naja haje*) and its predator, the Egyptian mongoose (*Herpestes ichneumon*) but at different sites within the ligand-binding domain of the α-1 subunit. N-glycosylation at positions 187 (mongoose) and 189 (cobra) impedes binding via steric hindrance due to the long carbohydrate chain preventing docking by the α-neurotoxins, rendering both species resistant (Asher, Lupu-Meiri, Jensen *et al.*, 1998; Takacs *et al.*, 2001; Takacs, Wilhelmsen & Sorota, 2004). Additionally, mutations to the proline subsite of the ligand-binding domain of the mongoose nAChR (194L and 197H), and testing of an artificial variant of the mouse sequence with a 194S mutation, result in decreased α-neurotoxin affinity (Kachalsky, Jensen, Barchan *et al.*, 1995). This is presumably due to changes in the conformation of the binding pocket.

Other resistant animals, including the honey badger (*Mellivora capensis*), hedgehogs (*Erinaceus concolor* and *E. europaeus*), and pig (*Sus scrofa*), have independently evolved amino acid substitutions from an aromatic residue to the positively charged arginine at position 187, which greatly reduces the affinity of α-bungarotoxin (Asher *et al.*, 1998; Barchan, Kachalsky, Neumann *et al.*, 1992; Drabeck, Dean & Jansa, 2015; Fuchs, Barchan, Kachalsky *et al.*, 1993), possibly due to electrostatic repulsion of the positively charged neurotoxins.

However, recent modelling has suggested that this mutation may impart resistance due to steric hindrance instead (Rahman, Teng, Worrell *et al.*, 2020).

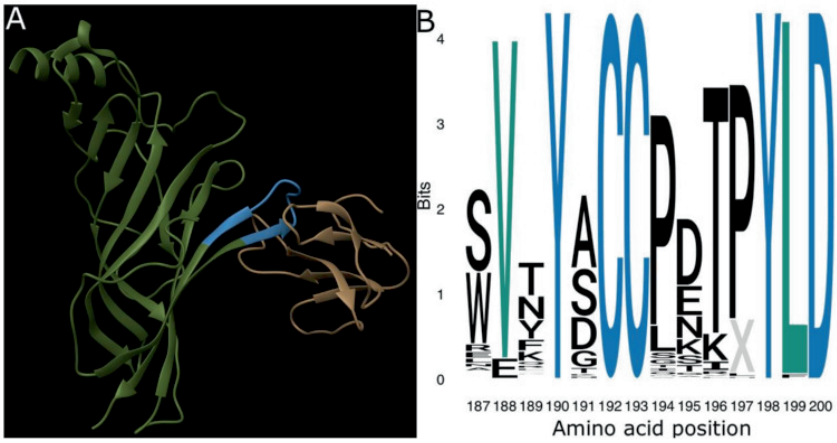


Figure 14. The ligand-binding domain of the nAChR. **A.** Ribbon model of α-bungarotoxin (brown) forming a complex with the ligand-binding domain (blue) on the extracellular domain of a single human α1-nAChR subunit (green). This structure is publicly available from the RCSB PDB under the ID 2QC1 (Dellisanti, Yao, Stroud et al., 2007a). B. Sequence logo showing the information value and amino acid content of the ligand-binding domain sequences in our dataset. Note the complete conservation of positions 190, 192, 193, 198, and 200 (blue) and strong conservation of positions 188 and 199 (teal) from (Khan, Dashevsky, Kerkkamp et al., 2020).

Table 11. Key sites and mutations that confer α-neurotoxin resistance.

Site	Mutation	Mechanism	Reference
187	NXS/T	Steric	(Barchan *et al.*, 1992)
	R	Steric	(Barchan, Ovadia, Kochva *et al.*, 1995)
189	NXS/T	Steric	(Takacs *et al.*, 2001)
194	L	Proline	(Kachalsky *et al.*, 1995)
	S	Proline	(Kachalsky *et al.*, 1995)
197	H	Proline	(Kachalsky *et al.*, 1995)

Given the convergence of these mutations across a diversity of resistant taxa, and in light of the trophic importance of snake venoms, we posit that α-neurotoxin resistance may be present in more species than is currently documented. In this study, therefore, we assess α1-nAChR sequences from 76 snake species for evidence of resistance mutations within the ligand-binding domain, with a particular focus upon the *N*-glycosylated asparagine form of steric hindrance. This is a far larger species sample, with a greater taxonomic range, than has previously been analysed. This allows us to look for multiple independent instances of evolutionary change and gain new insight patterns of resistance evolution. We examine amino acid sites associated with α-neurotoxin resistance (Table 11).

Material Methods

Ethics statement

All animal experimental procedures were conducted in accordance with local and international regulations. The milking of snakes for venom is not considered an animal experiment in accordance with the Experiments on Animals Act (Wet op de dierproeven, 2014), the applicable legislation in the Netherlands, and its implementation the European guidelines (EU directive no. 2010/63/EU). The milking was executed in a licenced establishment for the breeding and use of experimental animals, and subject to internal regulations and guidelines; advice was taken from the Leiden University Ethics Committee to minimise suffering. In the case of snake embryos used for DNA extractions, no license is required by Council of Europe (1986), Directive 86/609/EEC. DNA samples from the NIH in Pakistan were harvested under local regulations of the National Institute of Health Islamabad, Pakistan. No live animals in Australia were used; all samples studied were from existing tissue libraries (collected originally under University of Melbourne Animal Ethics approval number 03126).

Tissue samples

For the species examined, and the origins of the tissues samples see Table 12.

Table 12 Sequences included in this study, with accession numbers and species names. Key: 'Source' indicates the origin of the sequence or the DNA sample. The sequences determined by me de novo in this study and who sourced the DNA samples are listed in the column headed using the following abbreviations: BGF, Bryan G. Fry; FJV, Freek J. Vonk; HMIK, Harald M.I. Kerkkamp; JvT, Jory van Thiel; MAGdB, Merijn A.G. de Bakker; MAK, RMW, Roel M. Wouters, Muzaffar A. Khan and National Institute of

Health (NIH) Islamabad, Pakistan. The remaining sequences were obtained from NCBI (NCBI, National Center for Biotechnology Information, Bethesda, Maryland, United States).

Accession No.	Scientific Name	Common Name	Database	Tissue sample source
XM_018572442.1	*Nanorana parkeri*	High Himalaya frog	GenBank, NCBI	-
XM_002934601.5	*Xenopus tropicalis*	African clawed frog	GenBank, NCBI	-
XM_029605825.1	*Rhinatrema bivittatum*	Two-lined caecilian	GenBank, NCBI	-
XM_033946945.1	*Geotrypetes seraphini*	Gaboon caecilian	GenBank, NCBI	-
XM_030209957.1	*Microcaecilia unicolor*	Tiny Cayenne caecilian	GenBank, NCBI	-
XM_001514832.4	*Ornithorhynchus anatinus*	Platypus	GenBank, NCBI	-
XM_003763981.2	*Sarcophilus harrisii*	Tasmanian devil	GenBank, NCBI	-
XM_001376625.4	*Monodelphis domestica*	Gray short-tailed opossum	GenBank, NCBI	-
XM_007940110.1	*Orycteropus afer*	Aardvark	GenBank, NCBI	-
XM_023542827.1	*Loxodonta africana*	African savanna elephant	GenBank, NCBI	-
XM_004476894.2	*Dasypus novemcinctus*	Nine-banded armadillo	GenBank, NCBI	-

XM_006151116.1	*Tupaia chinensis*	Chinese tree shrew	GenBank, NCBI	-
XM_011991880.1	*Mandrillus leucophaeus*	Drill	GenBank, NCBI	-
NM_001039523.3	*Homo sapiens*	Human	GenBank, NCBI	-
XM_003478585.3	*Cavia porcellus*	Domestic guinea pig	GenBank, NCBI	-
XM_013028276.1	*Dipodomys ordii*	Ord's kangaroo rat	GenBank, NCBI	-
XM_004660327.1	*Jaculus jaculus*	Lesser Egyptian jerboa	GenBank, NCBI	-
XM_021649964.1	*Meriones unguiculatus*	Mongolian gerbil	GenBank, NCBI	-
U17016.1	*Erinaceus concolor*	Southern white-breasted hedgehog	GenBank, NCBI	-
XM_008138537.2	*Eptesicus fuscus*	Big brown bat	GenBank, NCBI	-
XM_006921218.1	*Pteropus alecto*	Black flying fox	GenBank, NCBI	-
XM_021075437.1	*Sus scrofa*	Pig	GenBank, NCBI	-
KR477832.1	*Mellivora capensis*	Honey badger	GenBank, NCBI	-
XM_003990883.5	*Felis catus*	Domestic cat	GenBank, NCBI	-
XM_007074557.2	*Panthera tigris*	Tiger	GenBank, NCBI	-

M93639.1	*Herpestes ichneumon*	Egyptian mongoose	GenBank, NCBI	-
XM_029932975.1	*Suricata suricatta*	Meerkat	GenBank, NCBI	-
MN337817	*Anilios bituberculatus*	Prong-snouted blind snake	Pet trade	FJV
MT274611	*Indotyphlops braminus*	Brahminy blind snake	Pet trade	BGF
MN337822	*Boa constrictor*	Common boa	Pet trade	BGF
MN337841	*Corallus hortulanus*	Garden tree boa	Pet trade	BGF
MN337819	*Aspidites melanocephalus*	Black-headed python	Pet trade	BGF
MN337856	*Malayopython reticuatus*	Reticulated python	Pet trade	BGF
XM_007444717	*Python bivittatus*	Burmese python	GenBank, NCBI	
MN337828	*Liasis mackloti*	Macklot's water python	Pet trade	BGF
MN337853	*Morelia spilota*	Carpet python	Pet trade	BGF
MN337818	*Acrochordus granulatus*	Banded file snake	Pet trade	BGF
MN337801	*Causus rhombeatus*	Rhombic night adder	Pet trade	FJV
MN337797	*Daboia russelii*	Russell's viper	NIH, Islamabad, Pakistan	MAK
GCA_000800605.1	*Vipera berus*	European adder	GenBank, NCBI	-

MN337798	*Echis carinatus*	Saw-scaled viper	NIH, Islamabad, Pakistan	MAK
MN337800	*Bitis gabonica*	Gaboon viper	Pet trade	BGF
MN337851	*Tropidolaemus subannulatus*	North Philippine temple pitviper	Pet trade	BGF
MN337844	*Deinagkistrodon acutus*	Chinese moccasin	Gifttierhause Eimsheim, Germany.	JvT & RMW
MN337836	*Trimeresurus albolabris*	White-lipped tree viper	Pet trade	BGF
MN337837	*Trimeresurus hageni*	Indonesian pit viper	Pet trade	FJV
XM_015815894.1	*Protobothrops mucrosquamatus*	Brown-spotted pit viper	GenBank, NCBI	
MN337854	*Bothrops asper*	Fer-de-lance	Gifttierhause Eimsheim, Germany.	JvT & RMW
MT262920	*Bothrops alternatus*	Urutu	Gifttierhause Eimsheim, Germany.	JvT & RMW
MN337838	*Agkistrodon bilineatus*	Cantil viper	Gifttierhause Eimsheim, Germany.	JvT & RMW
JPMF01213521.1	*Crotalus pyrrhus*	Speckled rattlesnake	GenBank, NCBI	-
LVCR01006207.1	*Crotalus horridus*	Timber rattlesnake	GenBank, NCBI	-

MN337852	*Crotalus vegrandis*	Uracoan Rattlesnake	Pet trade	JvT & RMW
MN337824	*Erpeton tentaculatum*	Tentacle snake	Pet trade	BGF
MN337825	*Homalopsis buccata*	Puff-faced water snake	Pet trade	BGF
MN337848	*Pseudoxenodon bambusicola*	Bamboo false cobra	Pet trade	BGF
MN337792	*Erythrolamprus poecilogyrus*	Yellow-bellied water snake	Pet trade	BGF
MN337846	*Philodryas baroni*	Baron's green racer	Pet trade	BGF
MN337832	*Oxyrhopus rhombifer*	Diamondback flame snake	Pet trade	BGF
MN337813	*Helicops leopardinus*	Leopard keelback	Pet trade	BGF
MN337842	*Ahaetulla prasina*	Asian vine snake	Pet trade	BGF
MN337847	*Platyceps florulentus*	Egyptian whip snake	Pet trade	BGF
MN337814	*Thrasops jacksonii*	Black tree snake	Pet trade	BGF
MN337815	*Dispholidus typus*	Boomslang	Pet trade	BGF
MN337811	*Thelotornis capensis*	Savanna vine snake	Pet trade	BGF
MN337850	*Trimorphodon biscutatus*	Western lyre snake	Pet trade	BGF
MN337810	*Oligodon cyclurus*	Cantor's kukri snake	Pet trade	BGF

MT262919	*Coelognathus radiatus*	Radiated ratsnake	Pet trade	BGF
JTLQ01052499	*Pantherophis guttatus*	Corn snake	GenBank, NCBI	
MN337833	*Pantherophis spiloides*	Grey rat snake	Pet trade	FJV
MN337849	*Stegonotus cucullatus*	Slaty grey snakes	Pet trade	BGF
MN337823	*Dasypeltis scabra*	Common egg-eating snake	Pet trade	BGF
MN337793	*Boiga irregularis*	Brown tree snake	Pet trade	BGF
MN337843	*Boiga dendrophila*	Mangrove snake	Pet trade	BGF
XM_032237666.1	*Thamnophis elegans*	Western terrestrial garter snake	GenBank, NCBI	-
MN337812	*Natrix natrix*	European grass snake	Pet trade	FJV
M26389.1	*Natrix tessellata*	Checkered water snake	GenBank, NCBI	
MN337835	*Pseudaspis cana*	Mole snake	Pet trade	BGF
MN337831	*Malpolon monspessulanus*	Montpellier snake	Pet trade	BGF
MN337834	*Psammophis mossambicus*	Olive grass Snake	Pet trade	BGF
MN337829	*Macrelaps microlepidotus*	Natal black snake	Pet trade	BGF
MN337840	*Atractaspis bibronii*	Bibron's stiletto snake	Pet trade	BGF

MN337839	*Atractaspis fallax*	False mole viper	Pet trade	BGF
MN337821	*Atractaspis microlepidota*	Small-scaled burrowing asp	Pet trade	BGF
MN337826	*Boaedon fuliginosus*	African house snake	Pet trade	FJV
MN337827	*Leioheterodon madagascariensis*	Malagasy giant hognose snake	Pet trade	BGF
MN337830	*Madagascarophis ocellatus*	Ocellated cat snake	Pet trade	BGF
MN337805	*Calliophis bivirgatus*	Blue Malaysian coral snake	Pet trade	BGF
MN337802	*Aspidelaps lubricus*	Cape coral cobra	Pet trade	FJV
AF077763.1	*Naja haje*	Egyptian cobra	GenBank, NCBI	
MN337806	*Naja kaouthia*	Monocled cobra	Pet trade	FJV
MN337807	*Naja naja*	Indian cobra	NIH, Islamabad, Pakistan	MAK
ETE71672.1	*Ophiophagus hannah*	King cobra	GenBank, NCBI	-
MN337804	*Bungarus caeruleus*	Common krait	NIH, Islamabad, Pakistan	MAK
MN337816	*Acanthophis rugosus*	Rough-scaled death adder	Gifttierhause Eimsheim, Germany.	JvT & RMW

XM_026696730.1	*Pseudonaja textilis*	Eastern brown snake	GenBank, NCBI	-
MN337809	*Oxyuranus microlepidotus*	Inland taipan	Pet trade	BGF
XM_026677744.1	*Notechis scutatus*	Mainland tiger snake	GenBank, NCBI	-
MN337808	*Hydrophis curtus*	Shaw's sea snake	Pet trade	BGF
MN337803	*Aipysurus mosaicus*	Mosaic sea snake	Weipa, Queensland, Australia	BGF
MN337831	*Malpolon monspessulanus*	Montpellier snake	Pet trade	BGF
MN337834	*Psammophis mossambicus*	Olive grass Snake	Pet trade	BGF
MN337829	*Macrelaps microlepidotus*	Natal black snake	Pet trade	BGF
MN337840	*Atractaspis bibronii*	Bibron's stiletto snake	Pet trade	BGF
MN337839	*Atractaspis fallax*	False mole viper	Pet trade	BGF

DNA was extracted from tissue samples preserved in 70% ethanol. The tissues were rinsed with 10% phosphate-buffered saline (PBS), then cut into small pieces and transferred to DNA lysis buffer containing 10% sodium dodecyl sulfate (SDS) and 10 µL /mL Proteinase K (ProtK) overnight with gentle shaking at 55 °C digital heat block (VWR International). After the incubation the buffer samples were centrifuged at high speed (20,238 rpm) for 15 min. The supernatant was mixed with 0.6 µL isopropanol to precipitate the DNA and then centrifuged at high speed. The resultant pellet was treated with 70%

ethanol, air dried and dissolved in RNA/DNA free water at 65 °C for 45-60 min.

Amplification and sequencing of the ligand binding domain of α-neurotoxin nAChR

Primers specific for the ligand-binding domain of the nicotinic acetylcholine receptor (nAChR) were designed based on the alignment of reference sequences of the following snake species: Egyptian cobra (*Naja haje*), Burmese python (*Python bivittatus*) and king cobra (*Ophiophagus hannah*). Primer sequences are shown in Supplementary File 4 and the amplicon sequences in Supplementary File 5. Successively, an amplicon of 400 bp of the ligand binding domain α-neurotoxin from the gene nAChR was amplified. PCR was performed in a volume of 25 µL mixture according to the instructions of manufacturer (Qiagen, Inc., California, USA). PCR reaction conditions included an annealing temperature of 65 °C for 10 s (-1/cycle). As a quality check, the PCR products were electrophoresed for 30 min, and visualised on gel documentation apparatus (Westburg, the Netherlands). The amplified PCR products of nAChR for all snake species were Sanger-sequenced in both directions by BaseClear B.V., the Netherlands. All sequences were submitted to The National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) and can be found under accession numbers Table 12.

Analysis of site-specific selection

Nucleotide sequences of the ligand-binding domain from other species were downloaded from NCBI. The relevant accession numbers are given in Table 12. The nucleotide sequences were translated into amino acids, manually aligned, and trimmed down to the 14 codons of the ligand-binding domain using AliView 1.18 (Larsson, 2014). A

phylogeny of all the species included in our dataset was compiled from a consensus generated by TimeTree.org and reconciled with taxon-specific phylogenies (Alencar, Quental, Grazziotin *et al.*, 2016; Betancur-R, Wiley, Arratia *et al.*, 2017; dos Reis, Inoue, Hasegawa *et al.*, 2012; Kumar, Stecher, Suleski *et al.*, 2017; Lerner & Mindell, 2005; Portillo, Stanley, Branch *et al.*, 2019; Prum, Berv, Dornburg *et al.*, 2015; Šmíd & Tolley, 2019; Zaher, Murphy, Arredondo *et al.*, 2019). The data set was separated into five major clades: Actinopterygii, Mammalia, Archelosauria, toxicoferan lizards, and Serpentes. The tree data are given in Supplementary File S4 . These were analysed using the FUBAR (Fast Unconstrained Bayesian Approximation) and MEME (Mixed Effects Model of Evolution) programs implemented in HyPhy (Hypothesis Testing Using Phylogenies) 2.220150316beta (Murrell, Moola, Mabona *et al.*, 2013; Murrell, Wertheim, Moola *et al.*, 2012; Pond, Frost & Muse, 2005).

Results and Discussion

We sequenced *de novo* the nAChR ligand binding domain of 66 snake species and obtained sequences for a further 11 species from The National Center for Biotechnology Information Table 12. A preliminary search for sites under positive selection was made independently using a smaller subset of the main sequence collection (Methods, Supplementary File S5). Positively selected sites inferred under posterior probability PP>0.95 were found (172, 177, 181, 187, 194 and 206). These positively-selected sites include sites 187 and 194, modifications of which are associated with toxin resistance.

In our analysis of the full dataset, we included a wide range of vertebrates for comparison with the snakes (Figure 15). This was in order to help us understand the significance of any signals of selection.

We identified a number of highly-conserved sites, which is interesting given that our dataset covers a broad taxonomic scope and contains an over-representation of resistant species (Figure 14B).

Conserved sites includes the tyrosine residues at 190 and 198 which are known to interact directly with ligands; and the cysteine doublet at 192-193 which is crucial to the structure of the ligand-binding domain (Kini & Evans, 1996). The conservation of these residues across such a diverse sampling of vertebrates suggests that they may be important to the physiological function of the nAChR. By contrast, sites 187 and 189, sites of known α-neurotoxin resistance mutations, are far more variable than 194 and 197. Even though most of the observed variation at these sites comes from mutations different from those that are known to produce resistance, we demonstrate that these α-neurotoxin resistance mutations are widespread among vertebrates. However, as we noted in Chapter 3, they are not found in any of the birds that we studied not even the snake specialists in the genus *Circaetus* (snake eagles) and *Sagittarius serpentarius* (secretary bird; Figure 13).

A number of species possess substitutions equivalent to those previously identified as offering α-neurotoxin resistance, via steric hindrance imparted by *N*-glycosylation of an asparagine as they possess the well-documented signature NXS/T motif Additionally, we find the 189-191NYS motif in all elapid snakes we examined, in addition to the *Naja* species in which it was originally characterised (Takacs *et al.*, 2001). Variants of the NXS/T motif were found in other snakes that we sampled, occurring within subfamilies Viperinae (5/6 species), Natricinae (3/3 species), Colubrinae (4/13 species), and Dipsadinae (1/5 species).

In our sequence analysis we also found that several species possess proline replacements at positions 194 and 197 identical to those that have been previously associated with resistance (Kachalsky *et al.*, 1995). The 194L mutation is particularly widespread, and was found in the yellow-bellied water snake (*Erythrolamprus poecilogyrus*), the dice snake (*Natrix tessellata*), the radiated ratsnake (*Coelognathus radiatus*), and most of the non-hydrophine elapids (6/7 species).

We also found the 194S mutation in the reticulated python (*Malayopython reticuatus*) and basal crotalines (3/12 species). No other snake species in our dataset possessed the 197H mutation found in the mongoose. The exact impact of these mutations is difficult to predict since the study that identified them suggested that there are complex patterns of interaction between mutations at these two sites, as well as between these mutations and those associated with steric hindrance resistance (Kachalsky *et al.*, 1995). Thus these results must be interpreted with caution. As mentioned above, even those specific substitutions which have been demonstrated to confer resistance in one taxon cannot confidently be stated to do so in others, especially mutations to amino acids which have never specifically been associated with resistance. For instance, α-neurotoxins have been found to bind the ligand-binding domain sequences of both the radiated ratsnake (Coelognathus radiates, which contains the 194L mutation) and Schlegel's Japanese gecko (*Gekko japonicus*, which contains the 194T mutations) with higher affinity than they do to other species tested (Harris, Zdenek, Debono *et al.*, 2020; Harris, Zdenek, Harrich *et al.*, 2020; Zdenek, Harris, Kuruppu *et al.*, 2019). These findings underscore the fact that not all substitutions at these sites confer resistance (e.g. (Dellisanti, Yao, Stroud *et al.*, 2007b), and that complex interactions, involving multiple amino acids, may be involved in conferring resistance.

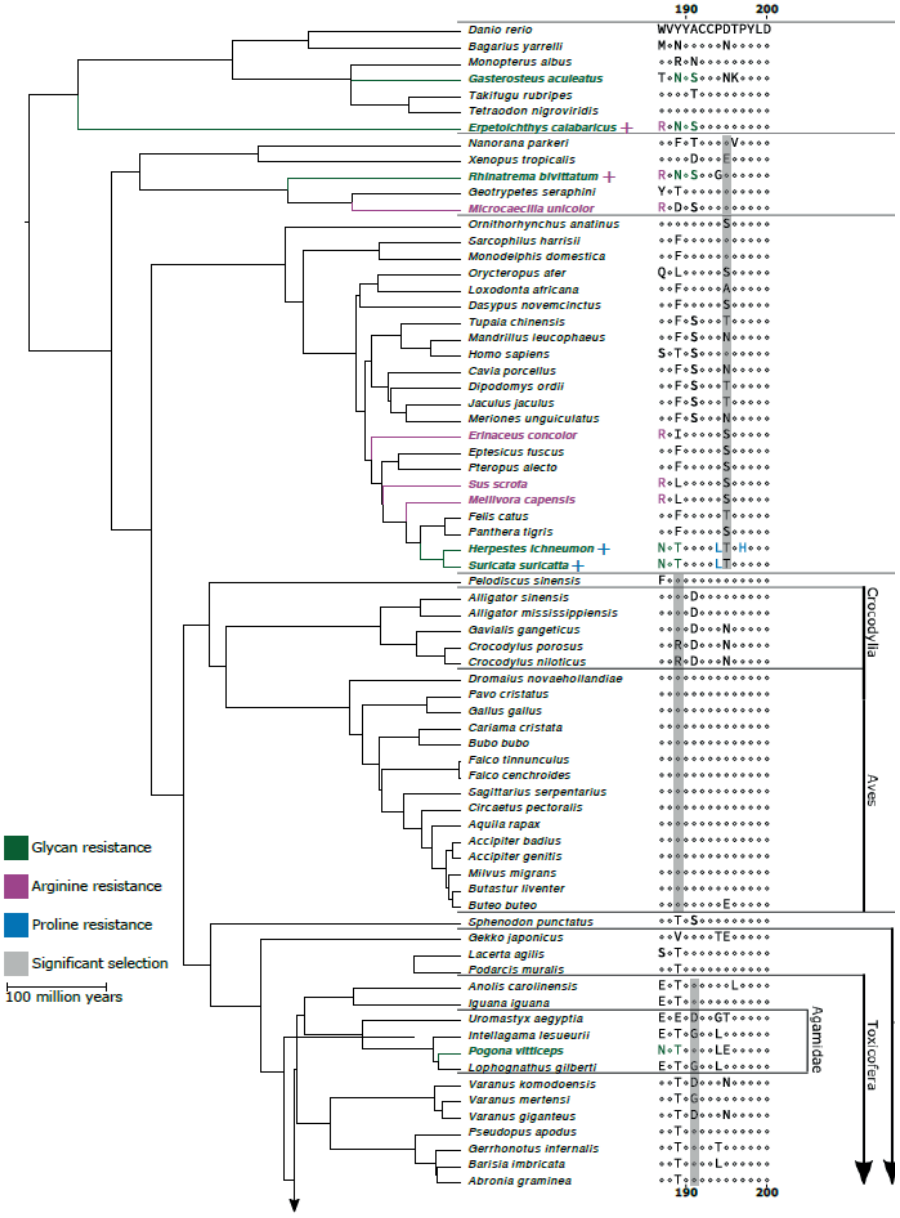
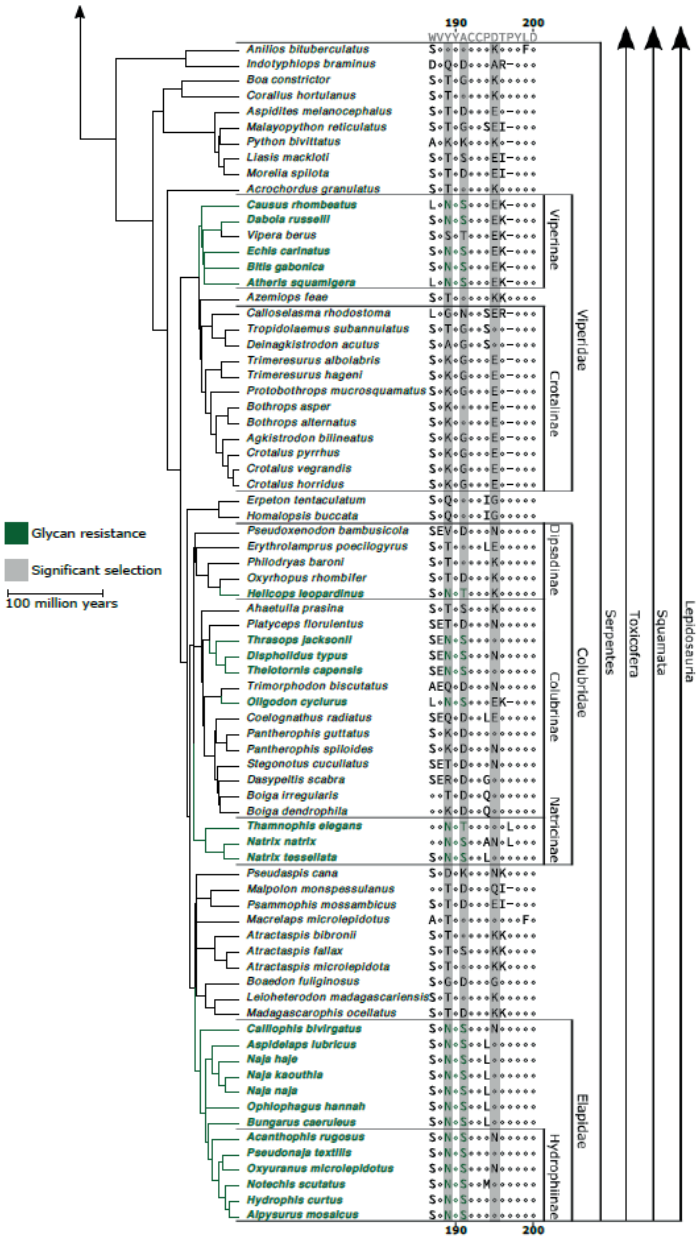

190
200
WVYYACCPDTPYLD
Danio rerio
Bagarius yarrelli
Monopterus albus
Gasterosteus aculeatus
Takifugu rubripes
Tetraodon nigroviridis
Erpetoichthys calabaricus
Nanorana parkeri
Xenopus tropicalis
Rhinatrema bivittatum
Geotrypetes seraphini
Microcaecilia unicolor
Ornithorhynchus anatinus
Sarcophilus harrisii
Monodelphis domestica
Orycteropus afer
Loxodonta africana
Dasypus novemcinctus
Tupaia chinensis
Mandrillus leucophaeus
Homo sapiens
Cavia porcellus
Dipodomys ordii
Jaculus jaculus
Meriones unguiculatus
Erinaceus concolor
Eptesicus fuscus
Pteropus alecto
Sus scrofa
Mellivora capensis
Felis catus
Panthera tigris
Herpestes ichneumon
Suricata suricatta
Pelodiscus sinensis
Alligator sinensis
Alligator mississippiensis
Gavialis gangeticus
Crocodylus porosus
Crocodylus niloticus
Dromaius novaehollandiae
Pavo cristatus
Gallus gallus
Cariama cristata
Bubo bubo
Falco tinnunculus
Falco cenchroides
Sagittarius serpentarius
Circaetus pectoralis
Aquila rapax
Accipiter badius
Accipiter genitis
Milvus migrans
Butastur liventer
Buteo buteo
Sphenodon punctatus
Gekko japonicus
Lacerta agilis
Podarcis muralis
Anolis carolinensis
Iguana iguana
Uromastyx aegyptia
Intellagama lesueurii
Pogona vitticeps
Lophognathus gilberti
Varanus komodoensis
Varanus mertensi
Varanus giganteus
Pseudopus apodus
Gerrhonotus infernalis
Barisia imbricata
Abronia graminea
Crocodylia
Aves
Agamidae
Toxicofera
Glycan resistance
Arginine resistance
Proline resistance
Significant selection
100 million years

Figure 15. Sites of positive selection in α1-nAChR ligand-binding domain in a wide range of vertebrate taxa. Topology constructed from the consensus of TimeTree.org and taxon-specific phylogenies (Alencar et al., 2016; Betancur-R et al., 2017; dos Reis et al., 2012; Kumar et al., 2017; Lerner et al., 2005; Portillo et al., 2019; Prum et al., 2015; Šmíd et al., 2019; Zaher et al., 2019). The most common amino acid sequence of the α1-nAChR ligand-binding is displayed for one species (Danio rerio) and differences from this sequence are displayed for all other species. Sites showing significant positive selection are highlighted in grey for the relevant clade. Green taxa and amino acids indicate resistance conferred through the glycosylated NXS/T motif, purple signify the 187R mutation, and blue indicates resistance granted by proline subsite mutations. Scale bar indicates 100 million years of branch length. Continued in next figure.



Continuation of **Figure 15**.

A number of mutations apparent in our dataset have not previously been discussed in the context of α-neurotoxin resistance. For instance, since the steric hindrance from *N*-glycosylation inhibits the binding of α-neurotoxins at positions 187 and 189, one might suspect that the steric hindrance created by arginine might similarly confer resistance to those species with a 189R mutation. These species the egg-eating snake (*Dasypeltis scabra*; Colubridae).

However, we find this unlikely since steric hindrance from the 187R mutation is imposed due to very specific interactions between the toxin and the ligand-binding domain and positions 187 and 189 interact with different parts of the toxin (Dellisanti *et al.*, 2007b; Rahman *et al.*, 2020). This is in contrast to the steric hindrance by N-glycosylation which, due to the large glycan emerging from the asparagine, presents a much larger obstacle to binding which can hinder the process from a wider variety of positions within the binding pocket of the nicotinic acetylcholine receptor.

We used signals-of-selection analyses to calculate the ratio of nonsynonymous (β) to synonymous (α) substitutions within the α1-nAChR nucleotide sequence. Non-synonymous mutations affect the biochemistry of the final gene product; synonymous mutations do not. A scarcity of non-synonymous mutations suggests that deviations from the ancestral state may be deleterious, and therefore selected against. Conversely, an overabundance of non-synonymous compared to synonymous mutations implies an adaptive process selecting for change or diversity, and this is a hallmark of evolutionary arms races. We therefore used this ratio to infer negative selection ($\alpha > \beta$), neutral

evolution ($\alpha = \beta$), and positive selection ($\alpha < \beta$) of sites within the nAChR sequence.

The analyses found several sites under significant positive selection (at the threshold of $p < 0.1$ for these conservative algorithms) within the ligand-binding domain (Figure 15). In the main analysis described here (Figure 15), we used Mixed Effects Model of Evolution (MEME) and Fast Unconstrained Bayesian Approximation (FUBAR) to analyse the following clades: Actinopterygii, Mammalia, Archelosauria, Toxicoferan lizards, and Serpentes. MEME is designed to detect sites that have undergone episodic diversification, whereas FUBAR is built to detect sites with more pervasive positive selection throughout their evolutionary history. Due to these differences, we would expect MEME to determine a greater number of sites as significant than would FUBAR. While there was no significant positive selection within Actinopterygii, Amphibia (position 195: MEME $p = 0.03$, FUBAR $p = 0.06$), Mammalia (position 195: MEME $p = 0.04$, FUBAR $p = 0.06$), Archelosauria (position 189, MEME $p = 0.01$, FUBAR $p = 0.18$), and Toxicoferan lizards (position 191, MEME $p = 0.06$, FUBAR $p = 0.07$) all have one site under positive selection. In Serpentes, three positions: 189 (MEME $p = 0.13$, FUBAR $p = 0.01$), 191 (MEME $p = 0.09$, FUBAR $p = 0.004$) , and 195 (MEME $p = 0.001$, FUBAR $p = 0.001$)

were found to be significant by at least one of the analyses. Both algorithms indicate that most remaining sites are subject to negative

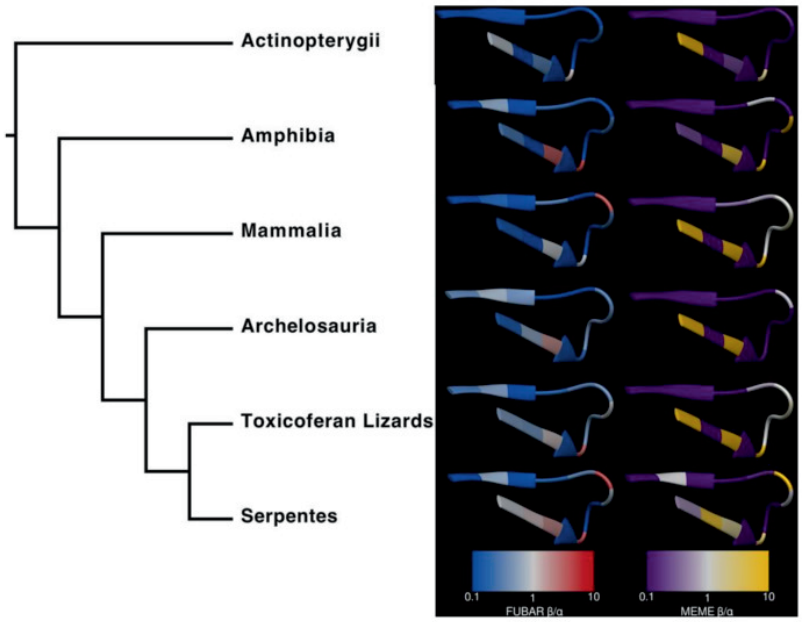


Figure 16. Amino acids in the α1-nAChR ligand-binding domains of snakes are subject to stronger and more pervasive positive selection than other taxa. Predicted surface of the ligand-binding domain (blue residues in Figure 13) coloured according to FUBAR β/α and MEME weighted β/α where red and yellow denote positive selection while blue and purple represent negative selection. This structure is publicly available from the RCSB PDB under the ID 2BG9; see also (Zouridakis, Giastas, Zarkadas et al., 2014).

selection (α > β); Sites that appear be nearly neutral (α ≈ β) in some clades include those at positions 192, 193, 198, 199, and 200, which are strongly conserved across our species. This is an artefact arising from extraordinarily strong negative selection, which eliminates all or

almost all mutations, including those that are non-synonymous, as discussed in (Dashevsky & Fry, 2018).

We shall now discuss those taxa not mentioned in previous chapters. Neither of the frog species included in our analyses possessed a resistance mutation, but two of the three caecilian species did. The resistant species are both South American which could be because of coral snakes (*Micrurus*) which are known to prey on caecilians specifically including the tiny Cayenne caecilian (Martins and Oliveira 1998). From our phylogeny it is impossible to be certain whether the 187R mutation is ancestral to all caecilians and further mutated to 187Y in the lineage leading to the Gaboon caecilian or whether it is a convergent mutation in the tiny Cayenne caecilian and two-lined caecilian. Since these lineages predate the evolution of elapids, our hypothesis predicts the latter scenario. Further research into these enigmatic amphibians will be necessary to confirm or deny this prediction. The significant positive selection at site 195 across the amphibians could allude to a possible association with resistance, though how mutations at this site might affect the binding of α-neurotoxins remains unclear.

We identified an additional species (the meerkat) that was previously not known to possess resistance. The meerkat is closely related to the mongoose and has a similar foraging strategy. This strongly suggests that the identical 187-189NVT sequence and the 194L mutations shared by these two taxa is a homologous trait that was present in their most recent common ancestor, which likely also preyed on venomous snakes. As with the amphibians, the consistent positive selection of site 195 across all mammals tested could be related to α-neurotoxin resistance, but this remains hypothetical until an actual effect or mechanism can be demonstrated.

Considering all the previously described resistance mutations, and the experimental evidence of resistance described in previous studies (*Eryx*, *Laticauda*, *Naja*, and *Natrix*) (Takacs *et al.*, 2001), our results suggest that the *N*-glycosylated asparagine form of α-neurotoxin resistance has evolved convergently at least six times within the snakes alone. The phylogenetic pattern provides evidence to suggest that these are independent origins of resistance rather than multiple losses. This is an extraordinary level of convergence of this very effective form of resistance.

We found mutations *N*-glycosylated asparagine form of α-neurotoxin resistance were particularly widespread in two of the major venomous snake families, Elapidae and Viperidae but also occurred within Colubridae. Within the elapids, the 189-191NYS mutation is present in all 13 species examined, but is not in other closely related snake families. This suggests that it evolved once in the common ancestor of the elapids, paralleling the explosive diversification of α-neurotoxins within this family (Fry, Wuster, Kini *et al.*, 2003). Within the viperids, only the Viperinae subfamily contains the *N*-glycosylated asparagine form of steric hindrance resistance (189-191NYS), which suggests that the selection pressure for resistance may postdate the divergence between these subfamilies. This leads us to posit that predation from ophiophagous elapids may have contributed to the evolution of this mutation given that the origin of elapids is thought to postdate the split between Viperinae and Crotalinae (Alencar *et al.*, 2016; Lee, Sanders, King *et al.*, 2016; Zaher *et al.*, 2019; Zheng & Wiens, 2016). Interestingly, the European adder (*Vipera berus*), a viperine, is the only species examined here with a reversal of the *N*-glycosylated form of steric resistance (NXS/T mutation). This adder has a very broad distribution across northern Eurasia, however it is found at relatively

high latitudes and is not sympatric with any elapid (Wallach, Williams & Boundy, 2017).

This reversal may therefore indicate that resistance mutations carry a fitness cost in a species that is no longer encountering α-neurotoxins. Such a scenario has been shown in several other cases of resistance to toxins (Carlo, Leblanc, Brodie Jr *et al.*, 2016; Ujvari *et al.*, 2015). Members of Colubridae are known to produce abundant α-neurotoxins within their venom which could lead to the evolution of autoresistance (Dashevsky *et al.*, 2018; Fry, Scheib, van der Weerd *et al.*, 2008; Pawlak, Mackessy, Fry *et al.*, 2006; Pla, Sanz, Whiteley *et al.*, 2017). Some of the taxa that possess resistance mutations are also sympatric with ophiophagous elapids, but in other cases such as Natricinae it is less clear whether there was sufficient overlap between ancestral populations to lead to predator-prey coevolution.

While additional mechanisms of resistance may have evolved, such as mutations to the proline subsite, this will require future functional testing to validate. All of the non-hydrophine elapids, except for the Malaysian blue coral snake (*Calliophis bivirgatus*), share the 194L mutation. This suggests that it may have evolved subsequent to the divergence of the Asian coral snakes, and reverted in the lineage that colonized Australia. Within the viperids, the Crotalinae subfamily has the proline replacement (194S).

It should be noted that the evidence linking this type mutation to α-neurotoxin resistance comes from a structure-function study of mammalian receptors which demonstrated these changes resulted in significant resistance (Kachalsky *et al.*, 1995). However, as the proline replacement mutations involve complex interplays between amino acids in the binding pocket, as opposed to the simple steric hindrance imposed by *N*-glycosylation of an asparagine, this mutation might not

confer resistance in the context of the other differences between the mammalian and crotaline sequences.

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