Proteomic and functional variation within black snake venoms (Elapidae: Pseudechis)


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ABSTRACT

Pseudechis (black snakes) is an Australasian elapid snake genus that inhabits much of mainland Australia, with two representatives confined to Papua New Guinea. The present study is the first to analyse the venom of all 9 described Pseudechis species (plus one undescribed species) to investigate the evolution of venom composition and functional activity. Proteomic results demonstrated that the typical Pseudechis venom profile is dominated by phospholipase A2 toxins. Strong cytotoxicity was the dominant function for most species. P. porphyriacus, the most basal member of the genus, also exhibited the most divergent venom composition, being the only species with appreciable amounts of procoagulant toxins. The relatively high presence of factor Xa recovered in P. porphyriacus venom may be related to a predominantly amphibian diet. Results of this study provide important insights to guide future ecological and toxicological investigations.

1. Introduction

The production of venom is considered to be metabolically ‘expensive’ (reviewed by Morgenstern and King, 2013), and this can create selection pressure for the ‘fine-tuning’ of venom to target specific prey (Jackson et al., 2013). For this reason, venom composition and activity can vary according to diet; For instance Gibbs et al. (2013) found that Sistrurus rattlesnakes which have more lizards (and fewer mammals) in their diet also had a higher proportion of CRISP toxins in their venom. Even more surprisingly, venom variation has been observed at the intraspecific level, as in Echis (Viperidae) and Pseudonaja (Elapidae) spp. (Barlow et al., 2009; Jackson et al., 2016; Rogalski et al., 2017). Venom is inevitably linked to an antagonistic evolutionary arms race with prey (Dawkins and Krebs, 1979; Casewell et al., 2013; Arbuckle, 2017), in which both predator and prey are continually experiencing selection to counteract adaptations of their natural enemies, as encapsulated in Van Velen’s (1973) concept of ‘Red Queen’ coevolution. Importantly, arms races have been linked to diversification of lineages and their traits (Ehrlich and Raven, 1964) and so the coevolutionary interactions between venomous snakes and their prey may have
contributed to the dramatic venom diversification that occurred in the colubroid (advanced) snakes.

Snake toxins can be classified into two broad categories: enzymes (e.g. phospholipases, serine proteinases, metalloproteinases, LAOOs,) and non-enzymatic toxins (e.g. three-finger toxins (3FTx), lectins, sarafotoxins, Kunitz peptides, CRISP) (Sunagar et al., 2013). However, the biological reality is not so binary since toxins such as phospholipase A2 (PLA2) have secondarily evolved novel non-enzymatic functions ranging from antiplatelet activity to neurotoxicity, with functional sites distinct from those used for the plesiotypic enzymatic function (Cull-Candy et al., 1976; Harris et al., 2000; Howell et al., 2014).

Australasia is a diversity hotspot for the Elapidae, being home to almost 50% of all species in this venomous snake family, many of which are endemic (Shine, 1995; Jackson et al., 2013). Throughout geological history, several ice ages have dropped the sea level by up to 100 m, exposing the Sunda and Sahul continental shelves (Barber et al., 2000; Rowe and Stegemann, 2009). These events facilitated dispersal and migration of many organisms between Asia and Australia, including elapid snakes (Wüster et al., 2005). Phylogenetic studies demonstrate that Australo-Melanesian elapids (Hydrophiinae) do not represent a Gondwanan group but arrived in Australasia only relatively recently (< 25 Mya) (e.g. Wüster et al., 2005; Sanders et al., 2008; Hsiang et al., 2015; Lee et al., 2016). At this time, only pythons and blind snakes were present among the local snake fauna and so the elapid snakes likely diversified (to > 100 extant terrestrial species) at least in part due to exploitation of empty niche space (McPeek and Brown, 2007); elapids are relatively agile and combined with their possession of venom this would have made them ecologically distinct snakes in the region. The clade is ecologically diverse and includes species with a range of body sizes, activity periods, and habitats, as well as many that are dangerous to humans (Sanders et al., 2008). Among all Australian elapids, five genera are considered the ‘big 5’ due to their substantial medical impact; these are Oxyuranus (taipans), Pseudonaja (brown snakes), Pseudechis (black snakes), Acanthophis (death adders), and Notechis (tiger snakes).
snakes).

*Pseudechis* Wagler, 1830 is a genus of nine described, plus one as yet undescribed, elapid species (Elapidae F. Boie, 1827). The genus ranges from < 1 m (e.g. *P. weigeli*) up to 3 m (*P. australis*) in length and all species are considered potentially dangerous (Ramasamy et al., 2005; Cogger, 2014). They are distributed throughout Australia, except for Tasmania (Georgieva et al., 2011), and two species are endemic to Papua New Guinea and the islands of Torres Strait (*P. papuanus, P. rossignolii*) (Wilson and Swan, 2003; Wüster et al., 2005). *P. australis* is particularly wide-ranging (across most of Australia), yet it displays extremely low levels of genetic diversity across its range consistent with a recent and rapid range expansion.

Most Australian elapid snake venoms are typified by being rich in 3FTx with phospholipase A2 (PLA2) toxins in lower amounts (Fry et al., 2003). However, previous studies have found *Pseudechis* venoms to be rich in PLA2 toxins, suggesting a relatively unusual venom, of toxicological and toxicological interest (Vaughan et al., 1981; Nishida and Tamiya, 1991; Fatehi et al., 1995; Laing et al., 1995; Viala et al., 2014; Pla et al., 2017). *Pseudechis* venoms are known to have strong myotoxic activity and antiplatelet action, which are mediated by PLA2 toxins (Geh et al., 1992; Lane et al., 2011).

This study analyses the venom composition and activity of all ten species of *Pseudechis* in order to investigate the evolution of the venom throughout the genus. Multiple samples are investigated for some species in order to enlighten on intraspecific/regional variation in venom composition and activity.

### 2. Materials and methods

#### 2.1. Species identification and venom collection

All venoms investigated were collected, milked, and delivered by Venom Supplies Pty Ltd. (Tanunda, SA, Australia), or part of the Venom Evolution Lab long-term research collection. Samples from a minimum of three adult individuals of the same species were pooled. Species and localities studied were: *Pseudechis australis* (Kulgera, NT, Mt. Isa, QLD, Eyre SA, Pt Hedland, WA), *Pseudechis butleri* (Yalgoo, WA), *Pseudechis colletti* (Longreach, QLD), *Pseudechis guttatus* (Glen Morgan, QLD), *Pseudechis pailsi* (Mt Isa, QLD), *Pseudechis papuanus* (Saibai Island, QLD), *Pseudechis porphyriacus* (Brisbane, QLD), *Pseudechis rossignolii* (Merauke, Irian Jaya), *Pseudechis sp.* unnamed (Daly River, Northern Territory), and *Pseudechis weigeli* (Kununurra, WA). Lyophilized venom was dissolved in MilliQ and filtered through a 0.45 μm pore size and 25 mm diameter filter (Agilent® Captiva Econo filter) to remove impurities that may have interfered with the analysis processes. The concentration of the filtered sample was then measured (Thermo Fisher Scientific® NanoDrop 2000) and aliquots were made and stored at -80°C until further analysis.

#### 2.2. Proteomics

##### 2.2.1. Liquid chromatography–mass spectrometry (LC–MS)

HPLC analysis of 25 μg crude venom was performed on a Nexera system (Shimadzu) using a Zorbax 300SB C18, 3.5 μm column (2.1 × 100 mm, Agilent) at a flow rate of 300 μL/min. The gradients adopted were: 2–40% Buffer B (90% acetonitrile) over 35 min, 40–98% Buffer B in 2 min, and left stable at 98% Buffer B for 2 min. Buffer A was
Fig. 3. Phylogenetics of the Pseudechis-specific clade of PLA2 toxins. Notechis scutatus PLA2 outgroup is not shown. Node values indicate posterior probabilities.
0.1% formic acid in water. The HPLC was directly connected to a DuoSpray™ ion source (ESI SCIEX) - TripleTOF 5600, operated in positive ion acquisition mode. Data were acquired for 46 min over the m/z range 350–2000 Da with a cycle time of 0.5 s. Raw results were analysed in Analyst® (SCIEX) and protein mass picks have been manually reconstructed. Subsequently, the total ion currents (TICs) were assessed in PeakView® 2.1 (SCIEX). The spectra and the protein masses of each species were then averaged to reproduce a single output per species. MSMS spot guide is available in Supplementary Fig. 1 and MSMS data available in Supplementary Table 1.

2.2.2. Electrophoresis

Electrophoresis SDS-PAGE and MS/MS were carried out as previously described by us (Ali et al., 2013a, 2013b; Ali et al., 2015).

2.2.3. Molecular evolution

Molecular evolution analyses using all available Pseudechis (set in Supplementary files) sequences were conducted as we have previously described (Koludarov et al., 2017) with customised protein structures were generated by using a representative sequence (Q45Z17) as input to the Phyre2 webserver.2017).

2.2.4. Bioactivity testing

Enzymology sPLA2 and Factor Xa assays were carried out as previously described (Cipriani et al., 2017; Debono et al., 2017).

Cytotoxicity assays were carried out as previously been described (Panagides et al., 2017). Raw data is available in Supplementary Table 2.

Fig. 4. Three dimensional structure of Pseudechis PLA2 toxin diversity coloured according to (A) AL2CO amino acid conservation score (conserved sites in teal and variable sites in orange), (B) FUBAR strength of persistent selection (sites under purifying selection in blue and sites under diversifying selection in red), and (C) MEME significance levels for episodes of diversifying selection during the evolution of the toxin family (moderately significant sites in dark green, highly significant sites in light green, and extremely significant sites in yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
2.2.5. Phylogenetic comparative analyses

All comparative analyses of the venom activities were conducted as previously described by us (Rogalski et al., 2017). Phylogeny used was as per Maddock et al. (2017) and Wüster et al. (2005). Analyses were implemented in R v3.2.5 (R Core Team, 2016) using the ape package for basic data manipulation (Paradis et al., 2004). Ancestral states of each functional trait (PLA2 activity, Factor Xa activity, and cytotoxicity on each cell line) were estimated via maximum likelihood with the contMap function in phytools (Revell, 2012). We then fit pGLS models using the caper package (Orme et al., 2013) to test the relationships between PLA2 activity and cytotoxicity on each cell line, and also to test whether cytotoxicity on the non-cancerous NFF cell line predicts cytotoxicity on the malignant melanoma MM96L skin cell line or the reciprocal.

3. Results and discussion

All species possessed PLA2 rich venoms, as revealed by LC/MS (Fig. 1) and 1D/2D gels (Fig. 2) showing a preponderance of components in the PLA2 characteristic 12–15 kDa range. In addition, 1D gels revealed significant amounts of snake venom metalloprotease (SVMP) (Fig. 2). MS/MS of 1D bands confirmed identity. Examination of the molecular evolution of the PLA2 toxins displayed evidence of considerable duplication and diversification (Fig. 3). The overall dN/dS value for those PLA2 sequences for which nucleotide data were available was 1.07, which indicates that the overall sequence coding for the mature protein has been subject to net neutral selection. However, the FUBAR and MEME methods detected a number of individual sites that most likely have been subjected to diversifying selection (Fig. 4). This suggests that these sites may be important in the co-evolutionary arms race between Pseudechis snakes and their prey and may be functionally valuable sites.

Venoms displayed significant variation in PLA2 enzymatic activity and also cytotoxicity. PLA2 enzymatic activity was not related to cytotoxicity on either cell line according to our pGLS analyses (MM96L: $t_{1,11} = -0.817$, $P = 0.431$; NFF: $t_{1,11} = -0.034$, $P = 0.974$; Fig. 5), suggesting that PLA2S are not key mediators of cytotoxicity in Pseudechis. Consequently, we suggest that cytotoxicity is most likely driven by toxic SVMPs (possibly in combination with PLA2) some of which have previously been found to kill cells (Casewell et al., 2015) and are abundant in the Pseudechis venoms examined here (Fig. 2).

Consistent with previously published studies, only P. porphyriacus displayed appreciable Factor Xa activity (Martin, 1893; Lane et al., 2011; Maddock et al., 2017) (Fig. 6). Since P. porphyriacus is the most basal member of the genus it appears that Factor Xa activity has been lost (or heavily reduced) once at the base of the clade containing all other Pseudechis (Fig. 6). Jackson et al. (2016) hypothesised that Factor Xa toxins may be more abundant in Australian elapid snakes which feed on ‘high-
metabolism’ prey, of which they considered frogs a potential example due to raised metabolism of calling males. The current study provides only mixed evidence for this hypothesis. *P. porphyriacus* has a broad diet but one containing more amphibians than other *Pseudechis* species previously studied (~60%) (Shine, 1987), consistent with the idea, but amphibians also comprise a relatively large proportion of *P. guttatus* diets (~40%) (Shine, 1987) and this species has no detectable fXa activity (Fig. 6). In addition, most species of *Pseudechis* opportunistically feed upon mammals (which undoubtedly possess high metabolic rates), and yet their venoms exhibit no fXa activity. Hence, one concordant datapoint and universally low fXa activity in the rest of the clade doesn’t provide strong evidence in support of the hypothesis, but neither does it provide a strong refutation as other selection pressures may dominate the evolution of fXa activity in this genus.

We initially found evidence that cytotoxicity on the two cell lines were positively related (pGLS: $t_{1,11} = 2.368$, $P = .037$), however this effect disappeared when *P. papuanus* was excluded (pGLS: $t_{1,10} = -1.468$, $P = .173$). Therefore the apparent relationship is driven only by the unusual venom of *P. papuanus* which has very low cytotoxicity on both cell lines compared to other members of the genus (Fig. 7). Interestingly, this lack of relationship between cytotoxicity to non-cancer and malignant melanoma cell lines suggests that *Pseudechis* venom is a promising candidate for biodiscovery of novel anticancer drugs as it appears to typically contain toxins that selectively attack cancer cells.

![Fig. 6. Ancestral state reconstruction of relative Factor Xa activity](image6.png)

![Fig. 7. Ancestral state reconstruction of relative cytotoxicity](image7.png)
enzymatic activity between the *P. australis* populations, there was extensive variation in activity across the genus. For instance, while two of the pygmy mulga species (*P. rossignolii* and *P. weigeli*) were among the venom with the most potent PL Ab enzymatic activity of all the venom tested, the other two (*P. sp. and *P. pailsei*) were among the weakest (Fig. 5). This is indicative of multiple rises and falls of PL Ab enzymatic driven function within this genus. While all species except *P. papuanus* displayed high levels of cytotoxicity on the human melanoma MM96L cell line, their effect on the healthy fibroblast (NFF) cell line was as variable as that of the PL Ab (Fig. 7). The lack of cytotoxic activity in *P. papuanus is in contrast to the high concentration of PL Ab in this study and a previous study (Williams et al., 2006), which reinforces the multifunctionality of PL Ab toxins, as they are clearly functioning as something other than cytotoxins in *Pseudechis*.

Despite the vast range of *P. australis*, the venom results correlate with the recent (late Pliocene - early Pleistocene) and rapid range expansion observed in phylogeographic analyses (Wüster et al., 2005) and Maddock et al. (2017) by reinforcing the taxonomic distinctiveness of these species. The closely related species *P. guttatus* and *P. collettii* also differed in their PL Ab activities, but were similar in their cytotoxicity profiles (again suggesting a lack of cytotoxic function of *Pseudechis* PL Ab). *P. porphyriacus* is the only species with FXa activity, corroborating the previous results of Lane et al. (2011) who demonstrated that at concentrations of over 100 ng/μl of *P. porphyriacus* venom, procoagulant activity of FXa overcomes the anticoagulant activity of the PL Ab.

Considering morphological data (Shine, 1987) and venom yield information (Cogger, 2000; Cogger, 2014) in the context of our results, it appears that over time, *Pseudechis* species have increased the complexity of the PL Ab component of their venom and increased their body size which enables them to inject a larger venom yield. This venom is consequently less toxic for a given quantity than that of other large size which enables them to inject a larger venom yield. This venom is one of the highest venom yields of any snake (Stiles et al., 1991). *Pseudechis australis* is also known for hanging on and chewing vigorously with its powerful jaws, thus driving the venom in deeper than simple fang length due to the compression of the flesh. This focus upon select toxins in the venom in tandem with a shift to a higher venom yield is in accordance with the ‘race to redundancy’ conjecture (Jackson et al., 2016), in which venom maintains only a few specific compounds, e.g. PL Ab toxins, that undergo positive selection to increase the intraclass variation of the toxin group in order to ensure the greatest success during prey subjugation.

The evolutionary pathways of *Pseudechis* seems driven by two major components: vacant ecotone occupations (Australia was a ‘snake-free continent’ (Wüster et al., 2005)) and rapid diversification at the species level subsequent to the evolution of the last common ancestor of *Pseudechis* (ten species appeared in < 8 million years (Wüster et al., 2005; Sanders et al., 2008)). Considering the virtual absence of FXa activity in all species other than *P. porphyriacus* findings suggest that the ancestral *Pseudechis* most likely expressed only low to moderate quantities of procoagulant toxins (FXa activity) in its venom, with a secondary increase in the lineage leading to *P. porphyriacus*, and secondary reduction/loss in other species. PL Ab have experienced substantial diversification across the clade, resulting in high interspecific variability of PL Ab activity. Collectively, this research has contributed to our understanding of the evolution of Australian snake venoms by considering evolutionary trends in venom composition and activity across the entire genus *Pseudechis*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2018.01.001.

Conflict of interest

The authors have no conflict of interest.

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