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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 A_2 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 toxinological investigations.

Keywords: venom evolution, *Pseudechis*, black snakes, diet, toxins, proteomic, enzymology, Oxyuraninae, PLA_2

INTRODUCTION

The production of venom is considered to be metabolically 'expensive' (reviewed by Morgenstern & 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 & 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 Valen's (1973) concept of 'Red Queen' coevolution. Importantly, arms races have been linked to diversification of lineages and their traits (Ehrlich & 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 A_2 (PLA_2) 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 meters, exposing the Sunda and Sahul continental shelves (Barber et al. 2000; Rowe et al. 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 & 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).

Pseudechis Wagler, 1830 is a genus of nine described, plus one as yet undescribed, elapid species (Elapidae F. Boie, 1827). The genus ranges from less than 1 meter (e.g. *P. weigeli*) up to 3 meters (*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. rossignoli*) (Wilson & 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 A_2 (PLA_2) toxins in lower amounts (Fry et al. 2003). However, previous studies have found *Pseudechis* venoms to be rich in PLA_2 toxins, suggesting a relatively unusual venom, of toxinological and toxicological interest (Vaughan et al. 1981; Nishida & 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 PLA_2 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.

MATERIALS AND METHODS

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 Headland, WA), *Pseudechis butleri* (Yalgoo, WA), *Pseudechis collettii* (Longreach, QLD), *Pseudechis guttatus* (Glen Morgan, QLD), *Pseudechis pailsei* (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 Econofilter) 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 -80C until further analysis.

Proteomics

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 x 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 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 sec. Raw results were analyzed in Analysts® (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 Figure 1 and MSMS data available in Supplementary Table 1.

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

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

Bioactivity testing

Enzymology $sPLA_2$ 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.

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 (PLA_2 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 PLA_2 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.

RESULTS AND DISCUSSION

All species possessed PLA_2 rich venoms, as revealed by LC/MS (Figure 1) and 1D/2D gels (Figure 2) showing a preponderance of components in the PLA_2 characteristic 12-15 kDa range. In addition, 1D gels revealed significant amounts of snake venom metalloprotease (SVMP) (Figure 2). MS/MS of 1D bands confirmed identity. Examination of the molecular evolution of the PLA_2 toxins displayed evidence of considerable duplication and diversification (Figure 3). The overall dN/dS value for those PLA_2 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 (Figure 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 PLA_2 enzymatic activity and also cytotoxicity. PLA_2 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: $t_{1,11} = -0.034$, $P = 0.974$; Figure 5), suggesting that PLA_2s are not key mediators of cytotoxicity in *Pseudechis*. Consequently, we suggest that cytotoxicity is most likely driven by toxic SVMPs (possibly in combination with PLA_2) some of which have previously been found to kill cells (Casewell et al. 2015) and are abundant in the *Pseudechis* venoms examined here (Figure 2).

Consistent with previously published studies, only *P. porphyriacus* displayed appreciable fXa activity (Martin 1893; Lane et al. 2011; Maddock et al. 2017) (Figure 6). Since *P. porphyriacus* is the most basal member of the genus it appears that fXa activity has been lost (or heavily reduced) once at the base of the clade containing all other *Pseudechis* (Figure 6). Jackson et al. (2016) hypothesised that fXa 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 (Figure 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: $t_{1,11} = 2.368$, $P = 0.037$), however this effect disappeared when *P. papuanus* was excluded (pGLS: $t_{1,10} = -1.468$, $P = 0.173$). 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 (Figure 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.

Although each venom possessed a similar generalised profile in being PLA_2 rich, there was extensive functional diversification between venoms. With the exception of the high conservation of relative PLA_2 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 amongst the venoms with the most potent PLA_2 enzymatic activity of all the venoms tested, the other two (*P. sp* and *P. pailsei*) were amongst the weakest (Figure 5). This is indicative of multiple rises and falls of PLA_2 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 PLA_2 (Figure 7). The lack of cytotoxic activity in *P. papuanus* is in contrast to the high concentration of PLA_2 in this study and a previous study (Williams et al. 2006), which reinforces the multifunctionality of PLA_2 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). The rapid expansion of the species range was probably facilitated by its generalised ecology, which allows it to occupy diverse ecosystems. Conversely, there was significant protein composition and functional variation between the species of pygmy mulga species *P. pailsei*, *P. rossignolii*, *P. sp* and *P. weigeli*, with even the closely related *P. pailsei*, *P. sp* and *P. weigeli* differing markedly in their PLA_2 and NFF potencies. This venom diversification correlates with the results of the genetic analyses of 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 PLA_2 activities, but were similar in their cytotoxicity profiles (again suggesting a lack of cytotoxic function of *Pseudechis* PLA_2). *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 PLA_2.

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 PLA_2 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 Australian elapids (Jackson et al. 2016; Lister et al. 2017), and we therefore suggest that *Pseudechis* have shifted toward a more quantitative rather than qualitative means for overpowering their prey. This is supported by the example of *P. australis*, which has 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. PLA_2 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 pathway 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 less than 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. PLA_2s have experienced substantial diversification across the clade, resulting in high interspecific variability of PLA_2 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*.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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REFERENCES

Ali, S.A., Baumann, K., Jackson, T.N.W, Wood, K., Mason, S., Undheim, E.A.B., Nouwens, A., Koludarov, I., Hendrikx, I., Jones, A., Fry, B.G., 2013a. Proteomic comparison of *Hypnale hypnale* (Hump-Nosed Pit-Viper) and *Calloselasma rhodostoma* (Malayan Pit-Viper) venoms. J. Proteom. 91, 338–343.

Ali, S.A., Yang, D.C., Jackson, T.N.W., Undheim, E.A.B., Koludarov, I., Wood, K., Jones, A., Hodgson, W.C., McCarthy, S., Ruder, T., Fry, B.G., 2013b. Venom proteomic characterization and relative antivenom neutralization of two medically important Pakistani elapid snakes (*Bungarus sindanus* and *Naja naja*). J. Proteom. 89, 15–23.

Ali, S.A., Jackson, T.N.W., Casewell, N.R., Low, D.H.W., Rossi, S., Baumann, K., Fathinia, B., Visser, J., Nouwens, A., Hendrikx, I., Jones, A., Fry, B.G., 2015. Extreme venom variation in Middle Eastern vipers: a proteomics comparison of *Eristicophis macmahonii*, *Pseudocerastes fieldi* and *Pseudocerastes persicus*. J. Proteom. 116, 106–113.

Arbuckle, K. 2017. Evolutionary context of venom in animals; in Malhotra, A. and Gopalakrishnakone, P. (eds) Evolution of Venomous Animals and Their Toxins (Springer Handbook of Toxinology series). Springer, Netherlands.

Barber, P.H., Palumbi, S.R., Erdmann, M.V., Moosa, M.K., 2000. Biogeography: a marine Wallace's line? Nature 406, 692–693.

Barlow, A., Pook, C.E., Harrison, R., Wüster, W., 2009. Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. Proc. R. Soc. Lond. B Biol. Sci. 276, 2443–2449.

Casewell, N.R., Wüster, W., Vonk, F.J., Harrison, R.A., Fry, B.G., 2013. Complex cocktails: The evolutionary novelty of venoms. Trends Ecol. Evol. 28, 219–229.

Casewell, N.R., Sunagar, K., Takacs, Z., Calvete, J.J., Jackson, T.N.W., Fry, B.G., 2015. Snake Venom Metalloprotease Enzymes in Venomous Reptiles and Their Toxins, Fry. B.G. editor. Oxford University Press. pp 347-363.

Cipriani, V., Debono, J., Goldenberg, J., Jackson, T. N.W., Arbuckle, K., Dobson, J., Koludarov, I., Li, B., Hay, C., Dunstan, N., Allen, L., Hendrikx, I., Kwok, H.F., Fry, B.G., 2017. Correlation between ontogenetic dietary shifts and venom variation in Australian brown snakes (*Pseudonaja*). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 197, 53–60.

Cogger, G.H., 2000. Reptiles and amphibians of Australia (6th ed.). Reed New Holland, Sydney, Australia.

Cogger, G.H., 2014. Reptiles and amphibians of Australia (7th ed.). CSIRO PUBLISHING.

Cull-Candy, S.G., Fohlman, J., Gustavsson, D., Lüllmann-Rauch, R., Thesleff, S., 1976. The effects of taipoxin and notexin on the function and fine structure of the murine neuromuscular junction. Neuroscience 1(3), 175–180.

Dawkins, R., Krebs, J.R., 1979. Arms races between and within species. Proc. R. Soc. Lond. B Biol. Sci. 205, 489–511.

Debono, J., Dobson, J., Casewell, N.R., Romilio, A., Li, B., Kurniawan, N., Mardon, K., Weisbecker, V., Nouwens, A., Kwok, H.F., Fry, B.G., 2017. Coagulating Colubrids: evolutionary, pathophysiological and biodiscovery implications of venom variations between boomslang (*Dispholidus typus*) and Twig Snake (*Thelotornis mossambicanus*). Toxins 9(5), 171.

Ehrlich, P.R., Raven, P.H., 1964. Butterflies and plants: a study in coevolution. Evolution 18(4), 586–608.

Fatehi, M., Rowan, E.G., Harvey, A.L., 1995. The effects of two phospholipase A_2 inhibitors on the neuromuscular blocking activities of homologous phospholipases A_2 from the venom of *Pseudechis australis*, the Australian king brown snake. Toxicon 33(12), 1633–1643.

Fry, B.G., Wüster, W., Ramjan, R., Fadil, S., Jackson, T., Martelli, P., Kini, R.M., 2003. Analysis of Colubroidea snake venoms by liquid chromatography with mass spectrometry: evolutionary and toxinological implications. Rapid Commun. Mass Spectrom. 17(18), 2047–2062.

Geh, S.L., Rowan, E.G., Harvey, A.L., 1992. Neuromuscular effects of four phospholipases A_2 from the venom of *Pseudechis australis*, the Australian king brown snake. Toxicon 30(9), 1051–1057.

Georgieva, D., Seifert, J., Ohler, M., Von Bergen, M., Spencer, P., Arni, R.K., Genov, N., Betzel, C., 2011. *Pseudechis australis* venomics: adaptation for a defense against microbial pathogens and recruitment of body transferrin. J. Proteome Res. 10, 2440–2464.

Gibbs, H.L., Sanz, L., Sovic, M.G., Calvete, J.J., 2013. Phylogeny-based comparative analysis of venom proteome variation in a clade of rattlesnakes (*Sistrurus* sp.). PLoS ONE 8(6), e67220.

Harris, J.B., Grubb, B.D., Maltin, C.A., Dixon, R., 2000. The neurotoxicity of the venom phospholipases A_2, notexin and taipoxin. Exp. Neurol. 161(2), 517–526.

Howell, J.M., Walker, K.R., Creed, K.E., Dunton, E., Davies, L., Quinlivan, R., Karpati, G., 2014. Phosphorylase re-expression, increase in the force of contraction and decreased fatigue following notexin-induced muscle damage and regeneration in the ovine model of McArdle disease. Neuromuscul. Disord. 24(2), 167–177.

Hsiang, A.Y., Field, D.J., Webster, T.H., Behlke, A.D.B., Davis, M.B., Racicot, R.A., Gauthier, J.A., 2015. The origin of snakes: revealing the ecology, behavior, and evolutionary history of early snakes using genomics, phenomics, and the fossil record. BMC Evol. Biol. 15(1), 87.

Jackson, T.N.W., Sunagar, K., Undheim, E.A.B., Koludarov, I., Chan, A.H.C., Sanders, K., Ali, S.A., Hendrikx, I., Dunstan, N., Fry, B.G., 2013. Venom down under: dynamic evolution of Australian elapid snake toxins. Toxins 5, 2621–2655.

Jackson, T.N.W., Koludarov, I., Ali, S.A., Dobson, J., Zdenek, C.N., Dashevsky, D., op den Brouw, B., Masci, P., Nouwens, A., Josh, P., Goldenberg, J., Cipriani, V., Hay, C., Hendrikx, I., Dunstan, N., Allen, L., Fry, B.G., 2016. Rapid radiations and the race to redundancy: an investigation of the evolution of Australian elapid snake venoms. Toxins 8(11), 309.

Koludarov, I., Jackson, T.N.W., Dobson, J., Dashevsky, D., Arbuckle, K., Clemente, C. J., Stockdale, E.J., Cochran, C., Debono, J., Stephens, C., Panagides, N., Li, B., Manchadi, M.R., Violette, A., Fourmy, R., Hendrikx, I., Nouwens, A., Clements, J., Martelli, P., Kwok, H.F., Fry, B.G., 2017. Enter the dragon: the dynamic and multifunctional evolution of Anguimorpha lizard venoms. Toxins 9(8), 242.

Laing, G.D., Kamiguti, A.S., Wilkinson, M.C., Lowe, G.M., Theakston, R.D.G., 1995. Characterisation of a purified phospholipase A_2 from the venom of the Papuan black snake (*Pseudechis papuanus*). Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol. 1250(2), 137–143.

Lane, J., Leary, M.A.O., Isbister, G.K., 2011. Coagulant effects of black snake (*Pseudechis* spp.) venoms and in vitro efficacy of commercial antivenom. Toxicon 58(3), 239–246.

Lee, M.S.Y, Sanders, K.L., King, B., Palci, A., 2016. Diversification rates and phenotypic evolution in venomous snakes (Elapidae). Royal Soc. Open Sci. 3(1), 150277.

Lister, C., Arbuckle, K., Jackson, T.N.W., Debono, J., Zdenek, C.N., Dashevsky, D., Dunstan, N., Allen, L., Hay, C., Bush, B., Gillett, A., Fry, B.G., 2017. Catch a tiger snake by its tail: differential toxicity, co-factor dependence and antivenom efficacy in a procoagulant clade of Australian venomous snakes. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 202: 39–54.

Maddock, S.T., Childerstone, A., Fry, B.G., Williams, D.J., Barlow, A., Wüster, W., 2017. Multi-locus phylogeny and species delimitation of Australo-Papuan blacksnakes (*Pseudechis* Wagler, 1830: Elapidae: Serpentes). Mol. Phylogenet. Evol. 107, 48–55.

Martin, C.J. (1893). On some effects upon the blood produced by the injection of the venom of the Australian Black Snake (*Pseudechis porphyriacus*). J. Physiol. 15, 380.

McPeek, M.A., Brown, J.M., 2007. Clade age and not diversification rate explains species richness among animal taxa. Am. Nat. 169, E97–E106.

Morgenstern, D., King, G.F., 2013. The venom optimization hypothesis revisited. Toxicon 63, 120-128.

Nishida, S., Tamiya, N., 1991. Tryptophan residues of phospholipase A_2 from the venom of an Australian elapid snake (*Pseudechis australis*). Toxicon 29(4-5), 429–439.

Orme, D., Freckleton, R., Thomas, G., Petzoldt, T., Fritz, S., Isaac, N., Pearse, W., 2013. Caper: Comparative Analyses of Phylogenetics and Evolution in R. R package version 0.5.2.

Panagides, N., Jackson, T. N., Ikonomopoulou, M.P., Arbuckle, K., Pretzler, R., Yang, D. C., Ali, S.A., Koludarov, I., Dobson, J., Sanker, B., Asselin, A., Santana, R.C., Hendrikx, I., van der Ploeg, H., Tai-A-Pin, J., van den Bergh, R., Kerkkamp, H.M.I., Vonk, F.J., Naude, A., Strydom, M.A., Jacobsz, L., Dunstan, N., Jaeger, M., Hodgson, W.C., Miles, J., Fry, B.G., 2017. How the cobra got its flesh-eating venom: cytotoxicity as a defensive innovation and its co-evolution with hooding, aposematic marking, and spitting. Toxins 9(3), 103.

Paradis, E., Claude, J., Strimmer, K., 2004. APE: Analyses of phylogenetics and evolution in R language. Bioinformatics 20, 289–290.

Pla, D., Bande, B.W., Welton, R.E., Paiva, O.K., Sanz, L., Segura, A., Wright, C.E., Calvete, J.J., Gutiérrez, J.M., Williams, D.J., 2017. Proteomics and antivenomics of Papuan black snake (*Pseudechis papuanus*) venom with analysis of its toxicological profile and the preclinical efficacy of Australian antivenoms. J. Proteom. 150, 201–215.

R-Core-Team, 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Available online: https://www.R-project.org/

Ramasamy, S., Fry, B.G., Hodgson, W.C., 2005. Neurotoxic effects of venoms from seven species of Australasian black snakes (*Pseudechis*): efficacy of black and tiger snake antivenoms. Clin. Exp. Pharmacol. Physiol. 32, 7–12.

Revell, L.J, 2012. Phytools: an R package for phylogenetic comparative biology (and other things). Methods Ecol. Evol. 3, 217–223.

Rogalski, A., Soerensen, C., Op den Brouw, B., Lister, C., Dashevsky, D., Arbuckle, K., Gloria, A., Zdenek, C.N., Casewell, N.R., Gutiérrez, J.M., Wüster, W., Ali, S.A., Masci, P., Rowley, P., Frank, N., Fry, B.G., 2017. Differential procoagulant effects of saw-scaled viper (Serpentes: Viperidae: *Echis*) snake venoms on human plasma and the narrow taxonomic ranges of antivenom efficacies. Toxicol. Lett. 280, 159–170.

Rowe, S.L., Stegemann, J.P., 2009. Microstructure and mechanics of collagen-fibrin matrices polymerized using ancrod snake venom enzyme. J. Biomech. Eng. 131, 61012.

Sanders, K.L., Lee, M.S.Y., Leys, R., Foster, R., Keogh, J.S., 2008. Molecular phylogeny and divergence dates for Australasian elapids and sea snakes (Hydrophiinae): evidence from seven genes for rapid evolutionary radiations. J. Evol. Biol. 21, 682–695.

Shine, R., 1987. The evolution of viviparity: ecological correlates of reproductive mode within a genus of Australian snakes (*Pseudechis*: Elapidae). Copeia 3, 551–563.

Shine, R., 1995. Australian snakes: a natural history. Cornell University Press.

Stiles, B.G., Sexton, F.W., Weinstein, S.A., 1991. Antibacterial effects of different snake venoms: purification and characterization of antibacterial proteins from *Pseudechis australis* (Australian king brown or mulga snake) venom. Toxicon 29(9), 1129–1141.

Sunagar, K., Jackson, T.N.W., Undheim, E.A.B., Ali, S.A., Antunes, A., Fry, B.G., 2013. Three-fingered RAVERs: rapid accumulation of variations in exposed residues of snake venom toxins. Toxins 5, 2172–2208.

Van Velen, L., 1973. A new evolutionary law. Evol. Theory 1, 1–30.

Vaughan, G.T., Sculley, T.B., Tirrell, R., 1981. Isolation of a hemolytic, toxic phospholipase from the venom of the Australian red-bellied black snake *(Pseudechis porphyriacus*). Toxicon 19(1), 95–101.

Viala, V.L., Hildebrand, D., Trusch, M., Arni, R.K., Pimenta, D.C., Schlüter, H., Betzel, C., Spencer, P.J., 2014. *Pseudechis guttatus* venom proteome: insights into evolution and toxin clustering. J. Proteom. 110, 32–44.

Williams, D., Wüster, W., Fry, B.G., 2006. The good, the bad and the ugly: Australian snake taxonomists and a history of the taxonomy of Australia's venomous snakes. Toxicon 48, 919–930.

Wilson, S., Swan, G., 2003. Reptiles of Australia. Princeton University Press, Princeton and Oxford.

Wüster, W., Dumbrell, A.J., Hay, C. Pook, C.E., Williams, D.J., Fry, B.G., 2005. Snakes across the Strait: Trans-Torresian phylogeography relationships in three genera of Australian snakes (Serpentes: Elapidae: *Acanthophis*, *Oxyuranus* and *Pseudechis*). Mol. Phylogenet. Evol. 34, 1–14.

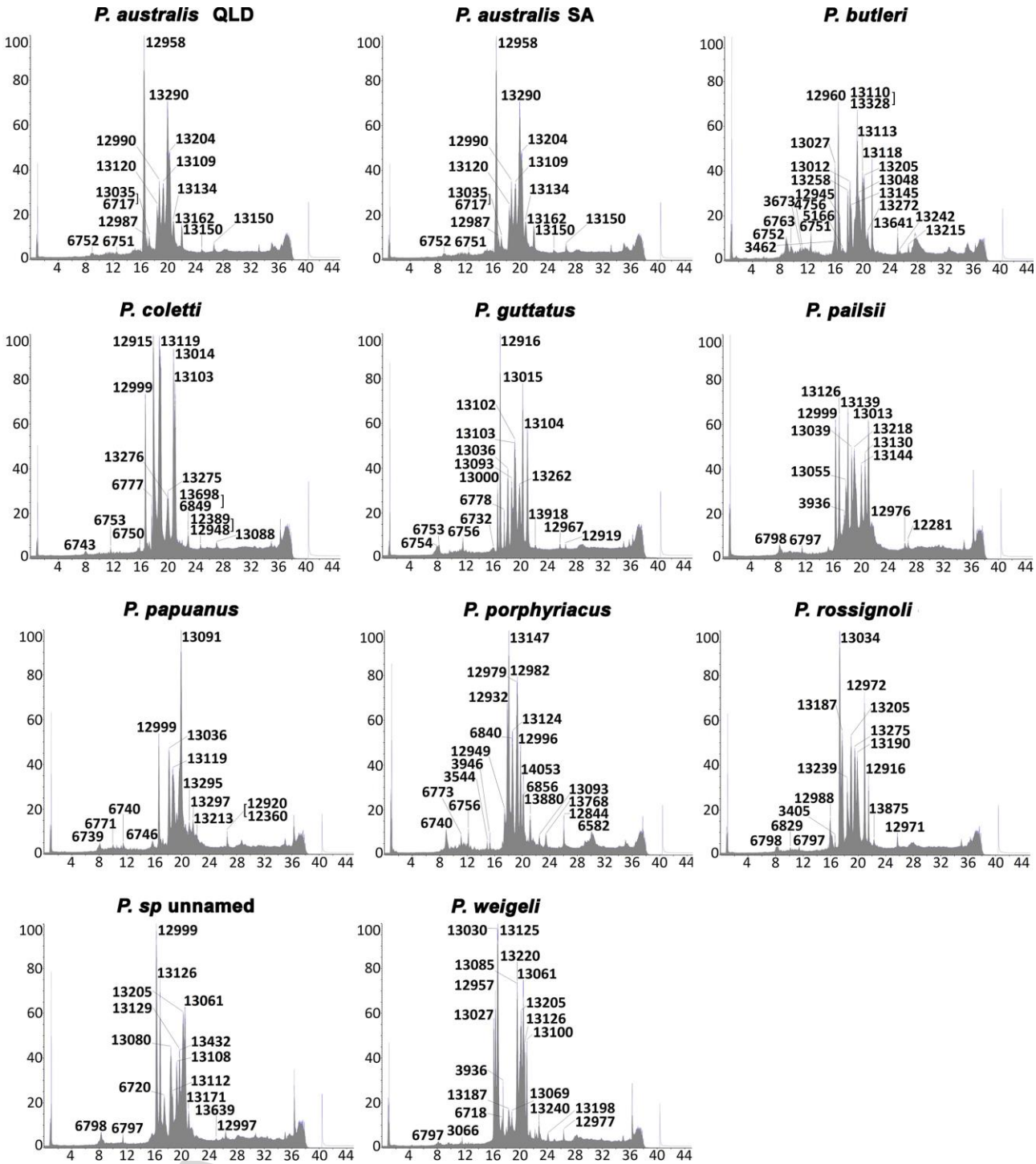

Figure 1: LC/MS comparison of Pseudechis venoms with reconstructed masses in Daltons above each peak.

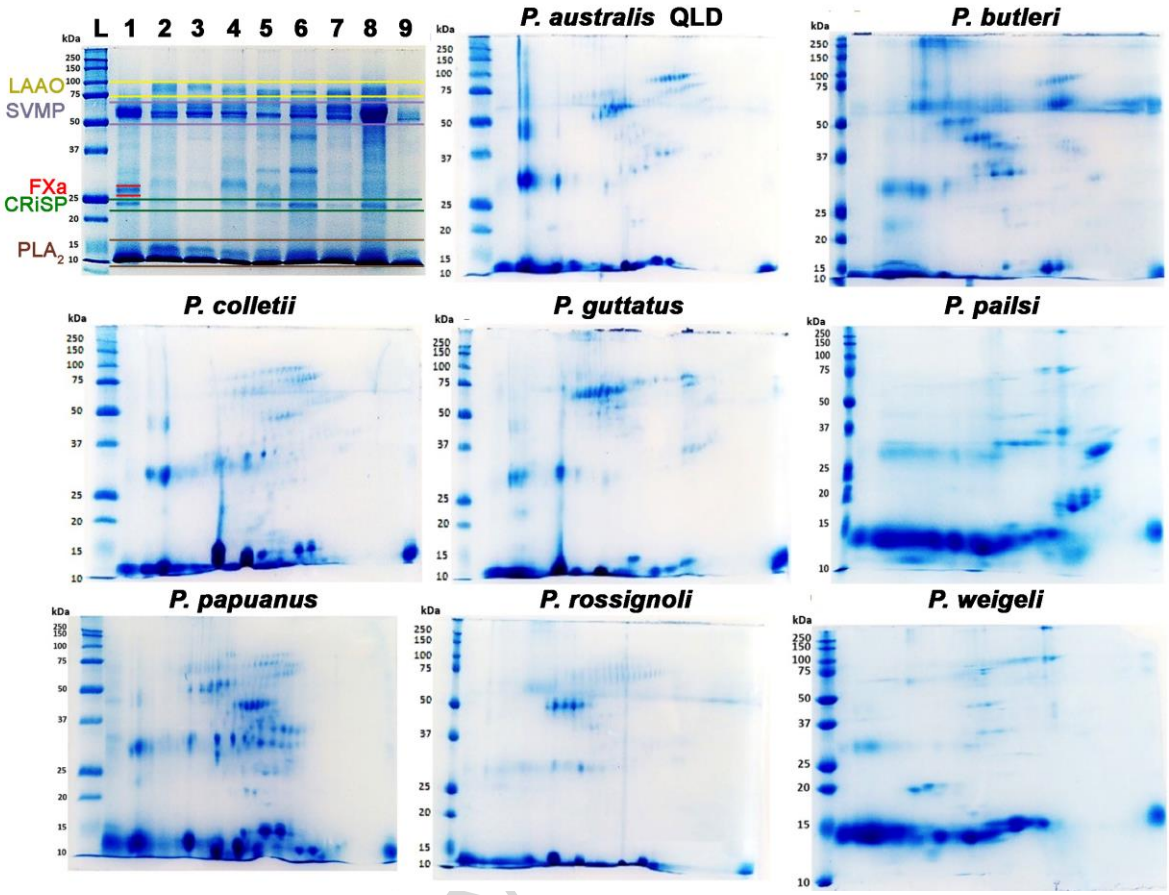

Figure 2: 1D and 2D SDS PAGE gel comparison of representative *Pseudechis* venoms. Molecular weight markers are shown for each. Lane 1 = *P. porphyriacus* 2 = *P. australis* (Eyre), 3 = *P. pailsi* (Mt.Isa), 4 = *P. australis* (Kulgera), 5 = *P. colletii*, 6 = *P., papuanus*, 7 = *P. guttatus*, 8 = *P. butleri*, 9 = *P. rossingnoli*.

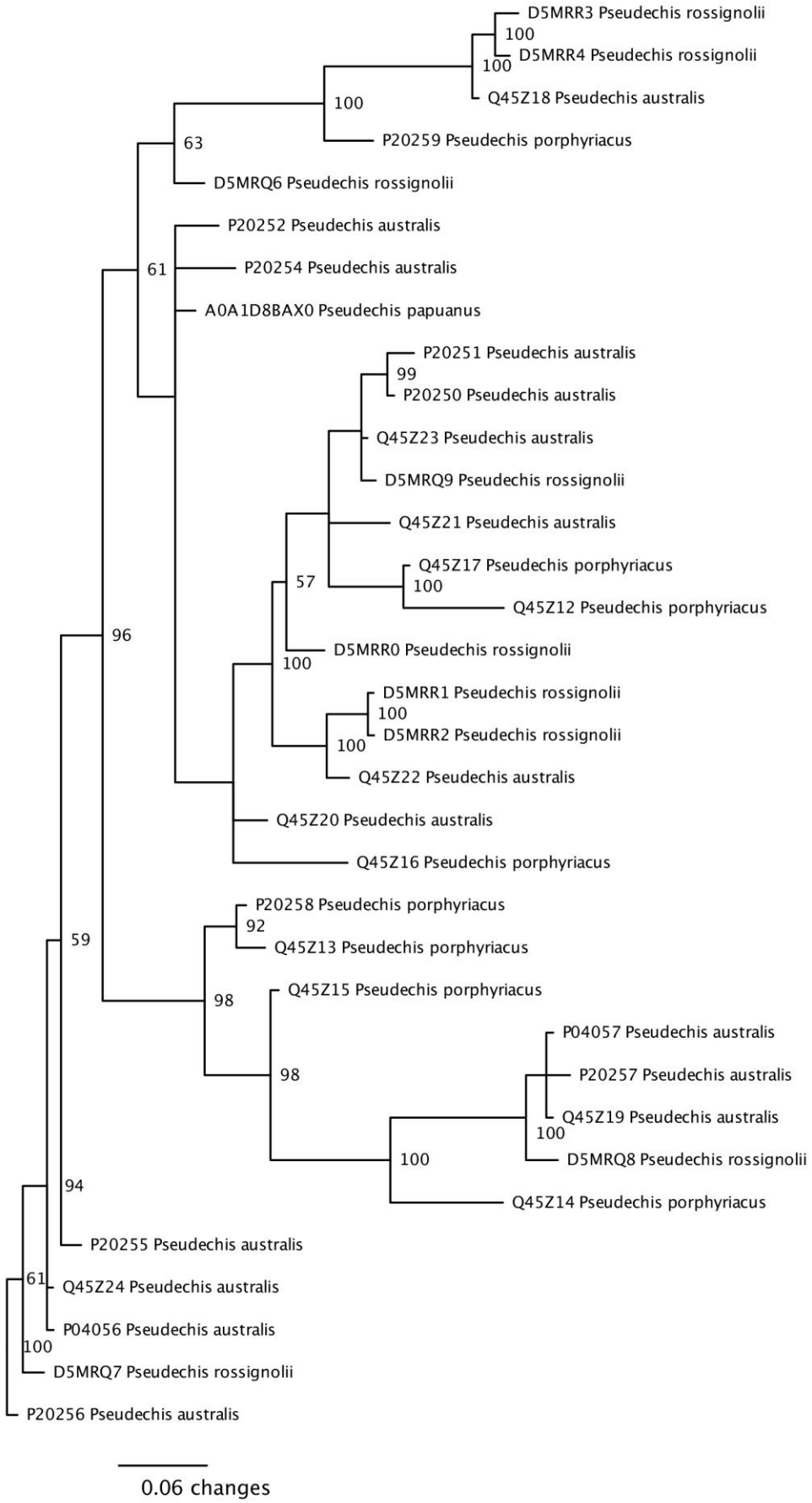

Figure 3: Phylogenetics of the *Pseudechis*-specific clade of PLA_2 toxins. *Notechis scutatus* PLA_2 outgroup is not shown. Node values indicate posterior probabilities.

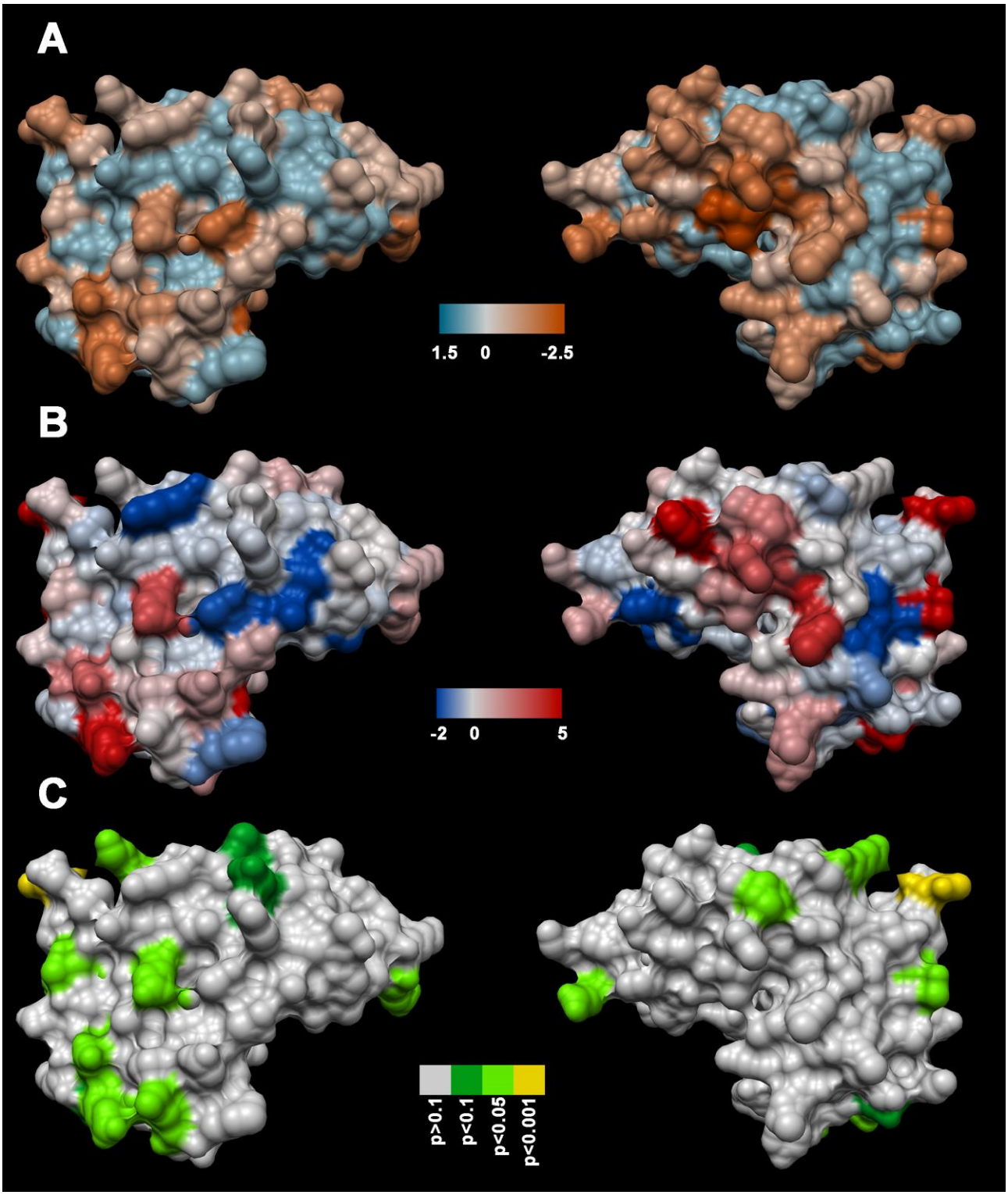

Figure 4: Three dimensional structure of *Pseudechis* PLA_2 toxin diversity coloured according to (A) AL2CO amino acid conservation score (conserved sites in teal and variable sites in orange), (B) FUBAR strength of persistent selection (sites under purifying selection in blue and sites under diversifying 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).

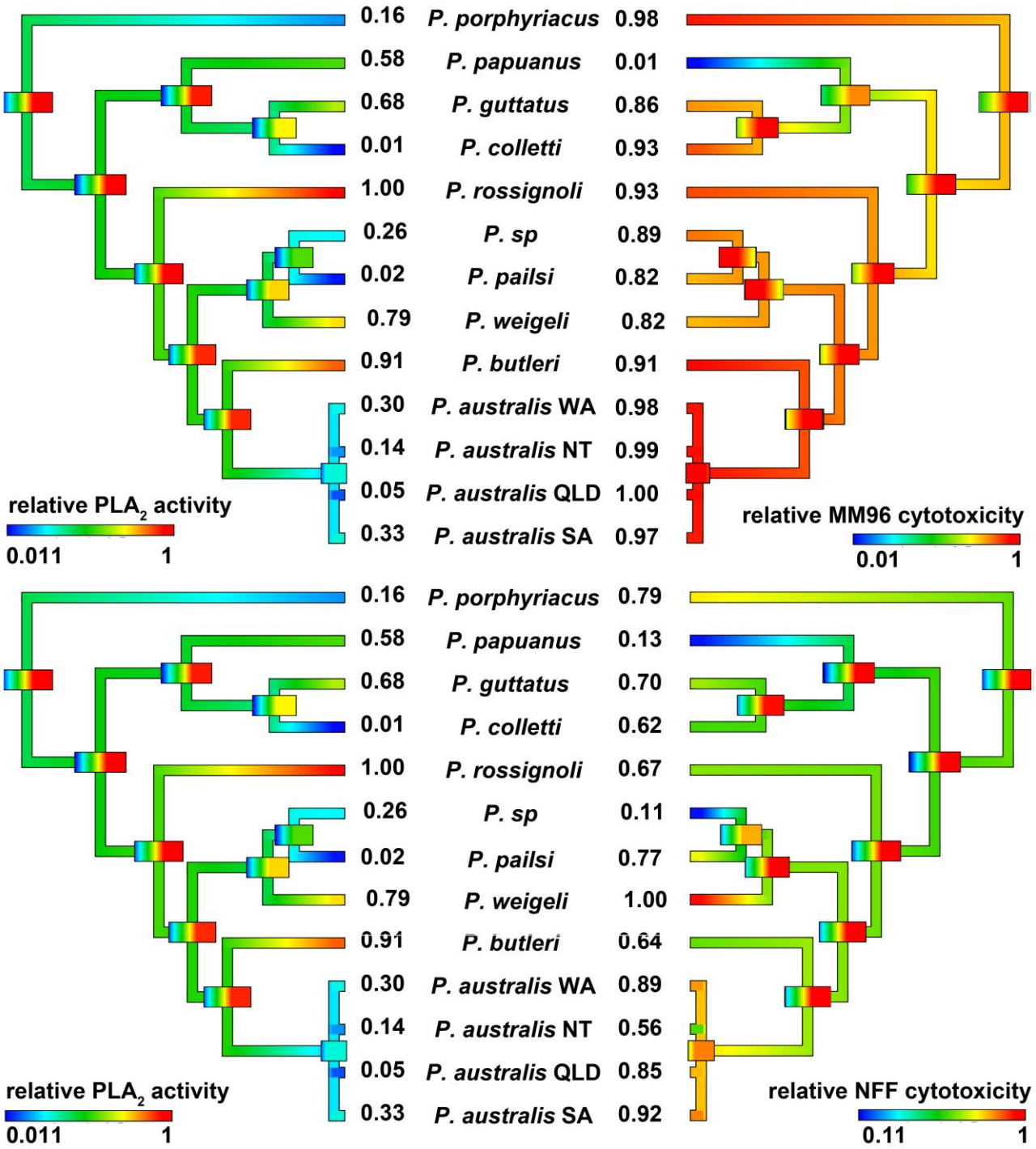

Figure 5: Ancestral state reconstruction of relative bioactivty, where warmer colours represent greater activity. Bars indicate 95% confidence intervals for the estimate at each node. Note that due to the high dynamicity of venom evolution the ranges quickly become broad as one moves down the tree. Phylogeny used was as per Maddock et al. (2017) and Wüster et al. (2005).

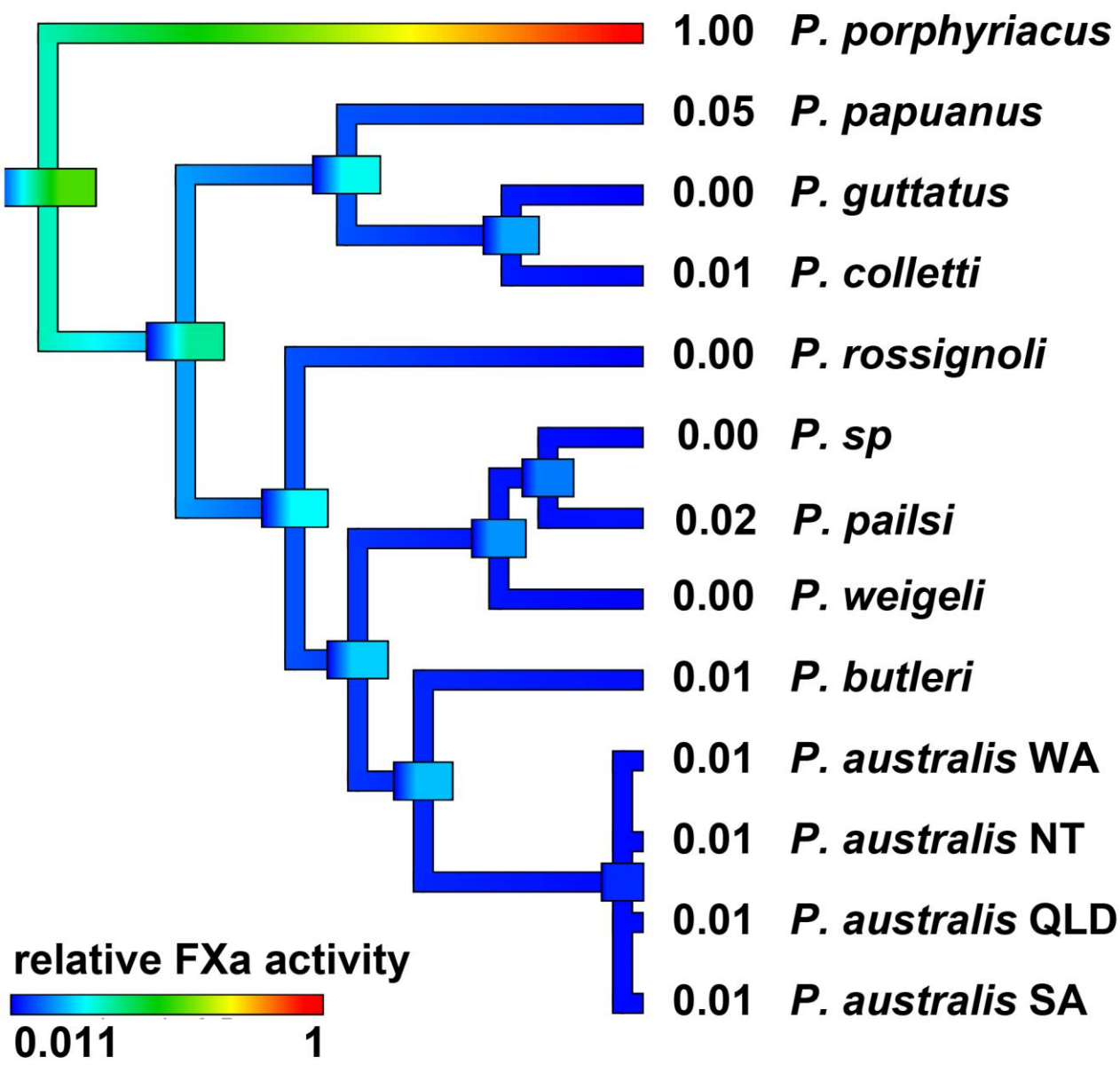

Figure 6: Ancestral state reconstruction of relative Factor Xa, where warmer colours represent greater activity. Bars indicate 95% confidence intervals for the estimate at each node. Note that due to the high dynamicity of venom evolution the ranges quickly become broad as one moves down the tree. Phylogeny used was as per Maddock et al. (2017) and Wüster et al. (2005).

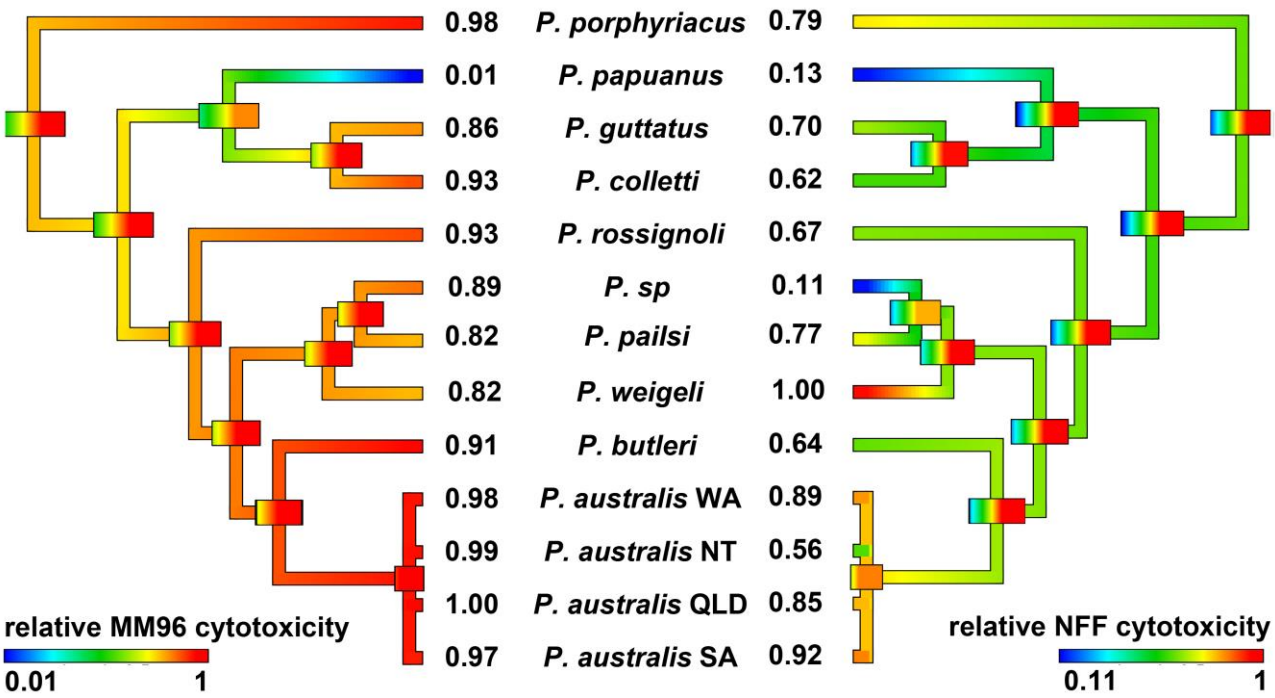

Figure 7: Ancestral state reconstruction of relative cytotoxicity, where warmer colours represent greater activity. Bars indicate 95% confidence intervals for the estimate at each node. Note that due to the high dynamicity of venom evolution the ranges quickly become broad as one moves down the tree. Phylogeny used was as per Maddock et al. (2017) and Wüster et al. (2005).