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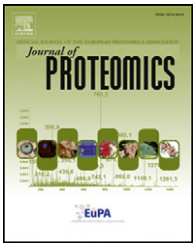

Pseudechis guttatus venom proteome: Insights into evolution and toxin clustering

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ABSTRACT

The Australian Elapidae spotted black snake *Pseudechis guttatus* venom proteome composition was analyzed by high throughput mass spectrometry. The crude venom proteins were decomplexed by 2D-PAGE and in-gel digestion peptides from 66 spot samples and analyzed by tandem mass spectrometry–LC-ESI-ion trap. Protein identification was performed combining PEAKS studio 7.0 and Mascot software. The analysis identified L-amino-acid oxidases, phospholipases A2, metalloproteases, nerve growth factors and ecto-5′-nucleotidases, and for the first time in this venom the components cysteine-rich secretory proteins similar to pseudechetoxin, phospholipase B and transferrin-like protein. The envenomation symptoms are in agreement with the identified components, but the present limitations of database information might impair the detection of toxin families, protein species and still unknown toxins. From the qualitative point of view, the similarity of this venom with the ones from other *Pseudechis* species could be assigned to recent speciation events.

Biological significance
Studies on the proteome of Australian Elapidae (Ancanthophiinae) are quite rare. In the present work we performed, using classic proteomic methods, a qualitative and partial analysis of the proteic components of *Pseudechis guttatus* venom. Although previous studies contributed to the knowledge of the major components of this venom, our study revealed some yet undescribed protein species, as well as new toxins, such as CRiSPs, phospholipase B, transferrin-like protein and ecto 5′-nucleotidase.

1. Introduction

Snake venoms are complex mixtures produced and secreted by specialized glands which contain a diversity of highly active proteins, peptides and other molecules interacting with prey homeostasis, used for defense and predation [1]. The diversity of bioactive molecules in venoms enabled the design of pharmacologically active substances, the development of

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more effective anti-venoms, the understanding of physiological pathways and evolutionary issues [1–3]. Venom proteomic analysis is an important approach to identify and catalog toxins from poorly studied venoms [4–6]. The venom system is essential for feeding, thus making it an important evolutionary selective force [7]. Mainly common body proteins are recruited to the venom arsenal of molecules and the diversification of families and introduced toxicity activities are driven by accelerated evolution [8–12].

The Elapidae snake *Pseudechis guttatus*, also known as blue-bellied or spotted black snake, is predominantly diurnal, and occurs in eastern Australia ranging from the southern Brigalow Belt in Queensland, south to around Forbes, New South Wales and feeds on prey like mammals, reptiles and frogs [13]. The Australian Elapidae have highly toxic venoms and some species are among the deadliest snakes in the world [13]. Although rare, accidents recorded in the literature due to bites by *P. guttatus* are considered dangerous, as its LD_{50} by subcutaneous injection in mice is 2.13 mg/kg and the average venom yield is 32 mg [13–16]. In humans, *P. guttatus* bites are similar in effects to *P. porphyriacus* bites, including generalized systemic features and local effects, such as marked swelling and pain at the bite site [14]. *P. guttatus* is considered medically important [13,17] thus an investigation of its venom composition is important to better understand envenomation by this snake and to prospect for new active pharmacological molecules [18], to obtain data for comparing the venoms of different species and provide insights into species biology and evolution. Given the limitation on venom sample we focused on non-quantitative high-throughput mass spectrometry identification of toxins from pooled venom, decomplexed by two-dimensional electrophoresis.

2. Materials and methods

Pooled venom from *P. guttatus* five captive individuals provided by Venom Supplies Pty. Ltd. (Tanunda, Australia) was used for this analysis. After milking, the venom was immediately freeze-dried and stored at −20 °C. Prior to use, the venom sample was dissolved in 9 M urea, 2% ampholytes and 70 mM DTT to a final venom concentration of 170 μg/ml. After incubation for 30 min at room temperature and centrifugation for 45 min at 15,000 g the supernatant was removed and frozen at −80 °C. The 2D-PAGE method was adapted slightly from previous works [19,20]. The spots were collected manually and in-gel Trypsin (Promega) digestion was performed according to the manufacturer's instructions. MS^2 protein identification by LC-ESI-ion trap analysis was performed on an Agilent 1100 LC/MSD-trap XCT series system, according to Meganathan et al. [20], with minor modifications. For protein identification, both PEAKS studio 7.0 [21] and MASCOT (www.matrixscience.com) software were used on a public database (Squamata proteins + Uniprot venom proteins from the animal toxin annotation program), results were concatenated using InChorus tool, and the identity was considered when significant score was achieved. All MS/MS assignments were manually revised for correctness as well as the quality of the mass spectra of peptides from near-threshold identification.

3. Results and discussion

3.1. Identified components

The 2D-PAGE displays components ranging from *pI* 3 to 10 and molecular masses from 14 to 119 kDa (Fig. 1). As expected, multiple horizontal trains of spots were observed indicating post-translational modifications (PTM). A total of 66 spots were collected (Fig. 1), 41 were assigned to the following protein families: phospholipase A_2 (PLA_2), metalloprotease (SVMP), L-amino acid oxidase (LAAO), cysteine-rich secretory proteins (CRiSP), venom nerve growth factor (VNGF), transferrin-like protein (TFLP), phospholipase B (PLB) and ecto 5′-nucleotidase (E5′N). The lack of specific transcriptomic and genomic data could be impairing the detection of further toxin variants or families. A summary is presented in Table 1 and further data such as peptide sequences are available in the supplementary material. The lack of pharmacological studies on this venom hinders correlation between venom components and venom effects. Hence, only some speculative comparisons can be made with previous studies with related species. Its venom activity is mostly haemolytic, coagulant, neurotoxic and includes local

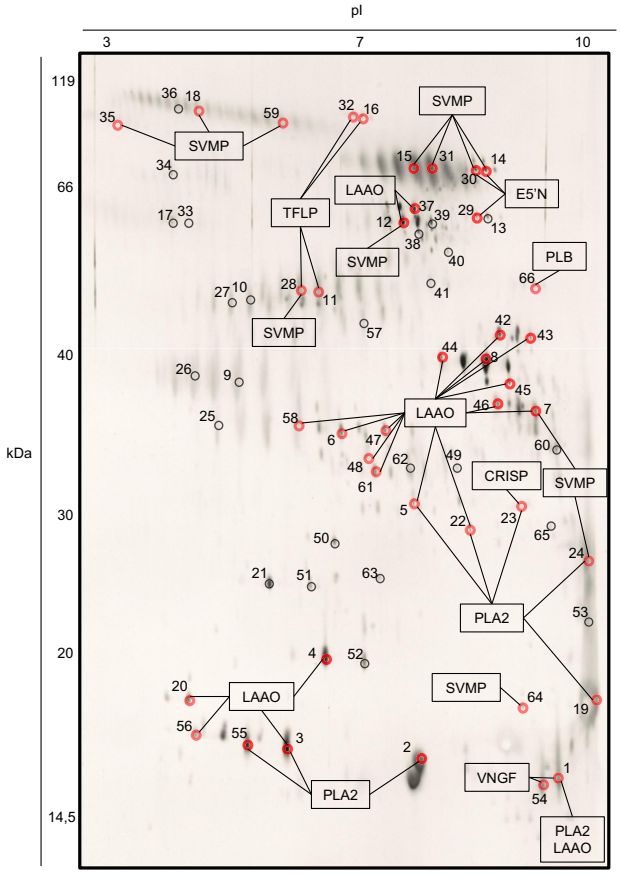

Fig. 1 – 2D-PAGE (*pI* and mass) of *Pseudechis guttatus* venom and the assigned proteins of spots (red circles). Grey circles are collected spots without positive match result. PLA2: phospholipase A2, SVMP: metalloprotease, LAAO: L-amino acid oxidase, CRiSP: cysteine rich secretory protein, TFLP: transferrin-like protein, PLB: phospholipase B, E5′N: ecto 5′ nucleotidase, VNGF: venom nerve growth factor.

effects, such as marked swelling and pain at the bite site, as observed in rare human accidents [15,16,22–24]. Our data support the pathological effects described which could be associated to the presence of PLA_2 variants inducing haemolytic disturbances and neurotoxic effects [25–28], SVMPs inducing haemostatic disturbing activity [29] and the generalized swelling could be explained by CRiSP inflammatory activity, as observed in *Naja atra* [30]. The *P. australis* CRiSP feature on smooth muscle contraction inhibition [31] should be investigated in this venom and also taken in account in future clinical reports. Other toxins identified in this study, such as LAAO, TFLP, E5′N, VNGF and PLB do not present a clear and so far well described activity on the prey and should be investigated further.

3.1.1. *Phospholipase* A_2

Acidic and basic PLA_2 spots were detected (Fig. 1). Snake venom PLA_2s display a broad range of activities including neurotoxic, myotoxic, cardiotoxic, anticoagulant and haemolytic effects [32]. The PLA_2 toxins described so far in the *Pseudechis* genus are known to present myotoxic, platelet inhibition, anticoagulant and neurotoxic effects [25–28] indicating that the neurotoxicity and haemolytic effect of this venom could both be associated with the presence of these enzymes.

3.1.2. *Snake venom metalloproteases SVMPs*

SVMPs were identified in three areas of the gel (Fig. 1). In addition to SVMP classic haemorrhagic activities described in many Viperidae, many other functions are also being studied such as fibrin(ogen)olytic activity, prothrombin activation, blood coagulation factor X activation, apoptotic activity, platelet aggregation inhibition, pro-inflammatory activity and blood serine proteinase inhibitor inactivation [29]. The biological activities of Elapidae SVMP have been poorly investigated. The SVMP from the *N. atra* venom do not have the haemorrhagic activity of Viperidae, but instead inhibits the complement pathways and also exhibits edema-inducing activity [33]. SVMP are actually classified according to their domain composition, where mature P-I has a zinc-dependent peptidase domain, P-II has an additional disintegrin domain at the C-terminal and P-III has, additionally to the P-II structure, a cysteine rich domain [29]. Our findings demonstrate the presence of P-III SVMP in these venom forming three main spot clusters (Fig. 1).

3.1.3. L-*amino acid oxidase LAAO*

The LAAO was identified in 25 out of 50 samples (Fig. 1). The wide spot distribution indicates the probable presence of isoforms, PTMs [34] and/or proteolytic degradation. The presence of LAAO in venom is thought to contribute to its toxicity mainly due to hydrogen peroxide production. Moreover, Suhr and Kim [35] and Torii et al. [36] were able to demonstrate LAAO induced apoptosis in vascular endothelial cells. On the other hand, the toxicity of LAAO from *Agkistrodon halys blomhoffii* could be associated to its anticoagulant activity by inhibiting the interaction between activated platelet integrin αIIb/β3 and fibrinogen [37] and by decreasing Factor IX procoagulant activity [38]. Those combined features may cause prolonged bleeding from the vessel wall at bite sites, but the detailed mechanisms to explain the activities on platelet aggregation, cell apoptosis and cytotoxicity are still unclear [39]. Interestingly, *P. australis* LAAO is known to have antimicrobial activity [40] and it might be recruited by the venom gland for adaptation against feeding pathogens [19] or venom apparatus infection.

3.1.4. *Minor components*

3.1.4.1. Cysteine rich secretory protein. The isolated spot 23 (Fig. 1) generated two peptides matching with Elapidae CRiSP sequences. These peptides are present in pseudechetoxin and pseudecin, two CRiSP from *P. australis* and *P. porphyriacus*, respectively [31]. Although CRiSP role remains unclear for most snake species, pseudechetoxin and pseudecin were shown to act as a smooth muscle contraction inhibitor and cyclic nucleotide-gated ion channel inhibitors [31], as well as a proinflammatory modulator in *N. atra* natrin [30]. Despite the high similarity between pseudecin and pseudechetoxin (96.6%), pseudecin effects are diminished [40], suggesting that the *P. guttatus* CRiSP could have either a different potency or function. Matsunaga et al. [42] described the N-terminal sequence from pseuguttin from the venom of *P. guttatus*, but its complete primary sequence is still unknown, and as our sequences did not align on an N-terminal portion, we are not able to confirm that we identified pseuguttin.

3.1.4.2. Venom nerve growth factor. Other two spot peptides matched to VNGF (Fig. 1). The spots are located at the bottom of the gel in agreement with usual VNGF theoretical mass (~13 kDa). However the spots *pI* (~9) do not correspond to the theoretical *pI* of *P. australis* VNGF (UniProt: NGFV1_PSEAU). The role of VNGF in the venom has not yet been elucidated, but its wide distribution in snake venoms suggests an important role for predation [43]. Common NGF is present in salivary gland of mammals, a tissue of probable common origin with the snake venom gland [44], therefore VNGF could be considered as a residual secretion.

3.1.4.3. Ecto 5' nucleotidase E5'N. A cluster of E5′N spots was identified (Fig. 1). Its role in venom is still not clear, nevertheless, the presence of free purines supports the potential of venom-induced hypotension and paralysis via purine receptors [45]. According to Hart et al. [46] E5′N can also inhibit platelet aggregation and Rodrigues et al. [47] additionally proposed that E5′N can be associated with PLA_2 activity when ATP is released from skeletal muscle by the myotoxic activity of PLA_2s stimulating purinergic receptors to enhance and spread the muscle damage.

3.1.4.4. Phospholipase B PLB. One isolated basic spot 66 (Fig. 1) generated two peptides covering 5% of its probable homologue, the PLB from the Elapidae *Drysdalia coronoides* [48] and *Crotalus adamanteus* PLB [49]. Common PLB are enzymes that catalyse the hydrolysis of glycerophospholipids. There are toxic variants already described in bees and snakes [50,51], such as the haemolytic PLB from the related species *Pseudechis colletti* [52]. Thus, PLB could explain in part, the haemolysis reported in *P. guttatus* envenoming [14].

3.1.4.5. Transferrin-like protein TFLB. Finally, TFLP was also identified. Although there is not much information on snake TFLP sequences, the pI matches the theoretical one described

Table 1 – Summary table of *Pseudechis guttatus* venom MS/MS identification on 2D-PAGE spots. Data generated by PEAKS + MASCOT (InChorus) analysis against reptile and toxin protein database.

Spot	Prot. family	Accession	Score (%)	Top hit description	Hit species	Coverage (%)	#Peptides	Peptide	m/z	Mass
1	VNGF	NGFV2_NAJSP	61.75	Venom nerve growth factor 2	*Naja sputatrix*	8	2	K.ALTM(+15.99)EGNQASWR.F	690	1379
								K.ALTMEGNQASWR.F	682	1363
								K.QYFFETK.C	482	961.5
	LAAO	OXLA_ECHOC	61.66	L-amino-acid oxidase	*Echis ocellatus*	2	1	R.EADYEEFLEIAK.N	729	1456
	PLA2	PA2_NOTSC	61.64	Phospholipase A2 II-5b (fragment)	*Notechis scutatus scutatus*	50	1	NLIQLSNM(+15.99)IK.C	595	1189
								NLIQLSNMIK.C	587	1173
		PA2BD_PSEAU	61.61	Basic phospholipase A2 PA-13	*Pseudechis australis*	8	1	R.GTPVDELDR.C	501	1000
		A6MJG6_HOPST	60.8	PLA2 Hs-4	*Hoplocephalus stephensii*	9	1	K.APYNQENWNIDTK.K	532	1592
2	PLA2	PA2A3_TROCA	84.29	Acidic phospholipase A2 3	*Tropidechis carinatus*	17	2	R.TPYNDANWNINTK.T	776	1550
								K.GGSGTPVDELDR.C	602	1202
3	LAAO	OXLA_OXYSC	62.2	L-amino-acid oxidase	*Oxyuranus scutellatus scutellatus*	5	2	K.TSADIVINDLSLIHQLPK.K	989	1976
								K.STTDLPSR.F	439	875.4
	PLA2	PA2BD_PSEAU	61.53	Basic phospholipase A2 PA-13	*Pseudechis australis*	8	1	R.GTPVDELDR.C	501	1000
4	LAAO	OXLA_OXYSC	87.16	L-amino-acid oxidase	*Oxyuranus scutellatus scutellatus*	9	4	K.FWEADGIHGGK.S	609	1216
								K.STTDLPSR.F	439	875.4
								K.TSADIVINDLSLIHQLPK.K	989	1976
								K.SGLTAAR.D	338	674.4
5	LAAO	OXLA_PSEAU	98.86	L-amino-acid oxidase	*Pseudechis australis*	13	7	K.YPVKPSEEGK.S	567	1133
								K.SDDIFSYEK.R	552	1102
								R.RIHFEPPLPPK.K	444	1330
								R.IHFEPPLPPK.K	392	1174
								K.FWEADGIHGGK.S	609	1216
								K.STTDLPSR.F	439	875.4
								R.IYFAGEYTASVHGWLDSTIK.S	753	2257
	PLA2	PA2A3_PSEAU	60.4	Acidic phospholipase A2 PA-3	*Pseudechis australis*	11	1	K.ATYNDANWNIDTK.T	763	1525
6	LAAO	OXLA_PSEAU	99.16	L-amino-acid oxidase	*Pseudechis australis*	14	8	R.EADYEEFLEIAK.N	729	1456
								K.DGWYVNLGPMR.L	654	1307
								K.DGWYVNLGPM(+15.99)R.L	662	1323
								K.YPVKPSEEGK.S	567	1133
								K.SASQLYR.E	413	823.4
								K.YDTYSTK.E	439	876.4
								K.SDDIFSYEK.R	552	1102
								K.RFDEIVGGFDQLPR.S	550	1648
								R.FDEIVGGFDQLPR.S	747	1492
		OXLA_OXYSC	99.01	L-amino-acid oxidase	*Oxyuranus scutellatus scutellatus*	8	5	K.YPVKPSEEGK.S	567	1133
								K.SASQLYR.E	413	823.4
								K.YDTYSTK.E	439	876.4
								K.SDDLFSYEK.R	552	1102
								R.VAYQTPAK.T	439	876.5
7	LAAO	OXLA_PSEAU	99.14	L-amino-acid oxidase	*Pseudechis australis*	12	7	K.DGWYVNLGPMR.L	654	1307
								K.DGWYVNLGPM(+15.99)R.L	662	1323
								K.YPVKPSEEGK.S	567	1133
								K.SASQLYR.E	413	823.4
								K.SDDIFSYEK.R	552	1102

(continued on next page)

Table 1 (*continued*)

Spot	Prot. family	Accession	Score (%)	Top hit description	Hit species	Coverage (%)	#Peptides	Peptide	m/z	Mass
								K.RFDEIVGGFDQLPR.S	550	1648
								R.FDEIVGGFDQLPR.S	747	1492
								R.RIHFEPPLPPK.K	444	1330
		OXLA_OXYSC	98.95	L-amino-acid oxidase	*Oxyuranus scutellatus scutellatus*	7	4	K.YPVKPSEEGK.S	567	1133
								K.SASQLYR.E	413	823.4
								K.SDDLFSYEK.R	552	1102
								R.VAYQTPAK.T	439	876.5
	SVMP	B5KFV3_PSEAU	61.39	Australease-1	*Pseudechis australis*	2	1	K.SVAVIHDHSKR.T	417	1248
8	LAAO	OXLA_PSEAU	99.19	L-amino-acid oxidase	*Pseudechis australis*	25	16	R.EADYEEFLEIAK.N	729	1456
								R.VVVVGAGMAGLSAAYVLAGAGHQVTLLEASER.V	1033	3096
								R.NEKDGWYVNLGPMR.L	560	1678
								K.DGWYVNLGPMR.L	654	1307
								K.DGWYVNLGPM(+15.99)R.L	662	1323
								K.YPVKPSEEGK.S	567	1133
								K.SASQLYR.E	413	823.4
								K.VIEELK.R	366	729.4
								K.VIEELKR.T	444	885.5
								K.YDTYSTK.E	439	876.4
								K.EYLIK.E	665	664.4
								K.SDDIFSYEK.R	552	1102
								K.RFDEIVGGFDQLPR.S	825	1648
								R.FDEIVGGFDQLPR.S	747	1492
								R.RIHFEPPLPPK.K	444	1330
								R.IHFEPPLPPK.K	588	1174
								R.IHFEPPLPPKK.A	435	1302
11	TFLP	Q1EL74_BOAFU	61.04	Transferrin	*Boaedon fuliginosus*	1	1	K.LFGSQGTQK.D	483	964.5
12	LAAO	OXLA_PSEAU	99.19	L-amino-acid oxidase	*Pseudechis australis*	21	17	R.EADYEEFLEIAK.N	729	1456
								R.NEKDGWYVNLGPMR.L	840	1678
								K.DGWYVNLGPMR.L	654	1307
								K.DGWYVNLGPM(+15.99)R.L	662	1323
								K.YPVKPSEEGK.S	567	1133
								K.SASQLYR.E	413	823.4
								K.YDTYSTK.E	439	876.4
								K.SDDIFSYEK.R	552	1102
								K.SDDIFSYEKR.F	630	1259
								K.RFDEIVGGFDQLPR.S	825	1648
								R.FDEIVGGFDQLPR.S	747	1492
								R.RIHFEPPLPPK.K	444	1330
								R.RIHFEPPLPPKK.A	366	1458
								R.IHFEPPLPPK.K	588	1174
								R.IHFEPPLPPKK.A	435	1302
								K.FWEADGIHGGK.S	609	1216
								K.STTDLPSR.F	439	875.4
								K.SGLTAAR.D	338	674.4
		U3EPI5_MICFL	95.02	L-amino acid oxidase 1c	*Micrurus fulvius*	5	3	K.SDDIFSYERR.F	644	1287

								K.YDTYSTK.E	439	876.4
								K.STTDLPSR.F	439	875.4
	SVMP	B5KFV2_PSEPO	61.42	Porphyriacase-1	*Pseudechis porphyriacus*	2	2	R.NDNAQLLTGIK.F	594	1186
								R.KRNDNAQLLTGIK.F	491	1470
		B5KFV3_PSEAU	59.7	Australease-1	*Pseudechis australis*	2	1	K.SVAVIHDHSKR.T	417	1248
14	E5′N	U3FYP9_MICFL	99.19	Ecto-5′-nucleotidase 1	*Micrurus fulvius*	21	11	K.LTILHTNDVHAR.V	464	1389
								K.FPILSANIRPK.G	419	1255
								K.IINVGSEK.V	430	858.5
								K.VGIIGYTTK.E	476	950.5
								K.LTTLGVNK.I	423	844.5
								K.IIALGHSGFK.E	522	1042
								R.QVPVVQAYAFGK.Y	654	1306
								K.ASGNPILLNK.S	514	1026
								R.HGQGTGELLQVSGIK.V	763	1523
								K.VLLPSFLAAGGDGYYMLK.G	958	1914
								K.VFPAMEGR.V	454	905.4
		U3T7C6_OVOOK	99.15	5′-nucleotidase (Fragment)	*Ovophis okinavensis*	15	7	K.ASGNPILLNK.D	514	1026
								K.VGIIGYTTK.E	476	950.5
								R.QVPVVQAYAFGK.Y	654	1306
								K.GDSSNHSSGNLDISIVGDYIK.R	727	2177
								K.IINVGSEK.V	430	858.5
								K.LTTLGVNK.I	423	844.5
								K.FMNSLR.Y	384	766.4
		V8NYW9_OPHHA	99.04	5′-nucleotidase (Fragment)	*Ophiophagus hannah*	25	6	K.VLLPSFLAAGGDGYYMLK.G	958	1914
								R.HGQGTGELLQVSGIK.V	763	1523
								K.HADKLTTLGVNK.I	433	1296
								K.IIALGHSGFK.E	522	1042
								K.VFPAMEGR.M	454	905.4
								K.LTTLGVNK.I	423	844.5
	SVMP	B5KFV2_PSEPO	55.29	Porphyriacase-1	*Pseudechis porphyriacus*	2	1	R.NDNAQLLTGIK.F	594	1186
15	SVMP	B5KFV2_PSEPO	61.55	Porphyriacase-1	*Pseudechis porphyriacus*	2	2	R.NDNAQLLTGIK.F	594	1186
								K.RNDNAQLLTGIK.F	672	1342
		E9JGG7_ECHCS	57.88	Metalloproteinase (Fragment)	*Echis carinatus sochureki*	2	1	R.SFAEWR.E	398	794.4
16	TFLP	Q1EL74_BOAFU	61.01	Transferrin	*Boaedon fuliginosus*	1	1	K.LFGSQGTQK.D	483	964.5
18	SVMP	B5KFV2_PSEPO	61.61	Porphyriacase-1	*Pseudechis porphyriacus*	2	1	R.NDNAQLLTGIK.F	594	1186
19	PLA2	PA2SC_AUSSU	84.35	Phospholipase A2 superbin c (Fragment)	*Austrelaps superbus*	48	2	NLIQLSNM(+15.99)IK.C	595	1189
								K.GGSGTPVDELDR.C	602	1202
								NLIQLSNMIK.C	587	1173
		Q45Z14_PSEPO	84.27	PLA-4	*Pseudechis porphyriacus*	11	2	R.GTPVDELDR.C	501	1000
								R.LTLYSWK.C	456	909.5
		PA2A7_AUSSU	84.26	Acidic phospholipase A2 S15-109	*Austrelaps superbus*	13	2	K.GGSGTPVDELDR.C	602	1202
								K.LTLYSWK.C	456	909.5
		PA2BB_PSEAU	83.72	Basic phospholipase A2 PA-11	*Pseudechis australis*	13	2	K.LTLYSWK.C	456	909.5
								K.ENYNIDTK.K	499	995.5
		PA2B_PSEAU	83.39	Basic phospholipase A2 PA-5	*Pseudechis australis*	17	2	K.ENWNIDTK.T	510	1018
								K.VHDDCYAEAGKK.G	668	1335
		PA2BD_PSEAU	82.53	Basic phospholipase A2 PA-13	*Pseudechis australis*	13	2	R.GTPVDELDR.C	501	1000
								NILQFR.K	396	789.4

(continued on next page)

Table 1 (*continued*)

Spot	Prot. family	Accession	Score (%)	Top hit description	Hit species	Coverage (%)	#Peptides	Peptide	m/z	Mass
20	LAAO	OXLA_NAJAT	67.75	L-amino-acid oxidase (Fragment)	*Naja atra*	4	2	K.FWEADGIHGGK.S	609	1216
								K.STTDLPSR.F	439	875.4
22	LAAO	OXLA_OXYSC	61.63	L-amino-acid oxidase	*Oxyuranus scutellatus scutellatus*	5	2	K.TSADIVINDLSLIHQLPK.K	989	1976
								K.STTDLPSR.F	439	875.4
		OXLA_PSEAU	52.21	L-amino-acid oxidase	*Pseudechis australis*	3	2	R.IHFEPPLPPK.K	588	1174
								K.STTDLPSR.F	439	875.4
	PLA2	PA2BD_PSEAU	57.6	Basic phospholipase A2 PA-13	*Pseudechis australis*	8	1	R.GTPVDELDR.C	501	1000
23	CRISP	R4FJJ5_9SAUR	60.94	CRiSP-Cac-1 (Fragment)	*Cacophis squamulosus*	4	1	R.GSIATPYK.S	419	835.4
		R4G2T2_DENDV	60.92	CRiSP-Den-3	*Denisonia devisi*	8	2	K.EIVDKHNALR.R	399	1194
								R.GSIATPYK.S	419	835.4
	PLA2	PA2BD_PSEAU	60.65	Basic phospholipase A2 PA-13	*Pseudechis australis*	8	1	R.GTPVDELDR.C	501	1000
		PA2BB_PSEPO	56.3	Basic phospholipase A2 pseudexin B chain	*Pseudechis porphyriacus*	7	1	K.ENYNINTK.T	498	994.5
24	PLA2	Q45Z14_PSEPO	84.13	PLA-4	*Pseudechis porphyriacus*	11	2	R.GTPVDELDR.C	501	1000
								R.LTLYSWK.C	456	909.5
	SVMP	B5KFV2_PSEPO	61.63	Porphyriacase-1	*Pseudechis porphyriacus*	3	2	R.YLQVK.K	326	649.4
								R.NDNAQLLTGIK.F	594	1186
28	TFLP	Q1EL74_BOAFU	60.6	Transferrin	*Boaedon fuliginosus*	1	1	K.LFGSQGTQK.D	483	964.5
	SVMP	B5KFV3_PSEAU	60.31	Australease-1	*Pseudechis australis*	2	1	K.SVAVIHDHSKR.T	417	1248
29	E5′N	U3FYP9_MICFL	99.04	Ecto-5′-nucleotidase 1	*Micrurus fulvius*	11	6	K.FPILSANIRPK.G	628	1255
								K.IINVGSEK.V	430	858.5
								K.VGIIGYTTK.E	476	950.5
								R.QVPVVQAYAFGK.Y	654	1306
								K.ASGNPILLNK.S	514	1026
								R.HGQGTGELLQVSGIK.V	762	1523
		V8NYW9_OPHHA	84	5′-nucleotidase (Fragment)	*Ophiophagus hannah*	11	2	R.HGQGTGELLQVSGIK.V	762	1523
								K.HADKLTTLGVNK.I	433	1296
30	E5′N	U3FYP9_MICFL	99.16	Ecto-5′-nucleotidase 1	*Micrurus fulvius*	16	9	K.LTILHTNDVHAR.V	464	1389
								K.FPILSANIRPK.G	628	1255
								K.IINVGSEK.V	430	858.5
								K.VGIIGYTTK.E	476	950.5
								K.LTTLGVNK.I	423	844.5
								R.QVPVVQAYAFGK.Y	654	1306
								K.ASGNPILLNK.S	514	1026
								R.HGQGTGELLQVSGIK.V	762	1523
								K.VFPAMEGR.V	454	905.4
								K.VFPAM(+15.99)EGR.V	462	921.4
		U3T7C6_OVOOK	98.97	5′-nucleotidase (Fragment)	*Ovophis okinavensis*	11	6	K.FMNSLR.Y	384	766.4
								K.FM(+15.99)NSLR.Y	392	782.4
								K.IINVGSEK.V	430	858.5
								K.VGIIGYTTK.E	476	950.5

								K.LTTLGVNK.I	423	844.5
								R.QVPVVQAYAFGK.Y	654	1306
								K.ASGNPILLNK.D	514	1026
	SVMP	B5KFV2_PSEPO	61.25	Porphyriacase-1	*Pseudechis porphyriacus*	2	1	R.NDNAQLLTGIK.F	594	1186
31	SVMP	B5KFV2_PSEPO	61.89	Porphyriacase-1	*Pseudechis porphyriacus*	5	4	R.NDNAQLLTGIK.F	594	1186
								R.KRNDNAQLLTGIK.F	491	1470
								R.YLQVK.K	326	649.4
								K.DEIKIEPEAK.V	586	1171
		A6XJS7_AUSSU	61.76	Asrin	*Austrelaps superbus*	3	3	R.NDNAQLLTGIK.F	594	1186
								R.KRNDNAQLLTGIK.F	491	1470
								R.YLQVK.K	326	649.4
		B5KFV8_RHING	61.68	Nigrescease-1	*Rhinoplocephalus nigrescens*	3	2	K.DIVAPPVCGNYFVER.G	840	1678
								K.YLQVK.K	326	649.4
		B5KFV3_PSEAU	59.33	Australease-1	*Pseudechis australis*	2	1	K.SVAVIHDHSKR.T	417	1248
								R.SFAEWR.E	398	794.4
		VM3A_NAJAT	54.15	Zinc metalloproteinase-disintegrin-like atrase-A	*Naja atra*	2	2	K.SFAEWR.A	398	794.4
								R.YLQVK.K	326	649.4
32	TFLP	Q1EL74_BOAFU	61.23	Transferrin	*Boaedon fuliginosus*	1	1	K.LFGSQGTQK.D	483	964.5
35	SVMP	B5KFV2_PSEPO	61.55	Porphyriacase-1	*Pseudechis porphyriacus*	2	2	R.NDNAQLLTGIK.F	594	1186
								R.KRNDNAQLLTGIK.F	491	1470
37	LAAO	OXLA_PSEAU	99.13	L-amino-acid oxidase	*Pseudechis australis*	15	9	R.EADYEEFLEIAK.N	729	1456
								K.DGWYVNLGPM(+15.99)R.L	662	1323
								K.DGWYVNLGPMR.L	654	1307
								K.YPVKPSEEGK.S	567	1133
								K.SASQLYR.E	413	823.4
								K.VIEELKR.T	444	885.5
								K.SDDIFSYEK.R	552	1102
								K.RFDEIVGGFDQLPR.S	825	1648
								R.FDEIVGGFDQLPR.S	747	1492
								K.STTDLPSR.F	439	875.4
42	LAAO	OXLA_PSEAU	98.39	L-amino-acid oxidase	*Pseudechis australis*	7	4	R.EADYEEFLEIAK.N	729	1456
								K.SDDIFSYEK.R	552	1102
								K.RFDEIVGGFDQLPR.S	550	1648

(continued on next page)

Table 1 (*continued*)

Spot	Prot. family	Accession	Score (%)	Top hit description	Hit species	Coverage (%)	#Peptides	Peptide	m/z	Mass
								R.FDEIVGGFDQLPR.S	747	1492
43	LAAO	OXLA_ECHOC	61.45	L-amino-acid oxidase	*Echis ocellatus*	2	1	R.EADYEEFLEIAK.N	729	1456
44	LAAO	OXLA_PSEAU	83.16	L-amino-acid oxidase	*Pseudechis australis*	4	2	R.EADYEEFLEIAK.N	729	1456
								K.SDDIFSYEK.R	552	1102
45	LAAO	OXLA_PSEAU	83.49	L-amino-acid oxidase	*Pseudechis australis*	4	2	R.EADYEEFLEIAK.N	729	1456
								K.SDDIFSYEK.R	552	1102
46	LAAO	OXLA_PSEAU	84.37	L-amino-acid oxidase	*Pseudechis australis*	4	3	R.FDEIVGGFDQLPR.S	747	1492
								K.RFDEIVGGFDQLPR.S	550	1648
								K.SDDIFSYEK.R	552	1102
47	LAAO	OXLA_PSEAU	98.4	L-amino-acid oxidase	*Pseudechis australis*	7	3	R.EADYEEFLEIAK.N	729	1456
								K.SDDIFSYEK.R	552	1102
								R.FDEIVGGFDQLPR.S	747	1492
48	LAAO	OXLA_PSEAU	66.65	L-amino-acid oxidase	*Pseudechis australis*	4	2	R.EADYEEFLEIAK.N	729	1456
								K.SDDIFSYEK.R	552	1102
		OXLA_ECHOC	61.62	L-amino-acid oxidase	*Echis ocellatus*	2	1	R.EADYEEFLEIAK.N	729	1456
54	VNGF	NGFV_PSEPO	57.04	Venom nerve growth factor	*Pseudechis porphyriacus*	3	1	K.QYFFETK.C	482	961.5
55	PLA2	PA2BD_PSEAU	61.58	Basic phospholipase A2 PA-13	*Pseudechis australis*	8	1	R.GTPVDELDR.C	501	1000
56	LAAO	OXLA_OXYSC	61.51	L-amino-acid oxidase	*Oxyuranus scutellatus scutellatus*	3	1	K.TSADIVINDLSLIHQLPK.K	660	1976
58	LAAO	OXLA_PSEAU	60.64	L-amino-acid oxidase	*Pseudechis australis*	2	1	R.EADYEEFLEIAK.N	729	1456
59	SVMP	B5KFV2_PSEPO	59.75	Porphyriacase-1	*Pseudechis porphyriacus*	2	1	R.NDNAQLLTGIK.F	594	1186
61	LAAO	OXLA_PSEAU	84.13	L-amino-acid oxidase	*Pseudechis australis*	4	2	R.FDEIVGGFDQLPR.S	747	1492
								K.SDDIFSYEK.R	552	1102
64	SVMP	B5KFV2_PSEPO	61.45	Porphyriacase-1	*Pseudechis porphyriacus*	2	1	R.NDNAQLLTGIK.F	594	1186
66	PLB	PLB_DRYCN	84.27	Phospholipase-B 81	*Drysdalia coronoides*	5	2	K.YGLDFSYEM(+15.99)APR.A	733	1464
								K.FTAYAINGPPVEK.G	704	1406

Table 2 – Comparison of already described toxins of *Pseudechis guttatus*, *P. porphyriacus* and *P. australis* venoms [19,26–28,40,41,57–66]. X represents toxins identified for the first time in *P. guttatus* venom and X refers to previously described toxin but not observed in this work.

Toxin	*P. porphyriacus*	*P. australis*	*P. guttatus*
CRiSP	x	x	X
PLA2	x	x	x
VNGF	x	x	x
Kunitz-type peptidase inhibitor	x	x	X
Natriuretic peptide	x	x	–
Waprin	x	x	–
TFLP	–	x	X
SVMP	–	x	x
LAAO	–	x	x
E5′N	–	x	x
Coagulation factor Xa	x	–	–
PLB	–	–	X

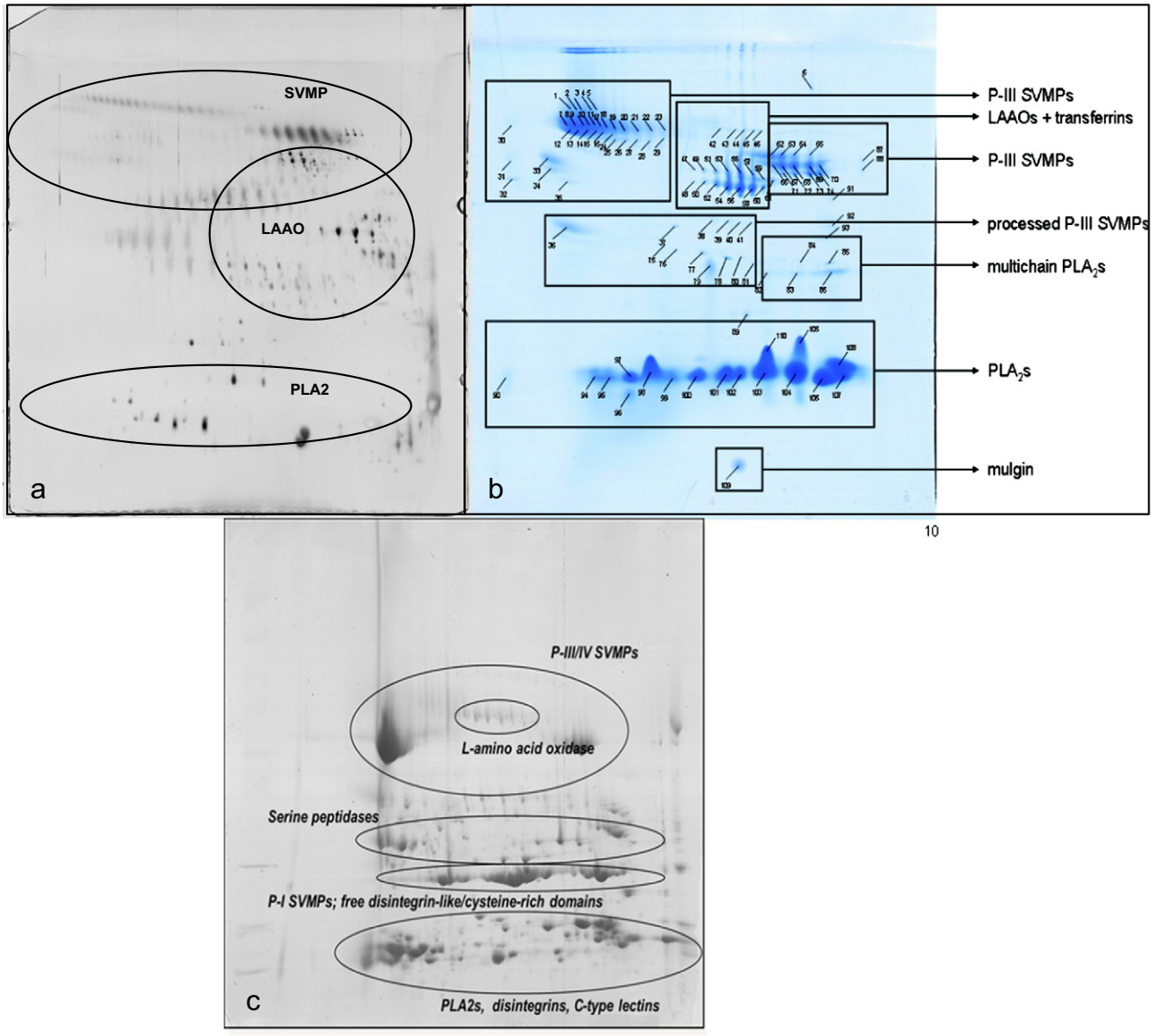

Fig. 2 – Venom clustering in *Pseudechis guttatus* (a), *Pseudechis australis* (adapted from [19]) (b) and *Bothrops jararaca* (c). PLA2: phospholipase A2, SVMP: metalloprotease, LAAO: L-amino acid oxidase.

for the snake *Lamprophis fulginosus* (pI 6.3) [53]. Originally, they are blood plasma proteins involved in iron delivery to the tissues [54] and its presence could be attributed to plasma extravasation as a consequence of mechanical damage during milking. Alternatively, it can be actually a component recruited by the venom gland as it was previously identified in the venom proteome of the related species *P. australis*, where an antimicrobial activity has been suggested [19].

3.2. *Pseudechis* genus venom composition

The genus *Pseudechis* (black snakes), family Elapidae and subfamily Acanthophiinae, is composed of eight other species (Uetz, P., editor, The Reptile Database, http://www.reptile-database.org, accessed February 06, 2014) such as *P. australis*, *P. porphyriacus*, *Pseudechis papuanus*, *P. colletti*, *Pseudechis butleri*, *Pseudechis rossignolii*, *Pseudechis weigeli* and *Pseudechis pailsei*, most known to be highly venomous species [13]. Except for the most studied species *P. australis* and *P. porphyriacus*, not much is known about their venom composition. Their phylogeny is still not well established, although congruent data [55,56] indicate that *P. porphyriacus* is basal in the genus, and that *P. colletti* and *P. guttatus* have diverged from *P. papuanus* ancestor recently during Pliocene, approximately 3 My ago. Molecular data from its venoms may be helpful for insights into evolutionary trends [3]. Table 2 summarizes some venom components comparison between the most studied species *P. australis*, *P. porphyriacus* and *P. guttatus* [19,26–28,40,41,57–66].

For the first time we describe CRiSP, PLB and TFLP in this venom. Especially, the CRiSP from *P. australis*, named pseudechetoxin, is a potent smooth muscle contraction inhibitor and cyclic nucleotide-gated ion channels inhibitors [40] and further efforts should be made to better characterize this toxin in *P. guttatus* venom. Elapidae SVMP activity is also poorly studied and deserves attention. Although future investigations will shed more light upon *Pseudechis* venom composition, interesting and unclear differences are observed. While the basal species *P. porphyriacus* has prothrombin activator coagulation factor Xa, also present in related genera as *Oxyuranus* and *Pseudonaja*, more recent species such as *P. australis* and *P. guttatus* lack it [57,62]. Does the loss of coagulation factor Xa represent an evolutionary trend in this clade?

The 2D gel proteomic analysis of *P. guttatus* venom revealed the presence of SVMP, PLA_2, LAAO, CRiSP, E5′N, VNGF, TFLP and PLB. This kind of analysis has proven to be very important in early stages of venom characterization [67,68]. Such toxin clustering has been performed, for instance, for *Bothrops jararaca* [69] and *Tityus serrulatus* venoms [70] through different methodological approaches, but the expected outcome of either research was to comprehend the molecular composition of the venoms and so to provide a better understanding of the envenomation processes, as well as setting basis for better phylogenetic relationships. Fig. 2 summarizes this idea. A classic 2D gel of *B. jararaca* venom is presented (lower panel) and is clustered according to published data [69]. The right panel shows a 2D gel of the *P. australis* venom [19], the most abundant snake of this genus on the Australian continent [13]. The middle panel presents the actual gel employed in this study with the resulting clustering derived from this work. Despite the obvious lack of genomic/proteomic information on *Pseudechis* when compared to *Bothrops*, one can observe that clustering does occur. For instance, PLA_2 distribution in those gels is virtually the same; however, the *P. guttatus* LAAO seems to be more relevant than in *P. australis*, since its cluster is larger and more independent of the SVMP cluster. SVMP's in the *Pseudechis* genus have a similar distribution.

4. Conclusions

In conclusion, we were able to present an in depth analysis of the composition of a pooled venom sample from the Australian Elapidae *P. guttatus*. By choosing this approach, we were able to identify novel toxins that were not previously described for *P. guttatus* (CRiSP, PLB and TFLP) as well as to describe the venom toxin clusters present in the 2D gel. We recommend the use of multiple bioinformatic software to improve results. The recent natural history of the genus supports the similarities observed in *Pseudechis* venom compositions and 2D gel pattern for PLA_2 and SVMP, but further studies must be carried on to assess the high molecular diversity and improve Australian Elapidae toxin databases and detect subtle differences between them. Other toxins identified in this study, such as LAAO, TFLP, E5′N, VNGF and PLB have not been directly associated with prey acquisition but their identification reinforce the need of a better biochemical and pharmacological study on these toxins.

Conflict of interests

There is no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.07.030.

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