JOURNAL OF PROTEOMICS 116 (2015) 106–113

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Extreme venom variation in Middle Eastern vipers: A proteomics comparison of *Eristicophis macmahonii*, *Pseudocerastes fieldi* and *Pseudocerastes persicus*

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ARTICLE INFO

Article history:
Received 25 June 2014
Accepted 6 September 2014
Available online 18 September 2014

Keywords:
Venom
Viper
2D gel
Proteomics
Antivenom
Toxin

ABSTRACT

Venoms of the viperid sister genera *Eristicophis* and *Pseudocerastes* are poorly studied despite their anecdotal reputation for producing severe or even lethal envenomations. This is due in part to the remote and politically unstable regions that they occupy. All species contained are sit and wait ambush feeders. Thus, this study examined their venoms through proteomics techniques in order to establish if this feeding ecology, and putatively low levels of gene flow, have resulted in significant variations in venom profile. The techniques indeed revealed extreme venom variation. This has immediate implications as only one antivenom is made (using the venom of *Pseudocerastes persicus*) yet the proteomic variation suggests that it would be of only limited use for the other species, even the sister species *Pseudocerastes fieldi*. The high degree of variation however also points toward these species being rich resources for novel compounds which may have use as lead molecules in drug design and development.

Biological significance
These results show extreme venom variation between these closely related snakes.
These results have direct implications for the treatment of the envenomed patient.
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http://dx.doi.org/10.1016/j.jprot.2014.09.003
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1. Introduction

Snake envenoming is a neglected tropical disease that causes up to 94,000 deaths worldwide each year [1]. Members of two families of snakes are predominately responsible for causing these deaths — *Viperidae* (viperid snakes or 'vipers') and *Elapidae* (elapid snakes). The venom composition of many viperid snakes has been well-studied over the past decade, with the recent advances in 'omic' technologies facilitating the characterization of the gene and protein compositions of the venom gland and secreted venom, respectively [2–5]. Characterizing the toxin composition of snake venoms is important for understanding the evolutionary basis of this trophic adaptation, identifying novel bioactive compounds and investigating the interaction between venoms and antivenoms [4,6–11].

Little is known about the venom composition of Middle Eastern viperid snakes of the genera *Eristicophis* and *Pseudocerastes* (Fig. 1). These genera are sister to one another and their clade is sister to that of all other Eurasian *Viperinae* snakes [12]. *Eristicophis* is a monotypic genus while *Pseudocerastes* includes *Pseudocerastes fieldi*, *Pseudocerastes persicus* and the recently described species *Pseudocerastes urarachnoides* [13].

Eristicophis macmahonii is a rare species found in the sandy deserts of Afghanistan, Iran and Pakistan [14–16]. It is known as Macmahon's viper, the leaf-nosed viper or the Asian sand viper [15]. A unique morphological feature of this snake is the butterfly-like shape of the rostral scale [16]. *E. macmahonii* is a fairly stout, dorsoventrally flattened viper, and reaches a maximum length of 72 cm [15]. It is reported to feed upon diverse variety of prey items, including lizards, rodents and birds [14–16]. While little is known about the venom of this species, it has been suggested that its potency may be comparable to that of the *Echis* saw-scaled vipers [15,16].

P. fieldi is widely distributed in the Middle East, including Israel, Syria, Jordan, Iraq, Saudi Arabia and parts of the Sinai [13,15–17]. This species inhabits open deserts with moderate scrub cover and scattered stones and reaches a maximum length of 89 cm. *P. fieldi* appears to be the dominant species of viperid snake wherever it is found. It occurs in a variety of habitats including basalt and limestone deserts (sometimes in association with wadi systems), rolling steppes with volcanic outcrops, mud flats [18], rocky slopes and mountainous country [19]. It prefers hard substrates and avoids true sand dunes. A diverse range of prey items, including lizards, rodents and birds, has been reported for this species.

P. persicus occurs in Iran, Afghanistan, Pakistan and Northern Oman. This species is allopatric with *P. fieldi* — for example, the Zagros Range in west Iran divides the distribution of the two congeners [13,17]. *P. persicus* has an average

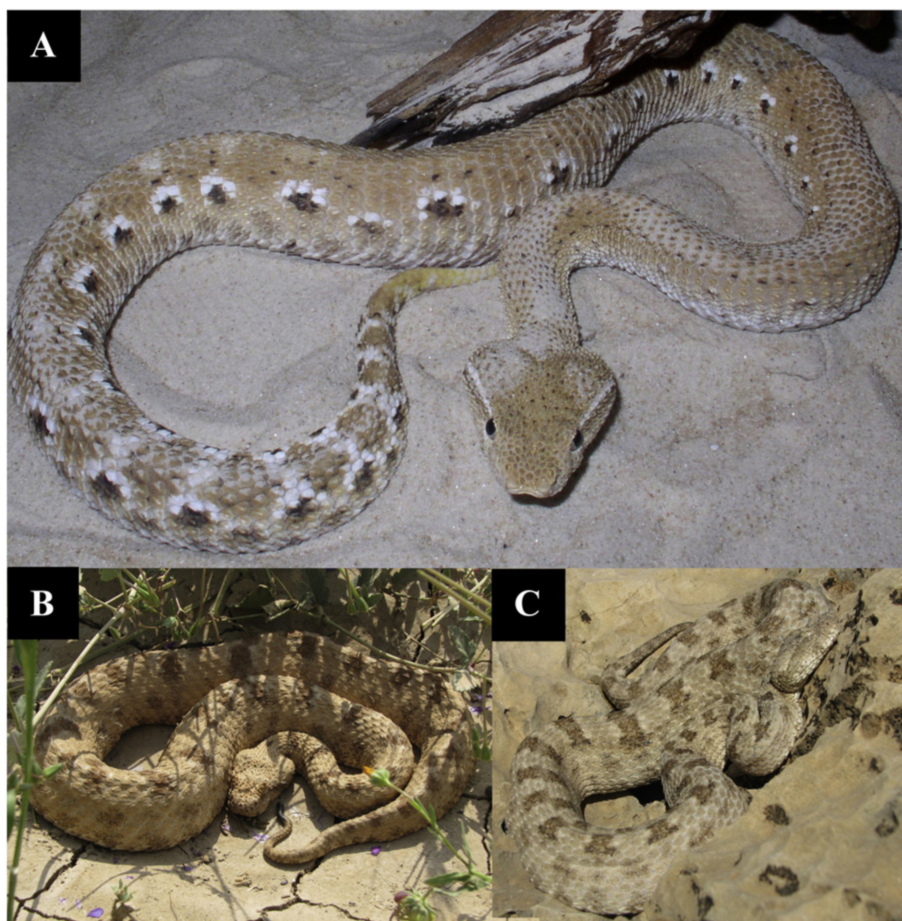

Fig. 1 – Species studied: A) *Eristicophis macmahonii*, B) *Pseudocerastes fieldi* and C) *Pseudocerastes persicus*.

total length of 70 cm, but a maximum length of 108 cm has been reported [14–16,20]. It occupies a variety of habitats including semi-desert, rocky and sandy grounds, rocky slopes and walls, limestone deposits, and flat plains with vegetation cover [14,20]. Like its sister taxon *P. fieldi*, *P. persicus* is primarily nocturnal but may be active during the day in cool weather [16]. Prey items reported for this species include arthropods [21] in addition to lizards, rodents and birds [14,16,20]. The abundance of feathers found in feces collected in the wild suggests a preference for birds over other food items [15]. *P. persicus* is sympatric with other viper species such as *Echis carinatus*, *Macrovipera lebetina*, *P. urarachnoides*, and the elapid snake *Walterinnesia morgani* (Fathinia personal observations).

The venoms of these three vipers have been poorly studied and reports of envenoming in humans are scarce. The clinical effects of *P. fieldi* and *P. persicus* envenomings in humans have not yet been reported, while a single report of a bite by *E. macmahonii* described symptoms including nausea, swelling, fatigue, stiffness, necrosis and a dry mouth [22]. There are however anecdotal reports of deaths from *E. macmahonii* bites. The venoms of *E. macmahonii* and *P. persicus* are potently coagulopathic [23–26]. Anticoagulant kunitz peptides have been isolated and characterized from *E. macmahonii* venom, although this toxin type appears to be absent from the venom of *P. persicus* [27,28]. Conversely, hypotensive natriuretic peptides have been isolated and characterized from *P. persicus* venom, a toxin type apparently absent in the venom of *E. macmahonii* [23,28].

Interestingly, the venom of *P. fieldi* appears to be substantially different from that of both *P. persicus* and *E. macmahonii*. This species has potently neurotoxic venom, which is rich in a novel PLA_2 complex that causes an irreversible neuromuscular blockade at the presynaptic site [29]. This heterodimeric complex consists of two subunits: A basic phospholipase (CBII) and an acidic phospholipase (CBI). CBI may protect the other subunits from non-specific binding and proteolytic activity, and the activity of CBII increases when CBI is present [30]. In addition to an apparent absence of the coagulopathic toxins characterized from its sister species, the venom of *P. fieldi* also appears to lack the L-amino acid oxidase (LAAO) enzyme that is a major component of the venom of some species of viperid snake [27,31].

While it is apparent that *P. fieldi* and *P. persicus* differ in venom function [17,24], the extent of this variation has not been established in terms of venom composition. Moreover, a broad characterization of the toxin composition of these two species and *E. macmahonii* remains to be elucidated. This study therefore aims to re-address this issue by using comparative proteomics to characterize and compare the toxin composition of these three largely unstudied and, potentially, medically important vipers. Such a study is long overdue as the potential capability of the Iranian polyvalent snake antivenom (Razi Vaccine & Serum Research Institute, Iran), used to treat *P. persicus* envenomings, at cross-neutralizing the venom of *P. fieldi* and *E. macmahonii* remains unknown. In addition to having potentially immediate implications for the treatment of envenomed patients, the characterization of toxins from unstudied venoms may also uncover novel compounds that may have potential uses for drug design and development [8,32].

2. Materials and methods

2.1. Snake venom collection

Leaf-nosed viper (*Eristocophis macmahonii*) venom was collected from 3 adult male snakes from the Nushki district (30.12°N 67.01°E) of Balochistan, Pakistan as described earlier [27,31,33]. For *P. fieldi* and *P. persicus* of unknown geographical breeding origin 3 adult male captives were milked by BGF. Venom samples were immediately centrifuged (8500 rpm for 30 min at 4 °C) to remove suspended cells. Supernatant(s) were subsequently filtered using 0.22 μm PVDF syringe filters (Millipore, USA) to remove insoluble materials. Samples were then lyophilized and stored at −86 °C.

2.2. 1D/2D PAGE analysis

For quick comparative analysis of snake venoms (~20 μg), 12% polyacrylamide gel (0.75 mm, 7 cm) electrophoresis (Mini-PROTEAN®3, Bio-Rad, USA) under dissociating (SDS) and dissociating and denaturing (SDS and DTT) conditions was performed. The gels were polymerized, run with a very well-established protocol [2,32] and stained by using 0.2% colloidal Coomassie Brilliant Blue G250 and destained in 1% acetic acid.

Two-dimensional gel electrophoresis was performed according to our established protocol [2]. Briefly, ~1 mg of venom sample(s) was directly solubilized in 300 μL of rehydration buffer (8 M urea, 100 mM DTT, 4% CHAPS, 110 mM DTT, 0.01% bromophenol and 0.5% ampholytes pH 3–10). Samples were centrifuged (14,000 rpm for 5 min at 4 °C), and the supernatant was applied to IEF gel strips (non-linear IPG pH 3–10 and 17 cm) for 24 h in passive rehydration. Venom components were focussed in a PROTEAN i12 IEF CELL (Bio-Rad, USA). The IEF running conditions were as follows: 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h and 8000 V for 98400 V/h. A constant current of 50 μA per strip at 20 °C was applied. After running IEF, IPG strips were equilibrated for 10 min in an equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2% DTT) followed by a second incubation for 20 min in an equilibration buffer that had DTT replaced with 2.5% iodoacetamide. IPG strips were briefly rinsed in SDS-PAGE running buffer (Tris–glycine, pH 8.3) and loaded on top of 12% polyacrylamide gels and covered with 0.5% (w/v) agarose. Second dimension gel fractionation was performed at 4 °C in 10 mA/per gel for 20 min followed by 30 mA/per gel for 4 h or until the bromophenol dye front was 1 cm from the base of the gel. Finally, gels were briefly washed in Milli Q water and stained in 0.2% (w/v) colloidal Coomassie Brilliant Blue G250 overnight and destained in 1% acetic acid (v/v).

Selected spots were subsequently picked from the gels and digested overnight (at 37 °C) using sequencing grade trypsin (Sigma, USA) as previously described [2]. Briefly, gel spots were washed with ultrapure water, destained (40 mM NH_4CO_3/50% acetonitrile) and dehydrated (100% acetonitrile). Gel spots were rehydrated in 10 μL of 20 μg/mL TPCK Trypsin and incubated at 37 °C overnight. Digested peptides were eluted by washing the gel spots for 20 min with each of the following solutions: 20 μL of 1% formic acid, followed by 5%

ACN/0.5% formic acid. The cleaved eluted peptides were pooled and further subjected for identification using LC–MS/MS analysis.

2.3. Whole venom shotgun sequence analysis

For shotgun sequencing, reduction and alkylation were performed by redissolving 3 μg of the whole crude venom in 50 μL of 100 mM ammonium carbonate. 50 μL of 2% iodoethanol/0.5% triethylphosphine in acetonitrile was then added to the redissolved samples. The reaction mixture was incubated for 2 h at 37 °C, before being dried by vacuum and re-suspended in 20 μL of 2.5% acetonitrile (ACN), and 1% formic acid (v/v). Additionally, 3 μg of reduced and alkylated sample was resuspended in 20 μL of 40 mM ammonium bicarbonate, before being incubated overnight (at 37 °C) with 750 ng of sequencing grade trypsin. Digestion was stopped by the addition of 1 μL of concentrated formic acid and only 5 μL (0.75 μg) of each sample was processed by LC–MS/MS [2].

2.4. Liquid chromatography–mass spectrometry

2D-gel spots and/or shotgun samples were analyzed by LC–MS/MS using an Agilent Zorbax stable bond C18 column (2.1 mm × 100 mm, 1.8 μm particle size, 300 Å pore size) at a flow of 400 μL/min and a gradient of 1–40% solvent B (90% acetonitrile, 0.1% formic acid) in 0.1% formic acid over 15 min or 4 min for shotgun and 2D-gel spots, respectively, on a Shimadzu Nexera UHPLC coupled with an AB SCIEX 5600 Triple TOF mass spectrometer (ABSciex). MS–MS spectra were acquired at a rate of 20 scans/s, with a cycle time of 2.3 s, and optimized for high resolution. MS2 spectra were searched against UniProt databanks using Proteinpilot v4.0 (ABSciex). Crude venoms were also analyzed by RP-HPLC LC–MS using an Agilent Zorbax 2.1 × 250 mm, 5 μm, 300 Å C18 column at a flow rate of 250 μL/min and a gradient of 1–60% B (90% acetonitrile, 0.1% formic acid) in 0.1% formic acid over 60 min on an Agilent LC coupled to an ABSciex Pulsar Q-TOF mass spectrometer with scanning between 400 and 2000 m/z and a cycle time of 1 s. Data processing was performed with the aid of the software Analyst QS (ABSciex). Data tables are shown in Supplementary Tables 1–6.

3. Results and discussion

In comparison with 1D-PAGE profiles the venom composition of *E. macmahonii* and *P. persicus* exhibited a number of similarities (Fig. 2A). Both venoms contain components of a broad range of molecular weight, with the majority between 10 kDa and 70 kDa in size. The 1D-PAGE profiles for *E. macmahonii* and *P. persicus* venoms therefore show some similarity to those previously described from other members of the *Viperinae*, such as the saw-scaled vipers of the genus *Echis* [34], the genus *Cerastes* [35], and the European vipers of the genus *Vipera* [36]. However, the 1D-PAGE profile of *P. fieldi* venom revealed marked differences to the venoms of other *Viperinae*, including its congener *P. persicus* and *E. macmahonii* (Fig. 2A). Noticeably, the vast majority of venom proteins are found at low molecular weights (~12–15 kDa), with only faint protein bands evident above 20 kDa in size, suggesting a low concentration of high molecular weight components.

The complexity of snake venom is more readily revealed by 2D-PAGE analysis than 1D-PAGE due to related toxin isoforms often sharing similar molecular weights but different isoelectric points [2]. Consequently, we investigated the venoms of *E. macmahonii* (Fig. 2B), *P. fieldi* (Fig. 2C) and *P. persicus* (Fig. 2D) using 2D-PAGE and identified the venom toxins present in each gel profile by in-gel tryptic digestion and shotgun sequencing using LC–MS–MS analysis (Table 1), with shotgun sequencing better able to detect small peptides.

The 2D-PAGE profile of *E. macmahonii* (Fig. 2B) revealed a relatively complex venom composition, with toxins distributed over a variety of molecular weights (as observed in the 1D-PAGE) and isoelectric points. The venom composition of *E. macmahonii* consisted of toxin families typically identified in viperid snake venoms, including enzymatic and non-enzymatic components. Enzymatic toxins recovered included snake venom metalloproteinases (SVMPs), kallikrein serine proteases, L-amino acid oxidases (LAAOs) and phospholipase A_2s (PLA_2s). Non-enzymatic components included disintegrins, lectins, cysteine-rich secretory proteins (CRiSP) and vascular endothelial growth factor (VEGF). Notably, considerable variation in isoelectric point was observed for many of these toxin families, including LAAOs, kallikrein, lectin and PLA_2s — the latter of which included both basic and acidic forms. These results are likely indicative of the presence of multiple related toxin isoforms, which are encoded by the same gene family. This isotypic diversity occurs in venom as the result of gene duplication events occurring over evolutionary time [11,37–39].

In contrast to the complex composition of *E. macmahonii* venom, venom of the closely related species *P. fieldi* appears relatively simple (Fig. 2C). As noted by 1D-PAGE analysis, relatively few mid-high molecular weight venom components were present in the 2D-PAGE profile, with only kallikrein, CRiSP and nucleotidases observed. Notably, for both kallikrein and CRiSP, and also the low molecular weight lectins: (i) the number of spots, (ii) the intensity of those spots and (iii) the range of their isoelectric separation were found to be much lower than in *E. macmahonii*. Perhaps most importantly, we identified no SVMPs, or disintegrins, in the venom of *P. fieldi*. These components are heavily implicated in causing pathologically important hemotoxicity in envenoming and are widely distributed in most viperid snake venoms, with SVMPs by far the most abundant toxin type in many species [3,40]. Their absence in the venom of *P. fieldi* suggests a lack of hemotoxic activity for this venom. A zymographic analysis, using 0.4% gelatin (Bloom 50) as substrate also confirmed the lack of gelatin-digesting metalloprotease activity (data not shown). In contrast, *P. fieldi* venom contains a wide diversity of PLA_2 enzymes, spreading across the isoelectric range displayed in the 2D-PAGE profile (Fig. 2C). These include the acidic and basic PLA_2s observed in *E. macmahonii* venom (although the protein bands for the basic forms are more extensive in *P. fieldi*), but also neutral PLA_2s that were unique to this species in the present study (Fig. 2). Moreover, the venom of *P. fieldi* was found to contain larger concentrations of kunitz peptides than present in other species, an observation that has been made previously [28].

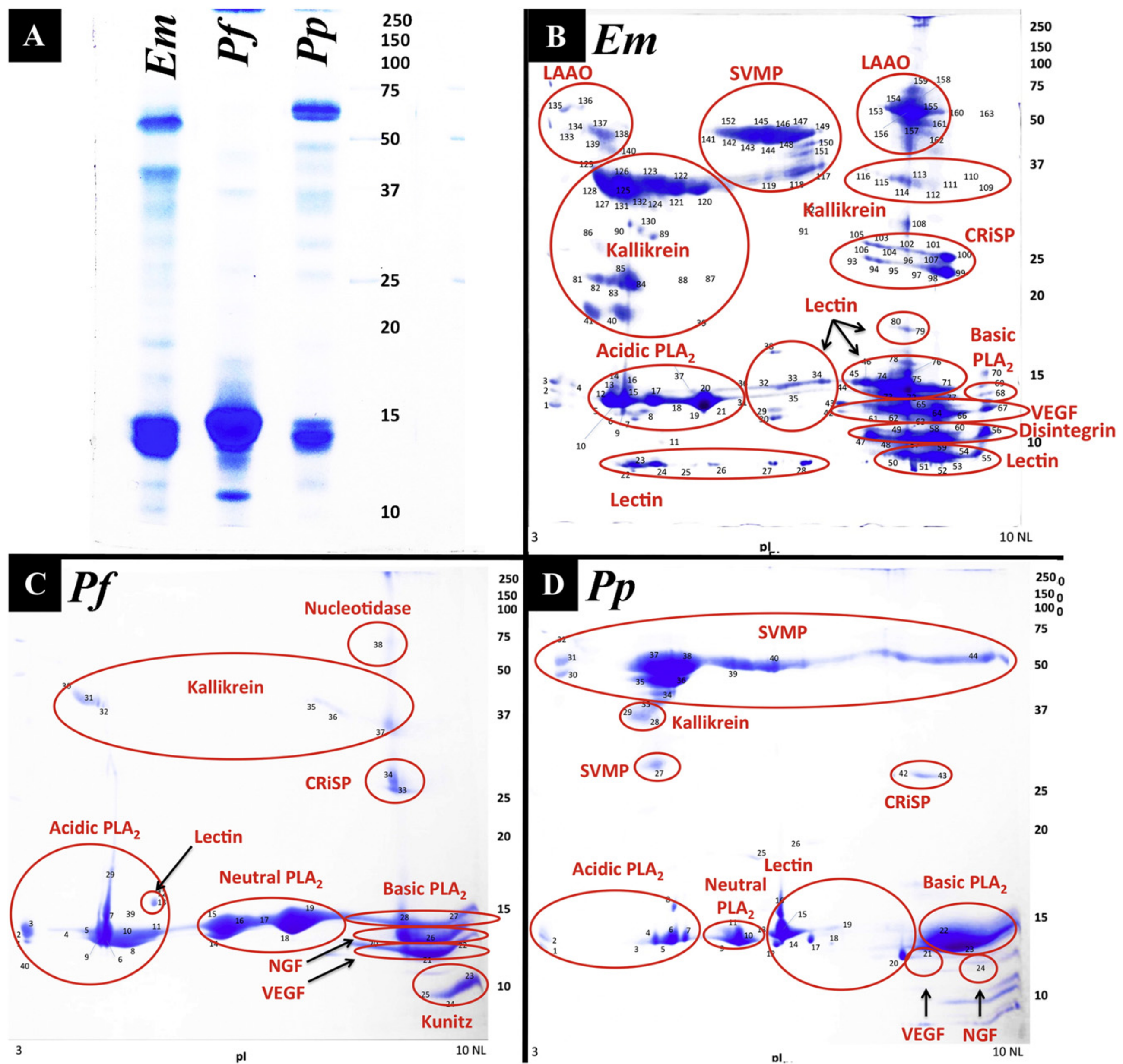

Fig. 2 – A) 1D gel comparison of *Eristicophis macmahonii*, *Pseudocerastes fieldi* and *Pseudocerastes persicus*, B) 2D gel of *Eristicophis macmahonii*, C) 2D gel of *Pseudocerastes fieldi* and D) 2D gel of *Pseudocerastes persicus*.

The venom composition of *P. persicus* appears to be intermediate in profile between that of *E. macmahonii* and *P. fieldi* (Fig. 2D). In the mid-high molecular weight region, similar to the same weight range of *E. macmahonii* venom, we identified a wide isoelectric range of SVMPs. In addition, a small number of kallikrein and CRiSP proteins that showed similarities to those identified from *P. fieldi* were also identified in this region. In the low molecular weight region *P. persicus* venom contained both the acidic and basic PLA_2 enzymes identified in *P. fieldi* and *E. macmahonii*. Neutral PLA_2s were present but in low amounts. Lectin toxins were also present and found to be more diverse and intensely stained than those observed in *P. fieldi*, but less so than those of *E. macmahonii*. Further similarities between *P. persicus* venom and that of its congener included the presence of nerve growth factor (NGF) and the absence of disintegrins and LAAOs, although both nucleotidases and kunitz peptides appear to be novel to *P. fieldi*. Notably, no evidence of natriuretic peptides, previously identified in the venom of *P. persicus* [23], was found here.

Variation in venom composition is a well-documented phenomenon that can occur at any taxonomic level, including inter- and intra-specifically, as well as ontogenetically [2,3,5,9,41–50]. This variation is largely thought to be dictated by the evolutionary history of toxin gene families, with the toxin gene composition present in the genome dictated by gene duplication and loss events occurring over evolutionary time in different species [11,51,52]. However, postgenomic factors, such as the up- or down-regulation of genes being transcribed and translated in the venom gland may also

Table 1 – Toxins detected by proteomics analyses.

	E. macmahonii		*P. fieldi*		*P. persicus*	
Toxin type	SG	G	SG	G	SG	G
CRiSP	✓	✓	✓	✓	✓	✓
Disintegrin	✓	✓			✓	
Kallikrein	✓	✓	✓	✓	✓	✓
Kunitz			✓	✓	✓	✓
LAAO	✓	✓	✓		✓	
Lectin	✓	✓	✓	✓	✓	✓
Natriuretic	✓					
NGF			✓	✓	✓	✓
Nucleotidase			✓	✓	✓	
Phosphodiesterase					✓	
PLA_2	✓	✓	✓	✓	✓	✓
SVMP	✓	✓	✓		✓	✓
VEGF	✓	✓	✓	✓	✓	✓

SG = shotgun; G = gel analysis; CRiSP = cysteine rich secretory protein; LAAO = L-amino acid oxidase; NGF = nerve growth factor; SVMP = snake venom metalloprotease; VEGF = vascular endothelial growth factor.

greatly affect the toxic composition of venom, particularly between closely related species or over the lifetime of the animal. Post-translational modifications are another great source of diversity [3,44]. Here we observe a major compositional change between the venoms of *P. fieldi* and *E. macmahonii* and moderate differences between those of *P. persicus* and *E. macmahonii* and the congeners of *P. fieldi* and *P. persicus*. From an evolutionary perspective, the venom of *E. macmahonii* seemingly represents the plesiotypic state as it, contains a wide range of toxic components similar to that observed in the venoms of other viperid snakes [2]. It is apparent that a broad shift in toxin composition occurs between the sister genera of *Eristicophis* and *Pseudocerastes*, which apparently involves a 'streamlining' of the toxin arsenal, including a reduction in kallikrein and lectin diversity and complete loss of LAAO and disintegrins. However, substantial changes also exist within *Pseudocerastes*, suggesting that *P. persicus* perhaps represents an intermediate condition between those of *E. macmahonii* and *P. fieldi* — emphasizing that the venom of *P. fieldi* likely represents the most apotypic condition, based on this study.

The most notable compositional changes observable in the venom of *P. fieldi* are the complete absence of SVMP toxins and the unique presence of neutral PLA_2 molecules (Fig. 2). These compositional changes are seemingly reflected in venom function, with *P. fieldi* venom exhibiting potent neurotoxic activity as the result of PLA_2 complexes [29], in contrast with the coagulopathic venoms of *E. macmahonii* and *P. persicus* [23–26]. Noticeably, other reports of viper venoms possessing neurotoxic activity via PLA_2 complexing, such as those observed for *Crotalus scutulatus scutulatus*, are also associated with a reduction in SVMPs [53]. This suggests that the evolution of neurotoxic PLA_2 toxins observed in viperid snakes may also be intrinsically associated with the parallel loss or down-regulation of SVMP toxins.

The forces shaping the observed adaptive change in venom composition are unclear due to the poor state of knowledge regarding the natural history of these three species. Since snake venoms are primarily used for prey capture, it has previously been suggested that variation in venom composition may be driven by adaptation for prey-specific venom toxicity [8,35,54]. However, from what little data exists, the species studied here all appear to be opportunistic ambush-feeding predators that occupy diverse ecological niches and feed as generalists on a variety of prey items. Further research, particularly ecological research, is therefore required to elucidate the driving forces and mechanisms underpinning the substantial variation in venom composition observed in this study.

Critically, the broad changes in venom toxin composition observed between these three Middle Eastern vipers raise questions relating to the potential validity of using the Iranian polyvalent antivenom raised against *P. persicus* venom for the treatment of snakebite by both *P. fieldi* and *E. macmahonii*. Variation in venom composition can undermine antivenom therapy, as antibodies directed against the venom of one species are unlikely to cross-react and neutralize toxin components that are unique to the venom of another species. The efficacy of antivenoms used to treat the bites of snakes with venoms not used for antivenom production can be very hard to predict, and is not necessarily predicted by the phylogenetic relatedness of the snakes in question due to the adaptive processes driving venom evolution [4,34,37,55,56]. Our evidence of considerable variation in venom composition between *P. persicus* and *P. fieldi*, coupled with the previous reports of notable differences in their venom functions, suggests that the treatment of *P. fieldi* envenomings with the Iranian polyvalent antivenom should be undertaken with great care. However, considering that an alternate treatment in the form of a *P. fieldi*-specific antivenom does not currently exist, envenomed victims would likely benefit from the future incorporation of *P. fieldi* venom into the immunizing mixture used for Middle Eastern antivenom production.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.09.003.

Acknowledgments

SAA was the recipient of postdoctoral fellowship (PDRF Phase II Batch-V) from Higher Education Commission (HEC Islamabad) Pakistan. BGF was funded by an Australian Research Council Future Fellowship and by the University of Queensland. TNWJ

was funded by an Australian Postgraduate Award. NRC was supported by a UK Natural Environment Research Council Fellowship.

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