Comparison of proteomic profiles of the venoms of two of the 'Big Four' snakes of India, the Indian cobra (*Naja naja*) and the common krait (*Bungarus caeruleus*), and analyses of their toxins.

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ABSTRACT: Snake venoms are mixtures of biologically-active proteins and peptides, and several studies have described the characteristics of some of these toxins. However, complete proteomic profiling of the venoms of many snake species has not yet been done. The Indian cobra (*Naja naja*) and common krait (*Bungarus caeruleus*) are elapid snake species that are among the 'Big Four' responsible for the majority of human snake envenomation cases in India. As understanding the composition and complexity of venoms is necessary for successful treatment of envenomation in humans, we utilized three different proteomic profiling approaches to characterize these venoms: i) one-dimensional SDS-PAGE coupled with in-gel tryptic digestion and electrospray tandem mass spectrometry (ESI-LC-MS/MS) of individual protein bands; ii) in-solution tryptic digestion of crude venoms coupled with ESI-LC-MS/MS; and iii) separation by gel-filtration chromatography coupled with tryptic digestion and ESI-LC-MS/MS of separated fractions. From the generated data, 81 and 46 different proteins were identified from *N. naja* and *B. caeruleus* venoms, respectively, belonging to fifteen different protein families. Venoms from both species were found to contain a variety of phospholipases A_2 and three-finger toxins, whereas relatively higher numbers of snake venom metalloproteinases were found in *N. naja* compared to *B. caeruleus* venom. The analyses also identified less represented venom proteins including L-amino acid oxidases,

cysteine-rich secretory proteins, 5'-nucleotidases and venom nerve growth factors. Further, Kunitz-type serine protease inhibitors, cobra venom factors, phosphodiesterases, vespryns and aminopeptidases were identified in the *N. naja* venom, while acetylcholinesterases and hyaluronidases were found in the *B. caeruleus* venom. We further analysed protein coverage (Lys/Arg rich and poor regions as well as potential glycosylation sites) using in-house software. These studies expand our understanding of the proteomes of the venoms of these two medically-important species.

Keywords: toxins, venomics, venom variation, ESI-LC-MS/MS, tryptic digestion, toxin profile.

1. Introduction

Snake bite is a serious medical problem in tropical and sub-tropical regions, including India. The annual number of human deaths due to snake bite in India is conservatively estimated at 35,000 to 50,000, and the majority of bites are inflicted by the Indian or spectacled cobra (*Naja naja*), common krait (*Bungarus caeruleus*), saw-scaled viper (*Echis carinatus*) and Russell's viper (*Daboia russelii*) (Warrell, 1999; Sharma *et al*., 2015), the so-called "Big Four" venomous snakes of India (although there is debate as to whether a fifth species, the hump-nosed viper [*Hypnale hypnale*] should be included as a "big fifth" species; Joseph et al. 2007; Simpson and Norris 2007). Such a notably high number of snake bite related deaths are due to the close interaction between human and snake populations as well as the high toxicity of these snake venoms. Snake venoms are complex mixtures of proteins, peptides and other molecules that are produced by specialized venom glands and used for prey acquisition as well as defense against potential predators (Stocker, 1990). The diversity of bioactive molecules in venoms has been used to design pharmacologically active substances as potential drug leads and to develop precise research tools for better understanding of mammalian physiological pathways (Stocker, 1990; Koh and Kini, 2012).

In this study, we have chosen to evaluate the composition of the venoms of two elapid snakes belonging to the "Big Four": *N. naja* and *B. caeruleus*. *Naja naja* is native to the Indian subcontinent and found throughout Pakistan, Sri Lanka, Bangladesh and Southern Nepal. *Bungarus caeruleus* is found from the West Bengal plains of India to the Sindh province of Pakistan; occurs through South India and

Sri Lanka; and is recorded from Afghanistan, Bangladesh, and Nepal. Although neurotoxicity, which leads to acute neuromuscular paralysis (potentially causing morbidity and mortality), is a common effect of envenomation by these elapid snakes (Seneviratne and Dissanayake, 2002; Bawaskar and Bawaskar, 2004; Ranawaka *et al*., 2013), other toxins present in these venoms may also determine the overall effect. Analysis of venom composition along with characterization of individual new toxins is needed to elucidate mechanisms of venom toxicity (Yang, 1996) in an attempt to correlate the two (a field now termed "toxicovenomics" (Calvete 2015; Lauridsen *et al*. 2016,2017). Venom composition exhibits intraspecific (seasonal, geographical and ontogenetic) variations in numbers and types of toxins making species-level characterization an interesting challenge (e.g. Chippaux *et al*. 1991; Daltry *et al*. 1996A,B; Alape-Girón *et al*. 2008; Calvete *et al*. 2009). Previous studies have demonstrated regional differences in the compositional, biochemical and pharmacological properties of *N. naja* venoms within India, and these differences correlate with the severity of pathogenesis (Mukherjee and Maity, 1998; Shashidharamurthy *et al*., 2002; Shashidharamurthy *et al*., 2010; Saikumari *et al*., 2015).

Relatively few proteomics studies of the venoms of either of these two dangerous elapids have been conducted. One previous study reported 26 proteins (with two or more associated peptide hits) from seven toxin families in the venom of a *N. naja* from Pakistan utilizing a combination of 2D gel electrophoresis and tryptic digest of crude venom followed by LC-MS/MS (Ali *et al*., 2013). Sintiprungrat *et al.* (2016) compared the venomics of *N. naja* from western India (pool of venom from Rajasthan and Gujarat) and Sri Lanka, and six major venom protein families were reported in venoms from both areas. These were the three-finger toxin (3FTx), phospholipase A_2 (PLA_2), cysteine-rich secretory protein (CRISP), snake venom metalloproteinase (SVMP), venom growth factor, and protease inhibitor protein families. Proteomic analysis of *N. naja* from eastern India revealed 43 proteins belonging to 15 venom protein families (Dutta *et al*., 2017; minimum one peptide used to call protein). Further, earlier reports on the characterization of thermostable peptides from *N. naja* venom (Vietnam) revealed 32 polypeptides and proteins belonging to the 3FTx, PLA_2, cobra venom factor (CVF), L-amino acid

oxidase (LAAO), CRISP, and SVMP protein families (Binh *et al*., 2010). A number of PLA_2s (Bhat *et al*., 1991; Bhat and Gowda, 1991; Rudrammaji and Gowda, 1998; Sudarshan and Dhananjaya, 2016), SVMPs (Jagadeesha *et al*., 2002), and other venom toxin components (Karlsson *et al*., 1971; Müller-Eberhard and Fjellström, 1971; Shafqat *et al*., 1990; Girish *et al*., 2004) have also been isolated and characterized from *N. naja* venom. During the revision of this manuscript, venomic analysis of *B. caeruleus* venom (pooled from Southeastern India and unspecified locales within India) was reported, with the observation that 68.2% of total proteins were PLA_2s, including β-bungarotoxins (Oh *et al*., 2017). Moreover, only a few protein toxins have been purified and characterized from *B. caeruleus* venom (Bon and Changeux, 1975; Moody and Raftery, 1978; Sharma *et al*., 1999; Singh *et al*., 2001, 2005; Mirajkar *et al*., 2005; More *et al*., 2010).

Improvement of proteomic techniques, including the development of high resolution mass spectrometry and the deposition of protein sequences in accessible databases, has allowed for more thorough snake venom profiling in shorter time frames (Calvete *et al*., 2007, 2009). Recent proteomic research has in turn spurred development of powerful methods for toxin characterization and immunological profiling of snake venoms (Pla *et al*., 2012; Gutiérrez *et al*., 2013). Hence, the information generated by such studies will potentially help in the production of more effective antivenoms and in the discovery of protein leads for the development of therapeutics (Vetter *et al*., 2011). In the present study we utilized three different proteomic strategies to characterize the venoms of two medically-important elapid snake species. The first strategy was one dimensional SDS-PAGE, which was used to separate the constituent proteins after which tryptic digestion of bands and electrospray tandem mass spectrometry (ESI-LC-MS/MS) of resulting peptides was used to identify them. The second strategy was tryptic digestion of crude venom followed by ESI-LC-MS/MS. The third strategy was gel filtration (GF) followed by ESI-LC-MS/MS of the separated fractions. By combining these three complimentary techniques, we have generated more comprehensive proteomic profiles of the venoms of *N. naja* and *B. caeruleus* than have been previously reported.

2. Materials and methods

2.1. Venoms

Lyophilized crude *N. naja* and *B. caeruleus* venoms were procured from the Irula Snake Catcher's Society, Tamil Nadu, India. The snakes were from within Tamil Nadu state, and venoms were pooled from a number of individuals. The venoms were reconstituted in water prior to analysis unless stated otherwise.

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of venoms

SDS-PAGE was performed in 12% polyacrylamide according to the method of Laemmli (1970). Crude venom samples (20 µg), each treated with non-reducing buffer were loaded in separate wells. The gels were stained with Coomassie blue and then destained by using 5% acetic acid in water. PageRuler™ prestained protein marker (Thermo Scientific, Waltham, MA, USA) was used for estimating molecular weight.

2.3. In-gel tryptic digestion

Each band from SDS-PAGE gels was cut and destained using 200 µl MeOH: 50 mM NH_4HCO_3 (1:1 v/v), and later dehydration was done using 200 µl ACN: 50 mM NH_4HCO_3 (1:1 v/v). Next, 200 µl of 100% ACN was added. The protein in the gel bands was reduced using 100 µl of 25 mM DTT in 50 mM NH_4HCO_3 for 20 min followed by alkylation in the dark at room temperature with 100 µl of 55 mM iodoacetamide (Sigma, St. Louis, MO, USA) in 50 mM NH_4HCO_3 for 15 min. Samples were then washed with 200 µl of 50 mM NH_4HCO_3 in 50% ACN for 15 min and dehydrated with 200 µl of 100% ACN at room temperature for 15 min. The gels were digested using 20 µl of 12 ng/µl trypsin (Trypsin Gold, Promega, Madison, WI, USA). Next 30 µl of 0.01% ProteaseMAX in 50 mM NH_4HCO_3 was added, and the sample was gently mixed and incubated at 37˚C for 3 h. After incubation, 0.5 µl TFA was added to inactivate trypsin. The samples were centrifuged at 12,000 rpm and supernatants were loaded onto an HPLC-MS/MS system.

2.4. Gel filtration chromatography of crude N. naja *and* B.caeruleus *venom*

Crude venoms (100 mg) were dissolved in 2.5 ml of 50 mM Tris-HCl (pH 7.4) and filtered through 0.2 mm nylon syringe filters. The clear venom filtrates (1 ml) were loaded onto a HiLoad 16/60 Superdex 200 pg gel filtration column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with 50 mM Tris-HCl (pH 7.4). Proteins were eluted at a flow rate of 1 ml/min with the same buffer using an Äkta Purifier HPLC system (GE Healthcare), and the elution was monitored at 215 and 280 nm.

2.5. In-solution tryptic digestion

Individual freeze-dried crude venom samples and gel-filtration fractions were reconstituted in deionized water (4 µg/µl). Each sample (50 µl) was mixed with 41.5 µl of 50 mM NH_4HCO_3 buffer, 1.0 µl DTT (for reduction), and 2.0 µl of 1% ProteaseMAX detergent (in 50 mM NH_4HCO_3) and incubated at 56°C for 20 min. Each sample was alkylated by adding 2.7 µl of 0.55 M iodoacetamide, and the mixture was incubated at room temperature (22-24°C) in the dark for 15 min. Trypsin (1.8 µg/µl in 50 mM acetic acid) was then added, and the samples were incubated at 37°C for 3 h. Next, 0.5 µl TFA was added and the mixture incubated at room temperature for 10 min. Samples were centrifuged at 10,000 rpm for 10 min, and supernatants were analyzed by HPLC-MS/MS.

2.6. Electrospray tandem mass spectrometry (ESI-LC-MS/MS)

The tryptic digests of individual samples obtained by in-gel or in-solution digestion (described above) were loaded onto an Accela LCQ Fleet ion trap mass spectrometer. Each 80 µl sample was injected onto a Hypersil Gold C18 column (50 x 2.1 mm, Thermo Scientific, Waltham, MA, USA) pre-equilibrated with 0.1% formic acid (buffer A). Elution was carried out at a flow rate 200 µl/min with a linear gradient of 100% ACN in 0.1% formic acid (buffer B). The gradient was set from 0 to 40% buffer B over 38 min and then from 40 to 80% over 18 min. The eluent from the LC column was directly fed to the mass spectrometer, and the system was set to positive ionization mode. Spectra were obtained with a scan range of 500 to 2000 m/z.

149 *2.7. Protein and peptide identification*

150 MS/MS spectra were analyzed using SEQUEST and Proteome Discoverer 3.1 software with a
151 false discovery rate of 0.01. The peptide fragments were assigned to protein families based on their
152 similarity to sequences in the Lepidosauria subset of the National Centre for Biotechnology Information
153 (NCBI) database. If at least two peptides were detected (with at least one being unique), protein species
154 were considered to be present in the sample. Only proteins with a SEQUEST score of 2.0 or higher were
155 utilized for analysis.

156 *2.8. Visualization of SEQUEST output*

157 The portions of the protein sequences covered by peptides detected via tandem mass spectrometry
158 were visualized using an in-house Perl script. The program generates figures showing the peptide
159 coverage, potential glycosylation sites and lysine (K) or arginine (R) rich and poor regions. As trypsin
160 lyses proteins directly after K or R residues, the regions that are too high or too low in presence of these
161 residues are either too small or too large (respectively) to be detected by MS based on the range
162 parameters set. K/R-rich and K/R-poor regions were defined as protein segments containing more than
163 two K/R within 3 to 5 residues or lacking K/R within 20 to 25 residues, respectively. The tryptic peptides
164 with 5 to 20 residues were considered as potentially detectable with the standard experimental parameters.
165 Although we did not specifically check for glycosylated peptides, their presence can cause failure in
166 peptide detection due to uncertainty pertaining to the mass of sugar moieties, they may lead to peptides
167 containing them being undetected. For this reason, we mapped potential glycosylation sites (predicted by
168 the presence of NXT/NXS, where X is any residue except a proline).

169 **3. Results and Discussion**

170 *3.1. Proteomics of SDS-PAGE gel bands*

171 The abundance of high and low molecular weight proteins in both crude venoms was shown by
172 SDS-PAGE (Fig.1). Visually, the banding patterns in the two venoms are distinct, with *N. naja* venom

appearing to have a greater number of large molecular weight proteins than *B. caeruleus* venom. Based on published molecular weights, the prominent bands indicate the potential proteins from various toxin families including 3FTx, PLA_2, CRISP, LAAO, and SVMP, among others, in both the venoms. The individual gel bands were sliced, pooled into four fractions (Fig. 1) and further subjected to in-gel trypsin digestion and HPLC-MS/MS. From the SDS-PAGE bands of *N. naja* venom, 25 total proteins belonging to ten toxin families were identified (Table S1). Likewise, from the *B. caeruleus* SDS-PAGE bands, we identified twenty three proteins from six different toxin families (Table S2).

3.2. Proteomics of venom by direct tryptic digestion

Crude venoms of *N. naja* and *B. caeruleus* were subjected to tryptic digestion and further analysis using HPLC-MS/MS. Forty different proteins from 13 toxin families were identified from the *N. naja* venom (Table S1). Similarly, from the *B. caeruleus* crude venom, we identified 43 different proteins from eight toxin families (Table S2).

3.3. Proteomics of gel-filtration fractions

The *N. naja* crude venom subjected to gel filtration chromatography yielded nine distinct protein peaks (Fig. 2). Within these peaks, 75 total proteins belonging to 14 toxin families were detected. In the *B. caeruleus* crude venom, seven distinct peaks were seen (Fig. 3) and within these peaks 34 total proteins from eight toxin families were observed. Identities of individual proteins are listed in Tables S1 and S2. Interestingly, gel filtration of *N. naja* venom yielded 36 proteins not identified using other techniques, but gel filtration of *B. caeruleus* venom yielded no unique peptides (Fig. 4).

3.4. Analyses of toxin families and isoforms

The complete analysis of the peptides obtained using combined approaches provided a comprehensive overview of the different protein families and species present in both *N. naja* (Fig 5) and *B. caeruleus* venoms (Fig. 6). The most widely used proteomic profiling workflow (Lomonte and Calvete, 2017) has some difficulty in eluting larger proteins of low abundance from C_{18} HPLC columns.

In this study, we have used complementary approaches so as to avoid such issues and to generate a more complete proteomic profile. The approaches using in-gel or crude venom tryptic digestion coupled with ESI-LC-MS/MS detected a similar number of proteins in both *N. naja* and *B. caeruleus* venoms. This might be due to the abundance of low to medium sized (in terms of molecular weight) proteins in both of the venoms. The presence of a larger number of high molecular weight proteins in *N. naja* crude venom compared to *B. caeruleus*, as observed in SDS-PAGE profiles, has resulted in 36 proteins being identified in *N. naja* venom using the third approach (in-solution tryptic digestion of gel-filtration fractions and ESI-LC-MS/MS) compared to none in *B. caeruleus* venom. The differences and similarities in protein profiles between the two species on examination of the proteomic results are discussed in detail.

3.4.1. PLA_2 enzymes

PLA_2 enzymes were found to be present in relatively high abundance and were represented overall by 17 and 19 proteins in *N. naja* and *B. caeruleus* venoms, respectively (Figs. 5 and 6). The members of this family induce a wide range of pharmacological effects including myotoxicity, neurotoxicity, hypotension, haemolysis, cardiotoxicity, antibacterial, coagulopathy, hemorrhage, edema, tissue damage and convulsion (Kini, 2003). Neurotoxic PLA_2 enzymes are among the most lethal toxic components in cobra and krait venoms (Tan *et al*., 1989; Yanoshita *et al*., 2006). Peptides belonging to a previously reported acidic PLA_2 and a group I PLA_2 were detected in both *N. naja* and *B. caeruleus* venoms (Tables S1 and S2). In the *N. naja* venom, peptides with sequences similar to several PLA_2 enzymes from the elapid species *N. sagittifera*, *N. atra*, *N. kaouthia*, *N. melanoleuca* and *Micropechis ikaheka* as well as some viperid species were detected (Table S1). In the *B. caeruleus* venom, peptides similar to several PLA_2 isoforms originally described from the elapid snakes *B. multicinctus, B. fasciatus, B. candidus, N. naja, N. sagittifera, Brachyurophis roperi, Laticauda colubrina, Pseudechis australis,* and *Cacophis squamulous* were detected (Table S2).

3.4.2. 3FTxs

We identified 24 proteins in the *N. naja* venom belonging to the 3FTx family and 14 in the *B. caeruleus* venom (Figs. 5 and 6). The 3FTxs have a distinctive structural arrangement composed of three β-stranded loops that protrude from a small hydrophobic globular core that makes them appear like a hand with three extending fingers. This protein scaffold is stabilized by four conserved disulfide bonds located in the globular core (Kini and Doley, 2010). Despite having similarities in structure, they are known to exert diverse activities such as neurotoxicity, cardiotoxicity, cytotoxicity, and anti-platelet effects (Kini and Doley, 2010). The 3FTxs detected in the *N. naja* and *B. caeruleus* venoms belong to the short-chain neurotoxin, long-chain neurotoxin, κ-neurotoxin, muscarinic toxin and orphan subfamilies (Tables S1 and S2). In both venoms, peptides identical to those from *N. naja* cytotoxin 7, long neurotoxin 1, and long neurotoxin 4; *N. oxiana* short neurotoxin 1; and *N. nivea* or *N. kaouthia* cytotoxin 2 were detected. (Tables S1 and S2). In *N. naja* venom, 3FTx peptides were obtained similar to sequences from the elapids *N. naja*, *N. kaouthia*, *N. mossambica*, *N. atra*, *N. nivea*, *N. annulifera*, *N. oxiana* and *N. annulata annulata* (Table S1). In the *B. caeruleus* venom, peptides similar to 3FTxs from the elapids *B. candidus*, *B. multicinctus*, *N. kaouthia*, *N. naja*, *N. melanoleuca*, and *N. atra* were identified (Table S2).

3.4.3. SVMPs

Analysis of the peptides obtained in our study revealed 13 SVMPs in the *N. naja* venom and five in the *B. caeruleus* venom (Figs. 5 and 6). SVMPs are organized into three broad classes- PI, PII (with subclasses a through e), and PIII (with subclasses a through d) based on differences in structure (Fox and Serrano, 2005, 2009, 2010). In addition to hemorrhagic activity, members of the SVMP family may also have fibrin(ogen)olytic activity, inhibit platelet aggregation, or be pro-inflammatory (Markland and Swenson, 2013). There is generally very little metalloproteinase content in elapid venoms (Li *et al*., 2004; Markland and Swenson, 2013) compared to viperids (Francischetti *et al*., 2004; Serrano *et al*., 2005; Cidade *et al*., 2006; Sanz *et al*., 2006). The SVMPs identified mostly belonged to the PIIIa subfamily, and peptides from zinc metalloproteinase disintegrins and other domains of SVMPs were found in both venoms (Tables S1 and S2). In *N. naja* venom, SVMP peptides showed similarity to sequences originally

from the elapids *N. kaouthia*, *N. atra*, *B. multicinctus*, *Hoplocephalus bungaroides*, *Micrurus fulvius* and *Drysdalia coronoides* as well as the viperid *Echis carinatus sochureki* (Table S1). SVMP peptides from *B. caeruleus* venom had similar sequences to those from the elapids *B. multicinctus*, *N. kaouthia*, and *Micrurus fulvius* (Table S2).

3.4.4. CRISPs/helveprins

A total of six CRISPs were identified in *N. naja* venom as compared to two in *B. caeruleus* venom, although they were the second largest group of non-enzymatic proteins found in both venoms (Figs. 5 and 6). The members of the CRISP family inhibit cyclic nucleotide-gated ion channels in photoreceptor and olfactory cells (Brown *et al*., 1999) and potassium activated smooth muscle contraction (Osipov *et al*., 2005; Ito *et al*., 2007). CRISP peptide sequences in the *N. naja* venom were similar to sequences from the elapids *N. atra* and *N. kaouthia* and from the viperids *Trimesurus gracilis* and *D. russelii* (Table S1). In the *B. caeruleus* venom, peptides with similar sequences to CRISPs originally identified from elapid *B. candidus* and *Ophiophagus hannah* were detected (Table S2).

3.4.5. LAAOs

A comparison of the number of LAAOs detected in both the venoms revealed six in the *N. naja* venom and two in the *B. caeruleus* venom (Figs. 5 and 6). LAAOs are homodimeric, high molecular weight enzymatic proteins (Tan and Fung, 2010) responsible for oxidizing hydrophobic L-amino acids thereby releasing α-keto acids, ammonia and H_2O_2 (Du and Clemetson, 2002; Dineshkumar and Muthuvelan, 2011). The product of this reaction, H_2O_2, causes edema formation; ADP or collagen-induced platelet aggregation inhibition or activation; apoptosis; and antibacterial, antiparasitic, anticoagulant, hemolytic and hemorrhagic effects (Suhr and Kim, 1996). LAAO peptides in the *N. naja* venom were similar to peptide sequences of proteins from the elapids *N. atra*, *Notechis scutatus*, and *Oxyuranus scutellatus* as well as the viper *D. russelii* (Table S1). In the *B. caeruleus* venom, LAAO peptides were similar to those from the elapid *B. multicinctus* (Table S2).

3.4.6. 5'-Nuclotidases (5'-NTDs)

There were two 5'-NTDs detected in the *N. naja* venom compared to one in the *B. caeruleus* venom (Figs. 5 and 6). Ubiquitous enzymes, 5'-NTDs are primarily responsible for causing disruption of physiological homeostasis in humans by inducing anticoagulant effects and by inhibiting platelet aggregation (Dhananjaya *et al*., 2006). They also act synergistically with other toxins to exert a more pronounced anti-coagulant effect during envenomation (Dhananjaya and D'Souza, 2010). Peptides of 5'-NTDs from *N. naja* and *B. caeruleus* venoms showed sequence similarity to 5'-NTD sequences from *Micrurus fulvius* (Table S1) and *Ovophis okinavensis* (Table S2), respectively.

3.4.7. Toxins specific to N. naja *venom*

There were four proteins belonging to the CVF family detected in the *N. naja* venom (Fig. 5). CVF is a complement-activating protein present in cobra venom. It is a three-chain protein that functionally resembles C3b, the activated form of complement component C3 (Vogel *et al*., 2004). Peptides from this protein family showed similarity to CVF sequences from *N. kaouthia* (Table S1).

Peptides belonging to two proteins of the poorly characterized toxin family of aminopeptidases were also found only in *N. naja* venom (Fig. 5). Aminopeptidases are exo-metalloproteases, and in mammals they are involved in normal physiological functions such as maintenance of blood pressure (Vajyapuri *et al*., 2010). Aminopeptidase peptides identified from *N. naja* venom showed similar sequence to those from the elapid *Micrurus fulvius* and the viperid *Crotalus horridus* (Table S1).

Two proteins belonging to the PDE family were identified in *N. naja* venom (Fig. 5). The findings correlate with the earlier reports that claim low levels of PDEs in elapid venoms and none in *Bungarus* sp. (Aird, 2002). The members of the PDE family are responsible for the successive release of 5'-mononucleotides from the 3' end of polynucleotides (Iwanaga and Suzuki, 1979) and for supplying nucleotide substrates for the activity of other toxins, such as 5-NTDs (Aird, 2002; Valério *et al*., 2003). PDEs are also reported to inhibit platelet aggregation and lower mean arterial pressure (Russell *et al*.,

1963). The detected PDE peptides showed similarity to sequences from the elapid *Micrurus fulvius* and the viperid *Macrovipera lebetina* (Table S1).

Two ohanin/vespryn proteins were identified in *N. naja* venom (Fig. 5). Ohanin identified from *O. hannah* venom was the first snake venom protein shown to induce hypolocomotion and hyperalgesia in experimental mice (Pung *et al*., 2005). The peptides identified from *N. naja* venom showed similarity to the isoforms from *N. kaouthia* and *O. hannah* (Table S1).

Only one SVSP was identified from the *N. naja* venom (Fig. 5). These proteins are widely distributed in viperid venoms but are found quite rarely in elapid venoms (Jin *et al*., 2007). SVSPs show fibrin(ogen)olytic activities and are not susceptible to hirudin, heparin, or other serine protease inhibitors, so they result in abnormal fibrin clots (Matsui *et al*., 2000). The identified sequences showed similarity to SVSPs from *N. atra* (Table S1).

The analysis also revealed peptides belonging to one previously reported VNGF protein from *N. naja* venom (Table S1). VNGFs isolated from the elapids have properties similar to those of nerve growth factor isolated from mouse submaxillary glands and are responsible for the survival and differentiation of neurons (Angeletti, 1970; Barbacid, 1995).

One previously reported isoform of Kunitz-type serine protease inhibitor (KPI) was identified in *N. naja* venom (Table S1). Several KPIs have been purified and characterized from elapid and viperid snake venoms (Shafqat *et al*., 1990). A wide variety of biological functions such as inhibition of serine proteases, blocking of ion channels, interference in blood coagulation, inflammation and fibrinolysis are exhibited by KPIs (Mukherjee *et al*., 2014).

3.4.8. Toxins specific to B.caeruleus *venom*

In *B. caeruleus* venom, peptides were detected for two hyaluronidase isoforms (Fig. 6). The members of this protein family cause degradation of hyaluronic acid in the extracellular matrix which is important in the diffusion of toxins from the site of a bite into the circulation. Hence, they are also called

318 "spreading factors" (Kemparaju and Girish, 2006). Hyaluronidases generate a wide range of metabolic
319 products through hyaluronic acid degradation (Kemparaju and Girish, 2006). Hyaluronidase peptides in
320 the *B. caeruleus* venom showed similarity to those from the viper *Bitis arietans* (Table S2).

321 One AChE was detected in the *B.caeruleus* venom (Fig. 6). These proteins play a crucial role in
322 cholinergic transmission and have been reported to occur at high levels in the venoms of elapid snakes,
323 particularly in *Bungarus* (Frobert *et al*., 1997). Identified peptides for this protein showed sequence
324 similarity to AChEs from *B. fasciatus* (Table S2).

325 In-depth analyses of identified proteins in *N. naja* and *B. caeruleus* venoms indicated that both
326 are rich in PLA_2s and 3FTxs. This correlates well with the severe neurotoxicity and cytotoxicity observed
327 in *N. naja* and *B. caeruleus* envenomations (Law *et al*., 2014). The other protein families, with relatively
328 low abundance, seem to have minor physiological effects on envenomated humans.

329 *3.5. Peptide coverage analysis*

330 The prediction of K/R rich or poor regions and potential glycosylation sites in proteins is critical for
331 a complete proteomic profiling, as it helps predict coverage to be expected based on any given technique.
332 Our in-house MS visualization program generated figures that show the overlapping of individual tryptic
333 peptides and the overall coverage in the identified proteins. Here, we have shown coverage data for toxins
334 from the PLA_2, 3FTx, CRISP, and SVMP families in both *N. naja* and *B. caeruleus* venom (Figures 7 and
335 8). Similarly, figures were generated for other toxin families (data not shown). The coverage and amount of
336 overlap of tryptic peptides were higher for PLA_2s and 3FTxs than for CRISPs and SVMPs in both *N. naja*
337 and *B. caeruleus* possibly due to a number of factors such as smaller sizes of 3FTxs and PLA_2s, tryptic
338 cleavage patterns (numbers and locations of K/R residues), and relative concentrations of each toxin in the
339 venom. In the *N. naja* venom potential glycosylation sites were observed in proteins belonging to the 3FTx,
340 CRISP, PLA_2 and SVMP families, although only 3FTx and SVMP proteins in *B. caeruleus* venom showed
341 the same possibilities.

4. Conclusion

Analysis of the proteomes of *N. naja* and *B. caeruleus* venoms using the complementary approaches of gel electrophoresis, liquid chromatography and mass spectrometry has generated an in-depth characterization of these venoms. We have identified 81 protein species from *N. naja* venom (a much greater number than previously reported in proteomic studies) and 46 from *B. caeruleus* venom. The detection of such a large number of venom proteins, including PLA_2s, 3FTxs and SVMPs, correlate well with clinical reactions reported in the literature. Hence, it can be concluded that using the three complementary strategies, rather than one can yield comparatively more information and give a better overall characterization of venom proteomes.

In this study we noted the presence of AChEs, PDEs, 5'-NTDs, and SVSPs specifically in *N. naja* venom as well as hyaluronidases and AChEs solely in *B. caeruleus* venom. This analysis clearly shows the differences in the toxin composition between the venoms and contributes further to the current knowledge of venom proteins in these species. To determine such variations in venom composition, analysis of samples from specific localities needs to be done, and that will help in designing more efficacious, region-specific anti-venoms.

The range of molecular sizes of proteins and peptides is a major obstacle in proteomic analysis of snake venoms. With the extensive developments in proteomic techniques, it has become possible to better elucidate the complete toxin profiles of snake venoms. As such, we have utilized complementary techniques to create an expanded understanding of the proteomes of the venoms of these two medically-important species.

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Fig.1. SDS-PAGE profiles of the crude venoms of *Naja naja* and *Bungarus caeruleus*. Lanes: 1, standard protein ladder; 2, *N. naja* crude venom; and 3, *B. caeruleus* crude venom. Abbreviations: 3FTxs, three finger toxins; CRISPs, cysteine-rich secretory proteins (helveprins); CVFs, cobra venom factors; KPIs, Kunitz-type serine protease inhibitors; LAAOs, L-amino acid oxidases; PLA_2s, phospholipases A_2; PDEs, phosphodiesterases; SVMPs, snake venom metalloproteinases; and VNGFs, venom nerve growth factors

Fig.2. Gel filtration chromatography profile of crude *Naja naja* venom. Crude venom (100 mg in 2.5 ml of 50 mM Tris-HCl, pH 7.4) was loaded onto a Superdex 200 column pre-equilibrated with the same buffer. Proteins were eluted with the same buffer at a flow rate of 1 ml/min and monitored at 215 nm. Insets indicate the snake venom protein families identified by tandem mass spectrometry in individual peaks. Numbers in parenthesis are the number of protein species identified for each family. Abbreviations: 3FTxs, three finger toxins; 5'-NTDs, 5'-nucleotidases; CRISPs, cysteine-rich secretory proteins (helveprins); CVFs, cobra venom factors; KPIs, Kunitz-type serine protease inhibitors; LAAOs, L-amino acid oxidases; PLA_2s, phospholipases A_2; PDEs, phosphodiesterases; SVMPs, snake venom metalloproteinases; and VNGFs, venom nerve growth factors.

Fig.3. Gel filtration chromatography profile of crude *Bungarus caeruleus* venom. Crude venom (100 mg in 2.5 ml of 50 mM Tris-HCl, pH 7.4) was loaded onto a Superdex 200 column pre-equilibrated with 50 mM Tris-HCl (pH 7.4). Proteins were eluted with the same buffer at a flow rate of 1 ml/min and monitored at 215 nm. Insets indicate the snake venom protein families identified by tandem mass spectrometry in individual peaks. Numbers in parenthesis are the number of protein species identified for each family. Abbreviations: AChEs, acetylcholinesterases; 3FTxs, three finger toxins; 5'-NTDs, 5'-nucleotidases; CRISPs, cysteine rich secretory proteins (helveprins); LAAOs, L-amino acid oxidases; PLA_2s, phospholipases A_2; and SVMPs, snake venom metalloproteinases.

Fig.4. Venn diagrams of the number of protein species obtained from A) *Naja naja* and B) *Bungarus caeruleus* venoms using three proteomic approaches. Differently shaded areas indicate overlap among the various techniques.

Fig.5. Relative abundance of venom protein families identified by ESI-LCMS/MS of crude *Naja naja* venom. Each section represents the number of protein species found within that specific toxin protein family, with relative percentage in parentheses. Abbreviations: 3FTxs, three finger toxins; 5'-NTDs, 5'-nucleotidases; CRISPs, cysteine-rich secretory proteins (helveprins); CVFs, cobra venom factors; KPIs, Kunitz-type serine protease inhibitors; LAAOs, L-amino acid oxidases; PLA_2s, phospholipases A_2; PDEs, phosphodiesterases; SVMPs, snake venom metalloproteinases; and VNGFs, venom nerve growth factors.

Fig.6. Relative abundance of venom protein families identified by ESI-LCMS/MS of *Bungarus caeruleus* crude venom. Each section represents the number of protein species found within that specific toxin protein family, with relative percentage in parentheses. Abbreviations: AChEs, acetylcholinesterases; 3FTxs, three finger toxins; 5'-NTDs, 5'-nucleotidases; CRISPs, cysteine rich secretory proteins (helveprins); LAAOs, L-amino acid oxidases; PLA_2s, phospholipases A_2; and SVMPs, snake venom metalloproteinases.

Fig.7. Sequence coverage of the major protein families in *Naja naja* venom. Alphanumeric designations on the left of each panel represent NCBI accession numbers. Panels: (A) 3FTxs, three-finger toxins; (B) PLA_2s, phospholipases A_2; (C) CRISPs, cysteine-rich secretory proteins (helveprins); and (D) SVMPs, snake venom metalloproteinases. Blue, black and brown regions show the lysine and arginine poor, medium and rich regions, respectively. Red, purple and green bars indicate tryptic peptides of low, medium and high score, respectively, based on SEQUEST analysis of the corresponding proteins. In addition, cyan regions in the protein sequence represent potential glycosylation sites.

Fig.8. Sequence coverage of the major protein families in *Bungarus caeruleus* venom. Alphanumeric designations on the left of each panel represent NCBI accession numbers. Panels: (A) 3FTxs, three-finger toxins; (B) PLA_2s, phospholipases A_2; (C) CRISPs, cysteine-rich secretory proteins (helveprins); and (D) SVMPs, snake venom metalloproteinases. Blue, black and brown regions show the lysine and arginine poor, medium and rich regions, respectively. Red, purple and green bars indicate tryptic peptides of low, medium and high score, respectively, based on SEQUEST analysis of the corresponding proteins. In addition, cyan regions in the protein sequence represent possible glycosylation sites.

Figure

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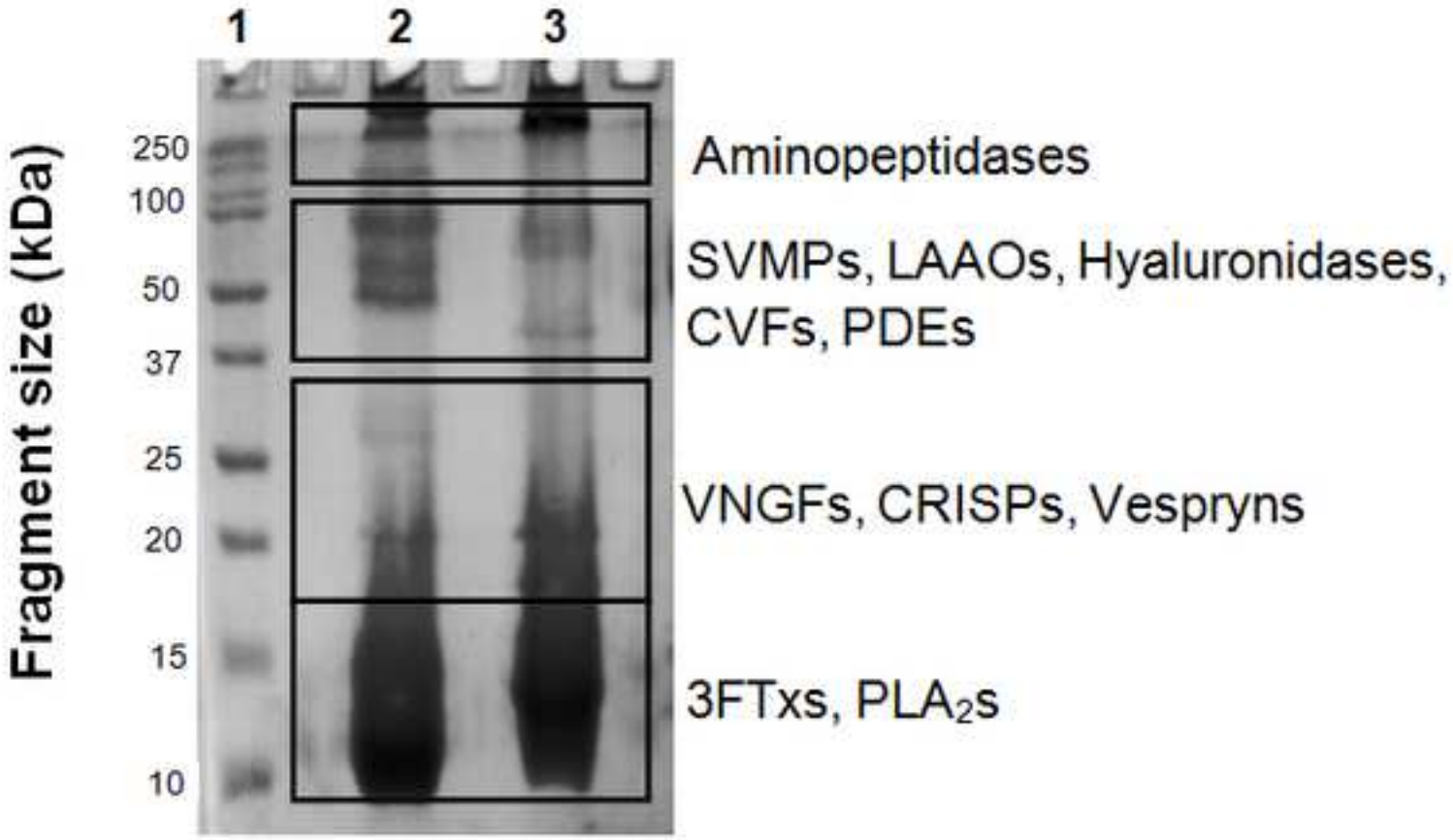

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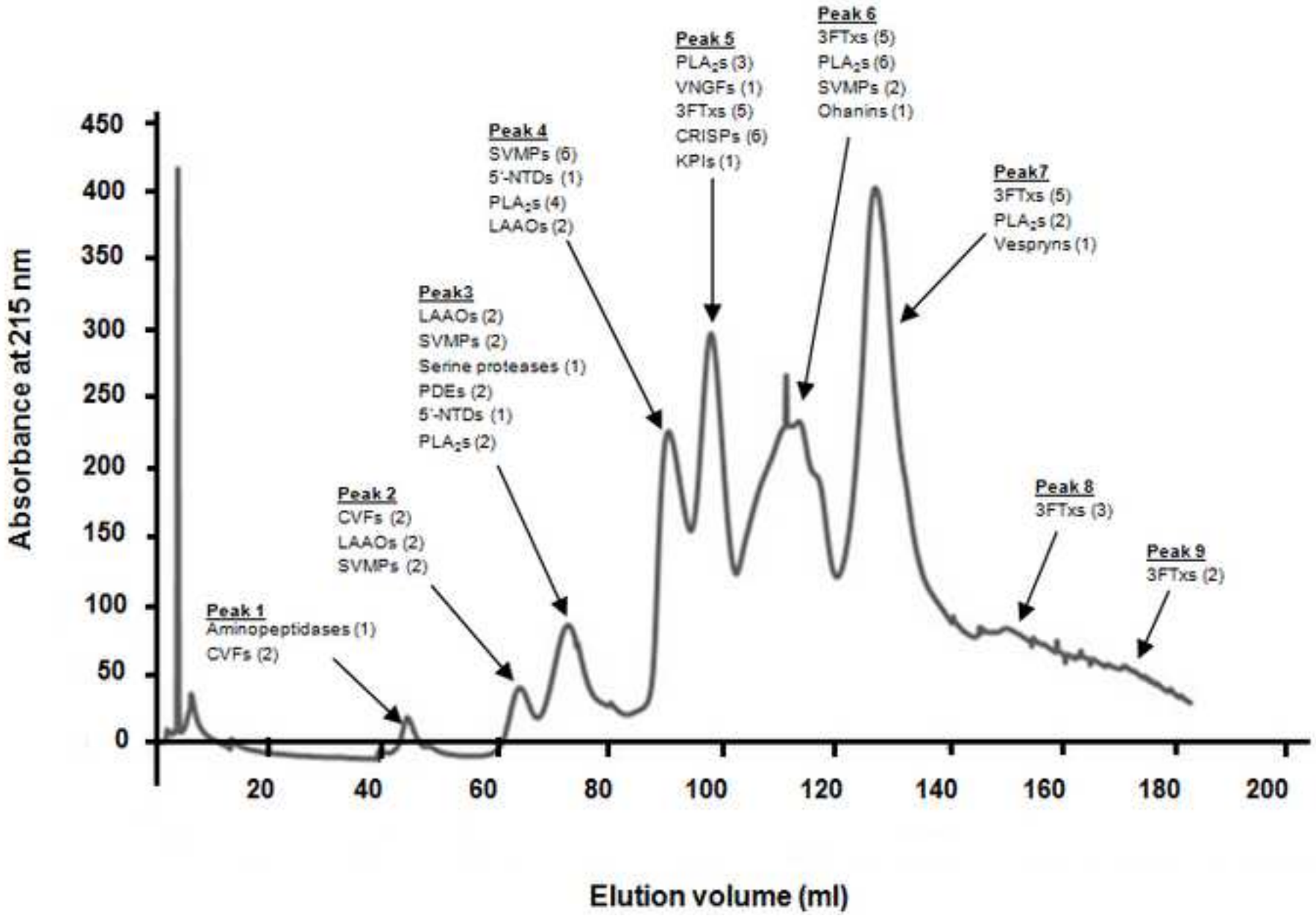

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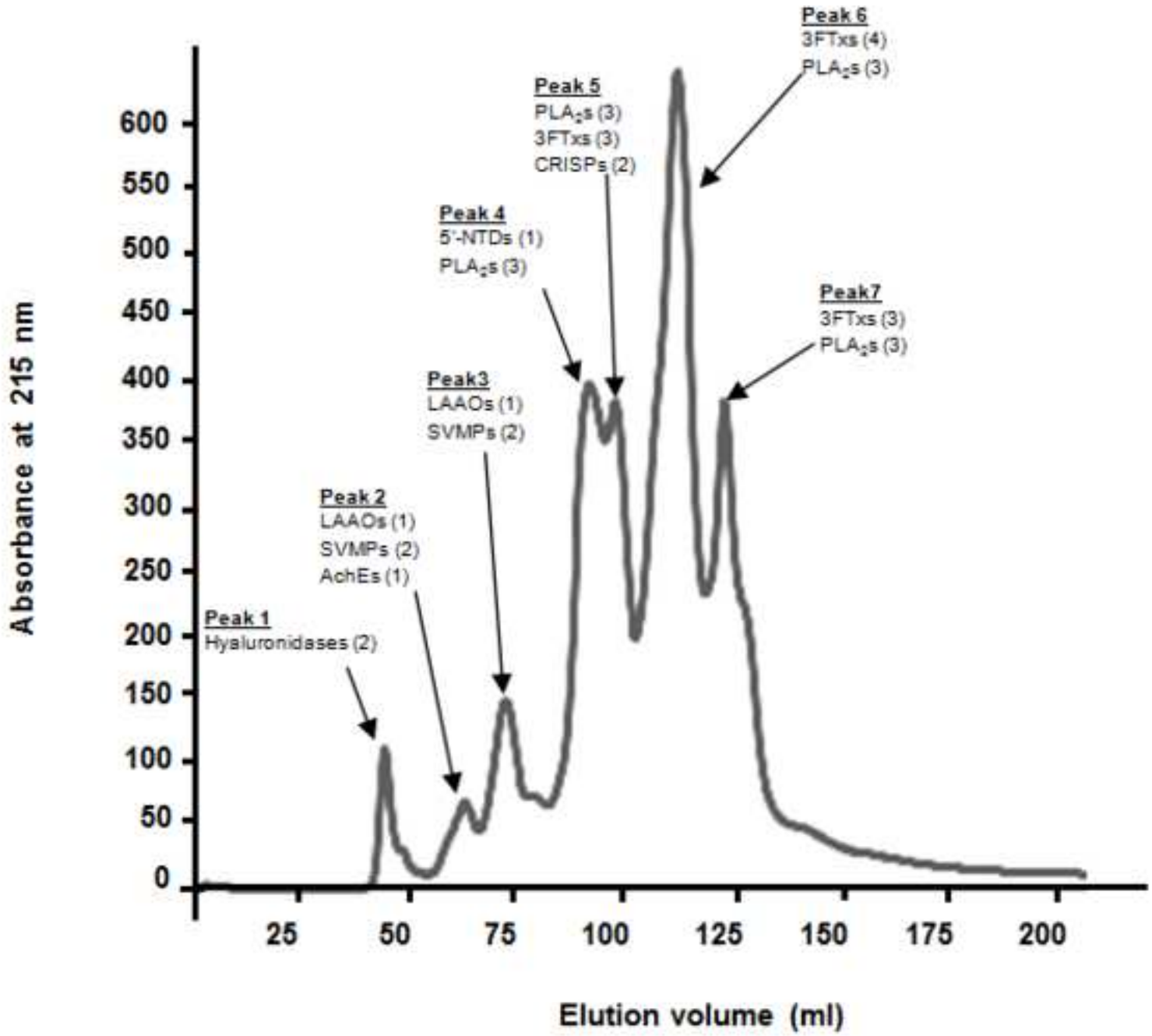

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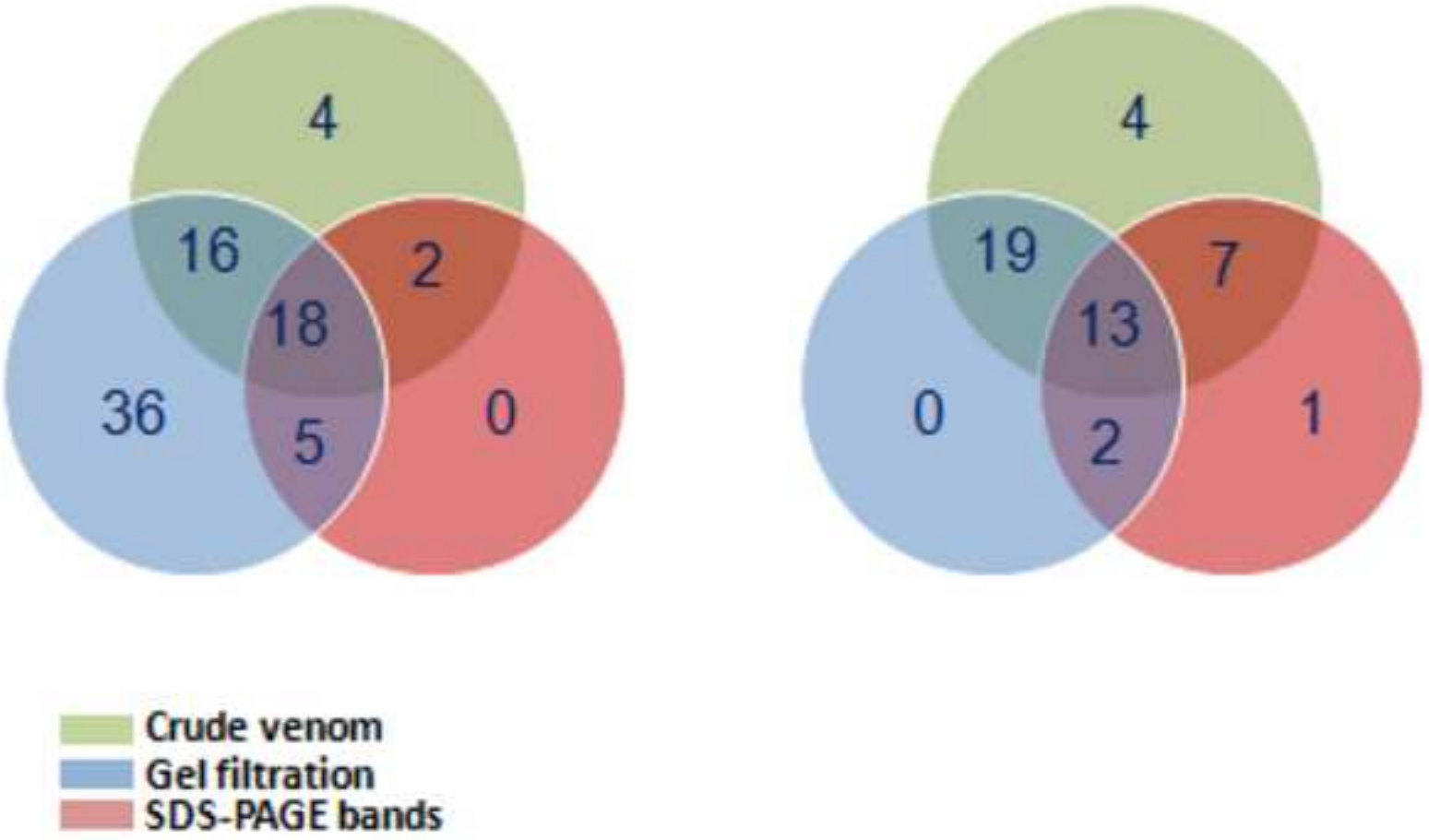

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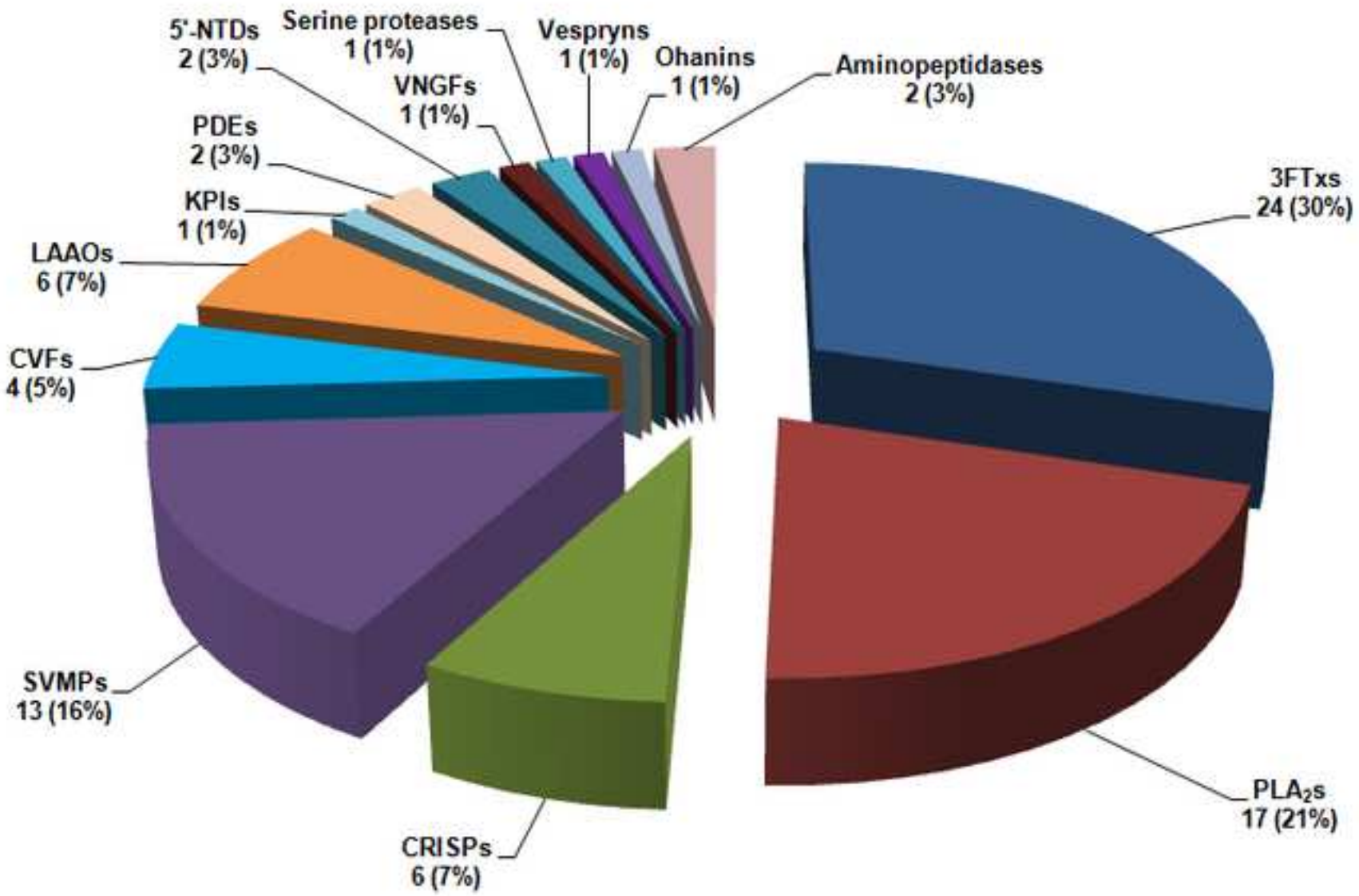

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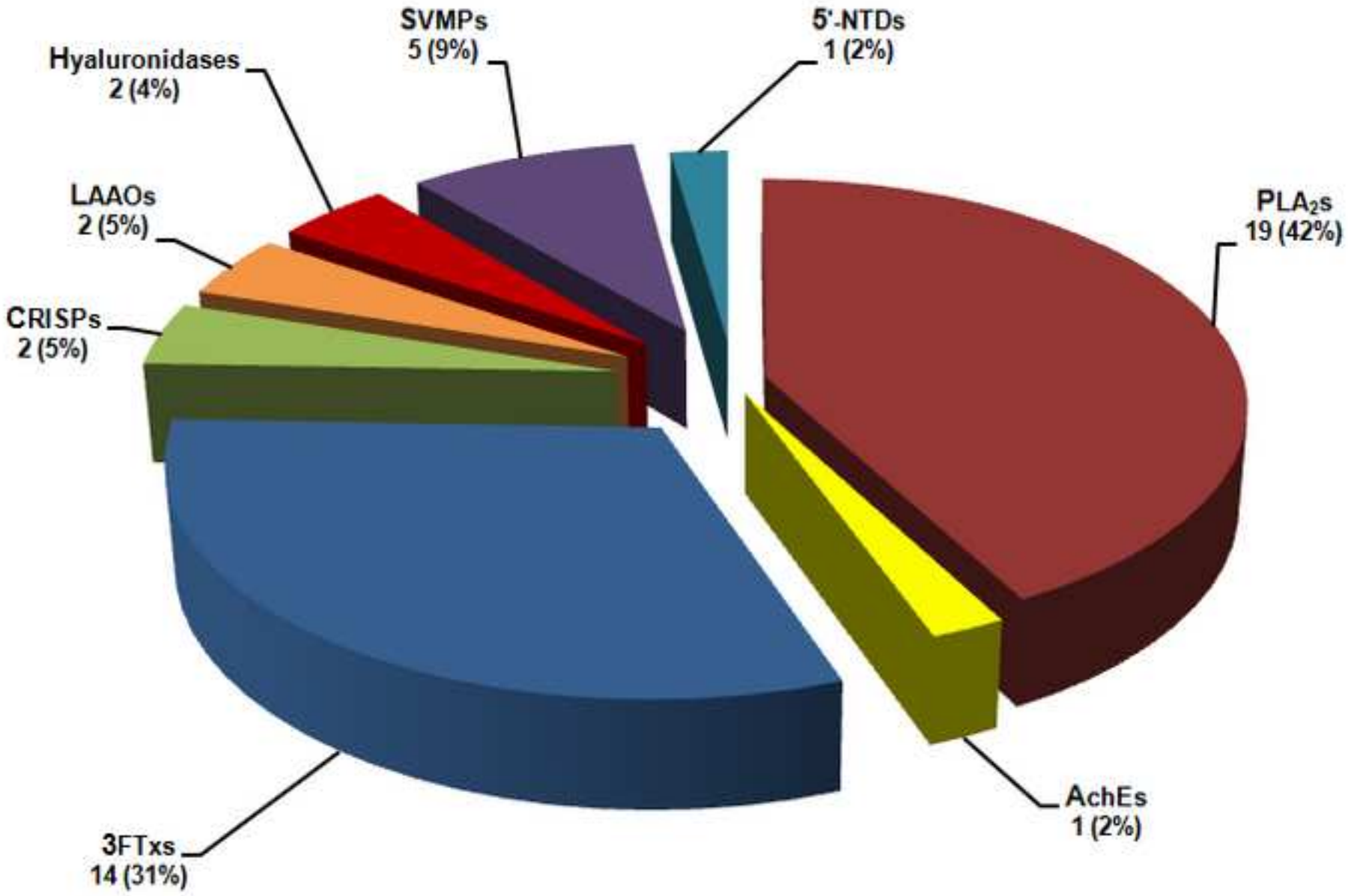

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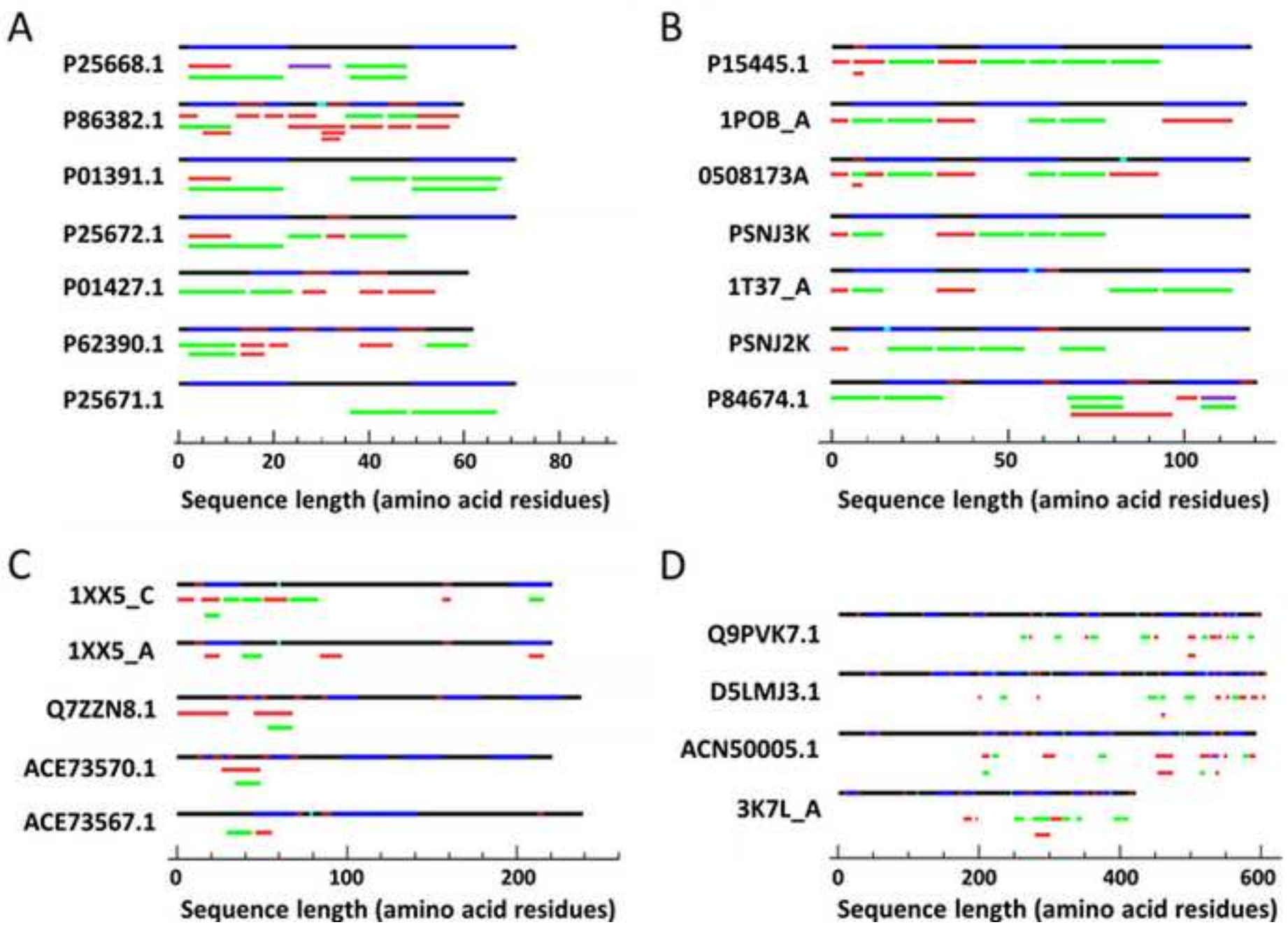

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