

Transcriptome-facilitated proteomic characterization of rear-fanged snake venoms reveal abundant metalloproteinases with enhanced activity



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ABSTRACT

High-throughput technologies were used to identify venom gland toxin expression and to characterize the venom proteomes of two rear-fanged snakes, *Ahaetulla prasina* (Asian Green Vine Snake) and *Borikenophis portoricensis* (Puerto Rican Racer). Sixty-nine complete toxin-coding transcripts from 12 venom protein superfamilies (*A. prasina*) and 50 complete coding transcripts from 11 venom protein superfamilies (*B. portoricensis*) were identified in the venom glands. However, only 18% (*A. prasina*) and 32% (*B. portoricensis*) of the translated protein isoforms were detected in the proteome of these venoms. Both venom gland transcriptomes and venom proteomes were dominated by P-III metalloproteinases. Three-finger toxins, cysteine-rich secretory proteins, and C-type lectins were present in moderate amounts, but other protein superfamilies showed very low abundances. Venoms contained metalloproteinase activity comparable to viperid snake venom levels, but other common venom enzymes were absent or present at negligible levels. Western blot analysis showed metalloproteinase and cysteine-rich secretory protein epitopes shared with the highly venomous Boomslang (*Dispholidus typus*). The abundance of metalloproteinases emphasizes the important trophic role of these toxins. Comprehensive, transcriptome-informed definition of proteomes and functional characterization of venom proteins in rear-fanged snake families help to elucidate toxin evolution and provide models for protein structure-function analyses.

1. Introduction

Snake venoms contain a variety of proteins and peptides that function primarily in prey immobilization and digestion, and secondarily as a mechanism of defense [1]. Venoms have allowed advanced snakes (Caenophidia) to transition away from the use of constriction (commonly seen in more basal “henophidian” snakes), and instead to rely on a chemical means of prey capture [2]. Among the Caenophidia, rear-fanged venomous snakes represent diverse, unique evolutionary lineages where a mechanically less complex venom delivery system has evolved, paralleling the more complex injection system of front-fanged snakes. Rear-fanged venomous snakes have a relatively low-pressure venom delivery system and lack the large venom storage reservoir (gland lumen) seen in front-fanged venomous snakes. In the rear-fanged snake venom gland, venom proteins are produced and typically stored intracellularly, and upon deployment, they are released more slowly into a main duct conducting venom to the base of rear maxillary teeth, which may be grooved or modified, but never hollow (hollow fangs are only seen in front-fanged venomous snakes) [3].

Rear-fanged snake venoms have remained largely unexplored, and this dearth of knowledge contrasts strongly with the extensive research on front-fanged snake venoms. It has been estimated that fewer than 3% of rear-fanged snake venom proteomes have been described [4]. Venom research has focused on elapid (cobras, kraits, mambas, and relatives) and viperid (vipers and pit vipers) venoms because these snakes produce significantly larger venom yields and are responsible for the vast majority of snake envenomations of humans [1]. Most rear-fanged venomous snakes are unable to deliver sufficient quantities of venom to produce systemic envenomation effects in humans, but at least five species (*Dispholidus typus*, *Thelotornis capensis*, *Rhabdophis tigrinus*, *Philodryas olfersii*, and *Tachymenis peruviana*) are believed to have caused human fatalities [5–9].

In general, rear-fanged snake venoms show lower complexity than those of front-fanged snakes, commonly manifesting only 20–40 protein spots on 2D sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D SDS-PAGE), while front-fanged snake venoms show considerably higher complexity, commonly displaying well over 100 protein spots [10]. Despite lower compositional complexity, rear-fanged venomous

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snakes sometimes exhibit front-fanged snake (elapid or viperid-like) venom phenotypes [11, 12]. Venom composition is likely closely linked to snake diet [13–17], with rear-fanged snake venoms producing examples of prey-specific neurotoxins [14, 18, 19], in addition to the examples of toxin taxon-specific receptor binding observed for elapids [20, 21]. In several cases, rear-fanged snake venoms have been documented to contain novel protein superfamilies with several distinct trajectories [14, 22–26].

The majority of venom protein superfamilies have representatives in both front-fanged and rear-fanged snake venoms [1, 6]. Some of the most prominent superfamilies include snake venom metalloproteinases (SVMPs), phospholipases A₂ (PLA₂S), serine proteinases, three-finger toxins (3FTxs), cysteine-rich secretory proteins (CRiSPs), proteinase inhibitors, and C-type lectins [1, 26]. Snake venom metalloproteinases are one of the most abundant components of viperid venoms [4] and are responsible for local and systemic hemorrhage often seen following viper envenomations [27, 28]. These metalloproteinases are zinc-dependent enzymes that consist of multiple domains thought to have evolved from early neofunctionalization of an ADAM-like (a disintegrin and metalloproteinase) ancestral sequence before the radiation of advanced snakes [29], and they may also serve a predigestive function during envenomation [30]. Myotoxic metalloproteinases have been observed in venoms of rear-fanged snakes, with proteolytic activity up to 25 times greater than that of some pitvipers [31–33]. There have been several SVMPs identified in rear-fanged snake venoms [10, 24, 34]. One of these metalloproteinases, alphosphinase, was characterized from the venom of *Borikenophis* {formerly *Alsophis*} *portoricensis* (Puerto Rican Racer), a New World rear-fanged snake [33]. Bites from *B. portoricensis* have been reported to cause edema and ecchymosis, likely from the SVMPs present in this venom [35, 36].

Ahaetulla prasina (family Colubridae; Asian Green Vinesnake), is native to large areas of southeast Asia. It is an arboreal snake with a diet of small, nestling birds, lizards, and frogs [37]. *Borikenophis portoricensis* is a rear-fanged “colubrid” snake (family Dipsadidae) native to numerous islands in the Caribbean. This is a ground-dwelling, diurnal snake with a diet consisting primarily of lizards (*Anolis* sp.) and *Eleutherodactylus* frogs [36, 38]. These snakes both have similar dietary preferences, but occupy rather different ecological niches: *Ahaetulla* is an elongate arboreal species found in broad regions of southeast Asia, while *Borikenophis* is a terrestrial predator ranging from dry scrub forests to lowland tropical forests in the Caribbean. They represent diverse model species in which to explore the adaptive significance of these two (potentially important) factors affecting venom composition. The present work explores venom gene expression in *A. prasina* and *B. portoricensis* venom glands and compares gene expression to venom proteome composition. By characterizing the venom gland transcripts, the venom proteome, and venom enzyme activity, a better understanding of toxin gene expression, venom composition, and the biological roles of rear-fanged snake venom proteins can be obtained, as well as identifying any potential human health hazards these snakes could pose [9, 39].

2. Materials and methods

2.1. Reagents

TRIzol reagent was purchased from Life Technologies (San Diego, CA, U.S.A.). Stranded mRNA-Seq kit and Library Quantification Kit (Illumina® platforms) were purchased from KAPA Biosystems (Boston, MA, U.S.A.). Agencourt AMPure XP reagent was from Beckman Coulter, Inc. (Brea, CA, U.S.A.). Novex Mark 12 unstained molecular mass standards, MES running buffer, LDS sample buffer, nitrocellulose membranes and precast 12% Bis-Tris NuPAGE electrophoretic gels were obtained from Life Technologies (San Diego, CA, U.S.A.). Pierce BCA protein assay kit was purchased from Thermo Fisher Scientific (Rockford, IL, U.S.A.). Phospholipase A₂ assay kit was purchased from Cayman Chemical Co (Ann Arbor, MI, U.S.A.). SAIMR Boomslang

antivenoms (South African Vaccine Producers, LTD.; batch Y00651, expiration March 2013) was a gift from the Sedgwick County Zoo, Wichita, KS, USA. All other reagents (analytical grade or better) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). All reagents and supplies used for molecular work were certified nuclease-free.

2.2. Venom glands and venom collection

Ahaetulla prasina (n = 4) imported from Indonesia (Bushmaster Reptiles) and *Borikenophis portoricensis* (n = 3) originating from Guana Island, British Virgin Islands were maintained in the University of Northern Colorado Animal Resource Facility in accordance with UNC-IACUC protocol #9204. One snake of each species was used for venom gland collection. Both snakes were adults, with *A. prasina* measuring 1000 mm snout-to-vent and weighing 150 g, and *B. portoricensis* measuring 580 mm snout-to-vent and weighing 75 g. Venom was manually extracted from rear-fanged snakes using the method of Hill and Mackessy (1997) with subcutaneous injections of ketamine-HCl (20–30 mg/kg) followed by pilocarpine-HCl (6 mg/kg) [40]. Adult *Crotalus viridis viridis* venom was obtained by manual extraction from a wild-caught specimen (Weld Co., Colorado, USA). All venoms were centrifuged at 9000 x g for 5 min, frozen at –80 °C, lyophilized, and stored at –20 °C until use. Four days post-extraction, when mRNA levels are highest [41], rear-fanged snakes were heavily anesthetized with isoflurane and euthanized via skull-cervical severing, and venom gland tissue was then collected. Tissue from each of the venom glands (right and left glands) from *A. prasina* was placed directly into TRIzol reagent for immediate RNA isolation. Gland tissue from *B. portoricensis* had been collected five years previously and had been stored in RNA-later at –80 °C before RNA was isolated for the current study. All procedures were approved by the UNC Institutional Animal Care and Use Committee (IACUC protocol 9204.1).

2.3. RNA isolation, library preparation and next-generation sequencing

RNA isolation was performed following the TRIzol reagent manufacturer's protocol with an additional overnight –20 °C incubation in 300 µL 100% ethanol with 40 µL 3 M sodium acetate. Total RNA from each species was resuspended in nuclease-free H₂O and poly-A+ RNA was selected from 4 µg of total RNA using KAPA Stranded mRNA-Seq kit oligo-dT beads. KAPA Stranded mRNA-Seq kit manufacturer's protocol for library preparation was followed for Illumina® sequencing. Products of 200–400 bp were selected by solid phase reversible immobilization using Agencourt AMPure XP reagent. PCR library amplification consisted of 14 cycles. Libraries were then checked for proper fragment size selection and quality using an Agilent 2100 Bioanalyzer. Library concentration was determined following KAPA Library Quantification Kit manufacturer's protocol, and each venom gland library was equally pooled and sequenced on an Illumina® HiSeq 2000 platform lane at the UC Denver Genomics core to obtain 100-bp paired-end reads.

2.4. Transcriptomics: Assembly, annotation and quantification

The quality of the sequenced reads was assessed using the Java program FastQC (Babraham Institute Bioinformatics, UK), and low-quality reads (Phred + 33 score < 30) and contaminating adaptor sequences were removed using Trimmomatic with a sliding window of 4 bps [42]. To obtain the best venom gland transcriptome assembly, two assembly approaches were used in combination with different k-mer sizes and assembly algorithms. A Trinity (release v2014-07-17) *de novo* assembly of paired-end reads was completed with default parameters (k-mer size 25) [43]. A second *de novo* assembly was completed with the program Extender (k-mer size 100) [44]. For Extender, reads were first merged with PEAR (Paired-End read mergeR v0.9.6; default parameters) if their 3' ends overlapped to create longer contiguous sequences [45]. The Extender assembly was performed specifying the

same parameters as previously used for other rear-fanged snake venom glands [11]. Contigs from the two assemblies were combined and BLASTx (executed using BLAST+ command line; minimum E -value of 10^{-4}) was conducted against a custom snake protein database, which was a collection of all identified squamate venom proteins, venom gland transcriptome toxin and non-toxin protein sequences, and all predicted proteins from the genomes of *O. hannah* and *Python bivittatus* [46–48]. All contigs that did not have hits against the custom squamate protein database were searched against the NCBI nr protein database with the same BLASTx parameters. To identify complete coding sequences (CDS), the resulting BLASTx output and all contigs were used as input files for the standalone ORFpredictor program [49]. Predicted CDS and protein sequences from contigs were clustered with CD-HIT to remove any redundancy from using multiple assemblers [50, 51]. Reads were aligned with Bowtie 2 and transcript abundances determined using RSEM (RNA-seq by Expectation-Maximization; v1.2.23) [52]. Transcripts below an FPKM abundance value of 1 were excluded from the analysis. Transcripts were identified as venom proteins after each was manually examined to determine if the resulting protein was full-length, shared sequence identity with a currently known venom protein, or contained a shared signal peptide sequence with other venom proteins in that superfamily.

2.5. Proteomics: liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Approximately 100 μ g of crude venom from each snake species was sent to Florida State University College of Medicine Translational Science Laboratory (Tallahassee, FL, U.S.A) for LC-MS/MS analysis on an LTQ Orbitrap Velos equipped with a Nanospray Flex ion source and interfaced to an Easy nanoLC II HPLC (Thermo Scientific). Crude venoms were digested using the Calbiochem ProteoExtract All-in-one Trypsin Digestion kit (Merck, Darmstadt, Germany) according to manufacturer instructions with LC/MS grade solvents. Peptide fragments were separated using a vented column configuration consisting of a 0.1×20 mm, 3μ m C_{18} trap column and a 0.075×100 mm, 3μ m C_{18} analytical column (SC001 and SC200 Easy Column respectively, Thermo Scientific). The elution gradient consisted of 5% buffer B (0.1% formic acid in HPLC grade acetonitrile) and 95% buffer A (0.1% formic acid) at 300 nL/min from the run start, to 35% B at 60 min, to 98% B from 63 to 78 min with a flow rate of 600 nL/min, and 5% B at 300 nL/min at 79 min. The mass spectrometer was operated in positive mode nanoelectrospray with a spray voltage of +2300 V. A “Top 9” method was used with precursor ion scans in the Orbitrap at 60 K resolving power and fragment ion scans in the linear ion trap. Precursor ion selection using MIPS was enabled for charge states of 2+, 3+ and 4+. Dynamic exclusion was applied for 60 s at 10 ppm. ITMS scans were performed using collision-induced dissociation (CID) at 35% normalized collision energy. MS/MS peptide spectra produced were interpreted using Mascot (Matrix Science, London, UK; version 1.4.0.288), Sequest (Thermo Fisher Scientific, San Jose, CA, U.S.A; version 1.4.0.288), and X! Tandem (theqpm.org; version CYCLONE 2010.12.01.1), assuming a trypsin digestion. The Mascot5_Trembl_bony vertebrate database and the Sequest and X! Tandem Uniprot Serpentes (A8570) databases were used for homology searches. Mascot was searched with a fragment ion mass tolerance of 0.8 Da and a parent ion tolerance of 10 ppm. Sequest and X! Tandem were searched with a fragment ion mass tolerance set to 0.6 Da and a parent ion tolerance of 10 ppm. Glu \rightarrow pyro-Glu of the N-terminus, ammonia loss of the N-terminus, Gln \rightarrow pyro-Glu of the N-terminus, carbamidomethylation of cysteines and carboxymethylation of cysteines were specified as variable post-translational modifications in X! Tandem. Oxidations of methionine, carbamidomethyl cysteine, and carboxymethyl cysteine were specified as variable post-translational modifications in Mascot and Sequest. Results were viewed and validated in Scaffold (Proteome Software Inc., Portland, OR, U.S.A; version 4.4.6), and protein identities

were accepted if they could be established at > 99.9% probability and contained at least one identified peptide. The normalized spectral abundance factor (NSAF) approach was used to quantify each venom protein superfamily [53, 54]. This approach was used for both spectra identified using a custom rear-fanged snake transcriptome reference (a combination of venom protein sequences obtained from the venom gland transcriptomes of *A. prasina* and *B. portoricensis* and all rear-fanged venom protein sequences available from NCBI, accessed May 2016) and for spectra identified using the Trembl_bony vertebrate and Uniprot Serpentes (A8570) databases.

2.6. One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using NuPage 12% Bis-Tris mini gels under reducing conditions (50 mM dithiothreitol final concentration). Samples and buffers were prepared according to the manufacturer. Crude venom (15 μ g from three individual *B. portoricensis* and four individual *A. prasina*) and 7 μ L of Novex Mark 12 unstained mass standard (Invitrogen, Inc., U.S.A) were added to lanes. The gel was run at 160 V, stained with 0.1% Coomassie Brilliant Blue R-250 overnight, destained (50/40/10, v/v, ddH₂O, methanol and glacial acetic acid) for two hours, and scanned.

2.7. Western blot

A second gel identical to that described above (but using 7 μ L of Novex Sharp pre-stained protein standard, and including two non-colubrid venoms, *Crotalus s. tzabcan* and *Ophiophagus hannah*) was transferred to nitrocellulose membrane as described previously [55]. After blocking with PBS-buffered 3% BSA (Sigma Fraction V) for 1 h at RT, the membrane was incubated with primary antibody (15 μ L SAIMR anti-*Dispholidus* antivenom in 15 mL PBS-buffered 3% BSA) overnight at 4 °C. The membrane was rinsed 3 \times with Tris buffered saline (TBS, 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4) and then secondary antibody (5 μ L rabbit anti-horse IgG conjugated with alkaline phosphatase) in 15 mL TBS was incubated with the membrane for 60 min at RT with gentle shaking. Membranes were then washed 4 \times with TBS, and alkaline phosphatase substrate (Roche BCIP/NBT, 5-bromo-4-chloro-3-indolyl-phosphate/Nitrotetrazolium Blue, prepared following the manufacturer's instruction) in 10 mL of MilliQ™ water was added. The color reaction was stopped with 20 mM disodium EDTA in PBS after ~5 min. Membranes were then rinsed in MilliQ™ water and photographed.

2.8. Enzyme assays

A Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, U.S.A) was used to determine the protein concentration for crude venom, using bovine immunoglobulin G as a standard. Metalloproteinase activity was determined using azocasein as a substrate with a 60 min incubation at 37 °C with 20 μ g of crude venom [56], and activity was expressed as $\Delta A_{342 \text{ nm}}/\text{min}/\text{per mg venom protein}$. L-amino acid oxidase activity was assayed according to Weissbach (1961) with 20 μ g of crude venom, and the activity was expressed as nmol product formed/min/mg protein [57]. Acetylcholinesterase activity was determined using 15 μ g crude venom, incubated with the acetylthiocholine iodide substrate in a cuvette at 37 °C [58]. Absorbance at 412 nm was taken every 10 s for 10 min and the linear portion of the graph was used to calculate specific activity (μ mole product formed/min/mg venom protein). Phosphodiesterase activity was assayed with 20 μ g crude venom using 1 mM bis-*p*-nitrophenylphosphate as substrate, following the protocol developed by Laskowski (1980) [59]; activity was reported as $\Delta A_{400 \text{ nm}}/\text{min}/\text{mg protein}$. Phospholipase A₂ activity was determined using a commercially available kit (Cayman Chemical Co.) as described by the

manufacturer, using 2 µg crude venom in 200 µL total volume. Absorbance was measured at 414 nm every minute for five minutes and activity was reported as µmole product formed/min/mg protein. Thrombin-like and kallikrein-like serine proteinase activity was assayed according to Mackessy (1993) with benzoyl-Phe-Val-Arg-paranitroaniline (thrombin-like activity) and benzoyl-Pro-Phe-Arg-paranitroaniline (kallikrein-like activity) [60]. These substrates were incubated with 20 µg crude venom and activity recorded as nmol of product formed/min/mg protein. All enzyme assays described above were performed in triplicate. Fibrinogenase activity was determined using 20 µg of crude venom incubated with human fibrinogen (final concentration 0.5 mg/mL) at 37 °C in a total volume of 200 µL for periods of 0, 1, 5, 10, 30, and 60 min; for the inhibition of zinc-dependent metalloproteinases, venom and fibrinogen were incubated after addition of EDTA (final concentration 5 mM EDTA) to venom solutions [61]. 20 µL of this reaction mixture (with and without EDTA) was removed at each time point and mixed with an equal volume of 4% SDS and 5% 2-mercaptoethanol, then heated in boiling water for 10 min. 5 µL aliquots were combined with 2 × LDS buffer, electrophoresed on a 12% NuPAGE Bis-Tris gel, stained with Coomassie Brilliant Blue overnight, destained for two hours, and imaged.

2.9. Data accessibility

Transcriptomic data has been submitted to the NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>). *Ahaetulla prasina*: Bioproject ID: PRJNA448629; Biosample accession: SAMN08861737; Sequence Read Archive code: SRP137038. *Borikenophis portoricensis*: Bioproject ID: PRJNA448628; Biosample accession: SAMN08861636; Sequence Read Archive code: SRP137035. All transcripts that resulted in complete translated venom proteins were submitted to GenBank with accession numbers MH232964 - MH233082.

3. Results and discussion

3.1. Venom gland transcriptomes

After Trimmomatic adaptor removal and low quality filtering of raw sequencing reads, 21,086,588 reads were used for the *A. prasina* venom gland transcriptome assembly and 19,395,248 reads for *B. portoricensis*. For *A. prasina*, the Trinity *de novo* assembly generated 148,863 contigs with an average length of 842 bases, and 523 contigs were assembled with Extender with an average length of 1341 bases. For *B. portoricensis*, the Trinity *de novo* assembly generated 123,411 contigs with an average length of 1243 bases, and 496 contigs assembled with Extender with an average length of 1440 bases. A total of 69 venom protein complete coding transcripts from 12 superfamilies were identified in the *A. prasina* venom gland transcriptome, and 50 complete venom protein coding transcripts from 11 superfamilies for *B. portoricensis*. Although the most abundantly expressed transcripts were toxins for both venom gland transcriptomes, a greater number of non-toxin transcripts were expressed in high abundance in the *A. prasina* venom gland transcriptome compared to that of *B. portoricensis* (Fig. 1).

The *A. prasina* venom gland transcriptome included transcripts from the following venom protein superfamilies, in order of abundance: SVMPs (P-III), 3FTxs, C-type lectins (CTLs), CRiSPs, ficolins, a phospholipase A₂ inhibitor, a Kunitz-type protease inhibitor (KUNs), acetylcholinesterases (AChEs), a waprin, a phospholipase B (PLB), a cobra venom factor (CVF), and a venom endothelial growth factor (VEGF) (Fig. 2A). Venom protein superfamilies identified in the *B. portoricensis* venom gland transcriptome included, in order of abundance, SVMPs (P-III), CTLs, CRiSPs, a natriuretic peptides (NP), a waprin, a PLB, a 3FTx, a VEGF, a ficolin, a 5' nucleotidase (NUC), and a phosphodiesterase (PDE) (Fig. 2B). The number of venom protein superfamilies identified is similar to previously reported rear-fanged snake venom gland transcriptomes [11]. This also demonstrates that in the case of *B.*

portoricensis, venom gland tissue kept at –80 °C in RNAlater for five years still has stable mRNA that can yield a complete venom gland transcriptome assembly.

For both *A. prasina* and *B. portoricensis* venom gland transcriptomes, metalloproteinase transcripts were the most highly expressed venom protein superfamily, making up 62% of toxin reads and 70% of toxin reads, respectively (Fig. 2). Metalloproteinases also had the highest number of toxin transcript isoforms, with 39 identified isoforms in the *A. prasina* venom gland (Supplemental Table 1) and 30 SVMP isoforms for *B. portoricensis* (Supplemental Table 2). The large majority of full-length metalloproteinase isoforms were assembled with Extender. It appears that for *B. portoricensis*, the most highly expressed SVMP isoforms have close expression values (Supplemental Table 2), whereas for *A. prasina*, SVMP gene expression is dominated primarily by several highly abundant SVMPs, with others expressed at lower levels (Supplemental Table 1).

Overall, both *A. prasina* and *B. portoricensis* SVMPs shared high sequence identities, and all *A. prasina* SVMP isoforms showed over 70% residue identity (Fig. 3A), and all *B. portoricensis* SVMP isoforms were over 75% identical (Fig. 3B). Alignments between the SVMPs from other rear-fanged venomous snakes, as well as SVMPs from the elapids *Naja atra* (Chinese cobra), *Bungarus multicinctus* (Many-banded krait), and *Hoplocephalus stephensi* (Stephens' banded snake), demonstrated > 69% amino acid sequence conservation to SVMP amino acid sequences in *B. portoricensis* (Fig. 3B). Several SVMP isoforms from the *B. portoricensis* venom gland shared 100% identity with the short amino acid sequence obtained from Edman degradation sequencing of alsophinase, the characterized P-III SVMP that is responsible for hemorrhagic and fibrinolytic activities of *B. portoricensis* crude venom [33] (Fig. 3B). SVMP_1 transcript isoform corresponds closely to the protein alsophinase since this is the most abundant SVMP transcript, and alsophinase was one of the most abundant SVMPs in the crude venom [33]; however, the sequence in the original report appears to be internal sequence rather than N-terminal sequence, perhaps because of an N-terminal pyroglutamate residue blocking Edman degradation sequencing that was not effectively removed (even following treatment with pyroglutamate aminopeptidase). Interestingly, in the case of at least three *B. portoricensis* SVMP transcripts, a substitution in the stop codon results in a 9-residue extension at the C-terminus (Fig. 3B, only two isoform examples shown). It is unknown how this C-terminus extension might affect metalloproteinase activity.

Metalloproteinases from both snakes were all classified as P-IIIs, which contain a disintegrin-like domain and a cysteine-rich domain, in addition to the metalloproteinase domain shared by all proteins in this superfamily [27]. Only SVMPs belonging to the P-III class have been identified in rear-fanged snakes, and they are the major venom toxins in some of these venoms [10, 33, 62–64]. Metalloproteinases are largely responsible for hemorrhagic activity secondary to degradation of basement membrane and adhesion proteins and can produce systemic symptoms upon envenomation [27, 28]. Rear-fanged snakes with significant levels of these venom proteins are potentially hazardous to human health [8, 9].

Other highly expressed toxin transcripts include CRiSPs, CTLs, and 3FTxs (*A. prasina*) (Fig. 2). Cysteine-rich secretory proteins are commonly found in many venoms, but their biological role in envenomation remains unclear [65]. C-type lectins are also unusual in that these transcripts are expressed in many venom glands, even though the proteins are not always components of the venoms [11, 46]. There was only one full-length 3FTx identified in the *B. portoricensis* venom gland transcriptome, unlike the *A. prasina* venom gland, which expressed 10 different 3FTx transcript isoforms that comprised 17% of toxin reads (Fig. 2).

Transcripts for 3FTxs did not show as high a level of amino acid sequence conservation compared to SVMPs. In the *A. prasina* venom gland transcriptome, 3FTx isoforms shared 34.8–98.9% sequence identity (Fig. 4). 3FTx sequences from the *A. prasina* venom gland were

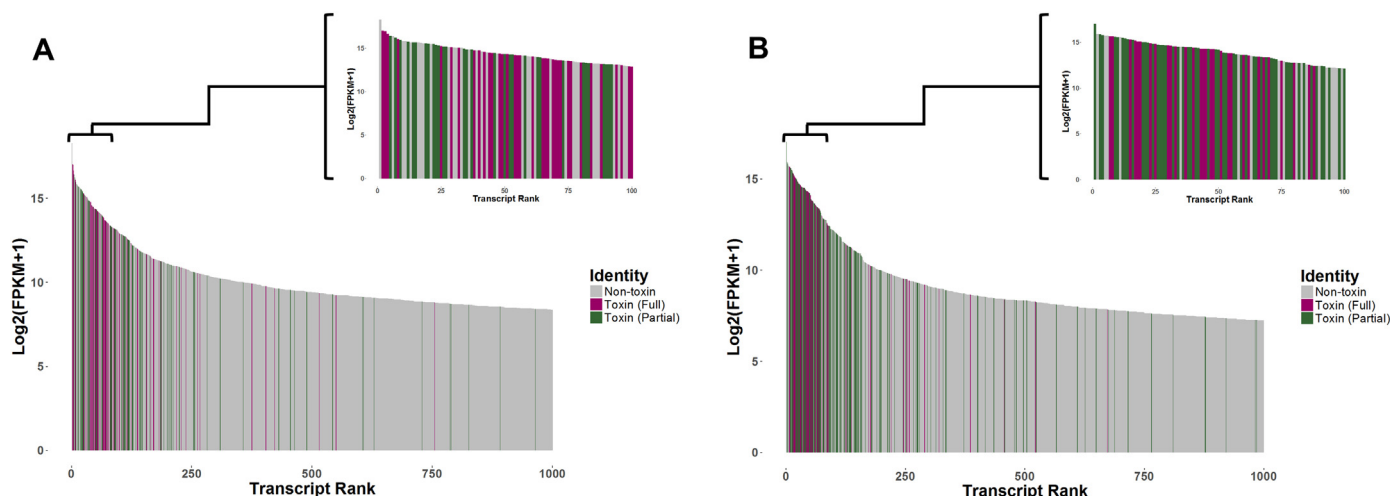


Fig. 1. Most abundant transcripts in rear-fanged snake venom gland tissue. Toxins (colored) comprise the majority of the most highly expressed genes in both *Ahaetulla prasina* (A) and *Borikenophis portoricensis* venoms (B). Insets in both show the top 100 expressed transcripts.

49% identical to 3FTxs identified in venom gland transcriptomes of *Dispholidus typus* (Boomslang) and *Thrasops jacksonii* (Jackson's Black Tree Snake), and species of cobras (*Ophiophagus hannah* and *Naja sputatrix*) (Fig. 4). The single 3FTx transcript from the *B. portoricensis* venom gland transcriptome, which exhibited an extended N-terminus, was most similar to 3FTxs from Asian Catsnakes (*Boiga* sp.) than to other known 3FTxs (Fig. 4).

3.2. Venom proteomes

Using Mascot, Sequest, and X! Tandem with public databases to map LC-MS/MS peptide fragments from *A. prasina* venom 7 venom protein superfamilies were identified, including SVMPs, cobra venom factors, PLBs, 3FTxs, a matrix metalloproteinase, CTLs, and a CRiSP (Fig. 5A). Only 71 peptide spectra were matched to these proteins, but a total of 906 peptide spectra matched venom proteins in the custom reference database. Six venom protein superfamilies were identified with the use of the custom reference from rear-fanged snake venom gland transcriptomes (Supplemental Table 3). These protein superfamilies

included SVMPs, CRiSPs, 3FTxs, a cobra venom factor, a PLB, and a CTL (Fig. 5B; those with abundances < 1% were grouped together in the “other” category).

Using the public databases, 8 venom protein superfamilies were identified in *B. portoricensis* venom. These included SVMPs, CTLs, 3FTxs, CRiSPs, matrix metalloproteinases, a PLA₂ inhibitor, a cobra venom factor (< 1%, excluded in the pie chart), and a C-type natriuretic peptide (Fig. 5C). Six venom protein superfamilies were identified in *B. portoricensis* venom using the custom rear-fanged snake transcriptome reference. These superfamilies include SVMPs, CTLs, 3FTxs, a CRiSP, a cobra venom factor and a PLB (Fig. 5D; those with abundances < 1% combined were excluded because the total was still < 1%). Without the custom reference, only 199 peptide spectra were assigned from *B. portoricensis* venom, whereas 1138 peptide spectra were assigned with the custom reference (Supplemental Table 4).

The use of larger public databases identified more venom protein superfamilies because the custom transcriptome reference (generated from species-specific venom glands and other rear-fanged snake venom

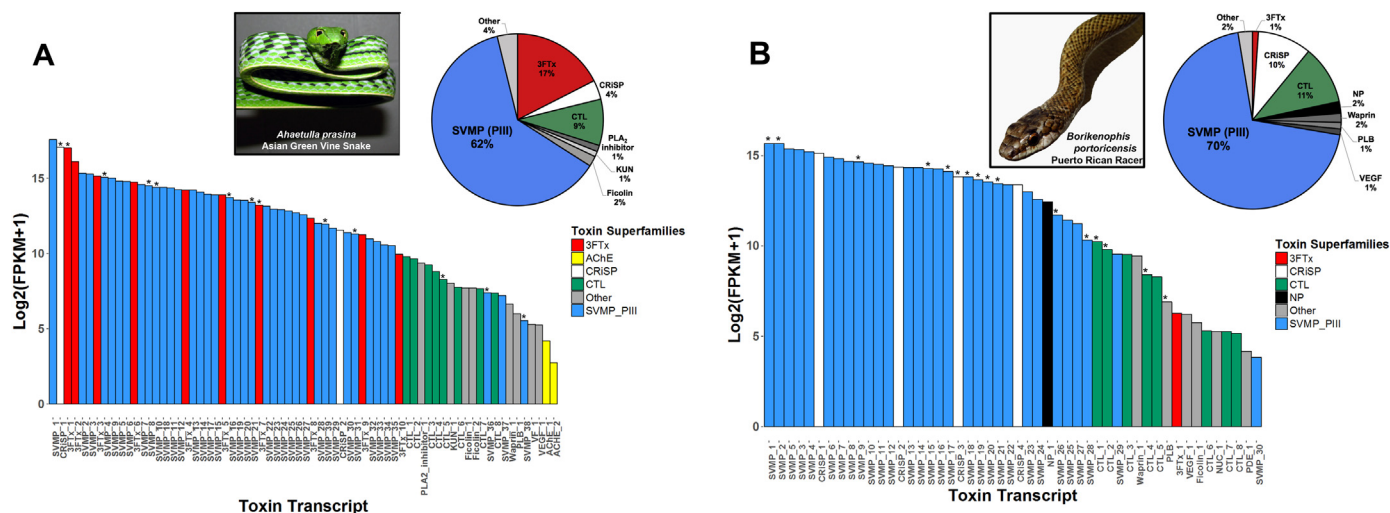


Fig. 2. *Ahaetulla prasina* (A) and *Borikenophis portoricensis* (B) venom gland transcriptomes show high expression of metalloproteinase transcripts. Venom peptides detected by MS/MS and assigned to a toxin transcript are marked with an asterisk. Inserts show the percentage of toxin reads for each venom protein superfamily, and those with < 1% abundances are grouped as “other”. Abbreviations: 3FTx = three-finger toxin, AChE = acetylcholinesterase, CTL = C-type lectin, CRiSP = cysteine-rich secretory protein, KUN = Kunitz-type protease inhibitor, NP = natriuretic peptide, NUC = 5’nucleotidases, PDEs = phosphodiesterase, PLA₂inhib = phospholipase A₂ inhibitor, PLB = phospholipase B, SVMP = snake venom metalloproteinase (P-III), VEGF = venom endothelial growth factor, and VF = cobra venom factor.

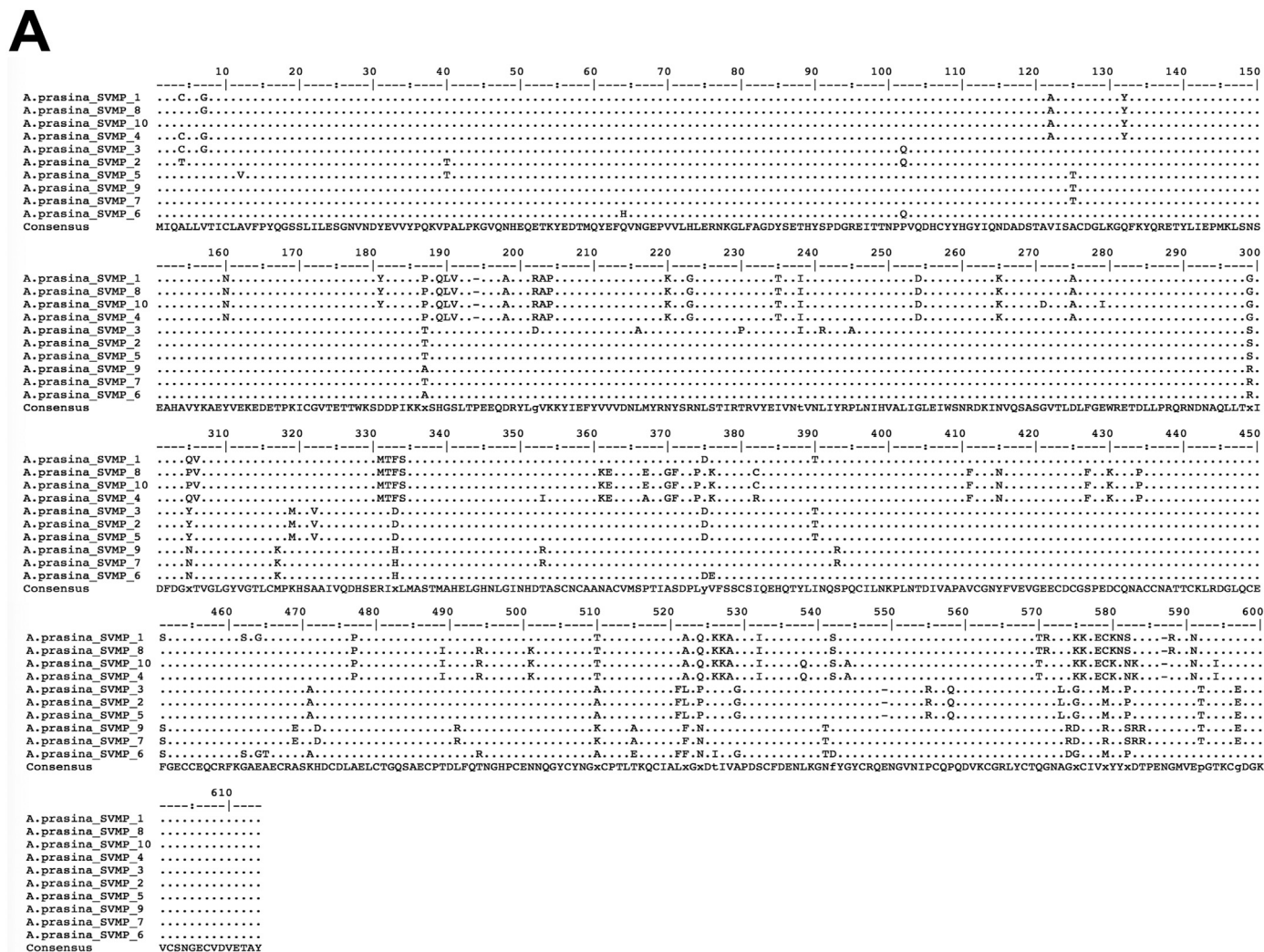


Fig. 3. Snake venom metalloproteinase (P-III) sequence alignments exhibit overall similarity in *Ahaetulla prasina* (A) and *Borikenophis portoricensis* (B) venom glands, and with other rear-fanged and elapid venomous snakes. Residues that are identical among isoforms and match with the consensus sequence are hidden, and residues that are different are shown. The partial amino acid sequence of the isolated and characterized metalloproteinase from *B. portoricensis* venom, alsophinase, is also included in the alignment. Other metalloproteinase sequences were obtained from rear-fanged snakes *Philodryas chamissonis* (AJB84503.1), *P. olfersii* (ACS74988.1), *Cerberus rynchops* (VM3_CERRY)) and elapids (*Naja atra* (VM3H_NAJAT), *Bungarus multicinctus* (VM3_BUNMU), and *Hoplocephalus stephensii* (ABQ01135.1).

gland transcriptomes) only included full-length (complete CDS) transcripts. It is possible that due to mRNA degradation or issues with venom gland transcriptome assemblies, some toxin transcripts in these venom glands were excluded [66]. This emphasizes the importance of using both species-specific transcriptome references and publicly available databases, in case of assembly and/or annotation oversight. However, the total number of peptide fragments/spectra mapped was far greater when species-specific transcriptome references were used. These also allowed better quantification of venom protein superfamilies in the venom with the label-free Normalized Spectral Abundance Factor (NSAF) method [53, 54], because fewer peptide spectra were excluded. In addition, pie charts generated for each venom protein family abundance using species-specific transcriptomes (Fig. 5B and Fig. 5D) better represented what was observed with SDS-PAGE (results detailed below).

The *A. prasina* venom proteome consisted predominately of P-III SVMPs (75%), with 3FTxs (13%) and CRiSPs (10%) observed in moderate abundance (Fig. 5B). Metalloproteinases (P-III) were also the most abundant venom protein for *B. portoricensis*, constituting 81% of the venom, followed by CTLs (13%), CRiSPs (3%) and 3FTxs (3%) (Fig. 5D). In addition to the differences in 3FTx abundances between

the two rear-fanged snakes, CTL abundances also varied, constituting only 1% of *A. prasina* venom, in comparison to 13% observed for *B. portoricensis*. It appears that for *A. prasina*, CTLs do not play a prominent role in envenomation. It is possible that these proteins might play a role in *B. portoricensis* envenomations, to which more extensive envenomation symptoms have been attributed [35], but the role of CTLs in colubrid venoms is not clear. Although CRiSP abundances also varied significantly between species, it is also unknown what role, if any, these venom proteins play in envenomation. CRiSP abundance was the primary disparity between venom gland transcriptomes and venom proteomes. This difference in CRiSP abundance was also observed for *Boiga irregularis* [12], although in the case of *B. portoricensis* there is an increased amount of transcript expression compared to CRiSP venom proteome abundance. Usually, the opposite trend is observed.

One of the more interesting venom proteins detected in these venoms was CVF. For *A. prasina* venom, 6% of the venom proteome was assigned to CVF using public database peptide matching. Cobra venom factors activate the complement system and have been noted as minor components of several elapid venoms [67, 68]. However, transcripts for CVFs have been observed for other rear-fanged snakes, such as *Leioheterodon madagascariensis* [69] and *Boiga irregularis* [11, 12]. It is likely

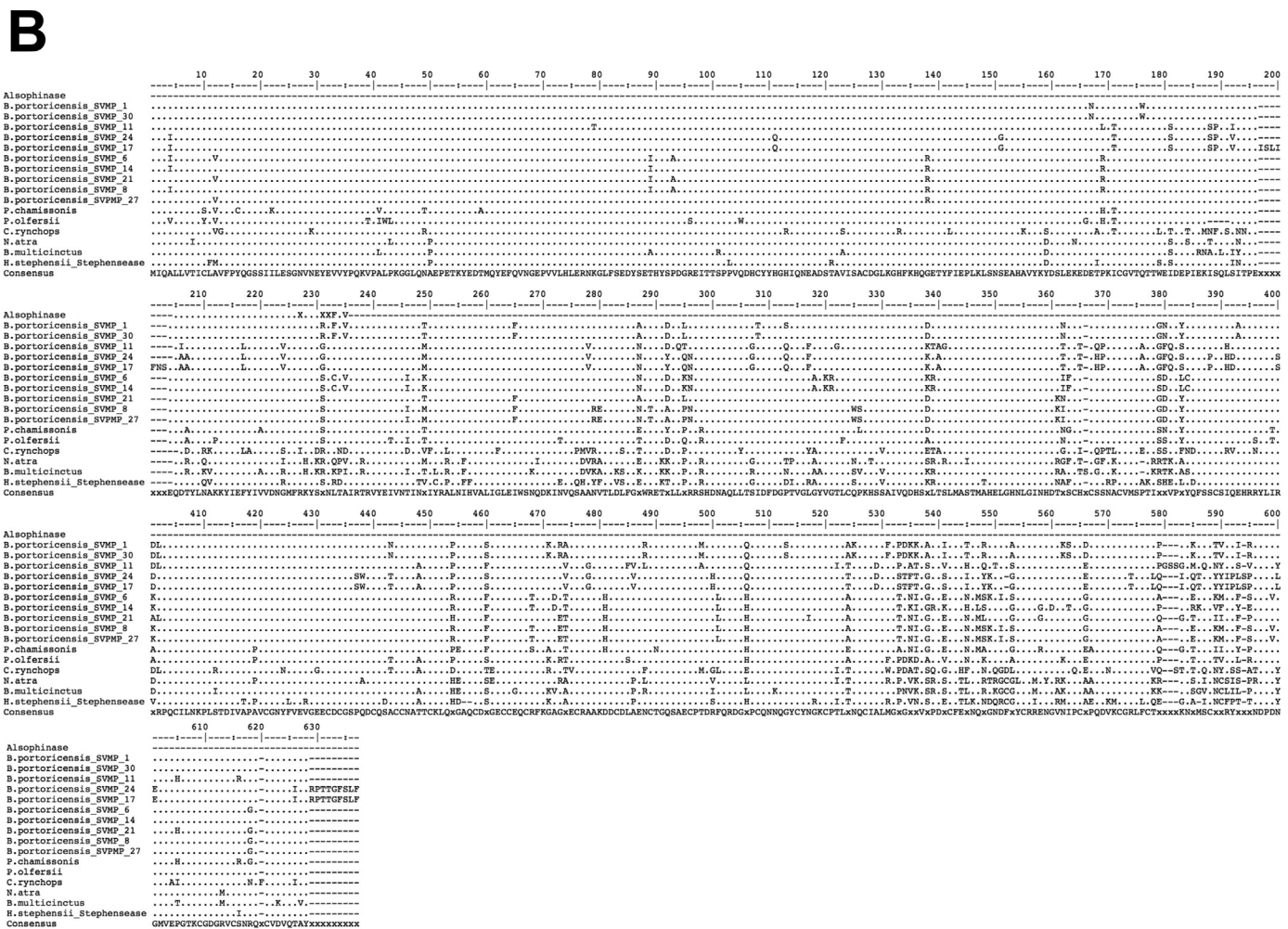


Fig. 3. (continued)

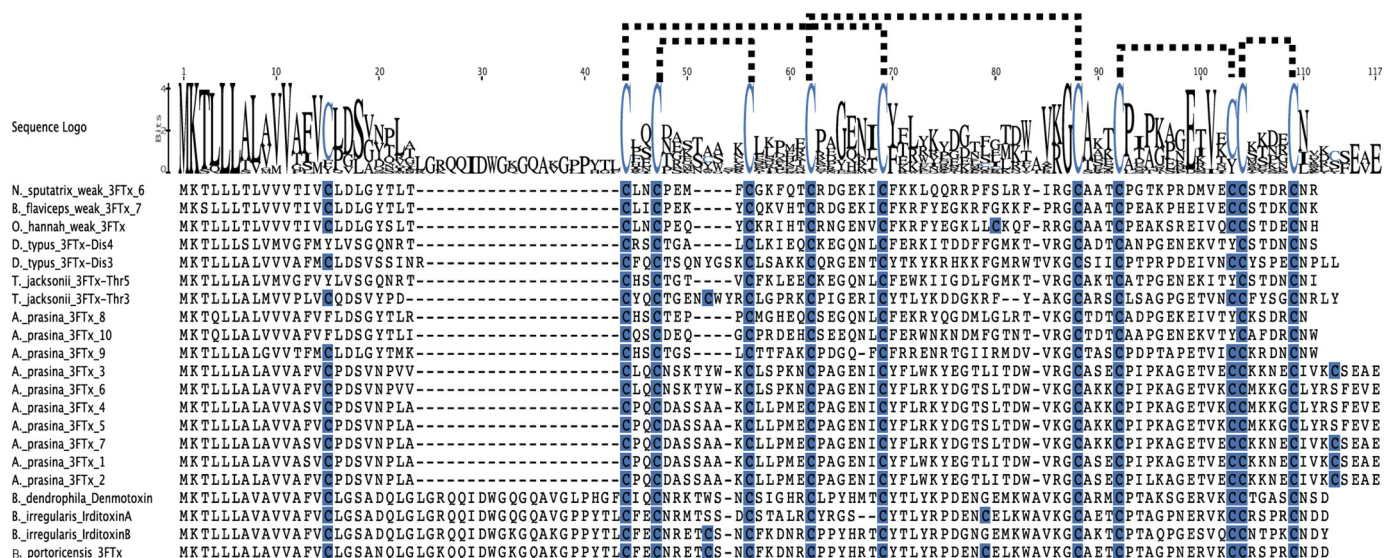


Fig. 4. Sequence alignment of three-finger toxin isoforms from rear-fanged snakes and elapids. Cysteines are highlighted due to the conserved three-finger toxin structure obtained from disulfide linkages between them. The five disulfide linkages characteristic of non-conventional 3FTxs are shown with dotted lines. Genbank accessions include: denmotoxin (Q06ZW0.1), irditoxin subunit A (A0S864.1), irditoxin subunit B (A0S865.1), *Thrasops jacksonii* (ABU68485.1 and 3SX5_THRJA), *Dispholidus typos* (ABU68483.1 and 3SX4_DISTY), *Ophiophagus hannah* (3NO24_OPHTA), *Naja sputatrix* (3NO26_NAJSP), and *Bungarus flaviceps* (ADF50022.1).

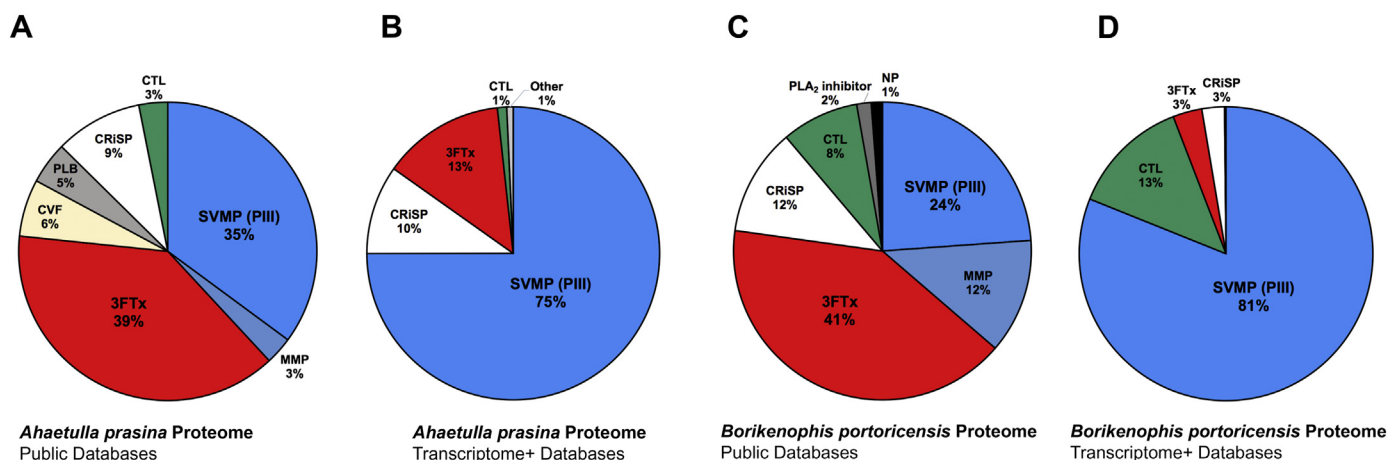


Fig. 5. Venom protein superfamily abundances for two rear-fanged snake venom proteomes, determined using publicly available databases and species-specific databases. Peptide mass fingerprinting was performed for *Ahaetulla prasina* venom with available public databases (A), and a species-specific venom gland transcriptome database (B), using search tools Mascot, Sequest, and X! Tandem. Peptide mass fingerprinting was also completed for *Borikenophis portoricensis* venom with public databases (C) and a species-specific venom gland transcriptome database (D). Venom protein superfamilies with abundances < 1% were grouped in the category “other”, unless total abundance was still below 1%, and then they were excluded. Abbreviations same as in Fig. 2.

that CVF makes up < 6% of *A. prasina* venom, but peptides belonging to this family of proteins share high identities [70] and therefore are matched better to public databases in comparison in venom proteins that exhibit lower conservation in sequence between species.

Only 13 of 69 transcripts (18%) were identified in the *A. prasina* venom proteome (Fig. 2A), a lower percentage than that observed for *B. portoricensis* (16 out of 50; 32%) (Fig. 2B), even though many more venom protein transcripts are present in the *A. prasina* venom gland. Similar ratios of transcripts and secreted protein products were reported for venom gland transcriptomes and proteomes of the rear-fanged snakes, *Hypsiglena* sp. and *Boiga irregularis* [11]. This is suggestive of translation-level regulation of transcript isoforms in a single venom protein superfamily, especially apparent in the case of metalloproteinases in these venoms. It is also possible that certain isoforms might have post-translational modifications or produce peptide fragments that are either too long or too short for MS detection. This is a considerable challenge associated with using crude venom for trypsin digestions and LC-MS/MS, a shotgun proteomics approach, instead of purified proteins or venom fractions where individual molecular masses can also be used to identify isoforms [12, 71, 72]. However, even taking into account these biases in MS/MS peptide detection, there is an absence of some SVMP isoforms. The peptide sequence NKGLFAEDYIET-HYSPDGR is not observed in *A. prasina* SVMP isoforms SVMP_17, SVMP_22, SVMP_31, SVMP_35, and SVMP_37, even though peptides in this same region are observed for the other isoforms, and this sequence does not exhibit glycosylation sites and would be of approximate size suitable for MS/MS detection.

Venoms from multiple individuals of each species, *A. prasina* and *B. portoricensis*, were subjected to reduced SDS-PAGE in order to visualize overall venom composition and observe intraspecific venom variation (Fig. 6A). For venoms of both species, bands of the expected mass range of SVMPs (PIII) were the most abundant, followed by CRiSPs, and 3FTxs (for *A. prasina*) or CTLs (for *B. portoricensis*). Western blot analysis of venoms using a primary SAIMR anti-*Dispholidus* monovalent antivenom demonstrated shared SVMP and CRiSP epitopes, with *B. portoricensis* venom showing a more intense reaction to *Dispholidus* antibodies (Fig. 6B). Venoms from multiple individuals of each species were found to show conserved banding patterns, indicating that one biological replicate for high-throughput proteomic and transcriptomic work provided a representative characterization of the venoms and transcriptomes. It has been noted that to obtain full characterization of a species' venom gland transcriptome, sequencing more than one individual is required [73]; however, use of one individual per species in

the current study reduced the number of sacrificed animals, and results from electrophoresis and enzyme assays (see below) of multiple individual venoms supported this strategy.

3.3. Venom biological activity

Both *A. prasina* and *B. portoricensis* venoms lacked PLA₂ activity, which is consistent with the lack of PLA₂ transcripts and proteins in venom glands and venom, respectively. Rear-fanged venomous snakes generally lack or exhibit very low PLA₂ activity, with few exceptions [34, 74, 75]. It appears that rear-fanged snake PLA₂s do not serve as vital a role in envenomation as they do for front-fanged venomous snakes, which consistently show high titers and diversity of venom PLA₂s, with varying pharmacological effects [4, 76, 77].

These venoms also lacked PDE, AChE, thrombin-like serine proteinase, and kallikrein-like serine proteinase enzyme activities. Very low levels of a PDE transcript were detected in the *B. portoricensis* venom gland transcriptome, but no peptides were assigned to venom PDE. A similar situation was observed for *A. prasina* and AChE, and low levels of AChE transcripts were expressed in the venom gland, but are either not translated or are present at such low concentrations in the venom that activity was not detectable. Serine proteinase transcripts or venom proteins were not found for either snake species. L-amino acid oxidase (LAAO) activity was absent from *B. portoricensis* venom; however, low levels of LAAO activity were detected for *A. prasina* (Table 1).

The most prominent enzyme activity was detected with azocasein (Table 1), a substrate that is degraded by snake venom metalloproteinases [36]. Both *A. prasina* and *B. portoricensis* venoms demonstrated metalloproteinase activity as high as that observed in some rattlesnake species, such as *Crotalus oreganus cerberus* (Arizona Black Rattlesnake), *Crotalus oreganus oreganus* (Northern Pacific Rattlesnake), and *Crotalus viridis viridis* (Prairie Rattlesnake), when the same amount of venom is used in the assay (20 µg) [30]. Detection of high SVMP activity levels was not surprising, given the abundances of both SVMP transcripts and proteins in these venom glands and venoms, respectively. Venom of *B. portoricensis* has previously been noted for high metalloproteinase activity, and one of the most predominate metalloproteinases from this venom has been isolated and characterized [33]. Metalloproteinase activity has not been explored for *A. prasina*, and SVMP activity observed for *A. prasina* venom is almost equal to that of *B. portoricensis* venom (Table 1).

Rear-fanged snake venom metalloproteinases have been reported to degrade fibrinogen subunits rapidly [32, 33]. Venoms from *A. prasina*,

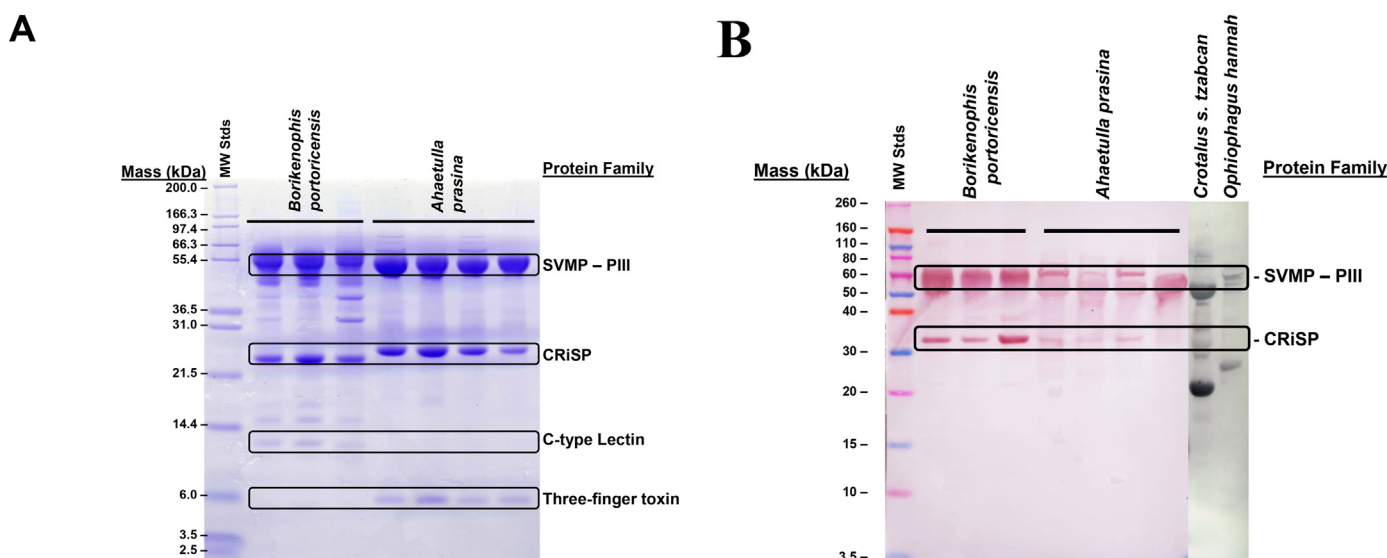


Fig. 6. Electrophoretic analyses of individual *Ahaetulla prasina* and *Borikenophis portoricensis* venoms. Reduced SDS-PAGE (A) of individual venoms demonstrate metalloproteinases (P-III) and CRiSPs predominate, with very little individual variation observed. Western blot of the same venoms (B) with primary SAIMR anti-*Dispholidus* antivenom show shared SVMPs and CRiSPs epitopes, and reactions are more intense for *Borikenophis*. Samples of *C. s. tzabcan* and *O. hannah* were run for comparison; anti-*Dispholidus* antibodies showed different patterns of epitope recognition, with some binding to P-III SVMP(s) in *Ophiophagus* venom.

Table 1

Enzyme activity of *Ahaetulla prasina* and *Borikenophis portoricensis* venoms.

	<i>Ahaetulla prasina</i> venom (n = 4)	<i>Borikenophis portoricensis</i> venom (n = 3)
Metalloproteinase activity ($\Delta A_{342nm}/min/mg$)	0.863 \pm 0.026	0.960 \pm 0.040
L-amino acid oxidase activity (nmol product/min/mg)	0.021 \pm 0.005	0

B. portoricensis, and *C. v. viridis* (a viperid species; positive control) showed fibrinolytic activity during 60 min incubations. Venom from *A. prasina* degraded the alpha subunit in less than a minute and in 5 min degraded the beta subunit (Fig. 7A). The gamma subunit was not degraded over the 60 min time course. *Borikenophis portoricensis* venom also degraded the alpha subunit of fibrinogen in less than a minute, consistent with reported activity of the metalloproteinase alophaenase in this venom [33]. The beta subunit was almost entirely degraded by 60 min, while the gamma subunit remained intact (Fig. 7A). For both *A. prasina* and *B. portoricensis*, fibrinolytic activity was inhibited by EDTA (data not shown), providing supporting evidence that SVMPs are responsible for this activity [33]. Surprisingly, even though *B. portoricensis* has been recognized as a snake responsible for human envenomations, resulting in localized pain and ecchymosis from metalloproteinases in this venom [35], and *A. prasina* venom has received little attention, it appears that *A. prasina* venom SVMPs have greater fibrinolytic activity.

Prairie Rattlesnake (*C. v. viridis*) venom degraded the alpha and beta subunits within 10 min, and the gamma subunit remained intact over the 60-min time course (Fig. 7B). Metalloproteinase inhibition with EDTA during *C. v. viridis* venom incubation with human fibrinogen resulted in slower alpha subunit degradation and beta subunit disappearance at the same rate (Fig. 7B). Viperid serine proteinases with fibrinolytic activity have been characterized, and are likely responsible for fibrinogen subunit degradation when metalloproteinase activity is inhibited [78–81]. It is striking to note the faster degradation rate of human fibrinogen by *A. prasina* venom metalloproteinases in comparison to a rattlesnake, *C. v. viridis*. Viper venoms generally have both fibrinolytic metalloproteinases and serine proteases, and bites typically result in serious envenomations [78, 82]. Bites from

Borikenophis have produced moderate swelling, necrosis, and ecchymosis, whereas bites from *Ahaetulla* are poorly documented, but not known to produce serious symptoms. The *in vitro* results obtained here indicate that there may be novel metalloproteinases present in *A. prasina* venom, perhaps with taxon-specific effects in non-mammalian prey, because this arboreal species is more likely to prey on lizards and birds.

Isoforms of SVMPs (P-III) have been identified that affect distinct coagulation mechanisms in humans, birds and small rodents, with some isoforms more specialized for rats or chickens [83]. Further, variations in SVMP (P-III) isoform expression have also been observed in rattlesnake populations that are locally adapted to prey [84]. SVMPs described in this study could provide interesting models for future work focused on structure-function relationships. Even SVMPs in elapid snakes, which are also of the P-III type, do not typically degrade the beta subunit of fibrinogen and only target the alpha subunit [85–87]. Both *A. prasina* and *B. portoricensis* have SVMPs that not only degrade both subunits, but do so even more rapidly than SVMPs found in some rattlesnake venoms. Venom metalloproteinases found in rear-fanged snakes provide insight into the evolution of this toxin family, mechanisms of action and biological roles of SVMPs, illustrating the importance of describing venom composition of poorly studied rear-fanged snakes and characterizing these toxins.

4. Conclusions

Rear-fanged snake venom gland toxin expression patterns appear to reflect the general pattern of enzymatic/hemorrhagic vs. small toxin/neurotoxic venom dichotomy that is observed between front-fanged viperid and elapid snake venoms [1, 11]. Venom gland transcriptomes and venom proteomes of *A. prasina* and *B. portoricensis* were both viperid-like, with high abundances of SVMP (P-III) transcripts and proteins. Several venom gland transcriptome assemblies have demonstrated that the most abundant toxin transcripts correlate with the most abundant venom proteins in the venom, and that post-transcriptional regulation contributes relatively little to the overall venom phenotype [88, 89]. However, other authors have observed a disparity in transcriptomic and proteomic abundance for some species of venomous snakes [90, 91]. In the current study, we found evidence supporting the former; however, there does appear to be translation regulation of

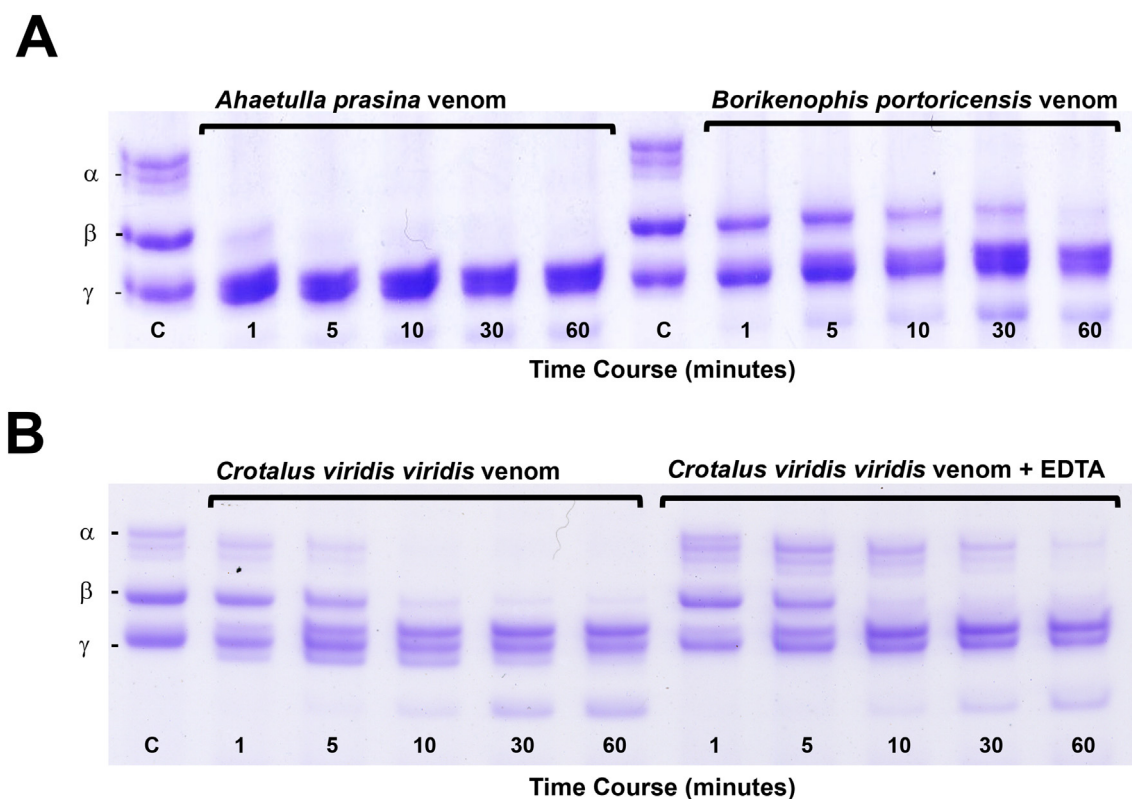


Fig. 7. Fibrinogen digest assay. *Ahaetulla prasina* and *Borikenophis portoricensis* crude venoms (20 μg) were incubated with human fibrinogen over a 60-min time course (A). The control, labeled C, was removed before the addition of any venom and shows all intact fibrinogen subunits (labeled α, β, and γ). *Crotalus viridis viridis* (20 μg) crude venom incubations with and without EDTA demonstrate the effects of active and inhibited metalloproteinase activity, respectively (B); EDTA abolished all fibrinogenolytic activity of *A. prasina* and *B. portoricensis* venoms (data not shown).

different isoforms in each venom protein superfamily.

The abundance of rapidly-acting metalloproteinases, comparable to those in rattlesnake and other highly venomous snakes, emphasizes the important role of venom proteins in facilitating prey capture *via* incapacitation and structural degradation (hemorrhage) for both front-fanged and rear-fanged snakes, regardless of the ecological niche occupied (arboreal *A. prasina* and terrestrial *B. portoricensis*). Venom proteins originating from rear-fanged snakes exhibit unique activities and are critical for studies of toxin evolution in the Colubroidea and of structure-function relationships.

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Significance

Characterizing venom gland transcriptomes, venom proteomes, and the enzymatic activities of rear-fanged snake venoms are critical steps necessary for unraveling the evolution of snake toxin superfamilies and elucidating the biological roles of components within these venoms. Currently, < 3% of rear-fanged snake venom proteomes have been described. We report the characterization of venom gland transcriptomes and venom proteomes for two species of rear-fanged snakes, *Ahaetulla prasina* (Asian Green Vine Snake) and *Borikenophis* {formerly *Alsophis*} *portoricensis* (Puerto Rican Racer). Integrated transcriptomics and proteomics revealed viperid-like proteolytic venoms, dominated by

P-III metalloproteinases. A custom database derived from the venom gland transcriptomes was necessary for the comprehensive identification of venom peptides and determination of protein superfamily relative abundances within each venom. Functional assays demonstrated that venom metalloproteinase activity was responsible for rapid degradation of human fibrinogen subunits, and a Western blot revealed shared metalloproteinase and CRiSP epitopes with the highly venomous rear-fanged snake *Dispholidus typus* (Boomslang). Quantification of venom metalloproteinase activity and abundance allows for the identification of potentially medically significant rear-fanged snake species and toxins, and emphasizes the importance of venom proteins in facilitating prey handling and predigestion for both front-fanged and rear-fanged snakes.

Conflict of interest statement

The authors state that no conflict of interest exists.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2018.08.004>.

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