Venoms of Colubrids

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Abstract

Advancements in high-throughput technologies in the field of venomics, coupled with the increasing emphasis on a combination of proteomic, transcriptomic, and genomic approaches, have resulted in the ability to generate comprehensive venom profiles for many species of snakes. Rear-fanged snake venom research has slowly progressed due to the difficulties obtaining crude venom and a lack of interest in snakes that only rarely are responsible for human morbidity and mortality. However, current research into rear-fanged snake venoms has demonstrated the existence of novel venom proteins and has provided insight into

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the evolution and origin of snake venom toxins within advanced snakes. These venoms still remain largely unexplored, and there exists within these venoms the potential to discover proteins of therapeutic significance or with unique characteristics. The majority of research conducted on these venoms has focused on protein chemistry and proteomic techniques (electrophoresis, enzymatic assays, liquid chromatography, and mass spectrometry), with fewer explorations of venom gland transcriptomes from expressed sequence tags (ESTs). Published research on rear-fanged snake genomes is not yet available, but such studies will provide insights into the evolutionary history of snake venom proteins and the regulation of toxin expression. Venom is a trophic adaptation, and as such, the evolution and abundance of venom proteins relates directly to prey capture success and organism natural history. Without this biologically relevant perspective, which considers the presence and evolution of rear-fanged venom proteins in terms of their biological significance to the organism, proteomic and genomic approaches could produce simply a list of proteins, peptides, transcripts, and genes.

Introduction

Snake venoms represent a critical innovation allowing advanced snakes (Caenophidian) to transition from a mechanical (constriction, as seen in Henophidians) to a chemical (venom) means of subduing prey (Kardong et al. 1997). The complex mixture of proteins and peptides which constitute a snake's venom contribute to multiple biological functions, including immobilizing, dispatching, and digesting prey (Mackessy 2010). However, rear-fanged venomous snakes are particularly interesting due to the fact that some species of these "colubrid" clades utilize constriction in addition to venom for facilitating prey capture. For example, venom from the brown tree snake (*Boiga irregularis*) contains a prominent heterodimeric three-finger toxin (3FTx) that is specifically toxic toward lizard and avian prey (Pawlak et al. 2009), and because this 3FTx is nontoxic toward mammalian prey, these snakes will instead constrict mammals (but not lizards; Mackessy et al. 2006). The specific receptor binding exhibited by 3FTxs originates from the accelerated accumulation of nucleotide substitutions within exons and the resulting changes to protein amino acid sequence and structure (Kini and Doley 2010; Sunagar et al. 2013), and in this case, a taxon-specific toxin has evolved. Venomic techniques combined with transcriptomics, genomics, natural history, behavior, and the recognition of venom as a trophic adaptation offer a powerful approach to unraveling the complex evolutionary history of venoms. This chapter considers the application of combined approaches toward the study of rear-fanged snake venoms.

Research centered on venom composition and individual protein characterization provides insight into the biological roles of venom compounds and

evolutionary relationships of venomous snakes as well as identifying compounds which evoke toxic symptoms resulting from snakebite and directly contributes toward production of more efficient antivenoms. Because front-fanged venomous snakes belonging to the families Elapidae and Viperidae produce significantly larger venom yields, and are responsible for the vast majority of human envenomations, venom research has primarily focused on species within these two families of snakes (Mackessy 2010). Rear-fanged venomous snakes, on the other hand, appear to exhibit a less derived venom delivery apparatus, produce significantly lower venom yields, and are generally perceived as nonthreatening to humans; as a result, they are generally understudied relative to the front-fanged snakes (Saviola et al. 2014). The large majority of rear-fanged venomous snakes are unable to deliver lethal quantities of these toxins or even enough toxins to result in systemic envenomations, but at least five species (*Dispholidus typus, Thelotornis capensis, Rhabdophis tigrinus, Philodryas olfersii,* and *Tachymenis peruviana*) are believed to have caused human fatalities (Kuch and Mebs 2002; Mackessy 2002; Prado-Franceschi and Hyslop 2002; but see Weinstein et al. 2013). Increasing awareness of severe, at times fatal, envenomations from rear-fanged snakes has led to a slowly growing interest in their venoms. In addition, advances in research techniques have resulted in a modest increase of data on individual toxins and on the composition and complexity of rear-fanged snake venoms.

Even though a single species may produce a venom with more than 100 protein components, snake venom proteins belong to a small number of enzymatic and nonenzymatic superfamilies. Some of these well-recognized venom protein families include phospholipases A_2 (PLA_2s), serine proteinases, snake venom metalloproteinases (SVMPs), three-finger toxins (3FTxs), proteinase inhibitors, and lectins (Mackessy 2010). These major venom protein families are found in almost all snake venoms, including many rear-fanged snake venoms (Mackessy 2002). In general, rear-fanged snake venoms show lower complexity than the venoms of front-fanged snakes, with upward of 40 expressed proteins visible following 2D SDS-PAGE (two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis), while front-fanged snake venoms show considerably higher complexity. In several cases, rear-fanged snake venoms have also been documented to contain novel protein superfamilies, thus providing a more comprehensive view of venom evolution (Ching et al. 2006; OmPraba et al. 2010; Pawlak et al. 2009). With the introduction of high-throughput proteomic and nucleic acid sequencing methods, detailed venom descriptive work encompassing the proteome, transcriptome, and genome is now possible with the relatively small amount of starting material obtained from rear-fanged snakes. These techniques, combined with protein biochemical characterizations and snake natural histories, will continue to elucidate the evolution and biological roles of rear-fanged snake venom components. The goal of this chapter is to provide a review of previous work, current research and methods, and future applications involving rear-fanged snake venoms.

Previous and Current Research on Rear-Fanged Snake Venoms

Classical Approaches

Since the mid-twentieth century, the study of snake venom toxinology has developed into a formalized scientific discipline. Originally, rear-fanged (opisthoglyphic) snakes were regarded as nonthreatening to humans, but the tragic deaths of herpetologists Karl Schmidt (due to envenomation from *D. typus*) and Robert Mertens (*Thelotornis capensis*) brought attention to the venomous potential of bites from rear-fanged snakes. These events initiated an increase in studies on these venoms (Weinstein et al. 2011), and the discovery that many rear-fanged venom secretions exhibit complex immunoidentity with numerous medically important viperid and elapid species (Minton and Weinstein 1987) further stimulated research endeavors into rear-fanged snake venoms. Some lethal venom components, such as 3FTxs, PLA_2 enzymes, and snake venom metalloproteinases (SVMPs), that were once thought to be found exclusively in elapid or viperid venoms now appear to be significantly abundant compounds in numerous species of rear-fanged snakes (Fry et al. 2003a; Mackessy 2002; Huang and Mackessy 2004; Peichoto et al. 2011). However, due to limited accessibility of specimens and low venom yields obtained during extractions, studies involving rear-fanged snake venoms have progressed slowly compared to the extensive work examining front-fanged snake venoms (Mackessy 2002). The now common utilization of anesthetics, such as ketamine hydrochloride, followed by a subcutaneous injection of the parasympathomimetic pilocarpine hydrochloride to stimulate venom secretion, has not only improved snake handling and safety for both the animal and handler but has also resulted in greatly increased venom yields (Rosenberg 1992; Hill and Mackessy 1997; Mackessy 2002). Since it is now possible to obtain sufficient quantities of venom, coupled with the continuing advancements in biochemical characterization and high-throughput proteomic, transcriptomic, and genomic techniques, it is now feasible to develop a much better understanding of the composition and complexity of rear-fanged snake venoms.

Traditional methods, such as the use of one- and two-dimensional gel electrophoresis, provide a quick and basic approach for identifying venom compounds present in crude venoms. For 1D SDS-PAGE (one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis), as little as 10–30 μg of crude venom separated in 12 % acrylamide precast gels provides a clear molecular fingerprint of potential venom compounds (Fig. 1), allowing for inter- and intraspecific comparisons of venom variation (e.g., Mackessy et al. 2006; Peichoto et al. 2012). Rear-fanged venoms that have been studied typically demonstrate a greater complexity in the higher molecular mass regions following SDS-PAGE (Peichoto et al. 2012). Two-dimensional gel electrophoresis provided additional venom compositional information in an analysis of South and North American opisthoglyphous snake species, allowing for the detection of multiple protein isoforms that shared similar molecular masses but differed in isoelectric points. Acidic proteins in the 30–40 kDa range, which would have been difficult to distinguish using only one-dimensional gel electrophoresis, showed differential expression in *Philodryas*

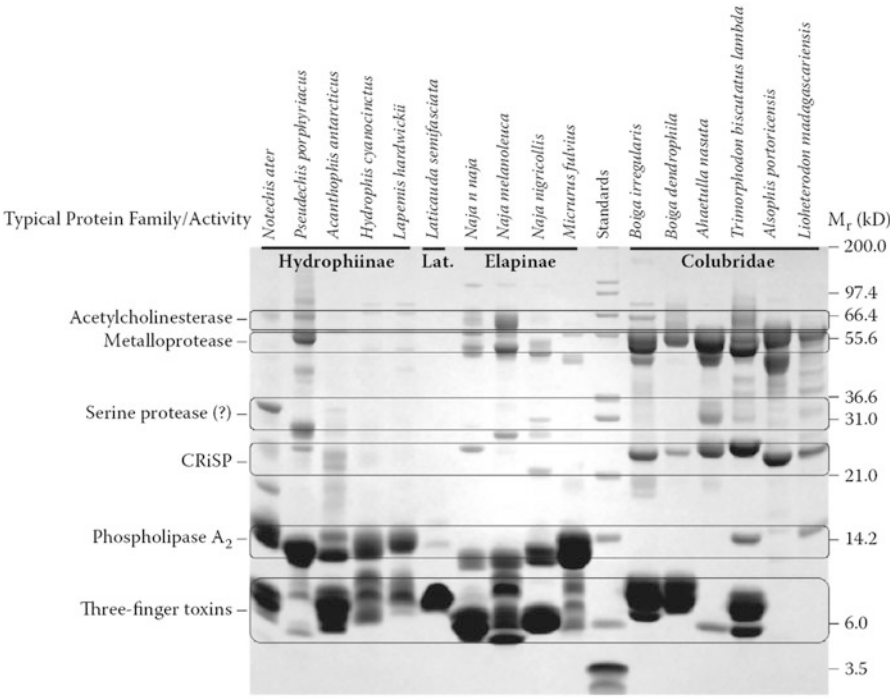

Fig. 1 One-dimensional SDS-PAGE of elapid and rear-fanged (Colubridae) snake venoms; 3–20 protein bands are visible. Note that proteins of the size of 3FTxs are shared among both clades and that SVMPs are commonly found as a major component of rear-fanged snake venoms but are less abundant in most elapid venoms (Reproduced from Mackessy (2010))

sp. and for *Trimorphodon biscutatus lambda* (Peichoto et al. 2012). The greater resolution provided by 2D gel electrophoresis is also an ideal fractionation method for in-gel trypsin digestion and mass spectrometry (MS) analysis. This technique allows for the identification of multiple protein isoforms that can exist in a venom protein superfamily (Fig. 2), such as the multiple matrix metalloproteinase isoforms found in the rear-fanged snake *Thamnodynastes strigatus* (Ching et al. 2012).

Whole-organism toxicity, another traditional venom characterization method, allows for identification of lethal doses (LD_{50}); this dosage reflects the amount of a substance required to kill half of the injected organisms within a 24-h period. Low LD_{50} values indicate the presence of potent (often neurotoxic) venom components, but it can be difficult to obtain enough material, especially purified individual venom components, from some rear-fanged snakes (Mackessy 2002). LD_{50} values have classically been determined using a mouse model because mice are easy to maintain, can be obtained in an array of essentially "reagent grade" strains, and are included in the diet of many venomous snakes (da Silva and Aird 2001). Because the diets of rear-fanged venomous snakes often encompass a broader range of prey taxa, species that regularly feed on nonmammalian prey may produce venom toxicity and other physiological data in mice that are not biologically relevant

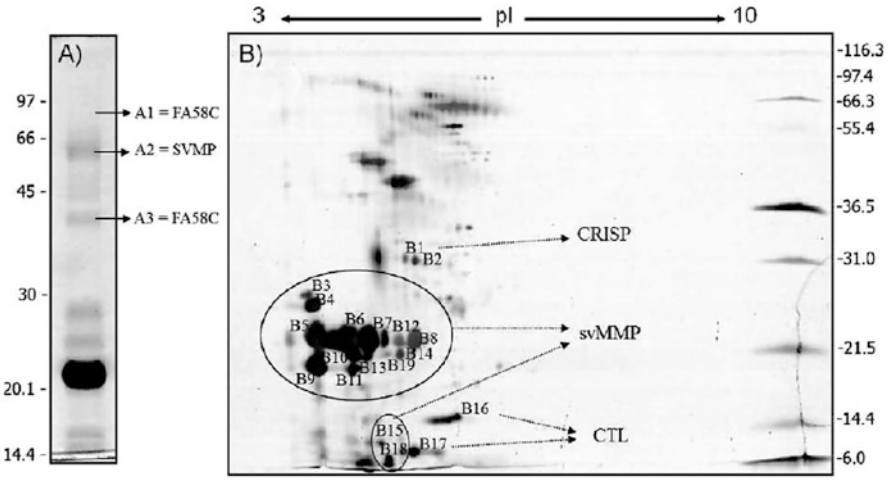

Fig. 2 1D SDS-PAGE (**a**) and 2D SDS-PAGE (**b**) of *Thamnodynastes strigatus* venom. Note the greater resolution of proteins in B – most proteins of this venom have an acidic pI (Reproduced from Ching et al. (2012))

(e.g., Pawlak et al. 2006, 2009). For example, NSA mice showed no adverse effects from the 3FTx irditoxin (from *B. irregularis* venom) at doses of at least 25 μg/g, whereas house geckos (*Hemidactylus frenatus*) and domestic chickens (*Gallus domesticus*) exhibited rapid flaccid paralysis, dyspnea, and increased respiratory rates at all doses tested, with an $LD_{50} < 0.55$ μg/g (Pawlak et al. 2009). This correlates closely with the diet of *B. irregularis*, which frequently feeds on birds and lizards, and demonstrates the importance of venom as a tropic adaptation and the need to acknowledge snake natural history when elucidating venom protein biological roles. LD_{50} values from mice alone (crude venom – 18–31 μg/g; Mackessy et al. 2006) would not have revealed the complexities of *B. irregularis* venom and would not have detected the presence of a prey-specific toxin.

Analyses of rear-fanged venoms by HPLC (high-performance liquid chromatography) size exclusion chromatography has revealed the presence of larger mass proteins, with acetycholinesterase and metalloproteinase activities limited to the first peaks, CRiSPs (cysteine-rich secretory proteins) found in the second peaks, followed by PLA_2s, and then 3FTxs when present (Peichoto et al. 2012). Ion exchange chromatography, especially cation exchange, has been shown to be an effective purification first step for 3FTxs present in rear-fanged snake venoms and was used successfully for the isolation of the 3FTxs fulgimotoxin and denmotoxin (Heyborne and Mackessy 2013; Pawlak et al. 2006). Anion exchange columns have been successful for purifying SVMPs from rear-fanged snake venoms (Weldon and Mackessy 2012). Reversed-phase (RP) HPLC is a common final polishing step for the removal of salts from size exclusion or ion exchange chromatography, but this method can result in denaturation of some venom proteins, in particular metalloproteinases and other enzymes.

RP-HPLC has also been utilized as a first step for descriptive venomics because this technique provides a clear image of crude venom complexity by separating protein isoforms and exhibiting relative abundance of venom protein superfamilies when combined with SDS-PAGE or MS (Calvete et al. 2009; Fry et al. 2003c; Pawlak et al. 2006). A combination of liquid chromatography and soft ionization mass spectrometry (LC/MS) has been used to analyze crude rear-fanged snake venoms, including species from Colubrinae, Homalopsinae, Natricinae, Psammophiinae, Pseudoxyrhophiinae, and Xenodontinae (Fry et al. 2003c). An advantage to this technique is that it can be performed with limited amounts of material. MS molecular masses and LC retention information can also provide an idea of represented venom protein superfamilies in a crude venom (Fry et al. 2003c). However, ion suppression with coeluting proteins is a problem with electrospray mass spectrometry (ESI-MS), and proteins of lower abundance can be overlooked.

For identifications of purified venom proteins, N-terminal sequencing (Edman degradation) has been frequently used. N-terminal sequencing and tandem MS for de novo sequencing can provide reliable amino acid sequences, and automated de novo sequencing tools are increasingly becoming more robust. However, identification of proteins from rear-fanged snake venoms can still be problematic given the limited amount of database information currently available for rear-fanged venom protein sequences. A postsynaptic neurotoxin was isolated in the rear-fanged Rufous beaked snake (*Rhamphiophis oxyrhynchus*) but lacked sequence homology to any previously identified snake venom toxin in the databases, making it difficult to determine what venom protein family this neurotoxin represented (Lumsden et al. 2007).

Venoms are composed of both enzymatic and nonenzymatic proteins, as well as small peptides and other organics (Mackessy 2010), and numerous enzyme assays have been developed for the detection of the major snake venom enzyme superfamilies. These assays include substrates that can indicate the presence of proteases (SVMPs and serine proteinases), acetylcholinesterases, PLA_{2s}, L-amino acid oxidases, hyaluronidases, and phosphodiesterases in rear-fanged snake venoms (Mackessy 2002). Proteolytic activity has been assayed for using several substrates, including casein yellow, azocasein, collagen, and fibrinogen (Sanchez et al. 2014). Zymogram gels, which are copolymerized with gelatin, have also been used to characterize rear-fanged snake venom proteins with proteolytic activity (general endoproteinase activity) (Hill and Mackessy 2000; Weldon and Mackessy 2010).

Using azocasein substrate, SVMP activity has been identified in many rear-fanged snake venoms, including the venoms of *Dispholidus typus*, *Philodryas* sp., *Hydrodynastes gigas, Hypsiglena torquata,* and *Alsophis portoricensis* (Hill and Mackessy 2000; Mackessy 2002; Peichoto et al. 2007; Weldon and Mackessy 2012). This list of species includes both New World and Old World rear-fanged snakes and is suggestive of potential local tissue damage and hemorrhage if bitten by these species (Peichoto et al. 2012; Sanchez et al. 2014). Currently, there are several rear-fanged SVMPs that have been further characterized, such as patagonfibrase from *Philodryas patagoniensis* and alsophinase from *Alsophis portoricensis*, both of which demonstrate alpha-fibrinogenolytic and hemorrhagic activities (Peichoto et al. 2007;

Weldon and Mackessy 2012). *Philodryas patagoniensis* venom has been reported to contain proteolytic activity greater than the venom of *Bothrops alternatus*, and the venom of *P. baroni* was reported to exhibit proteolytic activity 25 times greater than the activity reported for *B. jararaca* (Sanchez et al. 2014). Hemorrhagic SVMPs and serine proteinases are responsible for severe local inflammation and tissue necrosis in human envenomations, and significant bleeding has been reported from rear-fanged snake envenomations, likely due to the presence of these toxins (Weinstein et al. 2011). Assaying rear-fanged snake venoms for proteolytic activity, particularly SVMP activity, can be useful to predict the potential envenomation hazard these snakes could pose to humans.

Snake venom metalloproteinase classes differ in structure with regard to domain composition; P-Is have only the metalloproteinase domain, P-IIs have an additional disintegrin domain, and class P-IIIa-c have a metalloproteinase, disintegrin, and cysteine-rich domain, with P-IIId having an additional lectin domain (Fox and Serrano 2010). The only SVMPs to date that have been discovered in rear-fanged snake venoms have been of the P-III class, which have been characterized in several venoms, including *Dispholidus typus* and *Alsophis portoricensis* venoms, among others. Although full venomic analyses (protein digestion, followed by peptide mass fingerprinting) was not utilized to identify protein families in several of these studies, SVMP activity was detected using an azocasein substrate confirming the presence of SVMPs in these venoms. A combined proteomic and transcriptomic analysis of the venom of *Philodryas olfersii,* a rear-fanged venomous snake of South America with growing medical significance, revealed toxin similarities to those of snakes belonging to the family Viperidae, with the P-III class of SVMPs being the most abundant protein in the venom (Ching et al. 2006). P-III SVMPs are also the most abundant compounds in the venoms of *Thamnodynastes strigatus* (Ching et al. 2012) and of *Hypsiglena* sp. (McGivern et al. 2014). Both one- and two-dimensional gel electrophoresis further confirmed the presence of P-III SVMPs not only in *P. olfersii* but also in *P. patagoniensis, P. baroni,* and *Hypsiglena torquata texana* venoms (Peichoto et al. 2012). It is thought that during the evolution of a front-fanged venom system, the various domains observed in P-III SVMP were gradually lost. The P-I and P-II classes of SVMPs are currently only found in Elapidae and Viperidae venoms (Fox and Serrano 2010; Mackessy 2010).

Acetylcholinesterase activity has been reported in several rear-fanged snake venoms, with this activity being most prominent in venoms of *Boiga* species such as *B. irregularis* (Mackessy 2002; Mackessy et al. 2006). This acetylcholinesterase activity appears to be substrate specific as it lacks activity toward a butyrylcholine substrate (Mackessy 2002). Acetylcholinesterase activity is commonly detected in venoms with the use of the substrate acetylthiocholine that reacts with dithiobisnitrobenzoate to produce a colorimetric determination of activity. For the detection of PLA_2 activity, 4-nitro-3-(octanoyloxy) benzoic acid and egg yolk phosphatidycholine Type IV substrates have been used to assay activity in venoms of *Boiga dendrophila, Diadophis punctatus regalis, Dispholidus typus, Leptodeira annulata, Malpolon monspessulanus, Rhabdophis subminiata, Thelotornis capensis, Rhamphiophis*

oxyrhynchus, and *Trimophodon biscutatus lambda* (Hill and Mackessy 2000; Huang and Mackessy 2004). The PLA_2 trimorphin has been purified and characterized from *Trimorphodon biscutatus lambda* venom.

CRiSPs are also widespread in reptile venoms and exhibit a remarkable degree of sequence conservation (Heyborne and Mackessy 2010; Peichoto et al. 2009), and several members of this superfamily have been found to interact with different target proteins, such as cyclic nucleotide-gated ion channels as well as L-type Ca^{2+} and K^+ channels (Yamazaki and Morita 2004). The biological functions of many CRiSPs remain relatively unknown. A CRiSP isolated from the rear-fanged snake *Helicops angulatus* has been shown to exhibit robust neurotoxic activity that results in immediate respiratory paralysis in mice (Estrella et al. 2011), while patagonin, a CRiSP characterized from the venom of *P. patagoniensis,* was found to cause muscular damage (Peichoto et al. 2009). Rear-fanged venom CRiSPs appear to show the same conservation of structure and diversification of function as seen for 3FTxs.

In the absence of biochemical and biological assays, it can be difficult to predict the activity of venom proteins. Large venom protein superfamilies such as 3FTxs and PLA_2s can exhibit a diversity of activities, ranging from neurotoxicity as a result of specific receptor binding to general cytotoxicity resulting in tissue necrosis. Researchers should be careful assigning protein activity based solely upon sequence similarity to other proteins or if only limited biochemical assays have been conducted. In the case of PLA_2s, pharmacological effects may be dependent or independent of enzymatic activity; therefore, a biochemical assay focused only on PLA_2 enzymatic activity could overlook other pharmacological activities, such as neurotoxicity (Mackessy 2010). Many high-throughput venom descriptive techniques, such as those based on MS/MS (tandem mass spectrometry) data, are limited when it comes to evaluating structure-function variation within venom protein families. Venom proteins can share similar amino acid sequences and have different structural arrangements as a result of post-translational modifications or interactions between other venom proteins or substrates. It is also possible for toxins to have similar structural appearances but exhibit vastly different receptor targets or activities. An example of this is the diversity of biological activities exhibited by venom 3FTxs, which include neurotoxicity, enzyme inhibition, cardiotoxicity, cytotoxicity, ion channel blockage, and anticoagulation effects (Kini and Doley 2010).

Venoms of several rear-fanged snakes in the family Colubridae (*sensu stricto*) contain 3FTxs that maintain the same conserved three β-sheet stabilized loops (from which the name "three-finger toxin" originated) commonly seen in 3FTxs from elapid venoms. Several 3FTxs from rear-fanged snakes have taxon-specific receptor binding affinities which has not been observed for elapid 3FTxs. Denmotoxin, from venom of the rear-fanged mangrove cat snake (*Boiga dendrophila*), was the first prey-specific 3FTx identified and displayed potent postsynaptic neuromuscular activity by irreversibly inhibiting chick biventer cervicis nerve-muscle preparation twitches, but it induced much smaller and reversible inhibition of twitches in mouse hemidiaphragm nerve-muscle

preparations, suggestive of a bird-specific postsynaptic affinity (Pawlak et al. 2006). Irditoxin, a lizard- and avian-specific 3FTx from *B. irregularis*, was identified shortly after denmotoxin (Pawlak et al. 2009), and recently another prey-specific 3FTx, fulgimotoxin, was discovered in a New World rear-fanged snake, *Oxybelis fulgidus*, indicating that this phenomenon is not limited to Old World species and is likely more common in rear-fanged snake venoms (Heyborne and Mackessy 2013). Based on 1D SDS-PAGE and other data, 3FTxs are present in numerous venoms from rear-fanged snakes (Saviola et al. 2014).

More Recent Approaches

"First generation" venomics (e.g., Calvete et al. 2009) has been an exceptionally successful means to generate near-complete catalogs of venom proteins (Fig. 3), and this approach has also been applied to venoms of rear-fanged snakes. In recent years, the emergence of "omic" technologies has revolutionized venom research by integrating detailed high-throughput approaches to generate systematic venom studies involving whole genomes, transcriptomes, and proteomes (Calvete 2013). To date, a comprehensive approach, with (proteomics) MS/MS peptide sequencing of separated venom components (usually by RP-HPLC or 2D gel electrophoresis) combined with a species-specific venom gland transcriptome, has provided the most complete venom compositional coverage (Wagstaff et al. 2009; McGivern et al. 2014; Paiva et al. 2014; Goncalves-Machado et al. 2015). The change from 454 pyrosequencing to Illumina sequencing technology has also offered greater transcriptome coverage and depth (Rokyta et al. 2011; McGivern et al. 2014). MS/MS identification of peptide sequences relying on online protein sequence databases, such as the Mascot online server, can overlook unique isoform variations and can be unsuccessful at recognizing novel venom proteins if only small peptide fragments are used for protein identification. By generating a complementary transcriptome, MS/MS peptide sequences can be more precisely identified to the corresponding transcript, and translated transcripts will provide full protein sequences. Obtaining full sequences using only proteomic methodologies (such as N-terminal sequencing and MS/MS de novo sequence determinations from many peptide fragments) would otherwise be much more labor intensive and expensive.

Approaches to venom characterization have largely focused on mass spectrometry to generate complete venom profiles. The two primary MS methods for whole proteins include matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS and electrospray ionization MS (Kukhtina et al. 2000). These methods are frequently used to provide more accurate molecular masses for individual venom components and peptide fragments, and both allow for high-throughput analysis of complex samples. Mass spectrometric de novo sequence determination is especially of interest for protein sequences that have a blocked N-terminus, making it more difficult to determine the amino acid sequence from Edman degradation. Rear-fanged snake venom 3FTxs commonly have an N terminal

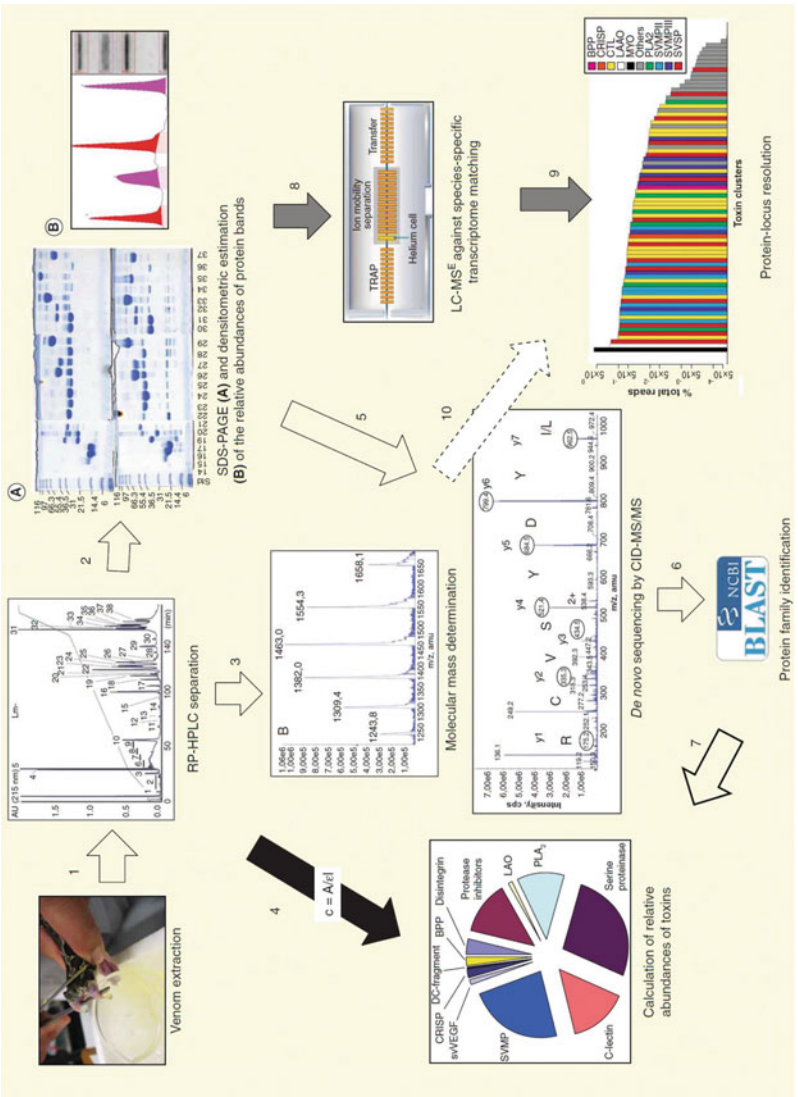

Fig. 3 An example of a venomics analysis of viper venom. Venom is fractionated using RP-HPLC and 1D SDS-PAGE; bands are digested and subjected to MS analysis (Reproduced from Calvete (2014))

pyroglutamate which must be removed prior to Edman sequencing (e.g., Pawlak et al. 2009; Heyborne and Mackessy 2013).

Top-down and bottom-up approaches are seen in proteomic literature regarding rear-fanged venomous snakes, where a top-down approach is done with intact venom proteins and a bottom-up approach is accomplished using proteolytic peptide mixtures. A top-down MALDI-TOF MS method using rear-fanged snake venom has revealed as many as 49 distinct protein masses (Peichoto et al. 2012). Top-down strategies allow for more complete characterization of protein isoforms and post-translational modifications (Han et al. 2008; Petras et al. 2015). Post-translational modifications found in rear-fanged snake venom proteins have yet to be studied in detail, and many opportunities exist for continued work using top-down MS methods.

A bottom-up approach, such as tandem MS performed on proteins digested with proteases such as trypsin (most commonly used), chymotrypsin, or Glu-C, generates a spectrum of fragmented singly charged peptide ions that can be matched to databases for protein identification (peptide mass fingerprinting) or can be used for de novo sequence determination (i.e., Chapeaurouge et al. 2015). Collision-induced dissociation (CID) is the most widely used MS/MS technique for this type of venom analysis. This technique creates a series of backbone fragmentations at the peptide bond, resulting in b- and y-fragment ions. MASCOT, SEQUEST, or other databases are searched using algorithmic comparisons of proteins derived from genomic sequencing or known protein amino acid sequences to identify unknown proteins based on their peptide fragment spectra.

However, post-translational modifications of venom proteins are not detectable when examining the venom genome or transcriptome, and discrepancies between the proteome and the transcriptome of a single species have been noted (Pahari et al. 2007; Sunagar et al. 2014). Translation blockages (Wang et al. 2010) are also not detectable based on transcriptome data alone, and therefore genomic and transcriptomic data do not fully represent the compounds that may constitute a species' venom. In addition, the increased sensitivity of transcriptomics results in all venom gland mRNA (messenger ribonucleic acid) being sequenced, and therefore it can be difficult to discern which transcript sequences are translated and secreted as venom proteins and which are simply endogenous cellular proteins. Adopting a combination of "omic" approaches allows for a species-specific transcriptome database of all potential venom protein components in a venom to be matched with MS/MS-generated peptide fragments from the crude venom proteome. Proteomics can also be used to check the accuracy of transcriptome assembly and translation. If peptide MS/MS sequences do not match a single species-specific transcriptome, it could be suggestive of erroneous contig (contiguous sequence from overlapping DNA reads representing a transcript) assembly, sequencing errors that resulted in a reading frame shift, or incorrect reading frame selection (Calvete 2014). Therefore, a combination of genomic, transcriptomic, and proteomic data is necessary to fully understand venom composition and evolution.

Currently, several rear-fanged genera, including *Boiga*, *Hypsiglena,* and *Philodryas*, are the best characterized venoms using a combination of proteomic

(general venom description and biochemical activity) and transcriptomic data. For *P. baroni, P. olfersii,* and *P. patagoniensis,* it has been shown that the majority of venom proteolytic activity is from SVMPs, with low levels of activity toward substrates for serine proteinases (Sanchez et al. 2014). The venom of *Boiga irregularis* was found to be dominated by 3FTxs (Fig. 4a), while that of *Hypsiglena* was dominated by SVMPs (Fig. 4b); the venom of *Boiga* appeared more "elapid like," while the venom of *Hypsiglena* was more "viper like" (McGivern et al. 2014). A combination of proteomics and transcriptomics is ideal for rear-fanged venomous snakes, particularly considering that these venoms are not well characterized

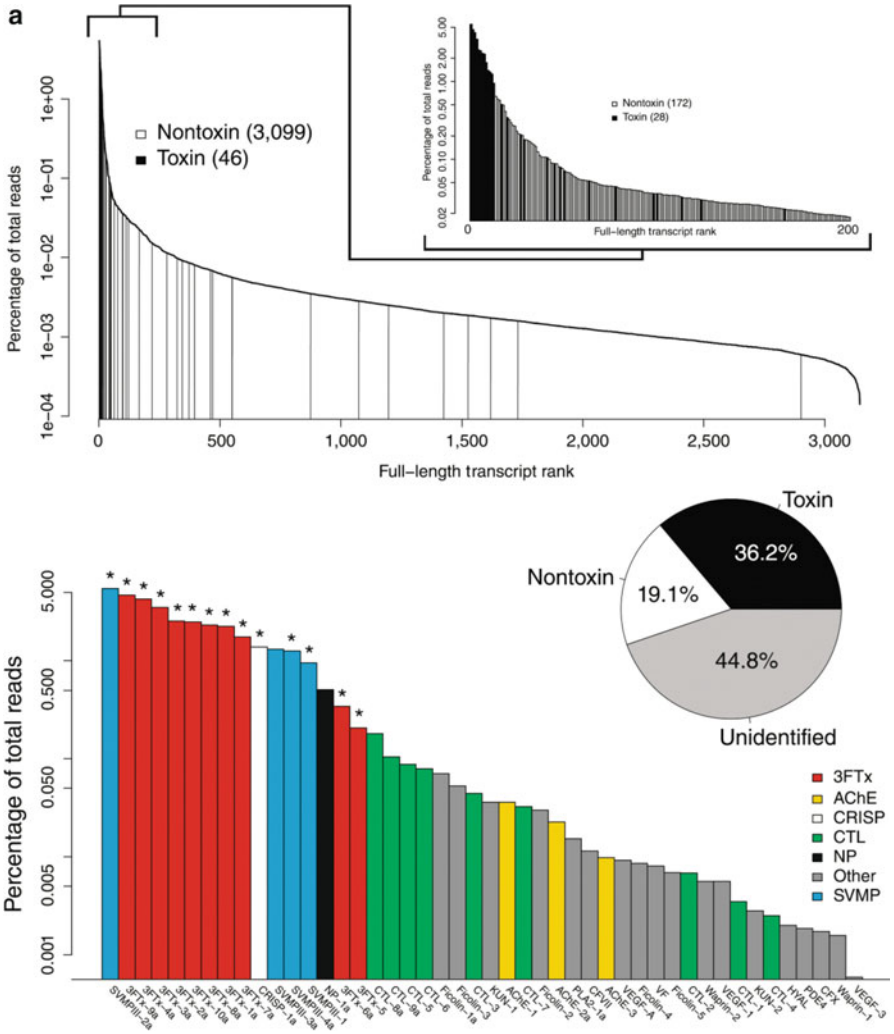

Fig. 4 (continued)

and have been observed to have novel protein superfamilies that would be missed using MS/MS peptide matching techniques alone (due to the lack of rear-fanged snake venom protein sequences in current databases). Genomic and transcriptomic data is becoming more readily available for venomous snakes (Rokyta et al. 2011, 2013; Vonk et al. 2013) and is enhancing the understanding of evolutionary relationships between venom compounds, and the snakes which produce them. As these databases grow, they will allow for the investigation of the multiple

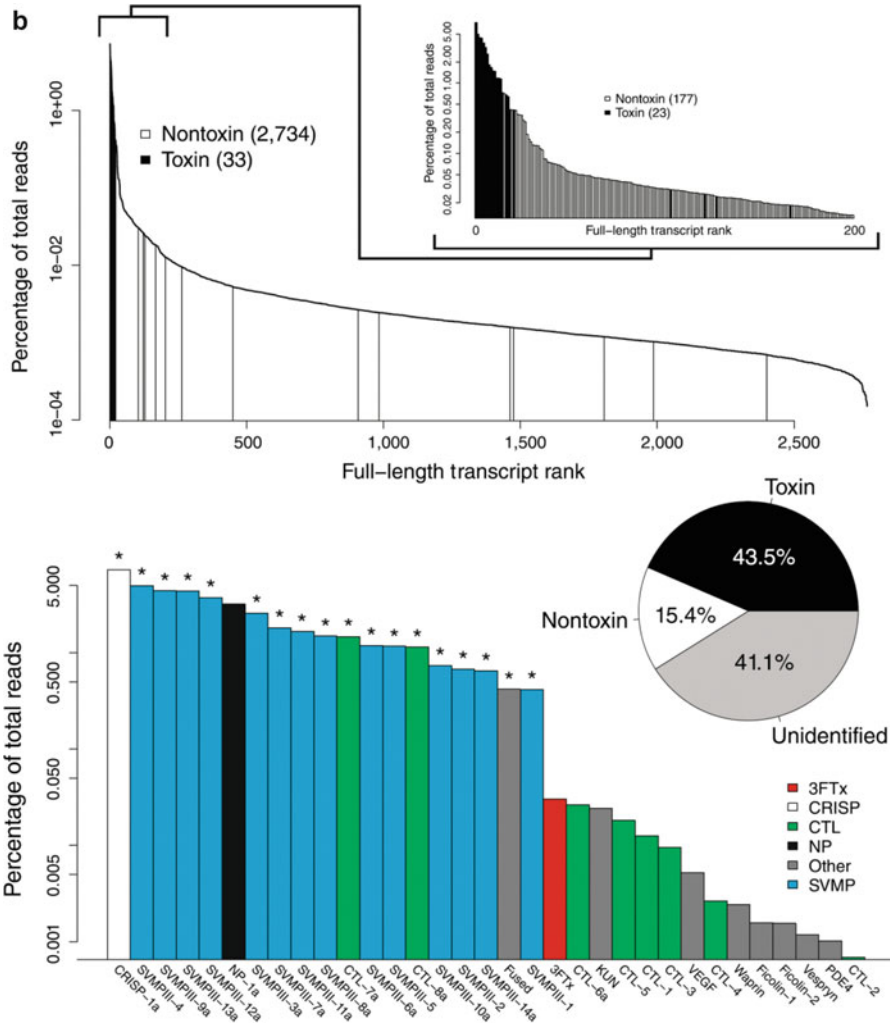

Fig. 4 Transcriptome of the venom gland of the brown tree snake (**a**) and of the desert night snake (**b**). The tree snake venom is rich in 3FTx transcripts, while the night snake transcriptome is dominated by (P-III) SVMP transcripts (Reproduced from McGivern et al. (2014))

levels of transcriptional and translational regulation of venom proteins that give rise to the variation that is seen in venom composition between species and even within individuals.

Venom gland transcriptome analyses are powerful for determining venom transcript expression, but genomic sequences can provide insight into venom gene transcriptional regulation (i.e., promoter sequences) and mechanisms resulting in venom protein diversity (i.e., alternative splicing events and/or gene dosage effects). A combined genomic and transcriptomic approach will also allow for splicing variations to be identified. Splicing variations allow for different functional proteins to be transcribed using the same exons, and this may help lead to binding to different receptors. New exons may also be inserted into the gene. Three-finger toxins in rear-fanged snakes have been found to have extended N-terminal segments compared to elapid and viperid 3FTxs, and for denmotoxin, this is the result of a newly inserted exon two. Currently, the function of this longer N-terminal region is unclear, but this is currently the only full 3FTx gene sequence for a rear-fanged snake and provides insight into additional mechanisms of evolution of these toxins (Pawlak and Kini 2008).

High-Throughput Proteomic Approaches to the Study of Snake Venoms

Protein chemistry methodologies have been utilized to examine snake venoms very early in the history of modern venom biochemical research. However, recent advances in proteomic techniques and the utilization of mass spectrometry have greatly expanded the understanding of venom composition, allowing for the field of venom proteomics (venomics) to flourish. The term "snake venomics" (see Fig. 3) was developed by Calvete and coworkers and has been important as a standardized venom characterization protocol and a semiquantitative estimation of venom protein relative abundances (see Calvete 2013 for a review). By using absorbance at 215 nm during the primary RP-HPLC separation (which roughly correlates with the abundance of peptide bonds), a percentage value can be assigned to each chromatographic peak, and in combination with a densitometric lane scan of SDS-PAGE run using individual chromatographic peaks, the relative percentage of different venom components that make up the overall crude venom can be determined (Calvete et al. 2009). Standard venomic protocols involve chromatographic (usually RP-HPLC) and electrophoretic techniques to separate crude venom proteins which are then digested into peptides with proteolytic enzymes (most commonly trypsin). Individual peptide ions can also be fragmented by collision-induced dissociation, with the resulting daughter fragment ions identified by manual inspection. This approach identifies venom proteins based upon multiple lines of evidence, including molecular mass, peptide mass matching, several peptide sequence identifications, and determination of the number of cysteine residues present. These data typically allow for the identification of most toxin classes found in snake venoms (Calvete 2014). These methodologies, incorporated with

biochemical and toxicological data, have allowed for a detailed examination of intraspecific, geographic, and ontogenetic venom variability primarily aimed at addressing the venom composition of dangerously toxic snakes of the families Elapidae and Viperidae (Calvete 2014; Calvete et al. 2009, 2012). Venomics also allows for identification of venom compounds that may be further examined for potential therapeutic value. Although the vast majority of venomic studies have included species that are of great medical significance, rear-fanged venomic studies are increasing and providing information on venom composition of these poorly known snakes.

Currently, only a few complete rear-fanged venomous snake proteomes are available, and most commonly, a bottom-up strategy is seen. There are primarily two bottom-up proteomic workflows. There is a "sort-then-break" approach, which includes performing protein fractionation and separation prior to protein digestion, followed by peptide analysis by peptide mass fingerprinting or de novo peptide sequence determination (Han et al. 2008). This workflow is seen in the venomic approach to venom profiling as mentioned above and was utilized with the venom of the rear-fanged snake *Thamnodynastes strigatus*. 2D gel electrophoresis was the method of separation before in-gel trypsin digestions and identification of individual protein spots using a MALDI Q-TOF (matrix-assisted laser desorption ionization quadrupole time-of-flight) Premier mass spectrometer (Ching et al. 2012). Also, several protein SDS-PAGE bands from the venoms of *Trimorphodon biscutatus lambda, Philodryas olfersii, Philodryas patagoniensis, Philodryas baroni,* and *Hypsiglena torquata texana* were also digested with trypsin and analyzed with MALDI-TOF/TOF (tandem matrix-assisted laser desorption ionization time of flight) to confirm the presence of PLA_2s, CRiSPs, and 3FTxs within some of these venoms (Peichoto et al. 2012).

An alternative is the "break-then-sort" approach, where protein digestion is performed without any prefractionation/separation and peptides are separated by multidimensional chromatography followed by tandem MS analysis (Han et al. 2008). This technique is referred to as "shotgun proteomics." Both methods are heavily reliant on the high-throughput advances in mass spectrometry, allowing for the identification of multiple peptide fragments to assemble an overall complete venom profile.

"Shotgun" methods involve the production of small sequence fragments of a greater whole that are identified and then assembled into a larger picture. In the case of shotgun venom proteomics, overall venom composition is determined from the identity of the fragmented peptide ions after a whole venom protein digestion. This approach was used to resolve the proteome of the rear-fanged dog-faced water snake (*Cerberus rynchops*) and resulted in the identification of a novel snake venom protein family (veficolins) that is speculated to induce platelet aggregation and/or initiate complement activation (OmPraba et al. 2010). Shotgun proteomics offers an alternative to venomics and can be particularly useful for rear-fanged snake venoms because they are typically much less complex than Elapidae or Viperidae venom proteomes. A shotgun technique can also be used on individual venom proteins; once a venom protein is purified, it can be digested with several different proteases,

and the resulting peptide fragments from different digestion libraries can be assembled to resolve the complete amino acid sequence (Bandeira et al. 2007).

Shotgun proteomics and venomics both offer insight into the complete composition of rear-fanged snake venoms and are high-throughput and sensitive techniques that can be done using relatively little starting material. These methods can also allow for rapid de novo elucidation of primary structure (amino acid sequence and post-translational modifications) of single peptides in a complex mixture or peptides derived by in-solution or in-gel proteolysis of larger proteins. Relative abundances of venom protein families can be estimated using these techniques; however, it is more difficult to determine relative abundances with shotgun venom proteomics. Shotgun proteomics results can be strongly biased, with portions of abundant proteins being overrepresented in many spectra and low-abundance protein spectra not being seen at all (Bandeira et al. 2007; Calvete 2014). The increased complexity of the generated peptide mixture requires highly sensitive and efficient separation. On the other hand, with venomics, it is also possible that during RP-HPLC separation, before electrophoresis and MS/MS sequencing, highly hydrophobic and/or large proteins may elute poorly and be absent or underrepresented in abundance (based upon chromatographic peaks and electrophoretic results).

Both of the above methods rely on the identification of peptide masses/ionization patterns. Peptide mass fingerprinting and de novo MS/MS sequence determination methods are high throughput, less labor intensive, and more cost effective than N-terminal sequencing, but a limitation to the reliance on peptide masses is that certain combinations of amino acids can have indistinguishable masses, therefore creating ambiguity. An example of this is the assignment of isobaric (Ile/Leu) or quasi-isobaric residues (Lys/Gln or Phe/Met-ox), although methods such as high-resolution Fourier transform ion cyclotron resonance or Orbitrap mass analyzers can be used to discriminate between quasi-isobaric residues (Calvete 2013). It can also be difficult to identify correctly all peptide sequences with peptide mass fingerprinting, especially those with unexpected modifications or from proteins that are absent from databases. Identification based on shared peptide sequences in databases often does not allow differentiation between isoforms, and snake venoms can have multiple different isoforms present, each with potentially different pharmacological activities (Calvete 2014; Kini and Doley 2010; Mackessy 2010). Complete amino acid sequences for large, unknown proteins from bottom-up methods is also not possible due to the incomplete recovery of a full tryptic peptide set. Although tryptic digestion followed by LC-CID-MS/MS (liquid chromatography collision-induced dissociation tandem mass spectrometry) is ideal for the identification of toxin classes, it does not provide information about the quaternary structure of individual toxins or toxin activities. After individual venom proteins are cleaved into peptide fragments, they can no longer be used for follow-up biochemical or pharmacological assays. In recent years, venomics and shotgun venom proteomics have become highly sensitive techniques to provide information obtaining to overall venom composition for a snake species, but biochemical and pharmacological assays are needed for complete venom protein characterization.

High-Throughput Transcriptomic and Genomic Approaches to the Study of Snake Venoms

The majority of colubrid venom studies have focused on the protein composition and enzymatic properties of these venoms, with relatively few published venom gland transcriptomes or venom protein transcripts. Although venom compositional and biochemical studies can help to infer clinical symptoms of envenomation and the biological roles of these proteins, venom protein transcripts can also be used to derive venom composition and predict protein activity. Transcriptomic studies can provide a starting point for proteomic methods when crude venom material is lacking or of low venom yield, commonly an issue with rear-fanged venomous snakes. A venom protein transcript can be translated to acquire an entire protein amino acid sequence, and this allows for identification of protein superfamilies and functional protein domains. Obtaining toxin transcripts can also assist in the assembly and completion of protein sequences where trypsin digests or N-terminal sequencing provides only partial sequence. Transcripts also provide information about the evolutionary history of venom protein superfamilies and can be used for the reconstruction of ancestral sequences. One can then explore questions such as the origin of venom and the mechanisms responsible for venom evolution and adaptation (Casewell et al. 2012, 2013). Transcript sequences are needed for positive selection analysis within protein superfamilies, because to establish protein amino acid sites under positive selection, protein transcripts must be used to determine where single-nucleotide polymorphisms are occurring and are resulting in nonsynonymous mutations (Sunagar et al. 2013).

Transcriptomic and genomic methods offer many exciting opportunities for future studies, and the cost of next-generation DNA sequencing is becoming more affordable. With rear-fanged snakes comprising several Colubroidea families and subfamilies, many novel venom transcripts likely exist and remain unexplored. Colubrid transcriptomes and genomes offer the opportunity to identify novel venom protein families and scaffolds and provide insight into the evolutionary histories of ubiquitous venom protein families (Ching et al. 2006; Fry et al. 2012; OmPraba et al. 2010).

Currently, complete venom gland transcriptomes have only been published for *Philodryas olfersii* (Ching et al. 2006), *Cerberus rynchops* (OmPraba et al. 2010), *Thamnodynastes strigatus* (Ching et al. 2012), and *Boiga irregularis* and *Hypsiglena* sp. (McGivern et al. 2014), and some venom transcript sequences from *Dispholidus typus, Telescopus dhara, Trimorphodon biscutatus, Liophis miliaris, Liophis poecilogyrus, Leioheterodon madagascarensis, Psammophis mossambicus,* and *Rhabdophis tigrinus* are also available (Fry et al. 2012). The venom gland transcriptome from *Cerberus rynchops* revealed a novel venom protein family, ryncolin, that was the first discovered venom protein to exhibit sequence similarity to ficolin (a mammalian protein with collagen-like and fibrinogen-like domains) (OmPraba et al. 2010). The venom gland transcriptome from *Thamnodynastes strigatus* was found to be largely composed of matrix metalloproteinases, unrelated to the metalloproteinases found in other Colubroidea

snake families (Ching et al. 2012). This was the first time that matrix metalloproteinases were identified as a prominent venom component and were discovered to make up the majority of *T. strigatus* venom (both in abundance of transcripts and proteins) (Ching et al. 2012). A combined RNA-seq (ribonucleic acid sequencing, completed with the generation of complementary deoxyribonucleic acid libraries [cDNA]) and mass spectrometry analysis of venom glands and venoms from two species indicated that there are very different venom compositional "strategies" present among rear-fanged snakes, reminiscent of differences seen between elapid and viperid species (McGivern et al. 2014). Other protein families identified in an analysis of several rear-fanged snake venom glands include lipocalin, phospholipase A_2 (type IIE), vitelline membrane outer layer protein, and ribonucleases (Fry et al. 2012). Besides identifying novel venom protein families, rear-fanged snake venom gland transcriptomes have provided sequences that have helped resolved venom protein evolutionary histories, such as the evolution of C-type natriuretic peptides throughout Colubroidae (Ching et al. 2006).

The number of rear-fanged snake venom transcripts and genes will certainly increase as sequencing technologies have become more available and affordable. Next-generation sequencing (NGS) allows for multiple venom gland transcriptomes to be sequenced in parallel and removes the need for tedious *E. coli* cloning procedures (Durban et al. 2011). The majority of current rear-fanged snake venom gland transcriptomes have been constructed by first selecting mRNA from gland tissue (usually from a gland removed 3–4 days after venom extraction), generating cDNA libraries by reverse transcription, cloning these sequences with the use of plasmid vectors and transformed *E. coli*, and then randomly picking clones to be sequenced with chain-terminating Sanger sequencing technology. This methodology can introduce bias into a study since smaller cDNA fragments have higher transformation efficiency, or transcripts could be partially expressed in *E. coli* with lethal effects (Durban et al. 2011). There is also the chance of missing transcripts that occur in low abundance if not enough colonies are selected. NGS techniques remove these biases and create larger sequence assemblies.

Given that there is still extensive proteomic work to be done to determine protein families that compose rear-fanged snake venoms, care must be taken when identifying "venom protein" transcripts from a rear-fanged snake venom gland transcriptome without proteomic evidence. It is probable that some transcripts for endogenous cellular proteins that are not secreted from the gland, and do not serve as functional venom components, will be encountered. Several published rear-fanged snake transcriptomes have provided proteomic evidence to support the translation and secretion of identified venom protein transcripts with the use of two-dimensional gel electrophoresis and/or HPLC separation and mass spectrometric analysis (Ching et al. 2006, 2012; McGivern et al. 2014; OmPraba et al. 2010), and until there is a better understanding of the venom protein families occurring in rear-fanged snake venoms, this approach should be a standard practice.

Ancestral venom proteins had diverse activities and performed physiological roles in a variety of tissues (Fry 2005). Recent analyses have determined that toxin

homologues are expressed in other tissues, suggestive that these "toxins" are either coexpressed in many tissues or are "reverse recruited" from the venom gland for other physiological roles in other tissues (Casewell et al. 2012; Reyes-Velasco et al. 2015; Hargreaves et al. 2014). It has been suggested that these genes are coexpressed in a variety of tissues and then following gene duplications are restricted to expression in the venom gland after transcriptional regulation changes within other tissues. Multiple tissue transcriptomes are critical in order to understand the evolutionary history of venom gene superfamilies and the events following venom gene duplication, subfunctionalization, and neofunctionalization within these gene families, as well as how these processes influence venom protein adaptability. Future tissue transcriptomes will also help to provide insight into the origin and evolution of venom proteins, because nontoxin homologues are needed to construct venom gene trees (Casewell et al. 2012). There is a need to look at more tissues to explore differential expression of venom gene homologues in other tissues and the possibility of "reverse recruitment"; current studies are limited in that only a handful of venom gene families, tissues, and snake species have been analyzed (Casewell et al. 2012; Hargreaves et al. 2014; Reyes-Velasco et al. 2015; Junqueira-de-Azevedo et al. 2015). Venom evolution is a very complex and dynamic system, and research in this area has applications for studies involving other proteins that experience accelerated evolution and novel functionality gain.

It is still largely unknown what mechanisms are responsible for the regulation of venom genes. Promoter regions, transcription factors, methylation, as well as other mechanisms of gene regulation remain largely unexplored in venomous snake genomes. Techniques such as ChIP-Seq (chromatin immunoprecipitation sequencing) and RIP-seq (RNA immunoprecipitation sequencing) are possible future approaches to determine the regulatory proteins binding to DNA and RNA involved in the transcription and translation of venom genes and transcripts. Multiple tissue transcriptomes provide the ability to observe these differential expression patterns.

Changes to DNA sequence can directly affect gene products and influence the evolution of a protein. Although protein sequences can provide information regarding potential structure and function, the coding gene sequence (CDS) can reveal hidden single-nucleotide polymorphisms (SNPs) or elucidate molecular evolutionary patterns. Venom gene nucleotide polymorphisms can have significant impacts on venom proteins; a dinucleotide deletion in a 3FTx gene resulted in the loss of neurotoxic activity in the marbled sea snake (*Aipysurus eydouxii*) (Li et al. 2005).

Venom genes experience increased nucleotide substitution rates, especially within exon regions, as compared to other protein-coding genes (Doley et al. 2009). Venom protein genes have the flexibility to accumulate mutations at an increased rate due to the presence of multiple gene copies resulting from gene duplications and subfunctionalization. If one sequence develops a detrimental mutation, other copies of the gene remain present and functional. This mechanism also allows for venom gene neofunctionalization. Venom multigene families have been identified as evolving by this "birth-and-death" gene model (Fry et al. 2003b; Vonk et al. 2013). Venom proteins typically possess a stable structural core maintained by multiple disulfide bonds, and nonsynonymous nucleotide

substitutions that alter nonstructural, surface-exposed residues can change protein-targeting interactions, such as targeting new receptor types (Doley et al. 2009). This allows for a venom protein family to develop multiple activities, and having multiple gene products provides a selective advantage over the optimization of a single gene product by allowing for evolutionary "experimentation."

There have been several mechanisms proposed to explain accelerated gene neofunctionalization rates within venom gene superfamilies, including accelerated segment switching in exons to alter targeting (Doley et al. 2009) and rapid accumulations of point mutations in exposed residues (Sunagar et al. 2013). Transposable elements have also been shown to produce protein diversity. The python genome was found to have a large abundance of retroelements in comparison to its size (Castoe et al. 2013), especially LINEs (long interspersed elements). LINEs have been associated with creating protein diversity by carrying along genetic material from transposition events and therefore resulting in additional exon segments. Transposable elements can contribute to nonhomologous recombination, one of the mechanisms responsible for generating gene duplications.

Recently, the first snake genomes, the Burmese python (*Python molurus bivittatus*) and king cobra (*Ophiophagus hannah*), were completed (Castoe et al. 2013; Vonk et al. 2013). These snake genomes provide genomic scaffolds that will facilitate assemblies and annotation for other snake genomes. Genomic sequencing also provides potential full venom protein sequences. Databases with genomic sequences can be translated into all possible reading frames and matched to resulting peptide fragments obtained by tandem mass spectrometry. Many proteomic techniques will therefore be aided by the addition of complete snake genomes and in combination with venom gland transcriptomes, these genomes will provide overall insight into venom protein expression and evolution. A complete genome of a rear-fanged venomous snake is currently unavailable, but once finished, this genomic information will be accessible to compare venom multigene families within different snake families. For example, in the case of the rear-fanged snake *Boiga dendrophila*, the full gene sequence of the venom 3FTx denmotoxin was found to exhibit unique gene organization compared to 3FTx gene sequences found in Elapidae. Three-finger toxins are usually composed of three exons and two introns; however, denmotoxin was found to have an additional exon. Exon shuffling is a general mechanism for the creation of new genes (Pawlak and Kini 2008), and in the case of SVMPs, loss of exon segments has contributed to the evolution of the P-II and P-I classes.

Conclusion and Future Directions

The Promise of Venomics, Transcriptomics, and Genomics

With the advancements in venom extraction methods for rear-fanged venomous snakes and in proteomic and molecular techniques, it is now possible to complete full analyses on rear-fanged snake venoms. This work will not only allow us to

identify compounds that constitute an entire venom for a species but will also provide a detailed description of venom composition of rear-fanged snakes that may be of medical significance with regard to snakebite. Further, rear-fanged venomics may provide insights into the utilization of these proteins for therapeutic drug development.

Proteomics, especially the high-throughput venomic and shotgun proteomic methods, have increased the sensitivity and speed at which a whole venom can be characterized and the abundances of individual venom protein families determined. Although the vast majority of rear-fanged snakes may be considered as nonthreatening to humans, proteomics allows identification of venom protein families which can further assist with examining the evolutionary relationships among venomous snakes and their toxins. For example, proteomic screening of the venom of *Thamnodynases strigatus* indicated that the most abundant protein family consisted of a new kind of matrix metalloproteinase unrelated to the traditional SVMPs documented in all families of venomous snakes (Ching et al. 2012). This same study further identified the presence of a lactadherin-like factor V/VIII C-terminal domain as a part of the proteome, in addition to well-known venom protein families such as SVMPs, C-type lectins, and CRiSPs. Rear-fanged snake venoms contain many of the same venom protein families that are found in medically significant venomous snakes, such as Viperidae and Elapidae, and as more transcript sequences are acquired and matched to corresponding purified proteins, in combination with biochemical and pharmacological assays, recurring venom protein domains can begin to be better defined and associated more directly with specific activities and molecular mechanisms of action (Fig. 5).

Transcriptomics in combination with proteomics offers the ability to specifically identify the abundance of each venom protein, even between isoforms, from MS/MS peptide spectra and provides greater sequence coverage than what can be accomplished using only de novo sequence determination methods. Comparisons between proteomes and transcriptomes also provide insight into the translational regulation of venom proteins. This "omic" approach was used to discover that microRNAs could potentially explain ontogenetic translational regulation. MicroRNAs produced in rear-fanged venom glands remain unexplored, though microRNAs have begun to be examined in Elapidae and Viperidae (Durban et al. 2013; Vonk et al. 2013). Transcriptomes assembled from a variety of snake body tissues as well as venom gland tissue of rear-fanged snake species will provide venom protein and nonvenom homologue transcript sequences. These sequences can then be used to construct gene trees in order to reveal complete venom protein evolutionary histories and identify amino acid sites under positive selection.

Genomics in combination with transcriptomics allows for the exploration of transcriptional regulation seen within venom protein families and the unique evolution of venom protein genes. Genomics has revealed the dynamic evolution and adaptation of the venom system, such as the massive and rapid expansion of venom gene families that correlates with their functional importance in prey capture (Vonk et al. 2013). The expansion of venom protein gene families may occur in response to an evolutionary arms race between venomous snakes and their prey.

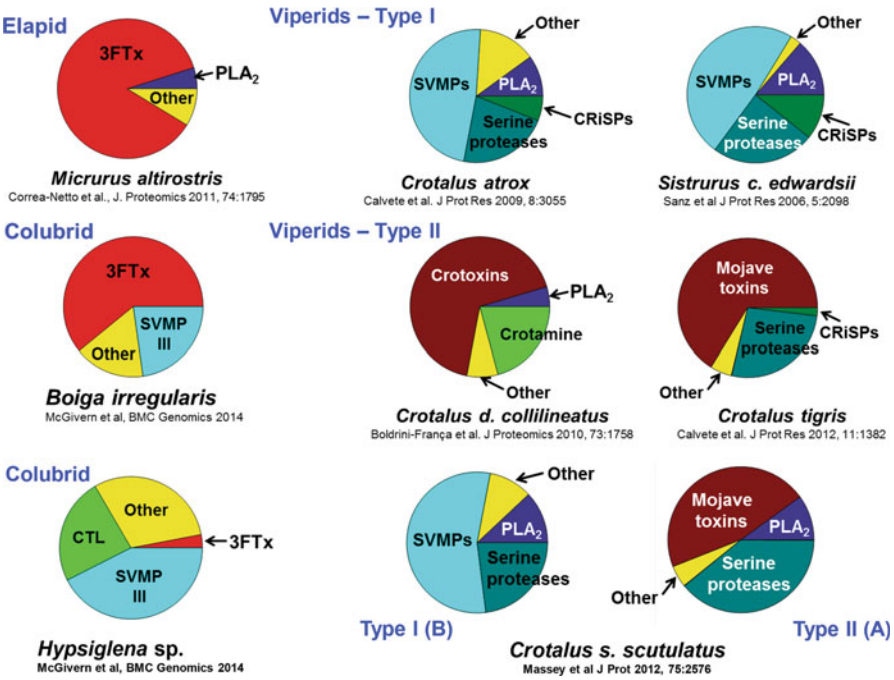

Fig. 5 Comparative proteomes and transcriptomes of elapid, colubrid, and viperid snakes. Note that potent toxins (3FTxs, crotoxins, Mojave toxins) dominate the venom profiles of the elapid, one colubrid (*Boiga*), and type II viperid venoms, while SVMPs dominate in venoms from the other colubrid (*Hypsiglena*) and type I viperid venoms. Pie charts were based on data in respective papers cited

Venom gene sequences are therefore ideal for studying accelerated patterns of evolution and the association between genotype and adaptive phenotypes.

The combination of proteomics, transcriptomics, and genomics in the study of rear-fanged snake venoms can provide a holistic approach to understanding venom protein evolution and regulation, which in turn impacts overall crude venom composition. However, it is also important to view the evolution and expression of rear-fanged venom proteins in terms of biological significance. Venom is a trophic adaptation, and as such, the presence and abundance of these proteins relates directly to prey capture and organism natural history. Without this biological perspective, proteomic, transcriptomic, and genomic approaches could simply generate a list of proteins, peptides, transcripts, and genes.

Forgotten Aspects of Understanding Venom Evolution

Snake venom prey-specific toxins provide an ideal model to study genotype-phenotype fitness interactions because the function of phenotypic variation (venom composition) can be related to the nature of the adaptation (prey preference

and susceptibility). At the molecular level, there is the evolution of protein catalytic/ligand-binding sites and targeting. At the organismal level, there are selection pressures brought on by prey availability, preference, and susceptibility to specific toxin effects. Therefore, the biological roles of venom proteins should be incorporated into high-throughput proteomic, transcriptomic, and genomic results aimed at understanding venom evolution.

Rear-fanged venomous snakes encompass several families and subfamilies of the Colubroidea, and collectively they include the largest number and diversity of venomous snakes. To explore the biological roles of rear-fanged snake venoms, or individual toxins within these venoms, it is important to use adequate toxicity models for assays that match the biology of the snake being studied. Understanding the diversity of venom components and their differential effects toward specific prey will facilitate a greater understanding of the selective mechanisms driving snake venom evolution and adaptation (Mackessy et al. 2006; O'Donnell et al. 2007). Future studies should take into account the interactions between the snake's venom and its natural prey, since toxicity is best defined within the context that it is being used. There is a need for inbred nonmammalian vertebrate species to be used as models for LD_{50} assays, as well as for viable nonvertebrate models of whole organism toxicity. Such models would be ideal for toxinologists interested in receptor-ligand evolution and positive selection of venom proteins involved in coevolutionary predator/prey arms races (Mackessy 2002). Some of these toxins may prove useful for understanding diversification and evolution of important ion channels, such as the nicotinic acetylcholine receptor; based on selective toxicity of *B. irregularis* venom and irditoxin, there appears to have been some form of coevolutionary adjustment between predator armaments (venom) and prey susceptibilities (receptor binding) which imparts particular effectiveness against specific prey types (Mackessy et al. 2006; Pawlak et al. 2009). The differential activities of these and other toxins could be exploited for the development as molecular tools for dissecting receptor-ligand binding interactions, and they may provide clues for their exploitation as therapeutics.

Potential for Drug Development from Venom Proteins and Peptides

A number of toxins have proved to be excellent research tools to decipher the molecular details of physiological processes, and several have led to the development of novel therapeutic agents (Lewis 2009; Takacs and Nathan 2014). Captopril, the first successful venom-based drug, was developed from bradykinin-potentiating peptides from the venom of the lancehead viper (*Bothrops jararaca*) and is still on the market as an antihypertensive drug. Other venom-based drugs include tirofiban (aggrastat) and integrilin (eptifabatide) that were both designed from the structure of snake venom disintegrins (Saviola et al. 2014; Vonk et al. 2011; Calvete et al. 2010). There are many rear-fanged venomous snake species that have venoms yet to be studied, providing an untapped source of proteins with novel activities for therapeutic development (Saviola et al. 2014).

Recently, five venoms from rear-fanged snake species were evaluated for potential antileishmanial activity. Exposure to relatively high levels of these rear-fanged snake venoms resulted in cytotoxicity toward cultured promastigote states of *Leishmania major* and venom of one species, *T. b. lambda*, showed significant cytotoxicity even at lower doses (Peichoto et al. 2011). Because rear-fanged snake venoms contain many of the same venom protein families as front-fanged venomous snakes, and because structural motifs of venom proteins are conserved but possess activities and specificities that may be highly variable, exploration of rear-fanged snake venom proteins could uncover some highly useful compounds. Anticoagulants in rear-fanged snake venoms include SVMPs, serine proteases, and phospholipase A_2 enzymes (Saviola et al. 2014), and initial analyses indicate that at least some may show higher specificities than homologues from front-fanged snake venoms (Weldon and Mackessy 2012). These variants provide opportunities to decipher the subtleties in functional sites in order to understand the plasticity of venom protein structure and function. Venom proteins can serve as templates for biomedical engineering and provide insight into selective receptor binding (Kini and Doley 2010), as is exhibited by several 3FTxs from rear-fanged snake venoms. Without the selectivity of alpha-bungarotoxin, a 3FTx from venom of the Many-banded krait (*Bungarus multicinctus*), the knowledge of the distribution of nicotinic acetylcholine receptors and neurotransmitter communication wouldn't have advanced to its current state. Because venom proteins originated from ancestral proteins that served cellular physiological (housekeeping) roles and often have exceptionally high binding specificities, they are ideal candidates for unraveling cellular signaling pathways and the resulting disruption of these pathways. Rear-fanged snake venoms provide a largely unexplored source of toxins, and with recent advances in proteomic, transcriptomic, and genomic approaches, there should be an increase in future research focused on these venoms.

Cross-References

- ▶ Applications of Snake Toxins in Biomedicine
- ▶ Shotgun Approaches for Venom Analysis
- ▶ Squamate Reptile Genomics and Evolution

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