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Interspecific and intraspecific venom enzymatic variation among cobras (*Naja* sp. and *Ophiophagus hannah*)

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ABSTRACT

The genera *Ophiophagus* and *Naja* comprise part of a clade of snakes referred to as cobras, dangerously venomous front-fanged snakes in the family Elapidae responsible for significant human mortality and morbidity throughout Asia and Africa. We evaluated venom enzyme variation for eleven cobra species and three *N. kaouthia* populations using SDS-PAGE venom fingerprinting and numerous enzyme assays. Acetylcholinesterase and PLA_2 activities were the most variable between species, and PLA_2 activity was significantly different between Malaysian and Thailand *N. kaouthia* populations. Venom metalloproteinase activity was low and significantly different among most species, but levels were identical for *N. kaouthia* populations; minor variation in venom L-amino acid oxidase and phosphodiesterase activities were seen between cobra species. *Naja siamensis* venom lacked the α-fibrinogenolytic activity common to other cobra venoms. In addition, venom from *N. siamensis* had no detectable metalloproteinase activity and exhibited an SDS-PAGE profile with reduced abundance of higher mass proteins. Venom profiles from spitting cobras (*N. siamensis*, *N. pallida*, and *N. mossambica*) exhibited similar reductions in higher mass proteins, suggesting the evolution of venoms of reduced complexity and decreased enzymatic activity among spitting cobras. Generally, the venom proteomes of cobras show highly abundant three-finger toxin diversity, followed by large quantities of PLA_2s. However, PLA_2 bands and activity were very reduced for *N. haje, N. annulifera* and *N. nivea*. Venom compositionalenzy analysis provides insight into the evolution, diversification and distribution of different venom phenotypes that complements venomic data, and this information is critical for the development of effective antivenoms and snakebite treatment.

1. Introduction

Snake venom contains many active peptides and enzymes, with multiple isoforms from gene duplications contributing to a diversity of protein activities within a single venom (Casewell et al., 2011, 2014; Gibbs and Rossiter, 2008; Mackessy, 2010a; Vonk et al., 2013). Neutral evolution (Aird et al., 2017; Mebs, 2001; Sasa, 1999), positive selection (Aird et al., 2015; Rokyta et al., 2011) and purifying selection (Sunagar and Moran, 2015; Sunagar et al., 2014) have all been suggested to be main drivers of venom protein gene evolution. Although it is well accepted that the key role of venom is to immobilize and digest prey, the direct and indirect processes resulting in venom variation (Aird et al., 2017; Casewell et al., 2014; Durban et al., 2017; Mackessy, 1988; Margres et al., 2017; Rokyta et al., 2015a; Zancolli et al., 2019) and the evolutionary origin of snake venom (Fry, 2005; Hargreaves et al., 2014; Reyes-Velasco et al., 2015) are still debated.

Venom compositional variation has been observed on multiple interspecific and intraspecific levels (Chippaux et al., 1991; Mackessy, 2010a). The biological significance behind venom variation is being actively explored, and more data is needed documenting and verifying factors contributing to the distribution, diversity and the genetic regulatory processes resulting in this variation. Currently, phylogenetic signal (Lomonte et al., 2014; Mackessy, 2010a; Sanz et al., 2006; Smith and Mackessy, 2016), age (Alape-Giron et al., 2008; Mackessy, 1988; Mackessy et al., 2006; Modahl et al., 2016), geographic locality (Massey et al., 2012; Rokyta et al., 2015b; Sunagar et al., 2014; Tan et al., 2015b) and diet (da Silva and Aird, 2001; Daltry et al., 1996; Li et al., 2005; Mackessy, 1988; Pawlak et al., 2006, 2009) are all known contributing biological factors.

Cobra (*Naja*) species belong to the snake family Elapidae and are

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medically significant snakes in Asia and Africa (Alirol et al., 2010; Mukherjee and Maity, 2002; Muller et al., 2012; Warrell et al., 2013), regions where vulnerability to snakebite is greatest (Longbottom et al., 2018). Due to various venom protein activities, symptoms of envenomation by cobras include diverse sequelae such as neurotoxicity, cardiotoxicity, cytotoxicity and hemolysis, and cobra venom is known to be quite variable in composition (Mukherjee and Maity, 2002; Tan and Tan, 1988). Cobra envenomation can result in rapid human mortality largely because of the abundance of postsynaptic neurotoxins within these venoms. Neurotoxic three-finger toxins (3FTxs) lead to rapid neuromuscular paralysis and respiratory failure (Laustsen et al., 2015), and these toxins bind with very high affinities to receptors, commonly the nicotinic acetylcholine receptors of skeletal muscle. In addition to 3FTxs, which can have many different pharmacologies (Kini and Doley, 2010), phospholipases A_2 (PLA_2s) are also abundant within cobra venoms (Doley and Mukherjee, 2003; Mukherjee and Maity, 2002; Petras et al., 2011; Shan et al., 2016; Tasoulis and Isbister, 2017). Phospholipases A_2s and cytotoxic 3FTxs result in tissue necrosis and can lead to various permanent disabilities (Chwetzoff et al., 1989; Mendez et al., 2011). Other cobra venom components include snake venom metalloproteinases (SVMPs), cysteine-rich secretory proteins (CRiSPs), L-amino acid oxidases (LAAOs), phosphodiesterases (PDEs), acetylcholinesterases (AChEs), nucleases (NUCs), hyaluronidases (HYAs), nerve growth factors (NGFs), and cobra venom factors (CVFs) (Tan and Tan, 1988; Tasoulis and Isbister, 2017; Utkin and Osipov, 2007; Vonk et al., 2013).

Transcriptomic and proteomic data from different cobra species have revealed significant venom compositional variation (Chang et al., 2013; Huang et al., 2015; Namiranian and Hider, 1992; Shan et al., 2016; Tan et al., 2015a.b, Tan et al., 2017a, Tan et al., 2019a). There are documented differences in cytotoxicity between spitting and non-spitting cobra species, suggestive of alternative evolutionary trajectories for venoms that provide a larger defensive role (Panagides et al., 2017). Venom variation within cobras has led to inconsistencies in antivenom neutralization, and the efficacy of an antivenom, even against a single species, can be quite variable over different parts of its range (Ali et al., 2013; Mendez et al., 2011; Sintiprungrat et al., 2016; Tan et al., 2015b). These results, as well as the high number of cobra envenomations across a vast geographical area, highlight the immense importance of effective antivenoms and medical supportive therapy in cobra bites. A more complete understanding of the variation in venom composition of numerous cobra species can facilitate development of better antivenoms and treatment regimes.

The aim of this study is to document interspecific and intraspecific venom compositional variation in a variety of cobras native to Asia and Africa. Because previous studies have documented proteomic and lethal toxicity differences in many cobra species, we instead focused on biochemical differences between the venoms (i.e., details of the venom phenotypes). Cobra venom composition and enzymatic activity was characterized and compared for eleven different cobra species that are common in Southeast Asia and Africa, including both non-spitting and spitting cobra species. Enzyme activities among cobra venoms was evaluated previously (Tan and Tan, 1988), but since this time, extensive taxonomic revisions of the genus *Naja* have occurred (Wallach et al., 2009; Wüster, 1996), and the current study provides a re-evaluation of enzymatic activity, with an additional focus on metalloproteinase and fibrin(ogen)lytic activity trends within these venoms. In addition, we compare spitting and non-spitting cobra venoms and demonstrate that enzyme activities such as metalloproteinases are lower in spitting cobra venoms.

2. Material and methods

2.1. Reagents

Precast NuPAGE 12% Bis-Tris mini Gels, LDS sample buffer, MES running buffer and Novex Mark 12 unstained molecular mass standards were all obtained from Life Technologies, Inc. (Grand Island, NY, U.S.A). Dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), azocasein, 4-nitro-3-(octanoyloxy) benzoic acid, acetylthiocholine iodide, L-kynurenine, bis-*p*-nitrophenylphosphate, human fibrinogen, and all other buffers and reagents for enzyme assays (analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A).

2.2. Venoms and animals

Pooled venom was obtained from captive adult spitting and non-spitting cobra species at the Kentucky Reptile Zoo (Slade, KY, U.S.A). Cobras originating from Asia included *Naja kaouthia* from three different localities (Malaysia, Thailand, and the Suphan Buri province of Thailand, where a significantly different color morph occurs), *Ophiophagus hannah*, *Naja siamensis* (spitting species), *Naja atra,* and *Naja naja.* Cobras originating from Africa included *Naja haje, Naja melanoleuca, Naja pallida* (spitting species), *Naja mossambica* (spitting species), *Naja annulifera* and *Naja nivea* (Fig. 1). Venom from each species or known different locality were pooled, lyophilized and kept frozen at −20 °C until used. Reconstituted venom concentrations were determined with a BCA assay kit (Thermo Fisher Scientific Inc., Carlsbad, U.S.A).

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

SDS-PAGE was performed on NuPage 12% Bis-Tris mini gels (Life Technologies, Inc., U.S.A) under both reduced (with 50 mM DTT) and non-reduced (without DTT) conditions to observe the relative molecular masses of venom components within different species. All samples and buffers were prepared based on manufacturer conditions. Venom (20 μg) for each species or locality was loaded into a lane, and 5 μL of Novex Mark 12 unstained mass standards was used for mass estimations. For reference, known *N. kaouthia* three-finger toxins (α-cobratoxin and a cytotoxic 3FTx, purified previously; Modahl et al., 2016) were also run. Gel electrophoresis was carried out at 175 V and gels were sequentially stained (0.1% Coomassie Brilliant Blue R250 overnight), destained (50/40/10, *v*/v, ddH2O: methanol: glacial acetic acid) for two hours and then imaged.

2.4. Enzyme assays

2.4.1. Phospholipase A_2 (PLA_2) assay

The PLA_2 assay was performed based on the method published by Holzer and Mackessy (1996). Venom (50 μg) was added to a total volume of 550 μL of 10 mM Tris-HCL, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0 buffer. After mixing vigorously, tubes were placed on ice for 3 min, and 50 μL of substrate solution (4-nitro-3-(octanoyloxy) benzoic acid, 3 mM) was added. Tubes were then incubated at 37 °C for 20 min. After incubation, tubes were placed on ice and 50 μL of triton X-100 solution (2.5% in ddH_2O) was added. Tubes were then vortexed and placed at room temperature for 10 min. Absorbance was recorded at 425 nm with specific activity expressed as nmole product/min/mg protein. Assays were conducted in triplicate for each sample.

2.4.2. Acetylcholinesterase (AChE) assay

The AChE assay was performed according to Ellman et al. (1961). Venom (3 μg in 7 μL), 3 μL substrate (acetylcholine iodide, 75 mM), 15 μL DTNB (10 mM) and 450 μL 100 mM diNa phosphate pH 8.0 buffer were combined within a cuvette held at 37 °C, and mixed well by pipetting. Absorbance was continuously recorded at 412 nm every 10 s for 5 min, and specific activity was expressed as μmole product/min/mg of venom.

2.4.3. Snake venom metalloproteinase (SVMP) assay

This assay was performed according to the method published by Aird and da Silva (1991). Briefly, 80 μg of venom was incubated for 30 min at 37 °C with 1 mg of azocasein substrate in a total volume of 1 mL 50 mM HEPES, 100 mM NaCl, pH 8.0 buffer. The solution was

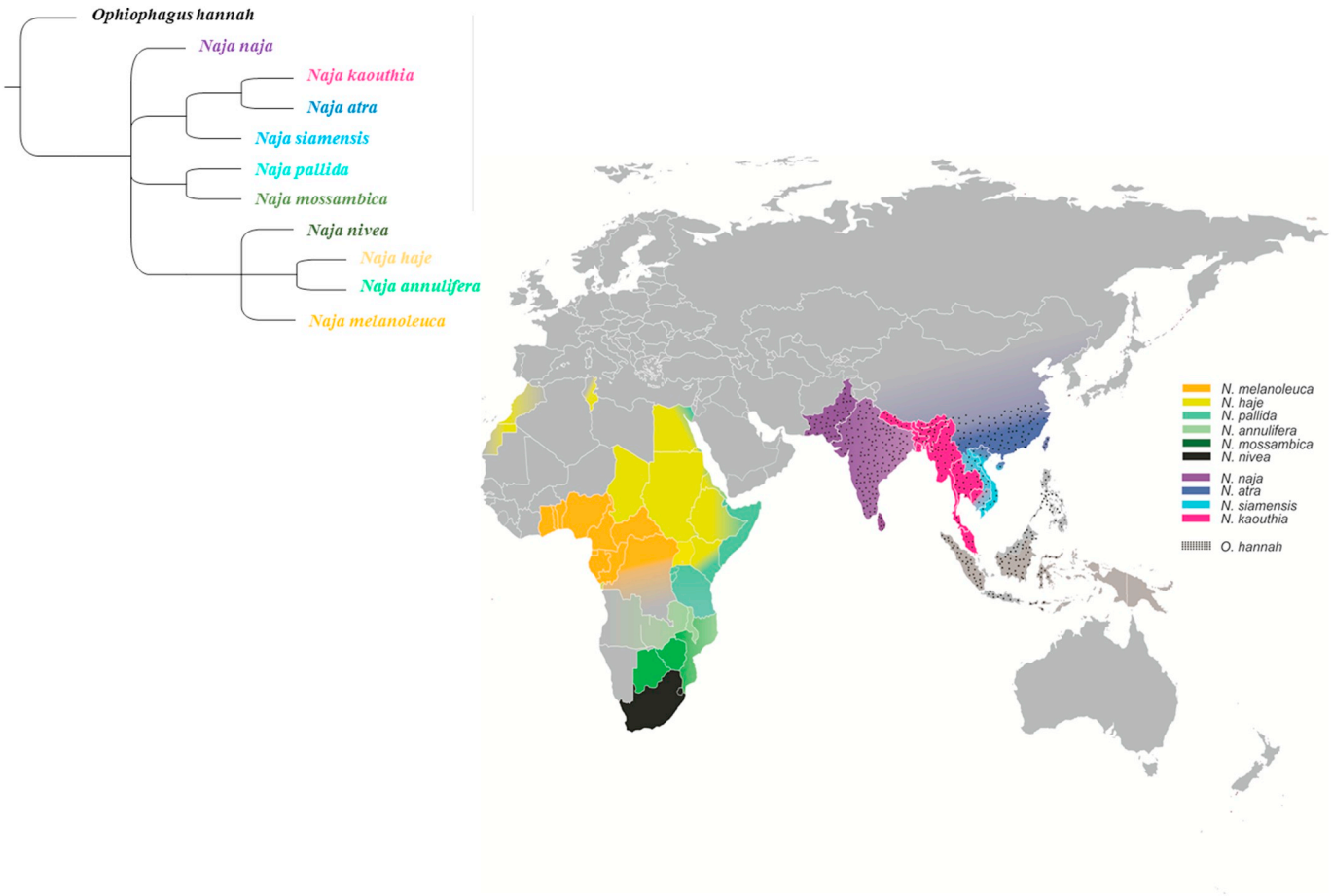

Fig. 1. Phylogenetic relationships and geographic ranges for *Ophiophagus hannah* and *Naja* species. Ranges obtained from the Reptile Database (Uetz et al., 2019), and the phylogeny modified from Wallach et al., 2009 to include only species in the current study.

then transferred immediately to ice and the reaction stopped with 250 μL 0.5 M trichloroacetic acid (TCA). Samples were centrifuged at 2000 rpm for 10 min, and absorbance recorded at 342 nm. Specific activity was expressed as $\Delta A_{342\ nm}$/min/mg venom and the assay was conducted in triplicate for each sample.

2.4.4. L-amino acid oxidase (LAAO) assay

LAAO activity was assayed as described by Weissbach et al. (1960). Briefly, 650 μL of buffer (50 mM HEPES with 100 mM NaCl, pH 8.0) and 80 μg of venom were added to each assay tube. After placing on ice for 5 min, 75 μL of L-kynurenine (1.04 mg/mL) was added and assay tubes incubated at 37 °C for 30 min. Assay tubes were then placed on ice and 750 μL of TCA (10% in ddH_2O) added to stop the reaction. After 10 min at room temperature, absorbance was taken at 331 nm. Specific activity was expressed as nmole product formed/min/mg protein and the assay was conducted in triplicate for each sample.

2.4.5. Phosphodiesterase (PDE) assay

This PDE assay was completed based on the method of Laskowski (1980). Buffer (100 mM Tris-HCL, pH 9.0 with 10 mM $MgCl_2$) was mixed with 80 μg venom in assay tube for a total volume of 225 μL. After 3 min on ice, 150 μL of substrate (1 mM bis-*p*-nitrophenylphosphate) was added and each tube incubated at 37 °C for 30 min. Following the incubation, assay tubes were transferred to ice and 375 μL termination solution (100 mM NaOH with 10 mM disodium EDTA) was added. After 10 min at room temperature, the absorbance of the mixture was recorded at 400 nm. Specific activity was expressed as Δ_{A400}/min/mg protein, and the assay was conducted in triplicate for each sample.

2.4.6. Fibrinogen digest assay

This assay utilized a modified form of a published method (Ouyang and Huang, 1979). One hundred μL of human fibrinogen (2 mg/mL 100 mM tris-HCl buffer, pH 8.0) was added to a 0.5 mL eppendorf tube and placed in a heating block at 37 °C for 3 min. Following this incubation, 20 μg of venom was added and mixed well. At different time points (0, 1, 5, 10, 30 and 60 min) 15 μL aliquots were removed and added to separate tubes containing 15 μL termination solution (4% SDS, 10% 2-mercaptoethaol, 20% glycerol). All aliquots were boiled for 5 min, and 5 μL aliquots were mixed with 2× LDS buffer, followed by gel electrophoresis on a 12% NuPAGE Bis-Tris gel as described above. This complete assay was then repeated following the addition of 5 mM EDTA (as a snake venom metalloproteinase enzyme inhibitor) to each assay tube.

2.5. Data analysis

An Analysis of Variance (ANOVA) was conducted using R (version 3.1.1) to test for significance differences in enzyme activities between cobra species. A Tukey HSD test was performed post-hoc and p-values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Cobra venom reduced and non-reduced SDS-PAGE profiles

Venom SDS-PAGE profiles for different cobra species were generated under reduced (Fig. 2A) and non-reduced (Fig. 2B) conditions. Approximately 9 to 17 protein bands were present for each cobra species with notable qualitative and quantitative differences. *Naja*

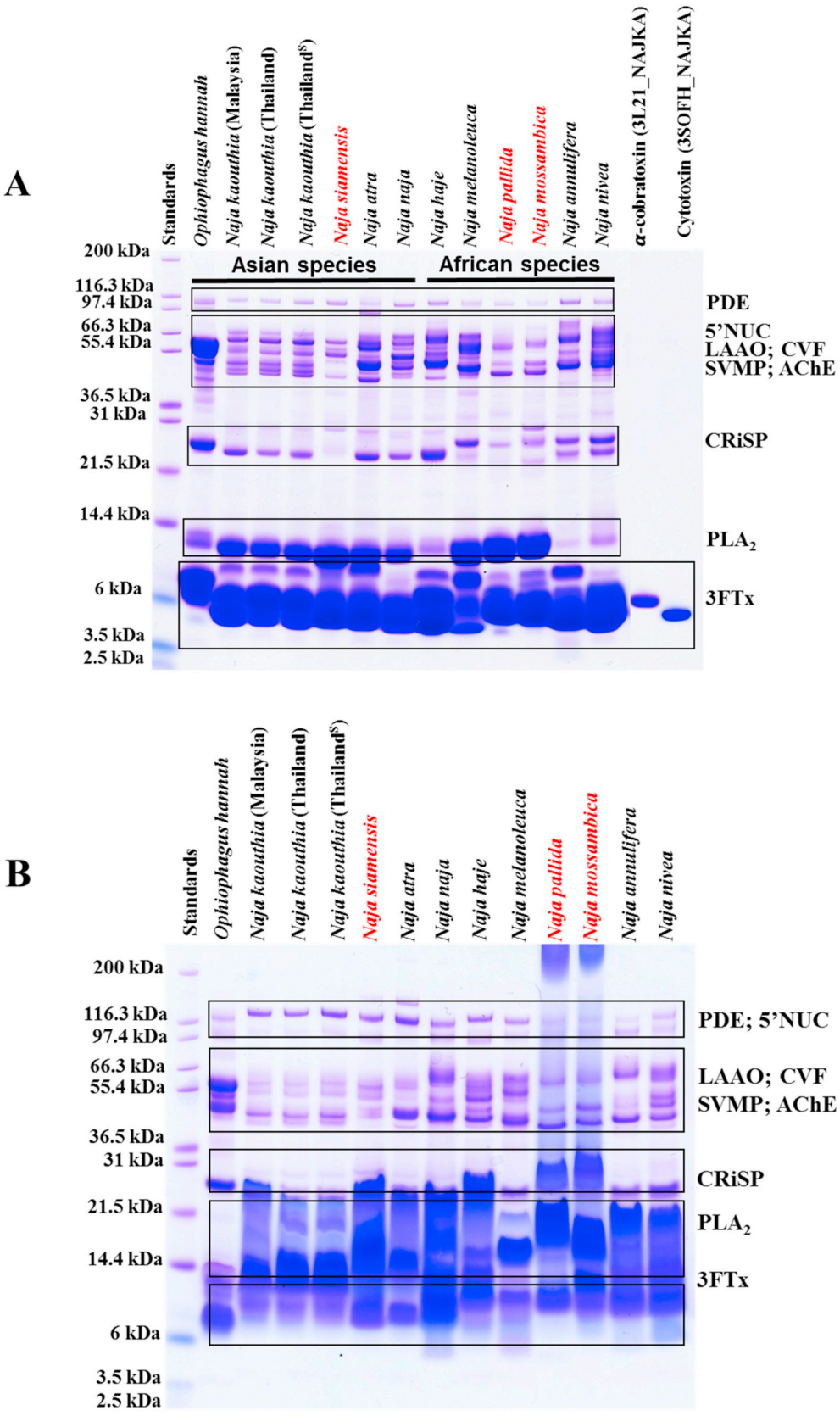

Fig. 2. SDS-PAGE profiles of cobra venoms. Crude venoms (20 μg) were visualized under reduced (A) and non-reduced (B) conditions. Cobra species highlighted in red are defensive spitting species, and these venoms show a reduction in number of higher molecular mass proteins. Molecular masses are indicated on the left, and putative venom protein identities are listed on the right. Abbreviations: 3FTx = three-finger toxin, 5'NUC = 5′ nuclease, AChE = acetylcholinesterase, CRiSP = cysteine rich secretory protein, CVF = cobra venom factor, LAAO = L-amino acid oxidase, PDE = phosphodiesterase, PLA_2 = phospholipases A_2, SVMP = snake venom metalloproteinase, and Thailand^S = Suphan Buri, Thailand. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pallida, N. mossambica and *N. siamensis* demonstrated lower venom complexity, exhibiting a lesser number and abundance of proteins of higher molecular mass compared to other cobra species (Fig. 2A). These three are spitting cobra species, suggesting that venoms of spitting cobras have evolved reduced venom complexity and specifically that lower molecular mass proteins have been retained. All cobra species had an abundance of proteins of lower molecular mass (approximately 5–9 kDa), and proteins within this range are likely 3FTxs, which are

known to occur in high abundance in cobra venoms (Modahl et al., 2016; Tasoulis and Isbister, 2017).

Based upon previous proteomic (LC-MS/MS) publications on cobra venoms, venom protein molecular masses corresponding to putative PDEs, 5'NUCs, LAAOs, CVFs, AChEs, SVMPs, CRiSPs, PLA_2s and 3FTxs were identified (Chang et al., 2013; Dutta et al., 2017; Lauridsen et al., 2017; Malih et al., 2014; Modahl et al., 2016; Tan et al., 2015b). Previously purified and MS/MS confirmed *N. kaouthia* α-cobratoxin (3L21_NAJKA) and a cytotoxic 3FTx (3SOFH_NAJKA) are also shown on the reduced gel to provide examples of cobra long-chain neurotoxic and cytotoxic

3FTxs, respectively; these are known common components in *N. kaouthia* venom (Modahl et al., 2016), and protein homologs appear to be present in many other cobra venoms. Overall, cobra venom profiles demonstrated a conserved pattern of highly abundant 3FTx, with band intensity variation between cobra species observed for higher molecular mass proteins (approximately 40–70 kDa), and PLA_2 and CRiSP isoforms.

3.2. Cobra venom enzyme activities

Cobra venoms were assayed for PLA_2, AChE, SVMP, LAAO, and PDE activities (Table 1). Significant differences between these activities were determined based on ANOVA results with a comparative post-hoc Tukey HSD test (Table 2). Venom enzymes that showed the greatest variation in activity for different cobra species were PLA_2s and AChEs.

Phospholipase A_2 enzymes within venoms exhibit a diversity of toxic and pharmacological effects, including neurotoxic, myotoxic, hemolytic, edematogenic, hyperalgesic, pro-inflammatory, hypotensive, platelet-aggregation inhibitory, anticoagulant and cytotoxic activities (Gutiérrez and Lomonte, 2013; Kini, 1997). Unless specific assays are performed, it is difficult to determine the biological roles provided by PLA_2s in cobra venoms, but they are likely toxic and digestive roles. The highest and the lowest PLA_2 activities were from *N. siamensis* (106.5 nmole product/min/mg) and *Naja nivea* (12.2 nmole product/min/mg) venoms, respectively. *Naja annulifera* and *Naja haje* venoms also demonstrated lower PLA_2 activity levels compared to other cobra species. Cobra species with lower levels of PLA_2 activity had corresponding lighter protein bands in the PLA_2 12–14 kDa range of their SDS-PAGE venom profiles (Fig. 2A). These same species of the subgenus *Uraeus*, *N. nivea, N. annulifera* and *N. haja*, were also recently reported to have venoms with low PLA_2 activity in comparison to cobra species of other subgenera, suggestive of a phylogenetic signal (Tan et al., 2019b). Malaysia and Thailand *N. kaouthia* localities were even observed to be significantly different in PLA_2 activity (Table 2). Tan et al. (2015b) also found the abundance of venom PLA_2s within Malaysia and Thailand *N. kaouthia* localities to be variable, 23.5% of the venom proteome for *N. kaouthia* from Malaysia and 12.2% of the Thailand *N. kaouthia* venom proteome.

Table 1
Cobra venom enzyme activities.

Species	PLA_2 (nmole product/min/mg)	AChE (μmole product/min/mg)	SVMP (absorbance/min/mg)	LAAO (nmole product/min/mg)	PDE (absorbance/min/mg)
O. hannah	24.52	1.26	0.09	15.46	0.71
N. kaouthia (M)	97.46	1.89	0.02	20.15	0.46
N. kaouthia (T)	61.50	0.72	0.02	25.30	0.37
N. kaouthia (T)^S	86.19	2.98	0.02	21.58	0.36
N. siamensis	106.49	2.02	0.00	23.75	0.29
N. atra	67.09	0.46	0.06	16.08	0.19
N. naja	48.00	0.21	0.04	35.99	0.54
N. haje	12.98	3.73	0.02	7.19	0.32
N. melanoleuca	45.68	4.12	0.08	38.43	0.34
N. pallida	74.75	1.19	0.03	23.60	0.32
N. mossambica	47.48	0.11	0.03	12.91	0.23
N. annulifera	13.67	4.82	0.04	10.77	0.42
N. nivea	12.21	1.71	0.08	6.15	0.29

Abbreviations: AChE = Acetylcholinesterase, LAAO = L-amino Acid Oxidase, PDE = Phosphodiesterase, PLA_2 = Phospholipases A_2, and SVMP = Snake Venom Metalloproteinase, M = Malaysia locality, T = Thailand locality, and ^{S} = Suphan buri, Thailand.

Acetylcholinesterases in cobra venoms catalyze the hydrolysis of ester bonds of the neurotransmitter acetylcholine and play a role in disruption of cholinergic transmission (Utkin and Osipov, 2007), likely contributing to the paralysis observed from cobra envenomations. For the AChE enzyme assays, *N. annulifera* had the highest activity (4.82 μmole product/min/mg). Lower levels of AChE activity were observed for *N. atra* (0.46 μmole product/min/mg), *N. naja* (0.21 μmole product/min/mg), and *N. mossambica* (0.10 μmole product/min/mg) in comparison to the other cobra species.

Metalloproteinases in cobra venoms have been found to inhibit platelet aggregation and blood coagulation (Fox and Serrano, 2010; Utkin and Osipov, 2007). All species showed low SVMP levels in comparison to other venomous snakes such as rattlesnakes (Mackessy, 2010c), with significant variation between cobra species ($p < 0.0001$). *Naja melanoleuca, N. nivea* and *Ophiophagus* showed the highest levels. However, no significant difference was observed in SVMP activity among *N. kaouthia* venoms from different localities, and *N. haje* was the most similar to *N. kaouthia* venom in SVMP activity (Table 2). Surprisingly, *N. siamensis* venom lacked detectable SVMP activity (Table 1).

L-amino acid oxidases in venoms catalyze the oxidative deamination of L-amino acids to α-keto acids, simultaneously producing ammonia and hydrogen peroxide (Izidoro et al., 2014). It was observed that *N. nivea* and *N. haje* have the lowest (6.15 and 7.19 nmole product/min/mg, respectively), and *N. melanoleuca* and *N. naja* had the highest (38.43 and 35.99 nmole product/min/mg, respectively) LAAO activity in their venoms, but overall only minor variations in LAAO activities were seen among cobra species (Table 2). Phosphodiesterases may participate in adenosine release which decreases prey blood pressure, leading to immobilization (Aird, 2002). For the PDE assay, *O. hannah* had significantly higher activity (0.71 $AU_{400\ nm}$/min/mg) than all *Naja* species. However, there was very little difference in PDE activity between most *Naja* species (Table 2).

Venom fibrinogen digest assays were performed in the absence and presence of EDTA (Fig. 3) for six of the cobra species that exhibited the highest and lowest venom SVMP activity. In the absence of EDTA, the α-chain of human fibrinogen was digested in less than one minute by *O. hannah* and *N. melanoleuca* venoms, in < 5 min by *N. atra* and *N. naja* venoms, and within 30 min by *N. mossambica* venom (Fig. 3A). There was no fibrinogen digestion observed for *N. siamensis* venom, and results for this assay highlight variations in α-fibrinogenase activity among cobra venoms.

Fibrinolytic enzymes break down fibrin clots and facilitate the spread of venom toxins throughout the circulatory system. Various fibrin(ogen)lytic enzymes have been identified in snake venoms, and they are usually metalloproteinases or serine proteases that preferentially (but not strictly) cleave either α- or β-chains of fibrinogen (Lu et al., 2005; Markland, 1998; Swenson and Markland, 2005). Fibrin (ogen)olytic serine proteases tend to cleave the β-chain, while most fibrin(ogen)olytic metalloproteases cleave the α-chain of fibrinogen (Lu et al., 2005). Several fibrin(ogen)lytic metalloproteinases have been purified and characterized from cobra venoms that are responsible for fibrinogen α-chain cleavage (Evans, 1981, 1984; Guo et al., 2007; Jagadeesha et al., 2002; Sun et al., 2007; Sun and Bao, 2010; Wei et al., 2006; Wijeyewickrema et al., 2007). These enzymes have been identified as metalloproteinases based on the inability of serine protease inhibitors, such as PMSF, to abolish the fibrin(ogen)lytic activity (Evans, 1981, 1984; Guo et al., 2007). However, metalloproteinases can be inhibited with EDTA, a metal ion chelator, and in the presence of EDTA, fibrinogen cleavage was not seen for any cobra species (Fig. 3B). These

Table 2
Tukey post-hoc comparisons of cobra venom enzyme activities.

	O. h	*N. k* (M)	*N. k* (T)	*N. k* (T)^{S}	*N. s*	*N. at*	*N. na*	*N. h*	*N. me*	*N. p*	*N. mo*	*N. an*	*N. ni*
PLA_2 activity													
O. h	–	****	****	****	****	****	*	NS	NS	****	*	NS	NS
N. k (M)	****	–	**	NS	NS	*	****	****	****	NS	****	****	****
N. k (T)	****	**	–	NS	***	NS	NS	****	NS	NS	NS	****	****
N. k (T)^{S}	****	NS	NS	–	NS	NS	**	****	***	NS	***	****	****
N. s	****	NS	***	NS	–	***	****	****	****	**	****	****	****
N. at	****	*	NS	NS	***	–	NS	****	NS	NS	NS	****	****
N. na	*	****	NS	**	****	NS	–	**	NS	NS	NS	**	**
N. h	NS	****	****	****	****	****	**	–	*	****	**	NS	NS
N. me	NS	****	NS	***	****	NS	NS	*	–	*	NS	*	**
N. p	****	NS	NS	NS	**	NS	NS	****	*	–	*	****	****
N. mo	*	****	NS	***	****	NS	NS	**	NS	*	–	**	**
N. an	NS	****	****	****	****	****	**	NS	*	****	**	–	NS
N. ni	NS	****	****	****	****	****	**	NS	**	****	**	NS	–
SVMP activity													
O. h	–	****	****	****	****	****	****	****	**	****	****	****	NS
N. k (M)	****	–	NS	NS	****	****	****	NS	****	****	NS	****	****
N. k (T)	****	NS	–	NS	****	****	****	NS	****	****	*	****	****
N. k (T)^{S}	****	NS	NS	–	****	****	****	NS	****	****	NS	****	****
N. s	****	****	****	****	–	****	****	****	****	****	****	****	****
N. at	****	****	****	****	****	–	****	****	****	****	****	****	****
N. na	****	****	****	****	****	****	–	****	****	****	****	NS	****
N. h	****	NS	NS	NS	****	****	****	–	****	****	NS	****	****
N. me	**	****	****	****	****	****	****	****	–	****	****	****	NS
N. p	****	****	****	****	****	****	****	****	****	–	NS	****	****
N. mo	****	NS	*	NS	****	****	****	NS	****	NS	–	****	****
N. an	****	****	****	****	****	****	NS	****	****	****	****	–	****
N. ni	NS	****	****	****	****	****	****	****	NS	****	****	****	–
LAAO activity													
O. h	–	NS	NS	NS	NS	NS	****	NS	****	NS	NS	NS	NS
N. k (M)	NS	–	NS	NS	NS	NS	****	**	****	NS	NS	NS	**
N. k (T)	NS	NS	–	NS	NS	NS	*	****	**	NS	**	**	****
N. k (T)^{S}	NS	NS	NS	–	NS	NS	**	**	***	NS	NS	*	***
N. s	NS	NS	NS	NS	–	NS	**	***	**	NS	*	**	****
N. at	NS	NS	NS	NS	NS	–	****	NS	****	NS	NS	NS	NS
N. na	****	****	*	**	**	****	–	****	NS	**	****	****	****
N. h	NS	**	****	**	***	NS	****	–	****	***	NS	NS	NS
N. me	****	****	**	***	**	****	NS	****	–	***	****	****	****
N. p	NS	NS	NS	NS	NS	NS	**	***	***	–	*	**	****
N. mo	NS	NS	**	NS	*	NS	****	NS	****	*	–	NS	NS
N. an	NS	NS	**	*	**	NS	****	NS	****	**	NS	–	NS
N. ni	NS	**	****	***	****	NS	****	NS	****	****	NS	NS	–
PDE activity													
O. h	–	****	****	****	****	****	****	****	****	****	****	****	****
N. k (M)	****	–	NS	*	****	****	NS	***	**	***	****	NS	****
N. k (T)	****	NS	–	NS	NS	****	****	NS	NS	NS	***	NS	NS
N. k (T)^{S}	****	*	NS	–	NS	****	****	NS	NS	NS	***	NS	NS
N. s	****	****	NS	NS	–	**	****	NS	NS	NS	NS	**	NS
N. at	****	****	****	****	**	–	****	***	****	***	NS	****	**
N. na	****	NS	****	****	****	****	–	****	****	****	****	**	****
N. h	****	***	NS	NS	NS	***	****	–	NS	NS	NS	*	NS
N. me	****	**	NS	NS	NS	****	****	NS	–	NS	**	NS	NS
N. p	****	***	NS	NS	NS	***	****	NS	NS	–	NS	*	NS
N. mo	****	****	***	***	NS	NS	****	NS	**	NS	–	****	NS
N. an	****	NS	NS	NS	**	****	**	*	NS	*	****	–	**
N. ni	****	****	NS	NS	NS	**	****	NS	NS	NS	NS	**	–

Abbreviations: AChE = Acetylcholinesterase, LAAO = L-amino Acid Oxidase, PDE = Phosphodiesterase, PLA_2 = Phospholipases A_2, and SVMP = Snake Venom Metalloproteinase, *O. h* = *Ophiophagus hannah*, *N. k* (M) = *Naja kaouthia* (Malaysia), *N. k* (T) = *Naja kaouthia* (Thailand), *N. s* = *Naja siamensis*, *N. at* = *Naja atra*, *N. na* = *Naja naja*, *N. h* = *Naja haje*, *N. me* = *Naja melanoleuca*, *N. p* = *Naja pallida*, *N. mo* = *Naja mossambica*, *N. an* = *Naja annulifera*, *N. ni* = *Naja nivea*, M = Malaysia locality, T = Thailand locality, and ^{S} = Suphan buri, Thailand.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.
**** $p < 0.0001$.

results strongly implicate that metalloproteinases are responsible for fibrin(ogen)lytic activity in these cobra venoms, which is in stark contrast to viperid venoms, which also contain abundant serine proteinases, many of which show activity toward fibrinogen and fibrin (Mackessy, 2010b).

Cobra venom metalloproteinases have been commonly described with masses of ~50–68 kDa (Evans, 1984; Guo et al., 2007; Jagadeesha et al., 2002; Sun et al., 2007), which is in the size range of PIII metalloproteinases (Fox and Serrano, 2010; Mackessy, 2010a). The abundance of proteins of this size range in the SDS-PAGE venom profiles are notably lower for *N. siamensis*, which has venom lacking fibrin (ogen)lytic and metalloproteinase activity, *N. mossambica*, which has

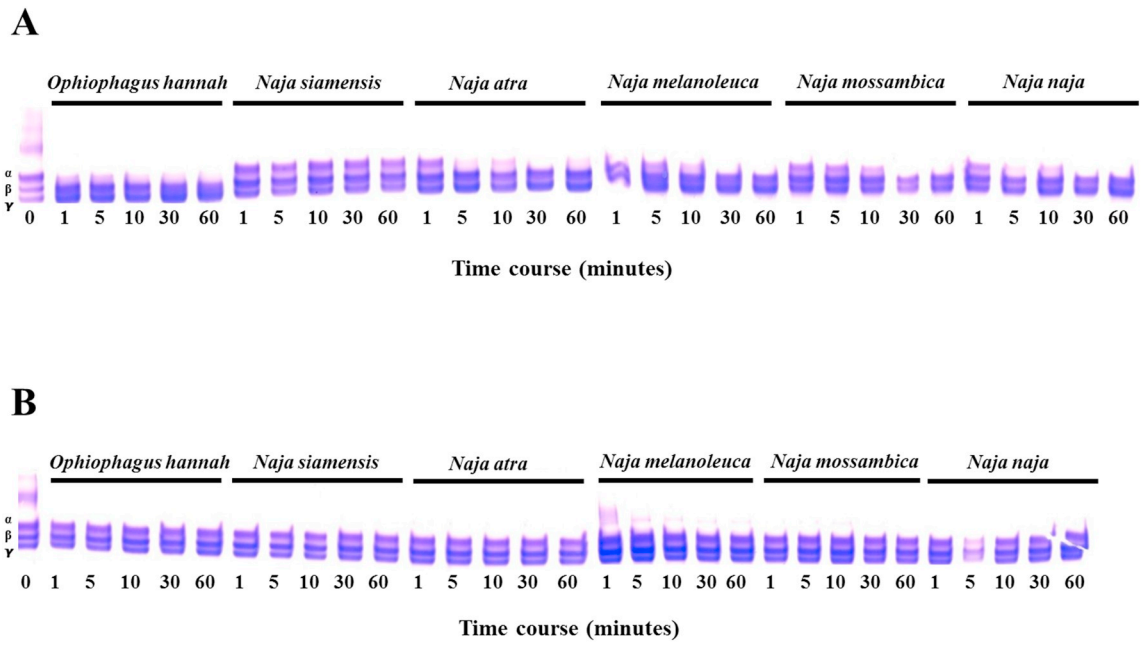

Fig. 3. Fibrinogen digest assay with venom from multiple cobra species. Crude venoms (20 μg) were incubated with human fibrinogen over a 60-min time course without EDTA (A) and with 5 mM EDTA (B); venom fibrinogenolytic activity was eliminated in the presence of EDTA. Minute 0 was before the addition of any venom and shows all intact fibrinogen chains. Fibrinogen chains are labeled: α, β and γ.

the least effective metalloproteinase fibrin(ogen)lytic activity of the cobras assayed, and *N. pallida* which also showed very low SVMP activity (Fig. 2A; Table 1). All three species are spitting cobras that spray venom in defense against predators. It has been previously noted that the evolution of increased cytotoxicity has coincided with the evolution of spitting as a defense in African cobras (Mendez et al., 2011; Panagides et al., 2017). This suggests that spitting cobras may have evolved venoms of reduced complexity and decreased enzymatic activity, with concomitant increases in venom proteins responsible for cytotoxicity, such as some PLA_2 and 3FTxs. It would be very interesting to evaluate venom profiles of *N. kaouthia* from West Bangal, India that exhibit spitting behavior (Santra and Wüster, 2017) in comparison to the non-spitting *N. kaouthia* utilized in the present study.

Small cytotoxic and neurotoxic proteins such as 3FTxs should diffuse very rapidly and quickly incapacitate vision, so these toxins would be more effective as defensive components of venom. Larger proteins, including most enzyme toxins, would diffuse more slowly and act less rapidly; in addition, they are likely more energetic to produce and take longer to replenish in the venom gland, and these larger venom proteins may also require chaperon proteins for proper folding, whereas smaller, compact toxins may not (Margres et al., 2013; Rokyta et al., 2012). In general, cobra venoms show high lethal toxicity (low LD_{50}s; Table 3), but there was no apparent correlation between toxicity and spitting tendency. It is possible that because spitting cobras also use their venoms defensively, and therefore, potentially require venom readily available, a streamlined, more effective venom with a reduced number of larger venom proteins, perhaps requiring less time to replenish between uses, has evolved among spitters. However, this study only included three spitting cobra species, and an analysis of venom from other spitting cobra species is needed to explore this hypothesis further.

Future studies should also examine non-enzymatic interspecific venom variation for these cobra species, especially documenting the differences in abundance and classes of 3FTxs within these venoms. There exist several classes of 3FTxs, including long- and short-chain neurotoxic 3FTxs, cytotoxic 3FTxs, non-conventional 3FTxs and others (Fry et al., 2003). Short-chain 3FTxs have been noted to be responsible for differences in antiserum efficiency (Tan et al., 2017b), and therefore explorations into cobra venom 3FTxs have direct implications in antivenom development. Further, by examining patterns of toxin evolution and diversification in a related clade of venomous snakes, it is possible to begin to unravel direct and indirect processes resulting in venom variation.

Table 3
Intraperitoneal lethality (IP LD50) of cobra venoms used in this study.

Species	IP $LD_{50(mg/kg)}$	Reference
Ophiophagus hannah	1.1	Danpaiboon et al., 2014
Naja naja	0.25	SPM – Unpubl. data
Naja kaouthia - Thai	0.25	SPM – Unpubl. data
Naja atra	0.62	Liu et al., 2010
Naja siamensis[a]	1.13	snakedatabase.org
Naja pallida[a]	2.0	Zhang et al., 2016
Naja mossambica[a]	0.083	Schweitz, 1984
Naja nivea	0.396	Mohamed et al., 1973
Naja melanoleuca	0.324	Kocholaty et al., 1971
Naja haje	0.12	Mohamed et al., 1973
Naja annulifera	0.5	Mohamed et al., 1980

[a] Spitting species.

4. Conclusions

Due to a complex mixture of peptides, proteins and enzymes in snake venoms, venomous snakebite can have a wide range of clinical manifestations, from tissue damage and necrosis to hematotoxicity and paralysis. Despite having similar classes of enzymes and peptides, multiple publications have demonstrated qualitative and quantitative venom compositional differences among cobra species (Chang et al., 2013; Mukherjee and Maity, 2002; Petras et al., 2011; Tan et al., 2015b, 2019b; Tan and Tan, 1988). Venom compositional variation can lead to differences in toxicity levels and antiserum neutralization efficiencies, as well as different individual responses, which all should be considered when designing suitable antisera and clinical treatments (Warrell et al., 2013).

Venom compositional variations for eleven cobra species from different geographic localities were evaluated by SDS-PAGE and enzyme assays, revealing considerable interspecific venom variation.

Acetylcholinesterase and phospholipase A_2 activities exhibited the most variability, including significant differences in intraspecific venom PLA_2 activity between *N. kaouthia* from Malaysia and Thailand localities. Venom metalloproteinase activity was also found to be significantly different for the majority of species, but identical for *N. kaouthia* populations. Only minor variations in venom L-amino acid oxidase and phosphodiesterase activities were seen between cobra species. Venom from the spitting cobra *N. siamensis* lacked metalloproteinase α-chain fibrin(ogen)lytic activity common to other cobra venoms, and venom from *N. mossambica*, another spitting species, exhibited a slower rate of α-chain fibrinogen degradation. SDS-PAGE venom profiles from spitting cobras (*N. siamensis*, *N. pallida*, and *N. mossambica*) all showed a reduction in higher molecular mass proteins, such as metalloproteinases. The evolution of venoms of reduced complexity and decreased enzymatic activity among spitting cobras may have resulted in greater ocular effects and a reduction in venom replenishment times, allowing for more frequent use. Documenting venom variation provides insight into the evolution of different venom phenotypes. Venom composition is a phenotypic adaptation that is the result of multiple genetic and environmental interactions, and results from this study emphasize how even venomous snake behavior, defensive spitting versus non-spitting cobras, can influence venom variation. Differences in venom composition leads to inconsistencies in antivenom neutralization, and this information is critical for designing more efficient region-based antivenoms and to better anticipate snakebite complications to venom proteins present.

Declaration of competing interest

The authors declare that they have no conflict of interest associated with this manuscript.

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