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Differential destructive (non-clotting) fibrinogenolytic activity in Afro-Asian elapid snake venoms and the links to defensive hooding behavior

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ABSTRACT

Envenomations by venomous snakes have major public health implications on a global scale. Despite its medical importance, snakebite has long been a neglected tropical disease by both governments and medical science. Many aspects of the resulting pathophysiology have been largely under-investigated. Most research on snake venom has focused on the neurological effects, with coagulotoxicity being relatively neglected, especially for venoms in the Elapidae snake family. In order to fill the knowledge gap regarding the coagulotoxic effects of elapid snake venoms, we performed functional activity tests to determine the fibrinogenolytic activity of 29 African and Asian elapid venoms across eight genera. The results of this study revealed that destructive (non-clotting) fibrinogenolytic activity is widespread across the African and Asian elapids. This trait evolved independently twice: once in the *Hemachatus/Naja* last common ancestor and again in *Ophiophagus.* Further, within *Naja* this trait was amplified on several independent occasions and possibly explains some of the clinical symptoms produced by these species. Species within the *Hemachatus/Naja* with fibrinogenolytic activity only cleaved the Aα-chain of fibrinogen, whereas *Ophiophagus* venoms degraded both the Aα- and the Bβ-chain of fibrinogen. All other lineages tested in this study lacked significant fibrinogenolytic effects. Our systematic research across Afro-Asian elapid snake venoms helps shed light on the various molecular mechanisms that are involved in coagulotoxicity within Elapidae.

Key contribution

Destructive fibrinogenolytic activity was found to be widespread across the African and Asian elapids. The molecular mechanism of this coagulotoxic venom activity was determined, with some species, to be cleavage of the Aα-chain of fibrinogen, whereas only *O. hannah* localities cleaved both the Aα- and the Bβ-chain of fibrinogen.

1. Introduction

Snakebite is a major neglected disease and public health problem in developing countries in the tropics and subtropics. Between 1.8 and 5.4 million snakebite victims are recorded annually, resulting in over 100,000 fatalities, which is likely a gross underestimations due to epidemiological record-keeping deficiencies in the most affected countries (Chippaux, 2017; Fry, 2018; Gutiérrez et al., 2006, 2010; Kasturiratne et al., 2008; Longbottom et al., 2018). As the burden of snakebite mostly affects agricultural workers in developing countries in Africa, Asia and Latin America, the socio-economic impact on local economies can be significant (Harrison et al., 2009). Unfortunately, the implications of snakebite on public health has long been neglected by many institutions, including governments, medical science and pharmaceutical companies and therefore has been categorized as neglected tropical disease. Fortunately, snakebite has recently been added back onto the list of neglected tropical diseases by the World Health Organization (WHO) in order to raise global awareness (Chippaux, 2017), and this issue has received substantial funding recently.

Snake venoms contain a large variety of toxic proteins and peptides that can destructively alter physiological systems and cause multiple local and systemic effects. Local venom effects can cause permanent injury and disfigurement, possibly inhibiting the ability of victims to work, while systemic venom effects relating to respiration and blood

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circulation can be life-threatening (Boyer et al., 2015; Harrison et al., 2009). The life-threatening snakebite effects observed from envenomations by Afro-Asia elapid snakes are mostly caused by neurotoxins and may lead to respiratory paralysis (Alirol et al., 2010). In combination with this impetus, and the difficulties in blood-related assays, the majority of snake venom research has largely focused the neurological effects.

Despite the potential for coagulotoxins to cause severe local and systemic hemorrhaging, which can result in death (Warrell et al., 1976), Afro-Asian elapid snake venom effects on blood coagulation have been relatively neglected in the literature. However, a study has recently shown that African spitting cobras are potently anticoagulant by competing for the binding of FVa to FXa, and also inhibiting thrombin, with these actions linked to the lethal hemorrhage seen clinically (Bittenbinder et al., 2018). As anticoagulation was a trait linked to defensive hooding and spitting behavior, this study suggested that anticoagulant may have a synergistic effect with cytotoxicity. The extent of activity and the modes of action of other anticoagulants have remained underinvestigated. Such investigations may shed further light on the possible role of anticoagulation in defensive components of hooding elapid venoms.

A key neglected area for anticoagulant actions of Afro-Asian elapid snake venom effects is direct actions upon fibrinogen, a pathophysiological action which has been linked to anticoagulation in a myriad of lizard and snake venoms (Coimbra et al., 2018; Debono et al., 2019; Dobson et al., 2017, 2019; Koludarov et al., 2017). The destructive cleavage of fibrinogen—the final zymogen in the blood coagulation cascade—has been shown to have independently evolved within the reptiles in two distinct toxin classes: kallikrein-type serine-proteases; and snake venom metalloproteases (SVMPs). These proteases are capable of cleaving either the Aα- or the Bβ-chain of fibrinogen, or both. Fibrinogenolysis may be directly anticoagulant through non-clotting (destructive) cleavage, or anticoagulant through a pseudo-procoagulant mechanism in which weak, unstable, clots are formed which readily break down, consuming clotting factors (Debono et al., 2019). Both actions lead to a decrease in viable fibrinogen levels and thus anticoagulant state (Fry et al., 2009; Gutiérrez et al., 2016; Isbister et al., 2010; Vaiyapuri et al., 2015). Fibrinogenolytic activity is well-characterized in viperid snake venoms but much less documented for elapid venoms (Li et al., 2004; Petras et al., 2011).

We aimed to fill part of the aforementioned knowledge gap by performing functional activity tests on 29 African and Asian elapid snake venoms to determine the type and extent of fibrinogenolytic activity across this clade. We present results that not only illustrate the mechanism of anticoagulant action of these venoms, but also reveal the relative potency of this anticoagulant venom action across this medically important clade.

2. Materials and methods

2.1. Venoms

In total, 29 snake venoms were tested: *Aspidelaps lubricus*, *A. scuttatus*, *Bungarus fasciatus*, *Dendroaspis. polylepis*, *Elapsoidea boulengeri*, *E. sundevallii longicauda*, *E. s. sundevallii*, *Hemachatus haemachatus*, *Naja annulata*, *N. annulifera*, *N. atra*, *N. haje*, *N. kaouthia*, *N. mellanoleuca*, *N. mossambica*, *N. naja*, *N. nigricincta*, *N. nigricollis*, *N. nivea*, *N. pallida*, *N. phillippinensis*, *N. samarensis*, *N. siamensis*, *N. sumatrana*, *Ophiophagus hannah* (Cambodia, Java, Malaysia and Thailand localities) and *Walterinnesia aegyptia*. Venom samples were sourced from the long-term research collection of the Venom Evolution Lab with the exceptions of *N. nigricincta*, and *N. samarensis* which were provided by FJV. Venoms were freeze-dried and stored at −80 °C until use. 1 mg aliquots of venom were reconstituted in deionized water, centrifuged (14,000 RCF at 4 °C for 10 min), and the supernatant was collected and its protein-concentration was measured using a ThermoFisher Scientific NanoDrop™ spectrophotometer. Working stocks of 1 mg/ml were made using 50% deionized water and 50% glycerol (> 99% Sigma-Aldrich) before being stored at −20 °C to reduce enzyme degradation. All venom work was undertaken under University of Queensland Biosafety Approval #IBC134BSBS2015.

2.2. Human fibrinogen

Human fibrinogen was reconstituted in isotonic saline solution to a concentration of 4 mg/ml, flash-frozen in liquid nitrogen, and stored at −80 °C until required.

Fibrinogen cleavage assays were performed as previously described (Dobson et al., 2017; Koludarov et al., 2017). Fibrinogen was defrosted for 5 min at 37 °C and transferred to 'primary' aliquots. Venom was then added to the fibrinogen at a 1:10 ratio. Subsequently, five 'secondary' aliquots were prepared, containing 10 µl buffer (5 µl 4× Laemmli sample buffer (Sigma-Aldrich, St. Louis, MO, USA) and 5 µl Milli-Q, 100 mM DTT. Then, at specific time points (0 min, 1 min, 5 min, 20 min and 60 min), 10 µl was taken from the primary aliquot and added to the secondary aliquot, pipette mixed and boiled for 4 min at 100 °C. The secondary aliquots were loaded onto SDS PAGE gels (recipe below) and run in 1× gel running buffer at room temperature at 90 V and subsequently 120 V until completion. The gels were then stained with coomassie blue.

For the preparation of 1 mM 12% SDS-PAGE gels, the following protocol was used: Resolving layer: 3.3 ml of Milli-Q, 2.5 ml of 1.5 M Tris-HCl buffer pH 8.8 (Tris – Sigma-Aldrich, St. Louis, MO, USA; HCl – Univar, Wilnecote, UK), 100 µl 10% SDS (Sigma-Aldrich, St. Louis, MO, USA), 4 ml 30% acrylamide mix (Bio-Rad, Herculas, CA, USA), 100 µl 10% APS (Bio-Rad, Hercules, CA, USA), 4 µl TEMED (Bio-Rad, Hercules, CA, USA). Stacking layer: 1.4 ml Milli-Q, 250 uL 0.5 M Tris-HCl buffer pH 6.8, 20 µl 10% APS (Bio-Rad, Hercules, CA, USA), 2 µl TEMED (Bio-Rad, Hercules, CA, USA). Running buffer was prepared as follows: 140 mM TRIS, 1.92 M glycine (MP Biomedicals), 1% SDS pH 8.3 (Sigma-Aldrich, St. Louis, MO, USA).

2.3. Phylogenetic comparative analyses

Scanned gel images were uploaded to ImageJ. After converting images to black and white and adjusting for brightness and contrast, control bands were selected and defined as the first lane (0 min). Subsequent bands were defined as lanes 2–8. Selected bands were plotted, and the area of each peak individually measured. The peaks thus represent band-intensity values, from which the degradation of the fibrinogen chains could be calculated as relative proportions of the control. The results were graphed using Prism 7.0 software (GraphPad Software Inc., La Jolla, CA, USA). Data are expressed as mean ± SD for each triplicate data point.

The phylogenetic tree used was based upon a previously published species tree (Lee et al., 2016) and manually recreated using Mesquite software (version 3.2) and then imported to Rstudio using the APE package (Paradis et al., 2004). Ancestral states were estimated over the tree for the fibrinogen cleavage trait using maximum likelihood in the contMap function of the R package phytools (Revell, 2012). We then fit PGLS models (Symonds and Blomberg, 2014) in caper (Orme et al., 2013) to test for phylogenetic relationships. As per previous studies using these methods (Lister et al., 2017; Rogalski et al., 2017) we used the phytools and PGLS scripts (Supplementary File 1).

3. Results

Our fibrinogen assays revealed differential cleavage patterns (representatives shown in Fig. 1). Other than the *Hemachatus/Naja* and *Ophiophagus* clades, none of the other species showed discernable effects upon fibrinogen. While species within the *Hemachatus/Naja* and *Ophiophagus* clades displayed the ability to degrade the Aα-chain, only

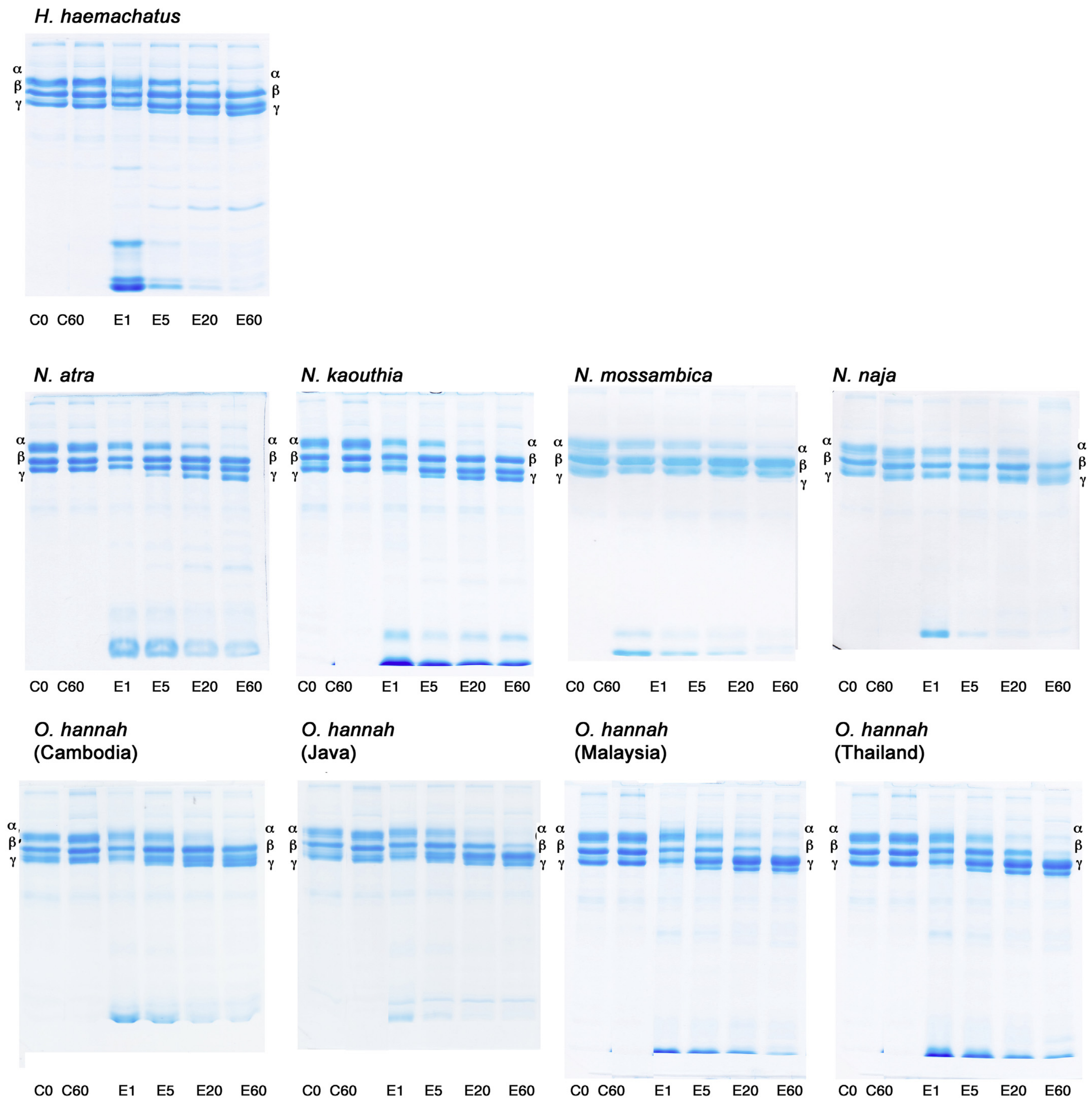

Fig. 1. Representatives of differential ability of Afro-Asian elapid venoms to degrade the Aα-, Bβ- and Yγ-chains of fibrinogen. Representative 1D-SDS page gel images showing the venom effects on fibrinogen over different time periods (1, 5, 20, 60 min, respectively). C0 and C60 represent 0 min and 60 min controls. Experiments were run in triplicate. Only *Ophiophagus* displayed ability to degrade the Bβ-chain.

Ophiophagus showed the ability to degrade both the Aα- and the Bβ-chains (Figs. 1–3). As previously observed in the activities of lizard and snake venoms, the cleavage of the Aα-chain occurred prior to the degradation of the Bβ-chain (Figs. 1–3) (Coimbra et al., 2018; Dobson et al., 2017; Dobson et al., 2019; Koludarov et al., 2017).

Phylogenetic ancestral state reconstruction of the venom effects revealed that fibrinogenolytic activity appears to have evolved on two separate occasions within the African and Asian elapid clade: *Hemachatus/Naja* clade and again in the *Ophiophagus* clade (Fig. 3). While all *Ophiophagus* variants displayed this trait, within *Hemachatus/Naja* it was amplified on several independent occasions: *H. haemachatus;* the *Naja* African spitting clade (*N. mossambica, N. nigricincta, N. nigricollis,* and *N. pallida*), *N. philippinensis* within the Asian spitting species; and also the Asian non-spitting clade of ornately marked species consisting of *N. atra, N. kaouthia,* and *N. naja*. (Fig. 3). In contrast to *N. philippinensis,* the other Asian spitting species studied (*N. samarensis, N. siamensis,* and *N. sumatrana*) did not display fibrinogenolytic activity.

4. Discussion

We performed functional activity tests to determine the type and

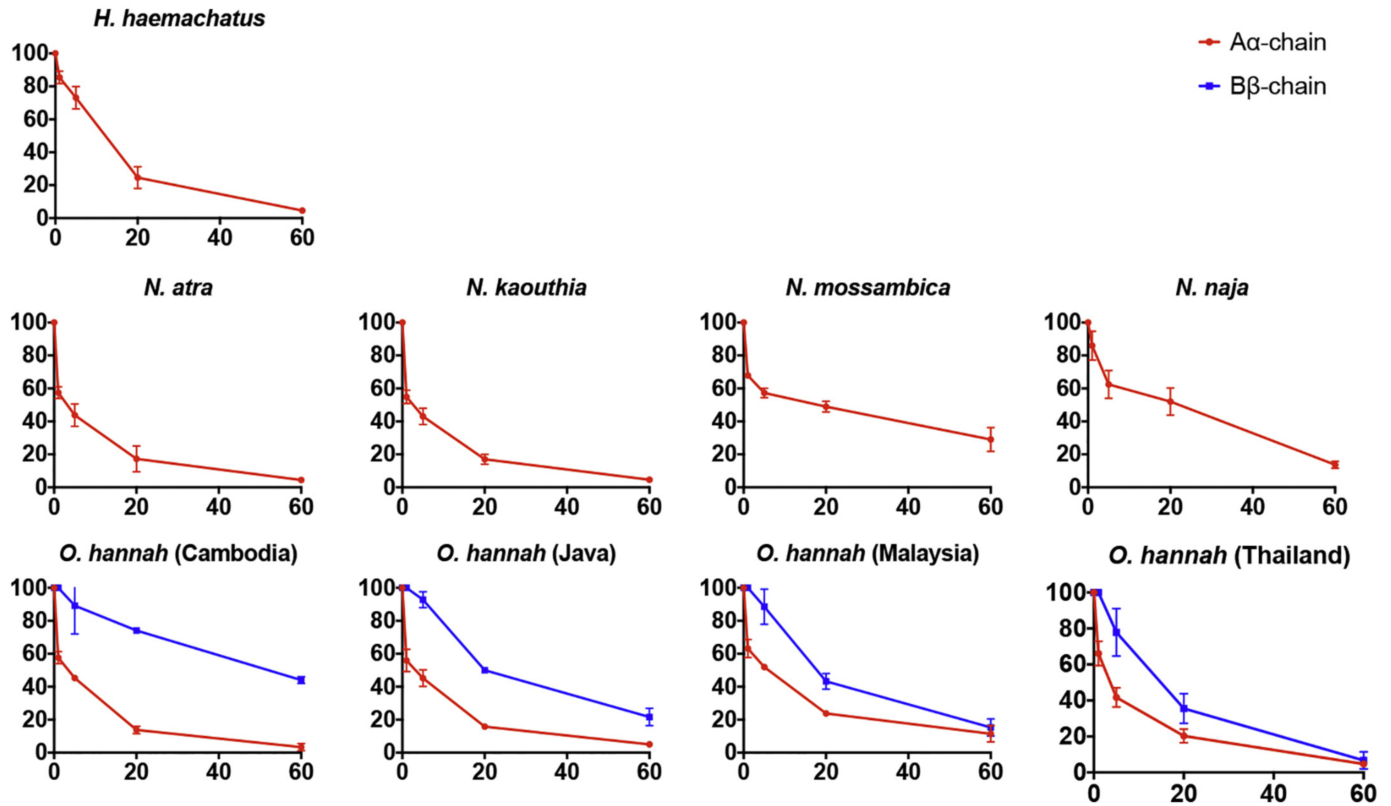

Fig. 2. Representative differential ability to degrade the Aα-chain and Bβ-chain of fibrinogen. Fibrinogenolytic activity was graphed to visualize the change in band density over time. X-axis: incubation time (min), Y-axis: ratio of intact fibrinogen. Experiments were run in triplicate. Only *Ophiophagus* displayed ability to degrade the Bβ-chain and therefore Bβ-chain was graphed only for this species.

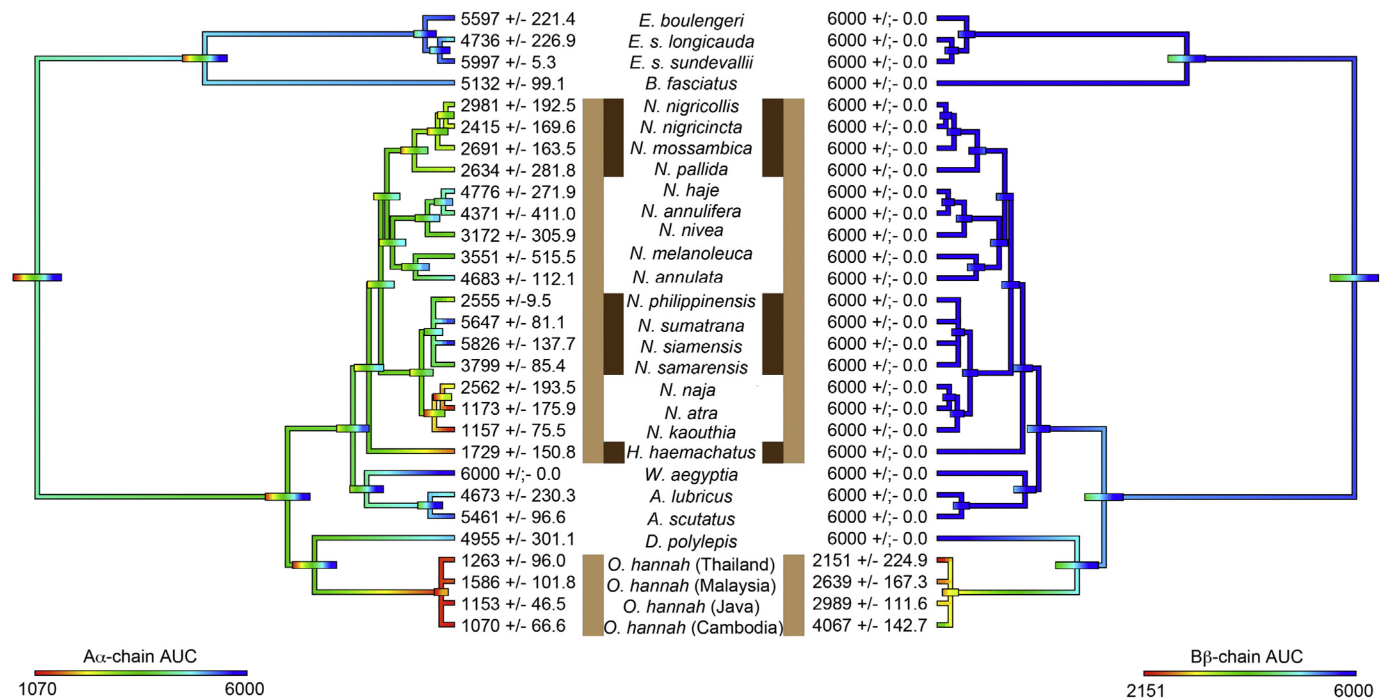

Fig. 3. Ancestral state reconstruction of fibrinogen cleavage. A reconstruction of the ancestral state of cleavage of the Aα-chain of fibrinogen, based on AUC (area under the curve) derived from calculated band-intensity values. Warmer colours represent smaller AUCs (higher fibrinogen chain cleavage activity). Phylogeny based upon (Lee et al., 2016; Wüster et al., 2007). Control value is 6000 AUC for no degradation of fibrinogen. Only *Ophiophagus* displayed ability to degrade the Bβ-chain. Light brown vertical bars show the two clades that have independently evolved hooding defensive behavior; dark brown vertical bars show the tree clades that have independently evolved spitting behavior (Bittenbinder et al., 2018). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

extent of fibrinogenolytic venom action across African and Asian elapid snake species. This study revealed that destructive (non-clotting) fibrinogenolytic activity is widespread across the hooding African and Asian elapids and amplified on several independent occasions (Fig. 3). This trait was not evident for non-hooding species.

Thus there was a clear separation of species into three types of fibrinogenolytic venom activity: 1) did not cleave the Aα-chain nor the Bβ-chain; 2) cleaved the Aα-chain (*Hemachatus/Naja*); or 3) cleaved both the Aα- and the Bβ-chain (*Ophiophagus*), (Fig. 3). Within the *Hemachatus/Naja* clade, the fibrinogenolytic activity was most potent in an Asian non-spitting clade of ornately marked species consisting of *N. atra, N. kaouthia,* and *N. naja*. These species only degraded the Aα-chain, leaving the Bβ-chain intact, even after 60 min. This Aα-chain cleavage activity was also amplified in African spitting cobra species (*N. mossambica*, *N. nigricincta*, *N. nigricollis*, and *N. pallida*) and in the other African spitting lineage *Hemachatus* but to a lesser extent (Fig. 3). Within the Asian spitting clade, only *N. philippinensis* displayed significant levels of Aα-chain cleavage, with the other Asian spitting species notably weaker by way of comparison.

As destructive cleavage of fibrinogen was a consistent feature of hooding species while not being a significant trait for non-hooding species, this is suggestive of a defensive role and possible synergy with the thrombin inhibition which is also a feature of the *Hemachatus/Naja* clade (Bittenbinder et al., 2018). Further, hooding has been linked to the evolution of potent defensive, pain-inducing cytotoxins (Panagides et al., 2017). It has been previously suggested that anticoagulation may have a synergistic role with cytotoxicity (Bittenbinder et al., 2018) and the documentation of fibrinogenolysis as an additional anticoagulant action is a rich area for future research into the link between defensive behavior, anticoagulation, and pain-inducing cytotoxicity.

While the selection pressures for use of this anticoagulant trait remains to be fully resolved, the results of this study indicate that the fibrinogenolytic function evolved independently in *Hemachatus/Naja* versus *Ophiophagus.* All localities of *Ophiophagus* were unique in displaying fibrinogenolytic activity on both the Aα- and the Bβ-chains. The fact that no other venoms displayed this ability, with *Hemachatus/Naja* only degrading the Aα-chain—and the species intervening between *Hemachatus/Naja* versus *Ophiophagus* lacked fibrinogenolytic activity—is strongly suggestive of not only independent evolutions of anticoagulant cleavage of fibrinogen, but that the two clades possess different toxin types with differential cleavage activities.

Fibrinogenolytic activity has been described for both kallikrein-type serine-proteases and snake venom metalloproteases (SVMPs) (Casewell et al., 2015; Vaiyapuri et al., 2015). These snake venom serine-proteases (SVSPs) are capable of cleaving either the Aα- and/or the Bβ-chains. For some SVSPs that have both α- and β-fibrinogenolytic activity, the specificity is directed to the Bβ-chain, only slowly degrading the Aα-chain (Kini and Koh, 2016; Matsui et al., 2000). SVMPs on the other hand are known for having their specificity directed towards the Aα-chain, with lower affinity towards the Bβ-chain (Kini and Koh, 2016; Markland, 1998; Matsui et al., 2000). Thus the Aα-chain fibrinogenolytic activity observed in the *Hemachatus/Naja* clade is most likely caused by SVMPs. These have been found in the venoms of various elapid species including within the *Naja* genus. Our findings are consistent with prior studies, which reported α-fibrinogenolytic activity by SVMPs with anticoagulant properties in a number of elapid species, including *N. atra*, *N. kaouthia*, *N. mossambica* and *N. nigricollis* (Evans, 1981; Guan et al., 2010; Jagadeesha et al., 2002; Kini and Evans, 1991; Kumar et al., 2010; Ward et al., 1996; Wei et al., 2006).

Across the *Ophiophagus* genus, similar levels of Aα-chain cleavage were observed. The main difference between the *Hemachatus/Naja* clade and the *Ophiophagus* clade lies within the ability of *Ophiophagus* venoms to degrade both the Aα- and the Bβ-chain (Fig. 3). This is also consistent with information from the literature, as no reports on β-fibrinogenolytic activity for any other elapid species except for *O. hannah* currently exist (Jin et al., 2007; Zhang et al., 1994). Our results indicate that the cleavage of Aα-chains took place first, followed by the degradation of the Bβ-chains (Fig. 1), which is similar to that seen for Viperidae venoms (Coimbra et al., 2018; Dobson et al., 2017) and lizard venoms (Koludarov et al., 2017). There may be a number of explanations for this observation: either 1) the cleavage site on the Bβ-chain is conformationally obscured by the Aα-chain; 2) the enzymatic affinity for the cleavage is higher for the Aα-chain; and/or 3) several venom enzymes are present in the venom with differing target specificity. To date, two different fibrinogenolytic toxins types have been isolated for *O. Hannah*- the first being a serine-protease capable of cleaving both the Aα- and the Bβ-chain, and the second being a metalloprotease that only cleaves the Aα-chain, leaving the Bβ-chain intact, even after prolonged incubation (Guo et al., 2007; Jin et al., 2007; Zhang et al., 1994).

While fibrinogenolytic enzymes are present in lower amounts in the species studied here relative to the very high levels seen in viper and lizard venoms (Coimbra et al., 2018; Debono et al., 2019; Dobson et al., 2017; Koludarov et al., 2017), the multiple amplifications of this trait seen here is strongly suggestive of positive selection pressures for this functional activity. Further, these toxins may potentiate the local and systemic bleeding seen clinically for some species, particularly for the African spitting species (Warrell et al., 1976; Tilbury, 1982; Rivel et al., 2016; Saaiman and Buys, 2018). Therefore, the pathological significance of fibrinogenolytic SVSPs and SVMPs in elapid venoms should not be overlooked.

In conclusion, this study determined the type and extent of destructive fibrinogenolytic activity across a substantial part of the African and Asian elapid snake clades. Our results provide further insight into the mechanism of anticoagulant venom activity and potency among these species, thereby aiding in filling knowledge gaps in this field. Future research should identify the toxins responsible for the cleavage of fibrinogen in the aforementioned species, determine the relative antivenom efficacy upon these toxins, and elucidate the potential synergistic actions between anticoagulant toxins and pain-inducing cytotoxins.

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Conflicts of interests

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2019.05.026.

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