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Clinical implications of differential antivenom efficacy in neutralising coagulotoxicity produced by venoms from species within the arboreal viperid snake genus *Trimeresurus*

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ABSTRACT

Snake envenomation globally is attributed to an ever-increasing human population encroaching into snake territories. Responsible for many bites in Asia is the widespread genus *Trimeresurus*. While bites lead to haemorrhage, only a few species have had their venoms examined in detail. We found that *Trimeresurus* venom causes haemorrhaging by cleaving fibrinogen in a pseudo-procoagulation manner to produce weak, unstable, short-lived fibrin clots ultimately resulting in an overall anticoagulant effect due to fibrinogen depletion. The monovalent antivenom 'Thai Red Cross Green Pit Viper antivenin', varied in efficacy ranging from excellent neutralisation of *T. albolabris* venom through to *T. gumprechti* and *T. mcgregori* being poorly neutralised and *T. hageni* being unrecognised by the antivenom. While the results showing excellent neutralisation of some non-*T. albolabris* venoms (such as *T. flavomaculaturs*, *T. fucatus*, and *T. macrops*) needs to be confirmed with *in vivo* tests, conversely the antivenom failure *T. hageni,* and the very poor results against *T. gumprechti* and *T. mcgregori,* despite being conducted in the ideal scenario of preincubation of antivenom:venom, indicates that the likelihood of clinically relevant cross-reactivity for these species is low (*T. gumprechti* and *T. mcgregori*) to non-existent (*T. hageni*). These same latter three species were also not inhibited by the serine protease inhibitor AEBSF, suggesting that the toxins leading to a coagulotoxic effect in these species are non-serine proteases while in contrast *T. albolabris* coagulotoxicity was completely impeded by AEBSF, and thus driven by kallikrein-type serine proteases. There was a conspicuous lack of phylogenetic pattern in venom variation, with the most potent venoms (*T. albolabris* and *T. hageni*) being distant to each other on the organismal tree, and with the three most divergent and poorly neutralised venoms (*T. gumprechti, T. hageni,* and *T. mcgregori*) were also not each others closest relatives. This reinforces the paradigm that the fundamental dynamic evolution of venom results in organismal phylogeny being a poor predictor of venom potency or antivenom efficacy. This study provides a robust investigation on the differential venom effects from a wide range of *Trimeresurus* species on coagulation, highlighting differential fibrinogenolytic effects, while also investigating the relative antivenom neutralisation capabilities of the widely available Thai Red Cross Green Pit Viper antivenom. These results therefore have immediate, real-world implications for patients envenomed by *Trimeresurus* species.

1. Introduction

Effective treatment for snake envenomation is becoming increasingly important, with a rise in reported snake bites worldwide (Fry, 2018; Gutiérrez et al., 2017, 2006; Kasturiratne et al., 2008). Human-snake conflicts are increasing due to a myriad of factors including human population expansion and urban development encroaching into snake territories, and an increase in snake activity periods due to climate change (Fry, 2018). In addition, the increase in exotic pets outside of zoological facilities also increases the risk of bites from exotic species of snake, which means that the issue of snakebite is no longer restricted to the developing world (Fry, 2018; Gutiérrez et al., 2017; Kasturiratne et al., 2008). Across Asia there are many species of highly venomous snakes, and populations of humans existing in close contact with these

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species is ever increasing. Pit vipers are responsible for a major proportion of envenomation due to large population densities of people that bring increased agriculture to regions overlapping with the pit viper habitat, coupled with poor snakebite management and education (Alirol et al., 2010; Chippaux, 1998).

Trimeresurus is a genus of pit viper that is widespread across much of Asia. *Trimeresurus albolabris* is a particularly commonly encountered species, and envenomations are considered medically significant with effects ranging from local blistering and necrosis, shock, to spontaneous systemic bleeding, defibrinogenation, thrombocytopenia and leucocytosis (Hutton et al., 1990). As a result, *T. albolabris* envenomation and venom composition has been the centre of much research focus (Chotenimitkhun and Rojnuckarin, 2008; Greene et al., 2017; Hutton et al., 1990; Lin et al., 2009; Muanpasitporn and Rojnuckarin, 2007; Peng et al., 1992; Pradniwat and Rojnuckarin, 2015; Rojnuckarin et al., 1999; Tan et al., 2017, 2012) and is the main *Trimeresurus* species targeted by available antivenoms, such as the monovalent Green Pit Viper Antivenin produced by the Thai Red Cross. However, in many of the regions where *Trimeresurus* species exist, a specific antivenom is lacking (Tan et al., 2017). Moreover, the venom composition and function of most other species within the genus have been comparably neglected, with a few exceptions such as *T. flavomaculatus*, *T. insularis, T. macrops, T. purpureomaculatus,* and *T. stejnegeri* (Clark and Davidson, 1997; Jones et al., 2019; Mitrakul, 1973; Rojnuckarin et al., 1999; Tai et al., 2004; Tan et al., 2017, 1994; Tan, 2010; Visudhiphan et al., 1989; Witharana et al., 2019; Wongtongkam et al., 2005).

Correct treatment for envenomation is crucial for patient care, to reduce both morbidity and mortality rates (Fry, 2018). Having a better understanding of the effects of such species' venom on the coagulation cascade can aid in this knowledge and improve current treatments available. Coagulotoxicity from *Trimeresurus* envenomations results in anticoagulant effects which clinically manifests into haemorrhaging, most commonly attributed to fibrinogenolytic activity, as seen for many pit-vipers (Bell, 1997; Debono et al., 2019a, b; Debono et al., 2018; Kolev and Longstaff, 2016; Longstaff and Kolev, 2015; Mosesson, 2005; Visudhiphan et al., 1989; Wolberg, 2007; Wolberg and Campbell, 2008). Fibrinogenolytic enzymes may act upon fibrinogen either directly induce an anticoagulant effect by destructive cleavage of fibrinogen, or indirectly by aberrant cleavage of fibrinogen, resulting in short-lived, weak clots in a pseudo-procoagulant manner, with both actions leading to a net anticoagulant state due to depletion of the levels of intact fibrinogen (Coimbra et al., 2018; Debono et al., 2019a, b; Debono et al., 2018; Dobson et al., 2018; Esnouf and Tunnah, 1967; Huang et al., 1992; Levy and Del Zoppo, 2006; Nielsen, 2016; Premawardena et al., 1998; Trookman et al., 2009; Zulys et al., 1989). This effect has been demonstrated in many other species of Asian pit viper (Dambisya et al., 1994; Debono et al., 2019a, b; Debono et al., 2018; Levy and Del Zoppo, 2006; Liu et al., 2011; Nielsen, 2016; Nolan et al., 1976; Zulys et al., 1989).

Understanding exactly how these anticoagulant effects arise and persist can drastically improve patient care and thus positive outcomes. Here we investigated the differential coagulotoxic effects of a total of 13 of the 37 known and described *Trimeresurus* species (Alencar et al., 2018, 2016) and ascertained the relative effectiveness of available monovalent Green Pit Viper antivenom in neutralising fibrinogenolytic actions for each species. Note: we follow Alencar et al., 2016 in considering *Trimeresurus* as the genus name for all species and do not subscribe to proposed division into several genera as proposed by Malhotra and Thorpe (2004), as the more inclusive definition of *Trimeresurus* renders these snakes as a well-defined monophyletic clade sharing ecological, evolutionary, and morphological features distinctive of these speices relative to all other Asian pit vipers. These venoms are evolving under unique selection pressures due to the species being unique amongst Asian pit vipers in being arboreal specialists and thus there is a high chance of prey escape. These venoms may also be diversification hot spots due to limited gene flow as a consequence of arboreality. Thus there may be real world implications of such venom variation for the envenomed patient as a consequence of differential efficacy by available antivenoms.

2. Methods

2.1. Venoms

A total of 13 venoms from the Asian pit viper genus *Trimeresurus* were investigated for their coagulotoxic effects (*T. albolabris, T. borneensis, T. flavomaculatus, T. fucatus, T. gumprechti, T. hageni, T. macrops, T. mcgregori, T. popeiorum, T. puniceus, T. purpureomaculatus, T. trigonocephalus, T. vogeli*), which were obtained from the long-term cryogenic collection of the Venom Evolution Lab. Pooled (N = 3 adult males for each), lyophilized venom samples were resuspended in deionized H_2O and protein concentrations (mg/ml) were determined using a ThermoFisher Scientific Nanodrop™ 2000c Spectrophotometer. Working stocks of 50% deionized water/50% glycerol (> 99%, Sigma-Alrich) for all venoms were prepared at 1 mg/ml and stored at −20 °C to preserve enzymatic activity and reduce enzyme degradation. All venom and plasma work was undertaken under the University of Queensland Biosafety Approval #IBC134BSBS2015.

2.2. Coagulation analyses on plasma and fibrinogen

The ability of the *Trimeresurus* venoms to affect clotting of plasma and/or fibrinogen was investigated using a Stago STA-R Max coagulation analyser as previously described (Debono et al., 2019a,b; Debono et al., 2018, 2017). Human plasma (3.2% citrate) was supplied by the Red Cross Blood Service (under Research Supply Agreement 18-03QLD-09), and all work was undertaken under University of Queensland Biosafety Approval #IBC134BSBS2015 (1/1/15) and Human Ethics Approval # 2016000256 (1/6/16). Human fibrinogen (4 mg/ml, Lot#F3879, Sigma Aldrich) was reconstituted to a concentration of 1 mg/ml in 150 mM $NaCl_2$, 50 mm Tris, pH 7.3, flash frozen in liquid nitrogen and stored at −80 °C until further use. Coagulopathic toxin effects were measured by a modified procoagulant protocol on a Stago STA-R Max coagulation robot (France) using Stago Analyser software v0.00.04 (Stago, Asniéres sur Seine, France). Plasma clotting baseline parameters were determined by performing the standardised activated Partial Thromboplastin Time (aPTT) assay (TriniCLOT APTT HS, Stago). In order to determine clotting times effected by the addition of varying venom concentrations, a modified clotting test was developed, in which 50 µl of venom (20 – 0.05 µg/ml) dilutions in STA Owren Koller Buffer (Stago Cat# 00360), 50 µLCaCl_2 (25 mM stock solution Stago Cat# 00367 STA), and 50 µl phospholipid (solubilized in Owren Koller Buffer adapted from STA C.K Prest standard kit, Stago Cat# 00597) in a final volume of 175 µl in Owren Koller Buffer was incubated for 120 s at 37 °C before adding 75 µl of human plasma or human fibrinogen (1.2 mg/ml final). Relative clotting was then monitored for 999 s or until plasma clotted (whichever was sooner). The calcium or phospholipid dependence was assessed by replacing calcium or phospholipid for Owren Koller Buffer, respectively. Inhibition of venom serine protease activity was determined using 4-benzenesulfonyl fluoride hydrochloride (AEBSF), a known serine protease inhibitor (Kuniyoshi et al., 2017; Torres-Bonilla et al., 2018; Xin et al., 2009; Yamashita et al., 2014) in the modified clotting assay. For this purpose, 25 µl of (diluted) venom, 50 µl $CaCl_2$ (5 mM final), 50 µl phospholipid, and 50 µl AEBSF (2 mM final) were incubated for 2 min at 37 °C before adding 75 µl of human fibrinogen (1.2 mg/ml final). As a control, fibrinogen clotting was measured using thrombin (stable thrombin from Stago Liquid Fib kit, unknown concentration from supplier (Stago Cat#115081 Liquid Fib)).

Change in Area Under the Curve (AUC) was used as a statistical representation to relative influence of cofactors (calcium or phospholipid) or the impact of antivenom. An increase in the AUC represents

Table 1
Inhibition assays.

FXa inhibition assay	Step 1. 50 µl 0.1 µg/ml venom (1 mg/ml 50% glycerol stock diluted with OK buffer) +50 µl 0.025 M calcium (Stago catalog # 00367) +50 µl phospholipid (Stago catalog #00597) +25 µl FXa (Stago catalog # 00311). Step 2. 120 s incubation. Step 3. Addition of 75 µl plasma.
Prothombinase complex inhibition assay	Step 1. 50 µl 0.1 µg/ml venom (1 mg/ml 50% glycerol stock diluted with OK buffer) +50 µl 0.025 M calcium +50 µl phospholipid +75 µl plasma Step 2. 120 s incubation. Step 3. Addition of 25 µl Factor Xa
FIXa inhibition assay	Step 1. 25 µl 0.1 µg/ml venom (2 mg/ml 50% glycerol stock diluted with OK buffer) +75 µl 0.025 M calcium (2:1 ratio of $CaCl_2$ + OK buffer) +50 µl phospholipid + 25 µl FIXa (Haematological technologies Cat# HCIXA-0050) Step 2. 120 s incubation. Step 3. Addition of 75 µl plasma
Thrombin inhibition assay	Step 1. 50 µl 0.1 µg/ml venom (1 mg/ml 50% glycerol stock diluted with OK buffer) +50 µl 0.025 M calcium +50 µl phospholipid +25 µl thrombin (Stago catalog # 00611). Step 2. 120 sec incubation. Step 3. Addition of 75 µl 4 mg/ml fibrinogen.

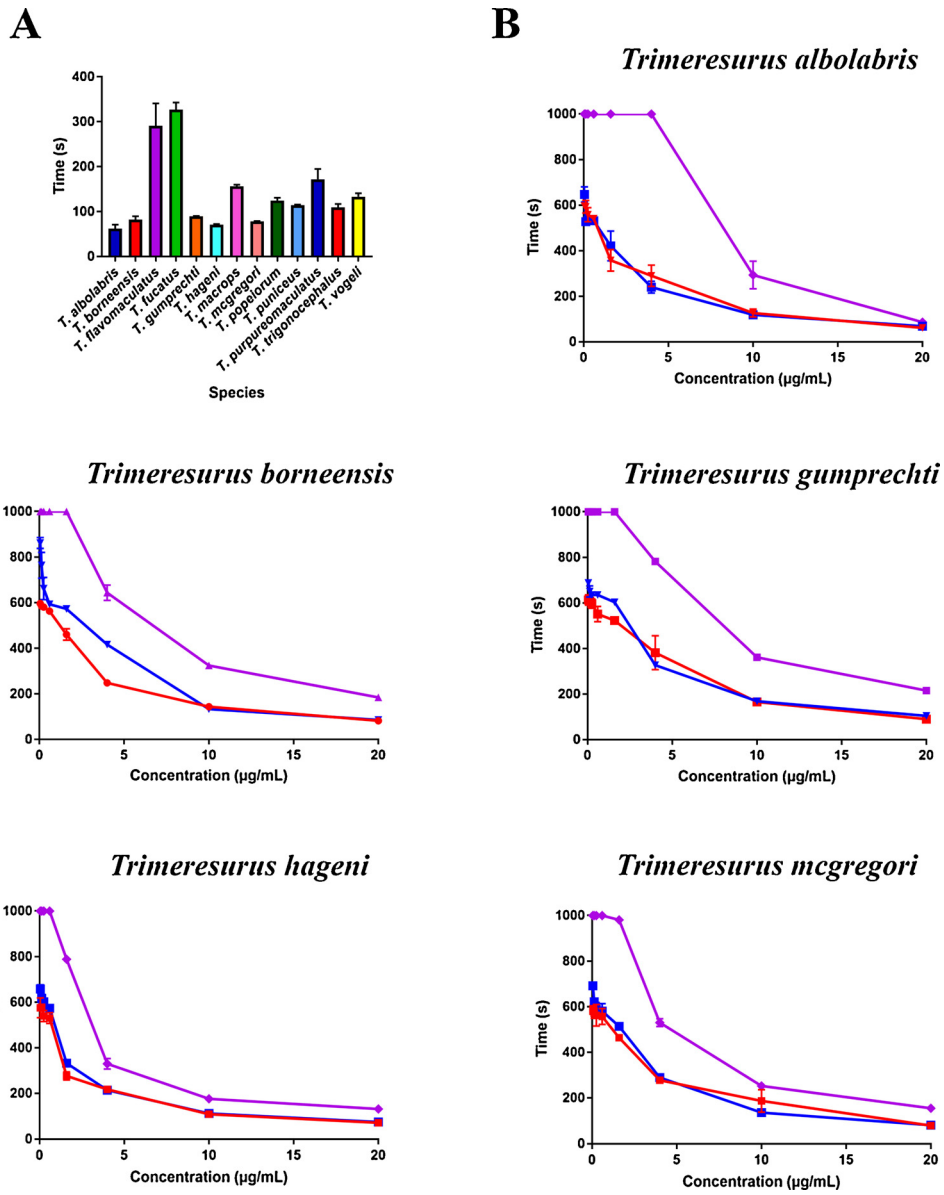

Fig. 1. A) Initial 20 µg/mL venom concentration clotting times on human plasma for all species included in study. B) 8-point dose-response curves of human plasma clotting activity with both calcium and phospholipid (red line), with just calcium (blue line), and with just phospholipid (purple line) for *T. albolabris, T. borneensis, T. gumprechti, T. hageni* and *T. mcgregori.* Clot time was measured until plasma had clotted for machine maximum of 999.99 s was reached. Y-axis = Clotting time (secs). Data points are N = 3 with standard deviations.

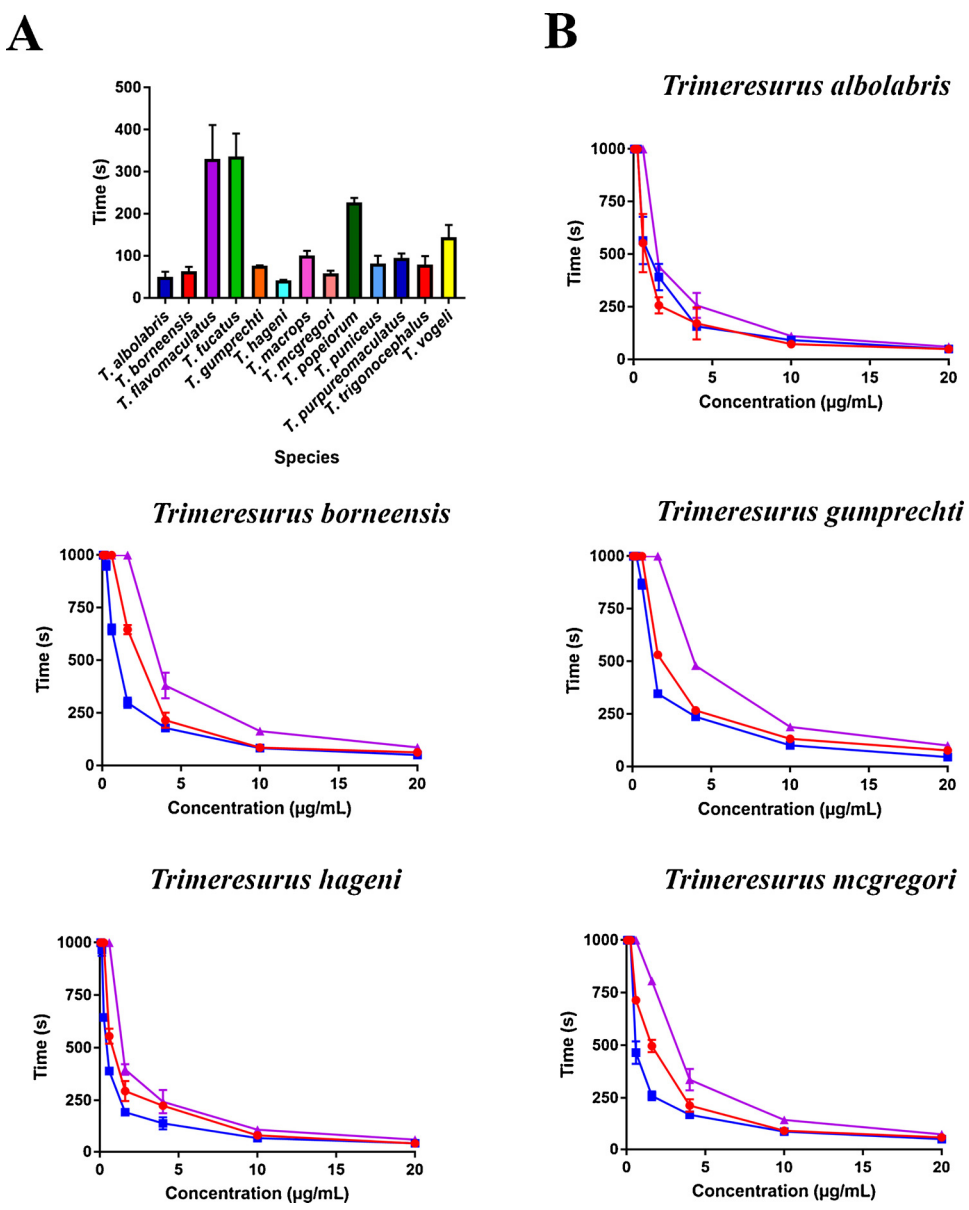

Fig. 2. A) Initial 20 µg/mL venom concentration clotting times on human fibrinogen for all species included in study. B) 8-point dose-response curves of human fibrinogen clotting activity with both calcium and phospholipid (red line), with just calcium (blue line), and with just phospholipid (purple line) for *T. albolabris, T. borneensis, T. gumprechti, T. hageni* and *T. mcgregori.* Clot time was measured until fibrinogen had clotted for machine maximum of 999.99 s was reached. Y-axis = Clotting time (secs). Data points are N = 3 with standard deviations.

longer clotting times compared to the optimum conditions (control). AUC values were calculated using the program GraphPad PRISM 8.0 (GraphPad Prism Inc., La Jolla, CA, USA) using the following formula: ΔX*(Y1 + Y2)/2. Each experimental condition AUC was divided by control AUC for each venom, and then 1 subtracted so that if there was no change, then this would have been a value of zero. The results were then multiplied by 100 in order to present them as % change.

2.3. Inhibition of FIXa, FXa or thrombin

Additional studies in which the inhibition of serine proteases FXa, prothrombinase complex formation, thrombin, or FIXa (working stock of 0.17 mg/ml so that 5 µg/ml of FIXa is in end concentration, Haematological technologies Cat# HCIXA-0050, Essex Junction VT USA), Table 1) by *Trimeresurus* venoms were performed using previously validated methods (Table 1) (Debono et al., 2019a, b; Debono et al., 2018; Youngman et al., 2018). All species were subjected to these assays. To identify the possible target in the clotting cascade which the venom was acting upon to result in the anticoagulant activity, either plasma or individual factors of the clotting cascade were incubated with the sample venom as the incubation step allows venom to bind and inhibit its target. Data was analysed calculating a shift away from the control for each assay using GraphPad PRISM 8.0 (GraphPad Prism Inc., La Jolla, CA, USA) as described above.

2.4. Thromboelastography analyses on plasma and fibrinogen

Venoms were investigated for their ability to clot or impede clotting of plasma and fibrinogen using a Thrombelastogram® 5000 Haemostasis analyser (Haemonetics®, Haemonetics Australia Pty Ltd, North Rdye, Sydney 2113, Australia) as previously described (Coimbra et al., 2018; Debono et al., 2019a, b; Debono et al., 2018; Oulion et al., 2018). Human fibrinogen (4 mg/ml,) was reconstituted in enzyme buffer (150 mM NaCl and 50 mM Tri-HCl (pH 7.3)). Briefly, 7 µl venom

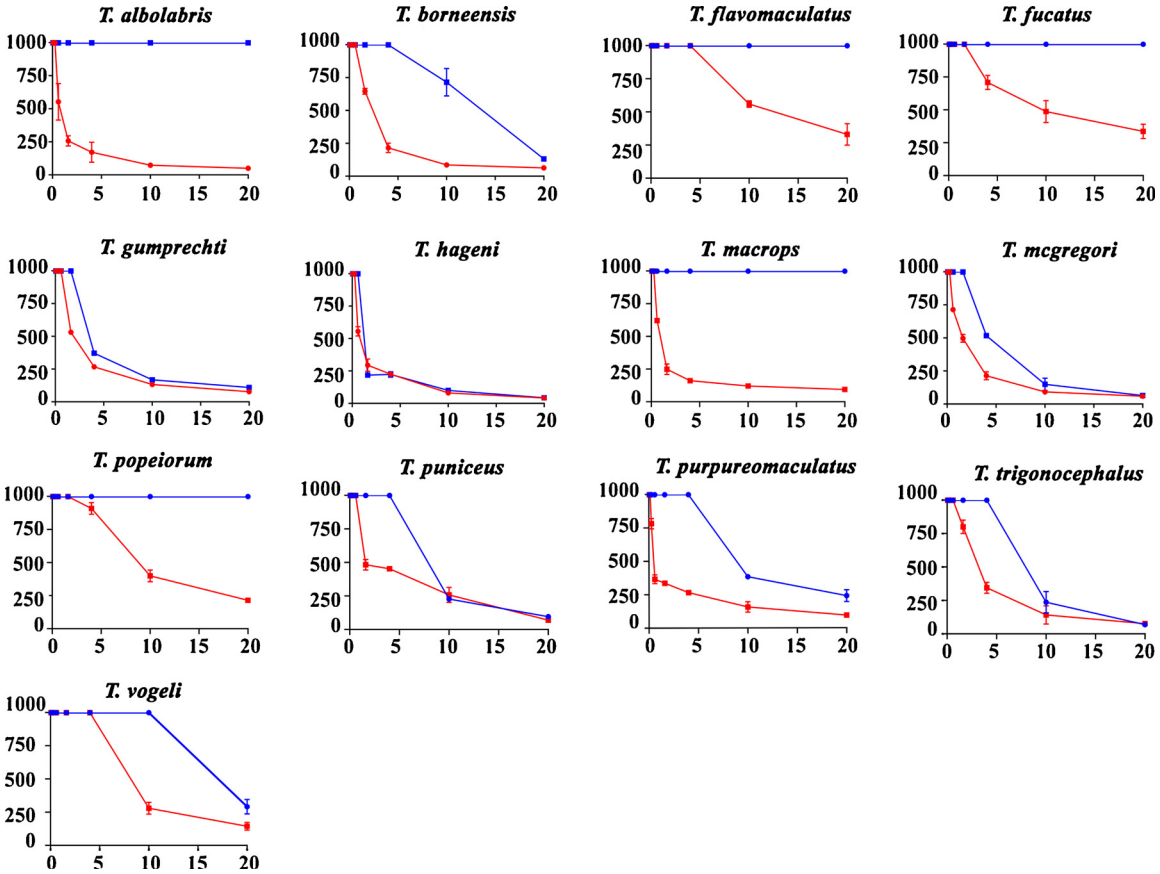

Fig. 3. Comparison of relative Thai Red Cross Green Pit Viper antivenom cross neutralisation across various species of *Trimeresurus* on fibrinogen (1.2 mg/mL final concentration). Fibrinogen + venom clotting curve = red line, venom + antivenom clotting curve = blue line. Clot time was measured until fibrinogen had clotted for machine maximum of 999.99 s was reached. X axis: final venom concentration (μg/mL), Y axis: clotting time in seconds. Values are averages of triplicates (single dilution measured three times) and standard deviation error bars are shown for each, although for most the error range is smaller than the line icon.

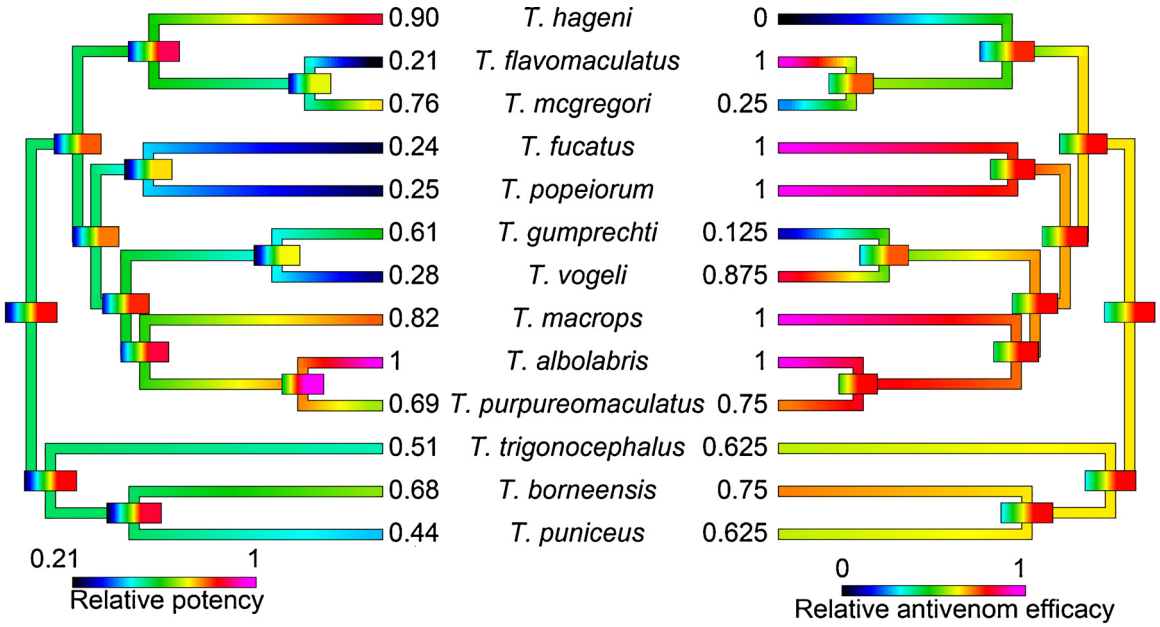

Fig. 4. Ancestral state reconstruction of relative fibrinogen pseudo-procoagulant clotting potency of *Trimeresurus* venoms and the relative Green Pit Viper antivenom neutralisation efficacy. Horizontal bars indicate 95% confidence intervals for the estimate at each node. Note: due to the dynamic nature of venoms, the bars rapidly become broad as one moves down the tree. PGLS analysis showed no statistical correlation between potency and antivenom efficacy ($p = 0.1751$). Organismal phylogeny was based upon (Alencar et al., 2016; Malhotra and Thorpe, 2004).

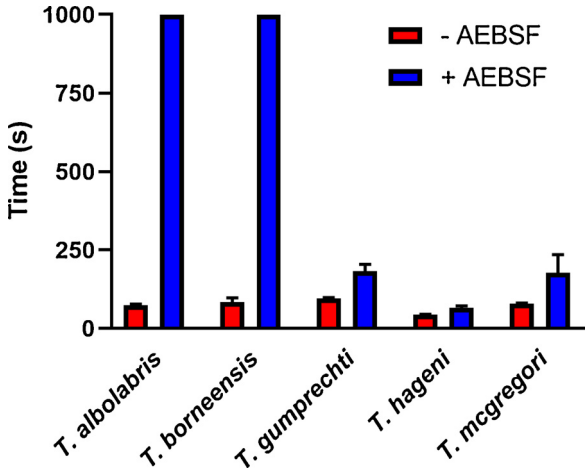

Fig. 5. Inhibition of SVSPs by AEBSF (2 mM) against 20 µg venom from pseudo-procoagulant species performed on a Stago STA R Max. Clotting time was measured against 4 mg human fibrinogen with the addition of cofactors calcium and phospholipid. Red columns = venom without 2 mM (end concentration) inhibitor, blue columns = venom with 2 mM (end concentration) inhibitor incubated together for 20 min. at 37 °C. Clot time was measured until fibrinogen had clotted for machine maximum of 999.99 s was reached. Y axis = clot time (s), X axis = venom species. N = 3.

working stock (1 mg/ml) or 7 µl thrombin as a positive control (stable thrombin from the Stago Liquid Fib kit, unknown concentration from supplier, (Stago Cat#115081 Liquid Fib)), 72 µl $CaCl_2$ (25 mM stock solution, Stago Cat# 00367 STA), 72 µl phospholipid (solubilized in Owren Koller Buffer adapted from STA C.K Prest standard kit, Stago Cat# 00597), and 20 µl Owren Koller Buffer (Stago Cat# 00360) was combined with 189 µl fibrinogen or human plasma and run immediately for 30 min. to allow for ample time for clot formation. An additional positive control of 7 µl Factor Xa (unknown concentration from supplier, Liquid Anti-Xa FXa Cat#253047, Stago) was also incorporated for plasma only. If no clot was formed for a venom in the fibrinogen assay by 30 min, 7 µl thrombin (stable thrombin from Stago Liquid Fib kit, unknown concentration from supplier (Stago Cat#115081 Liquid Fib)) was added to ascertain if destructive cleavage of fibrinogen had occurred due to the venom.

Parameters obtained from thromboelastography analysis were: SP = split point, time taken until clot begins to form (mins); R = time to initial clot formation where formation is 2 mm+ (mins); A = amplitude of detectable clot (mm); MRTGG = maximum rate of thrombus generation (dsc, dynes/cm^2/s); TMRTG = time to maximum rate of thrombus generation (min); and TGG = total thrombus generation (dynes/cm^2).

2.5. Fibrinogenolysis analysis

Fibrinogenolytic activity was assessed as previously described (Debono et al., 2019a, b; Debono et al., 2018). In brief, fibrinogen (1 mg/ml reaction concentration, 150 mM NaCl / 50 mM Tri-HCl (pH 7.3), Lot#F3879, Sigma Aldrich, St. Louis, Missouri, United States) was incubated with venom (10 µg/ml reaction concentration) for 60 min. at 37 °C, and fibrinogen cleavage was assessed at several time points (1, 5, 10, 15, 20, 30, 45 and 60 min.) using SDS-PAGE analysis under reducing conditions followed by staining with Coomassie Brilliant Blue R-250. The visualized protein fragments were quantified using ImageJ software (V1.51 r, Java 1.6.0_24, National Institutes of Health, Bethesda, Maryland, USA) and analysed using GraphPad PRISM 7.0 (GraphPad Prism Inc., La Jolla, CA, USA).

2.6. Fibrinolysis

The ability of venoms to actively lyse clots was investigated following methods described previously (Debono et al., 2019a,b; Debono et al., 2018). Varying concentrations of venom (1–0.1 µg/µl) were tested either with or without the addition of tissue plasminogen activator (tPA, Sekisui Diagnostics, Lexington, MA, USA). Briefly, tissue factor (TF, Innovin, Siemens, USA) and phospholipid vesicles (PCPS, 75% phosphatidylcholine and 25% phosphatidylserine, Avanti Polar Lipids, Alabama, USA) were incubated at 37 °C for 1 h in HEPES buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, 0.1% BSA (Bovine Serum

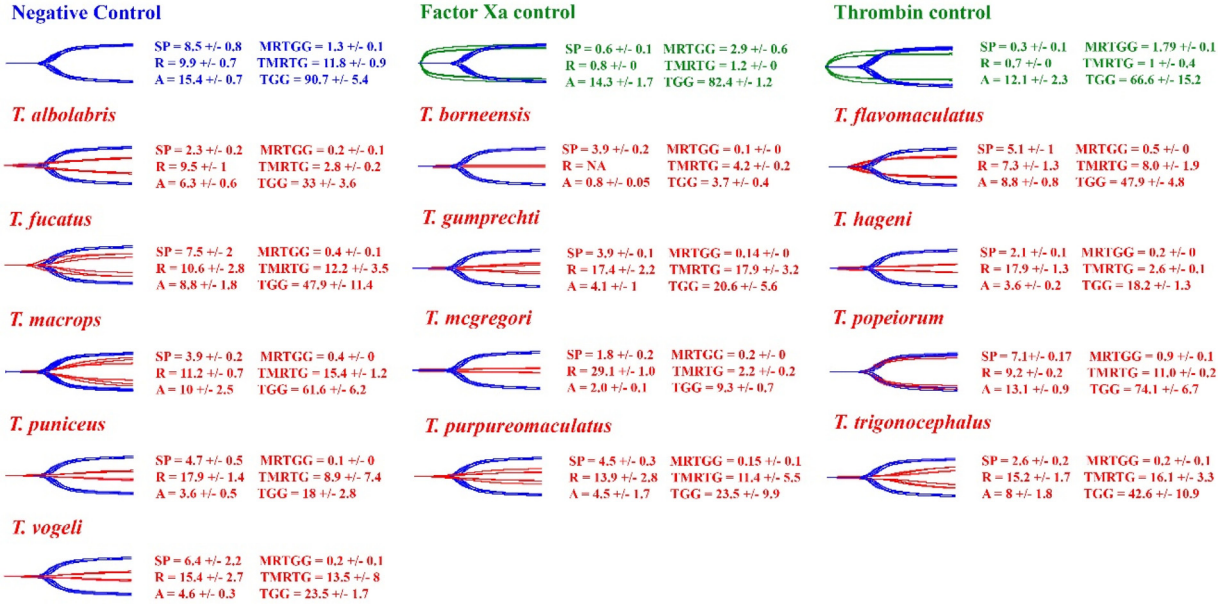

Fig. 6. Overlaid thromboelastography traces showing effects of venoms ability to clot plasma relative to spontaneous clot control where species cleave plasma in a clotting manner to form weak clots. Blue traces = spontaneous clot controls, green traces = thrombin induced clot or Factor Xa induced clot, red traces = samples. SP = split point, time taken until clot begins to form (mins). R = time to initial clot formation where formation is 2mm+ (mins). A = amplitude of detectable clot (mm). MRTGG = maximum rate of thrombus generation (dsc, dynes/cm^2/s). TMRTG = time to maximum rate of thrombus generation (min). TGG = total thrombus generation (dynes/cm^2). Overlaid traces are N = 3 for each set of control or experimental conditions. Values are N = 3 means and standard deviation.

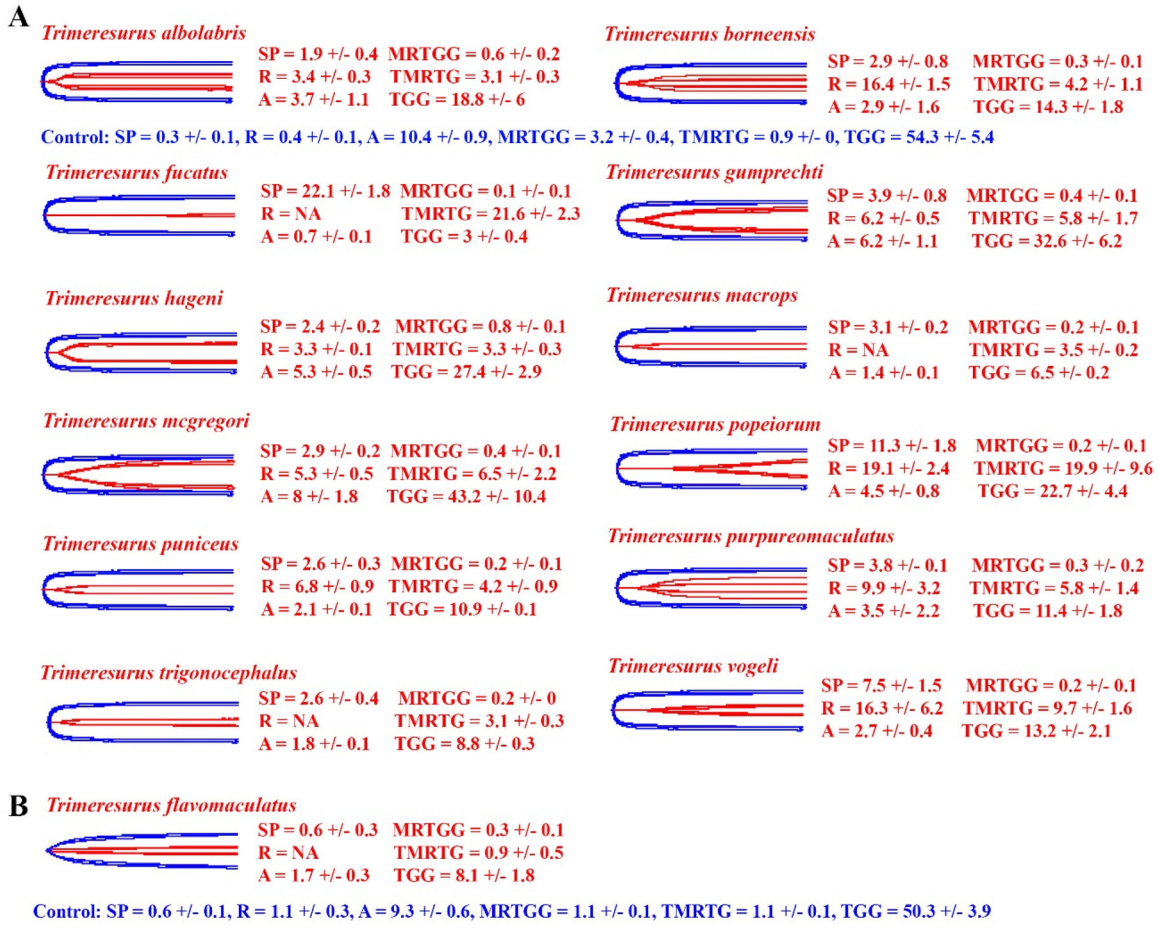

Fig. 7. Overlaid thromboelastography traces showing effects of venoms A) ability to clot fibrinogen relative to thrombin control; or B) test for the ability to degrade fibrinogen for species which did not clot in (A) whereby thrombin was added at the end of the 30 min runs to test for intact fibrinogen. Blue traces = thrombin clot controls, red traces = samples. SP = split point, time taken until clot begins to form (mins). R = time to initial clot formation where formation is 2mm+ (mins). A = amplitude of detectable clot (mm). MRTGG = maximum rate of thrombus generation (dsc, dynes/cm^2/s). TMRTG = time to maximum rate of thrombus generation (min). TGG = total thrombus generation (dynes/cm^2). Overlaid traces are N = 3 for each set of control or experimental conditions. Values are N = 3 means and standard deviation.

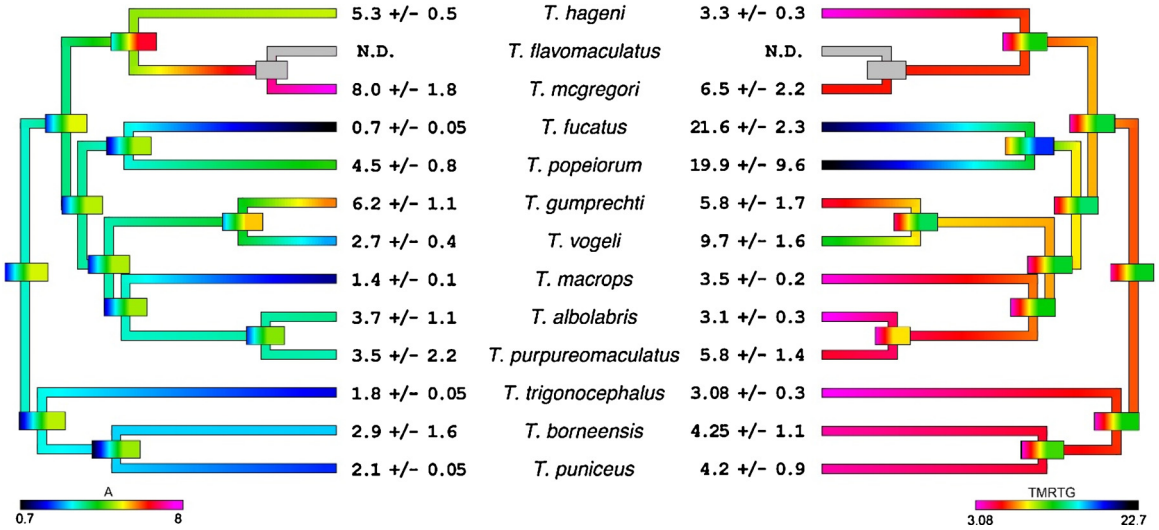

Fig. 8. Ancestral state reconstructions of sampled *Trimeresurus* species tested for their ability to clot fibrinogen using thromboelastography (Fig. 7). Heat map illustrates the relationship between clot strength (amplitude (A)) *versus* speed of action (Time to Maximum Rate of Thrombus Generation (min) (TMRTG)). Cooler colours are smaller amplitudes and longer time taken to generate thrombus, while warmer colours are larger amplitudes and shorter time taken to generate thrombus. PGLS analysis showed no statistical correlation between speed of reaction and clot size formed on fibrinogen (p = 0.3753). Values are N = 3 means and standard deviation. Organismal phylogeny was based upon (Alencar et al., 2016; Malhotra and Thorpe, 2004). *T. flavomaculatus* is in gray because it did not clot fibrinogen within the assay time period.

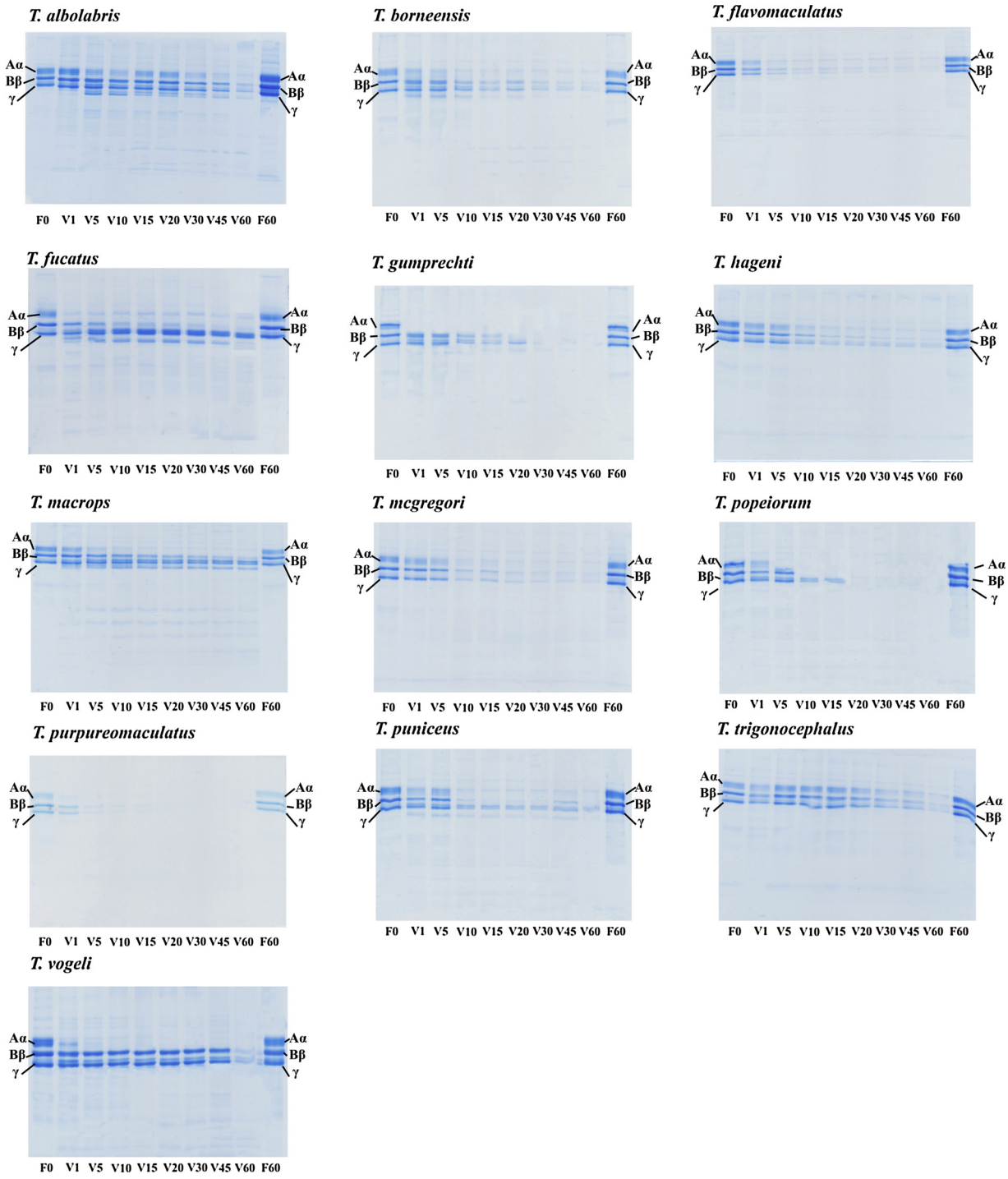

Fig. 9. 1D SDS PAGE time dependent fibrinogen chain degradation (α, β or γ) by venom at 0.1 μg/μL concentration at 37 °C over 60 min. F = fibrinogen at 0 min. or 60 min. incubation controls, V = venom at 1, 5… 60 min incubation.

Albumin). To the TF/PCPS mixture (1.8 pM/3 μM final), CaCl2 (17 mM final), tPA (37.5 units/ml reaction concentration, diluted in 20 mM HEPES, 150 mM NaCl, 0.1% PEG-8000, pH 7.5), venom (50% v/v), and plasma (50% v/v, prewarmed at 37 °C) were added. The fibrin clot formation and the subsequent lysis was monitored by measuring the absorbance at 405 nm for every 30 s. during 3 h at 37 °C in a SpectraMax M2e microplate reader. The onset of clot formation was defined as the time point at which the turbidity increased (delta absorbance > 0.04); the clotting time was the time from the start of the assay to the onset of clot formation. The clot lysis time was the interval between the clear to turbid transition (defined as the midpoint between the onset of clot formation and the maximum turbidity) and the turbid to clear transition; the latter was determined by a sigmoidal fit of the turbidity plots using GraphPad PRISM 8.0 (GraphPad Prism Inc., La Jolla, CA, USA).

2.7. Antivenom efficacy assessment

The monovalent antivenom effects on all 13 *Trimeresurus* crude venoms was investigated using fibrinogen clotting assays as described above. The monovalent Green Pit Viper Antivenin (*Trimeresurus albolabris*) (Lot #TA00119, Expiry date: 15th Jan 2024) was purchased

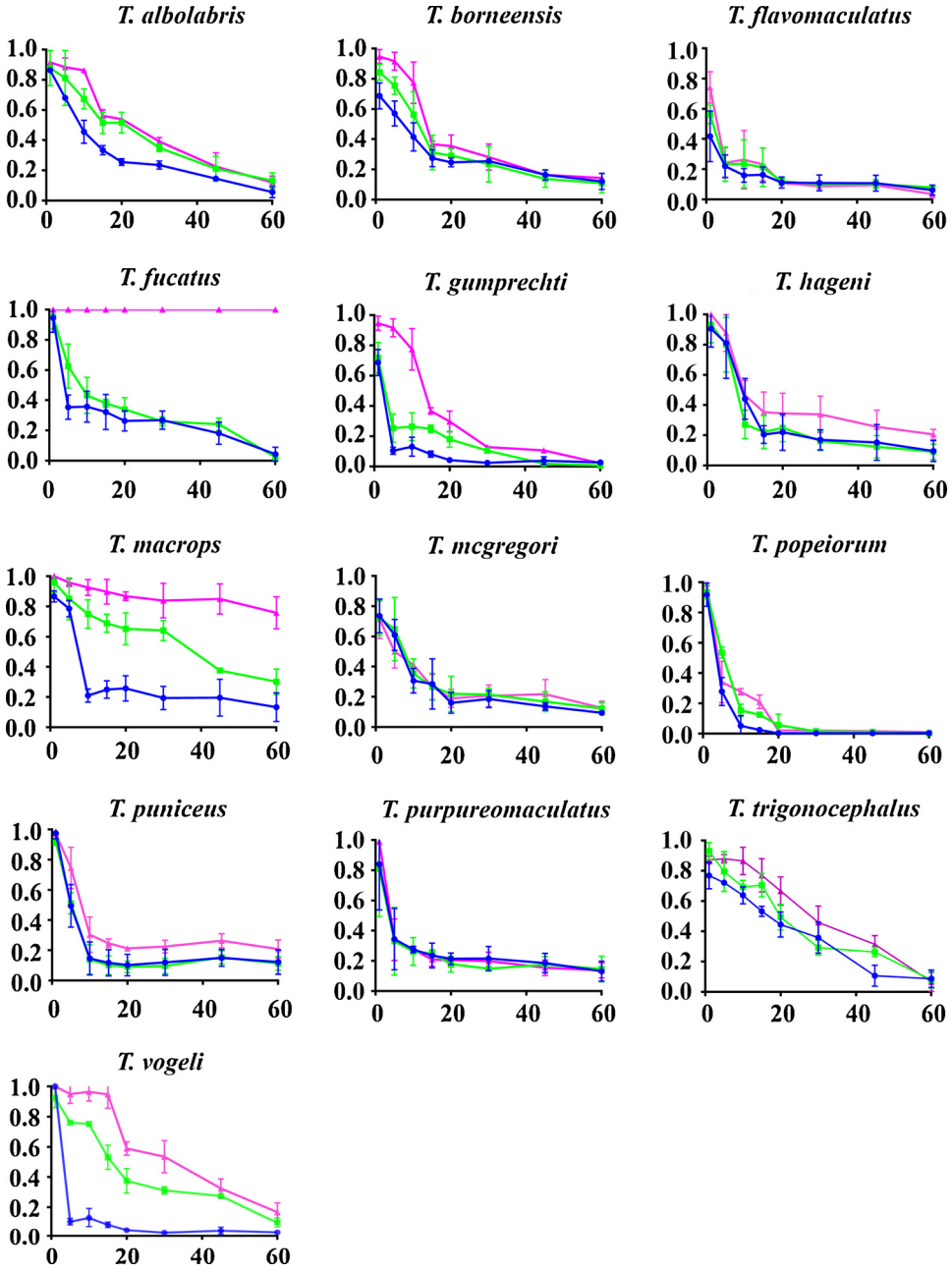

Fig. 10. Relative cleavage of alpha (blue), beta (green) or gamma (pink) chains of fibrinogen. X-axis is time (min), y-axis is percentage of intact chain remaining. Error bars indicate standard deviation and N = 3 means.

from Queen Saovabha Memorial Institute, The Thai Red Cross Society, Bangkok, Thailand. One vial of dry antivenom was reconstituted in 10 ml provided saline solution (using manufacturers guide) and centrifuged at 12000 rpm on an Allegra™ X-22R Centrifuge (Lot#982501, Beckman Coulter, Brea, CA, USA) for 10 min at 4 °C, upon which the supernatant was removed, filtered (0.45 µm Econofltr PES, Agilent Technologies, Beijing, China), aliquoted, and stored at 4 °C. For each experiment, a working stock of 5% antivenom and 95% Owren Koller Buffer was prepared, and the final antivenom concentration for each reaction was 0.5%. Briefly, 25 µl of antivenom diluted in STA Owren Koller Buffer, 50 µl of venom (20 – 0.05 µg/ml) dilutions in STA Owren Koller Buffer, 50 µl $CaCl_2$ (5 mM final), and 50 µl phospholipid in a final volume of 175 µl in Owren Koller Buffer was incubated for 120 s at 37 °C before adding 75 µl of human fibrinogen (1.2 mg/ml final). Relative clotting was then monitored for 999 s or until plasma clotted (whichever was sooner). Note that antivenom does not clot fibrinogen and that a control was performed to rule out any additional effects antivenom has on fibrinogen, in which antivenom was substituted into the above outlined protocol in replacement of a venom sample.

2.8. Phylogenetic comparative analyses

The phylogenetic tree used was based upon a previously published species tree (Alencar et al., 2016; Malhotra and Thorpe, 2004) 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 for all traits using maximum likelihood as implemented in the contMap function of the R package phytools (Revell, 2012). As in previous studies with these methods (Lister et al., 2017; Rogalski et al., 2017), we used the phytools script shown in Supplementary File 1. We used Phylogenetic Generalized Least Squares (PGLS) models (Symonds et al., 2014) in caper (Orme et al., 2013) to

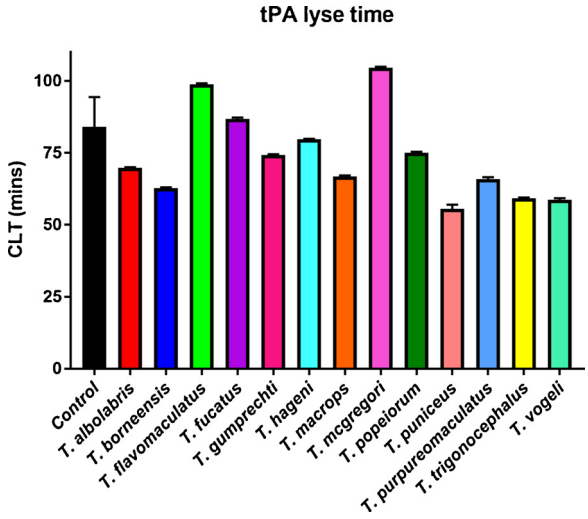

Fig. 11. Clot Lysis Time (CLT) for each species in the presence of tPA at 500 ng/μL venom concentrations (other concentrations not shown). Control is indicated in black. Columns are averages of triplicates and error bars given for each. Bars which are lower than the control lysed the plasma clot quicker than under normal tPA clot lyse conditions.

test for relationships. Phytools and PGLS scripts are detailed in Supplementary File S1.

3. Results

3.1. Coagulation analyses on plasma

The ability of the *Trimeresurus* venoms to affect clot formation of human plasma was investigated. Using an initial concentration of 20 μg/ml venom, all the venoms displayed the ability to clot plasma quicker than the negative control (spontaneous clotting of recalcified plasma: 584 ± 35 s) with five species being particularly potent: *T. albolabris* (62 ± 9 s), *T. borneensis* (82 ± 8 s), *T. gumprechti* (90 ± 1 s), *T. hageni* (71 ± 2 s), and *T. mcgregori* (78 ± 1 s) (Fig. 1A). For these five venoms, their relative dependency on the cofactors calcium and phospholipid was assessed in further detail (Fig. 1B–F). A pronounced shift in the area under the curve (AUC) was observed for the clotting activity obtained in the absence of calcium (AUC shift of 172 ± 0% for *T. albolabris,* 75 ± 11% for *T. borneensis,* 102 ± 1% for *T. gumprechti,* 75 ± 0% for *T. hageni,* and 85 ± 1% for *T. mcgregori*). In contrast, the absence of phospholipid was negligible for most species (AUC shift of 4 ± 2% for *T. albolabris*, 4 ± 2% for *T. gumprechti,* 5 ± 0% for *T. hageni,* and 6 ± 5% for *T. mcgregori*), except for *T. borneensis* (22 ± 1%).

3.2. Coagulation analyses on fibrinogen

Consistent with the effects on whole plasma, all venoms displayed the ability to clot fibrinogen (Figs. 2A and 3). To gain further insight into the biochemical mechanisms by which the five most potent *Trimeresurus* venoms (*T. albolabris, T. borneensis, T. gumprechti, T. hageni* and *T. mcgregori*) induce fibrin clot formation in plasma, an assessment of the cofactor-dependent fibrinogen clotting activity was undertaken (Fig. 2B–F). Again, the venoms were most dependent upon calcium (AUC shift of 32 ± 0% for *T. albolabris,* 104 ± 4% for *T. borneensis,* 89 ± 0% for *T. gumprechti,* 77 ± 0% for *T. hageni,* and 92 ± 0% for *T. mcgregori*) than phospholipid (AUC shift of 12 ± 7% for *T. albolabris,* 35 ± 2% for *T. borneensis,* 26 ± 1% for *T. gumprechti,* 38 ± 1% for *T. hageni,* and 30 ± 0% for *T. mcgregori*; Fig. 2B–F), corroborating our findings obtained for plasma clotting.

Monovalent antivenom from *T. albolabris* (Thai Red Cross Green Pit viper antivenin) was tested against all 13 *Trimeresurus* species included in this study for its neutralising capabilities on fibrinogen clotting. *Trimeresurus albolabris* was completely neutralised even at the highest venom concentrations, as too were *T. flavomaculatus*, *T. fucatus*, *T. macrops* and *T. popeiorum*. Other species were variably neutralised by the antivenom, with *T. gumprechti* and *T. mcgregori* particularly poorly and *T. hageni* virtually non-existently (Fig. 3). There was no phylogenetic signal of antivenom efficacy such as *T. purpureomaculatus*, the closest relative of *T. albolabris,* much less well neutralised than *T. albolabris*. Similarly, *T. flavomaculatus* and *T. mcgregori*, two species who are each other's closest relatives, were diametrically opposed in their relative neutralisation (Fig. 4). There was also no correlation between potency and antivenom efficacy (PGLS: $p = 0.1751$), with the most potent venom (*T. albolabris*) neutralised the best, while the least neutralised venoms (*T. hageni*) was also one of the most potent venoms (Fig. 4).

To ascertain the relative contribution of snake venom serine proteases (SVSPs) to fibrinogen clotting, the effect of AEBSF (a well-characterised serine protease inhibitor (Torres-Bonilla et al., 2018; Xin et al., 2009; Yamashita et al., 2014)) was investigated. The fibrinogen clotting activity of both *T. albolabris* and *T. borneensis* venom was completely inhibited by incubation with 2 mM AEBSF, indicated by an undetectable fibrinogen clotting time in the presence of the inhibitor (> 999 s; Fig. 5). These observations suggest that SVSPs are responsible for the coagulotoxic effects upon fibrinogen for *T. albolabris* and *T. borneensis* venoms, On the other hand, the fibrinogen clotting activity of the *T. gumprechti*, *T. hageni*, and *T. mcgregori* venoms was only partially inhibited upon AEBSF incubation (Fig. 5). These observations suggest that SVSPs are only partially contributing to the coagulotoxic effects upon fibrinogen by the *T. gumprechti*, *T. hageni*, and *T. mcgregori* venoms and that metalloproteases are responsible for the majority of the fibrinogen clotting effects of these venoms. These three venoms were also the most poorly neutralised by the antivenom, which is consistent with the differential biochemistry underlying the same fibrinogen clotting function.

3.3. Thromboelastography (plasma)

The clot strength of venom-induced clotted plasma was investigated in the presence of calcium and phospholipid using thromboelastography (Fig. 6). While the clot strength and speed of action varied between species, all clots produced were extremely small and weak relative to the control plasma clots, and therefore the action is pseudo-procoagulant, not a true procoagulant activity. However, *T. popoeirum* venom was the only species that did not clot the plasma directly, producing a trace very similar to that of the spontaneous control clot, indicative of actions upon clotting enzymes in addition to actions upon fibrinogen.

3.4. Thromboelastography (fibrinogen)

Next, the direct clotting effects of the *Trimeresurus* venoms upon fibrinogen were assessed, again using thromboelastography in the presence of calcium and phospholipid (Fig. 7). As with the plasma tests, the results revealed significant variation between the venom-induced clots in both speed of action and the relative clot strength. Consistent with the previous observations on plasma clot strength, all clots formed were weak relative to the thrombin-induced control. All *Trimeresurus* species tested (with the exception of *T. flavomaculatus*) clotted fibrinogen within the initial 30 min in a pseudo-procoagulant manner indicated by a reduction in clot strength relative to the thrombin control. *Trimeresurus popoeirum* was very slow in the action upon fibrinogen, which is consistent with its inability to clot plasma before spontaneous plasma clotting occurred (see section above). In the case of *T. flavomaculatus,* which did not display any ability to directly clot fibrinogen within the initial 30 min, thrombin was subsequently added

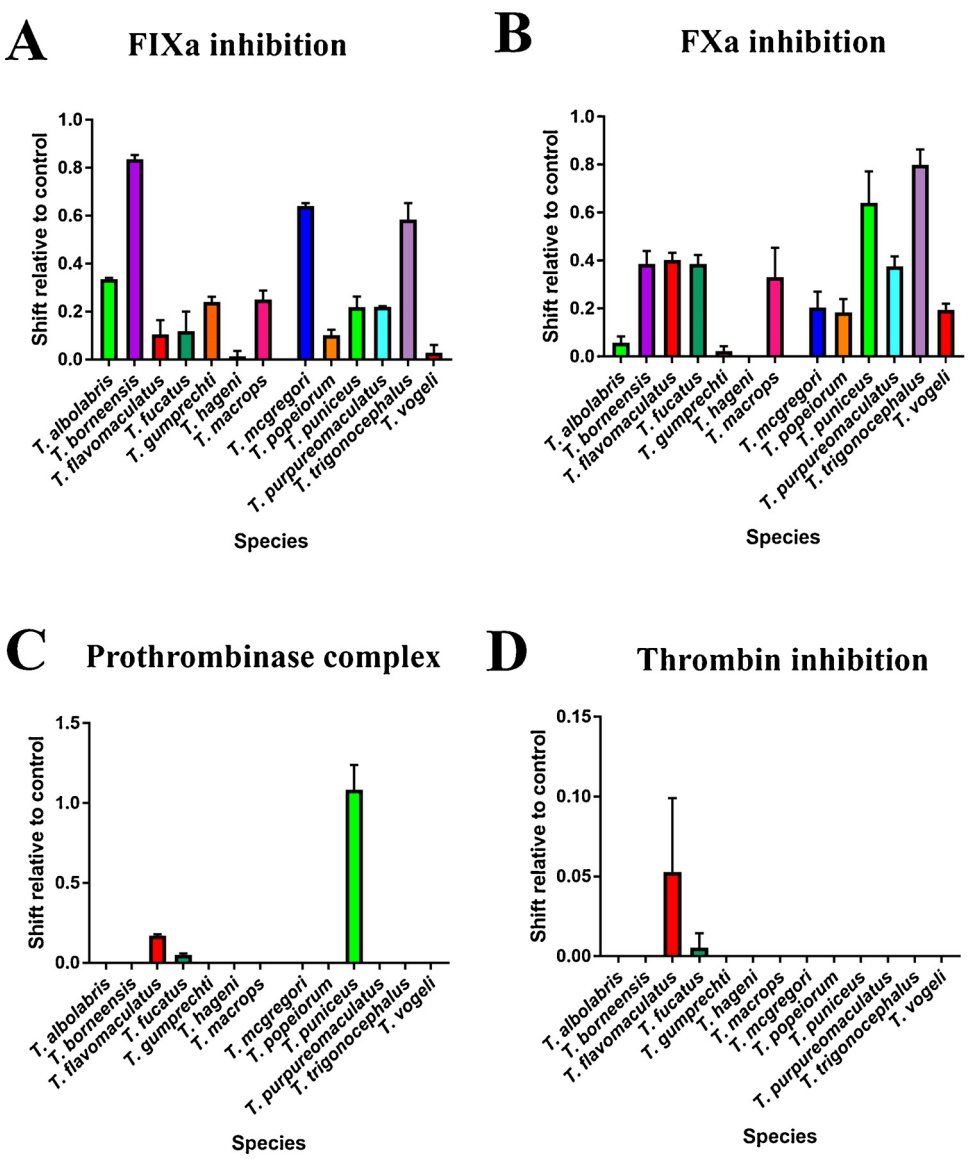

Fig. 12. Clotting factor inhibition panel showing the relative inhibitory effects of *Trimeresurus* venom on preventing the activation of: A) FIXa, B) FXa, C) Prothrombin to thrombin (Prothombinase complex) and D) Thrombin inhibition. For A) venom was incubated with FIXa for 2 min. before adding plasma, with clot time then immediately measured. For B) venom was incubated with human plasma for 2 min before adding Factor Xa with clot time then immediately measured. For C) and D) venom was incubated with FXa or thrombin for 2 min. before adding plasma or fibrinogen, with clot time then immediately measured. Data points are N = 3 displayed as a shift away from the control with standard deviations (no shift from control values would be a zero value).

with the aim of generating a clot to test for destructive cleavage of fibrinogen by the venom. Indeed the thrombin-induced clot displayed a greatly reduced clot strength in comparison to the thrombin control in the absence of venom, which is consistent with destructive (non-clotting) cleavage of fibrinogen by *T. flavomaculatus* venom (Fig. 7).

Consistent with the extreme variance in speed of action and clot strength, PGLS analysis confirmed that there is no statistical correlation between the speed of reaction and the strength of the fibrin clot produced ($p = 0.3753$) (Fig. 8). This indicates that the strength of the clot (A) is independent of the time to maximum thrombus generation (TMRTG). Interestingly, *T. mcgregori*, which produced the strongest clot (A = 8 ± 1.8, TGG = 43.2 ± 10.4) registers at mid-range in terms of speed of clot formation (SP = 2.9 ± 0.2, MRTGG = 0.4 ± 0.02), while one of the fastest to produce a clot, *T. trigonocephalus*, (SP = 2.6 ± 0.4, MRTGG = 0.2 ± 0.02) produces one of the weakest clots (A = 1.8 ± 0.05, TGG = 8.8 ± 0.03).

3.5. Fibrinogen chain degradation

In order to determine the specific fibrinogen chain cleavage by the various *Trimeresurus* venoms, time-dependent assays were conducted to illustrate effects upon each chain (Figs. 9 and 10). A wide variation in pattern emerged, with many of the venoms displaying the ability to cleave the alpha chain (with the exception of *T. gumprechti* and *T. popeiorum*). In most venom samples, the alpha chain was initially quite rapidly degraded within the first 5 min, followed by the beta chain. *T. albolabris, T. borneensis, T. flavomaculatus, T. hageni, T. mcgregori, T. purpureomaculatus, T. trigonocephalus* and *T. vogeli* also cleaved the gamma chain, thereby displaying the ability to cleave all three chains, with partial degradation of the gamma chain noted for *T. puniceus*. Some species (*T. flavomaculatus* and *T. purpureomaculatus*) degraded all three chains the quickest, however this ability was not congruent with their ability to clot plasma (Figs. 1 and 6) or fibrinogen (Figs. 2 and 7), indicative of at least some cleavage being destructive rather than clot forming. Other species, however, which did display an ability to clot plasma in a timely manner did not necessarily cleave all three chains rapidly (*T. albolabris*), nor did they cleave both alpha and beta (*T. gumprechti*). This suggests notable variation in destructive chain degradation leading to *versus* systematic degradation of chains leading to clot formation (albeit with weak and unstable fibrin structures), but both effects leading to a net depletion of fibrinogen levels.

3.6. Fibrinolysis

The ability of the venoms to actively lyse plasma clots was investigated in the presence and absence of tPA (tissue plasminogen

activator) (Fig. 11). Although plasma clots were unable to be directly lysed by any of the venoms, some species increased the ability of tPA to induce fibrinolysis. These species (*T. albolabris, T. borneensis, T. gumprechti, T. hageni, T. macrops, T. popeiorum, T. puniceus, T. purpureomaculatus, T. trigonocephalus* and *T. vogeli*) were able to shorten the clot lysis time (CLT) as compared incubations in the absence of venom.

3.7. Inhibition of FIXa, FXa or thrombin

Investigations to determine the sites of potential venom-dependent enzyme inhibition proceeded in a stepwise manner in order to ascertain specific sites of action : i) incubation of venom with FIXa followed by the addition of plasma to determine the venom's ability to directly inhibit FIXa; ii) incubation of venom with FXa followed by the addition of plasma to determine the ability of the venom to directly inhibit FXa; iii) incubation of venom with plasma followed by the addition of FXa to determine the effects of the venom on prothrombinase complex formation; iv) incubation of venom with thrombin followed by the addition of fibrinogen to determine direct inhibition of thrombin.

The results were as follows (Fig. 12): i) incubation with FIXa revealed a wide variation in the level of inhibitory activity of the venoms with some species displaying stronger inhibitory effects than others (*T. borneensis* and *T. mcgregori*) (Fig. 12A); ii) a wide variation in FXa inhibition was notable across the genus. (Fig. 12B); iii) incubation of the venom with plasma followed by the addition of FXa did not result in a prolonged clotting time and thus potential inhibition of prothrombinase, with the exception of *T. puniceus* venom which also showed inhibition towards FXa (Fig. 12C); iv) no substantial inhibition of thrombin-dependent fibrinogen cleavage was observed following incubation with *Trimeresurus* venom (Fig. 12D). However, as these enzymes work as a cascade, the cumulative effect of the inhibition may strongly contribute to the anticoagulant actions of these venoms in addition to actions upon fibrinogen.

4. Discussion

Species of *Trimeresurus* are wide spread throughout the Asian continent. As arboreal specialists, they are under extreme selection pressure due to high prey escape potential combined with prey variation. Arboreality also restricts gene flow, which in turn creates a diversification hot spot for many species to flourish and for venom to diverge. Such venom diversification within a genus can have real world implications for the treatment of envenomed patients due to differential coagulopathy and therefore a variable response to antivenom.

Despite species *Trimeresurus* species being very similar ecologically and morphologically, this study has revealed that their venoms are extremely functionally diverse and therefore capable of producing divergent clinical effects that are variably neutralised by antivenom. We observed coagulotoxic effects of the venom on plasma and fibrinogen of pseudo-procoagulant cleavage of fibrinogen to produce weak, unstable, short-lived fibrin clots. Such venom-mediated proteolysis of fibrinogen results in a depletion of fibrinogen levels, thus leading to a net anticoagulant state. The anticoagulation was potentiated by inhibition of clotting enzymes.

These laboratory findings are consistent with the haemorrhagic clinical manifestations of envenomation by *Trimeresurus* species (Chan et al., 1993; Chotenimitkhun and Rojnuckarin, 2008; Cockram et al., 1990; Greene et al., 2017; Hutton et al., 1990; Rojnuckarin et al., 2007, 1999; Tan et al., 2017; Visudhiphan et al., 1989; Witharana et al., 2019; Wongtongkam et al., 2005). In particular, the venoms have been shown clinically to produce rapid and pronounced decrease in fibrinogen levels leading to a net anticoagulant state (Collet et al., 2000; Longstaff and Kolev, 2015; Ryan et al., 1999), which is consistent with the results presented here. These effects are similar to that produced by other Asian pit vipers (Debono et al., 2019a, b; Debono et al., 2018).

Previous analysis of fibrinogen degradation on a very limited range of *Trimeresurus* species only provided qualitative, descriptive data and did not quantify the rates of fibrinogen chain cleavage (Jones et al., 2019). Further, this prior study did not attempt to determine the effect fibrinogen cleavage had upon clotting or the ability of antivenom to neutralise these pathological effects. In addition, prior studies also only measured antivenom binding through the use ELISA or Western Blot, which would reveal only simple antivenom binding to venom proteins, with no kinetic information, and, crucially prior studies did not ascertain whether the antivenom was effective in neutralising the venom's pathological functions(Jones et al., 2019; Tan et al., 2017). In contrast, in this study we not only quantified the effects upon fibrinogen but also investigated the ability of the Thai Red Cross Green Pit Viper Antivenin to neutralise the coagulotoxic effects of the 13 species of *Trimeresurus* investigated here. As expected, since the antivenom is made using *T. albolabris* venom, this species was completely neutralised even at the highest venom concentration. *Trimeresurus borneensis, T. flavomaculatus, T. macrops* and *T. popeiorum* were also extremely well neutralised. Other venoms were differentially neutralised by the antivenom, with notably poor effects upon *T. gumprechti,* and *T. mcgregori* and no detectable effect upon *T. hageni.* There was not, however, a phylogenetic pattern in regards to which species were well neutralised or poorly neutralised, which is consistent with the rapid molecular evolution characteristic of predatory venoms (Casewell et al., 2013).

In addition to investigating the effects upon fibrinogen by the venoms, this study also ascertained inhibition of clotting enzymes by the venoms. *Trimeresurus trigonocephalus* displayed the most pronounced inhibition of FXa, while *T. borneensis* displayed the largest inhibitory effect on FIXa. The inhibition of clotting enzymes would have a synergistic relationship with the depletion of fibrinogen levels, thereby potentiating the net anticoagulant effect. Indeed, anticoagulation is a major clinical feature in patients envenomated by *Trimeresurus* species (Chotenimitkhun and Rojnuckarin, 2008; Cockram et al., 1990; Greene et al., 2017; Hutton et al., 1990; Rojnuckarin et al., 2007, 1999; Rojnuckarin et al., 1998). Fibrinogenolytic effects have frequently been reported leading to swelling, excessive bleeding and defibrination. Antivenom has been reported as being effective for *T. albolabris* and *T. macrops* envenomations (Chan et al., 1993; Hutton et al., 1990; Rojnuckarin et al., 2007; Yang et al., 2007), which is consistent with the results we obtained for both species in this study. Haemorrhagic shock would be further potentiated by the anticoagulant mechanisms we have identified in this study working synergistically with metalloprotease enzymes that damage the vascular wall (Escalante et al., 2011; Gutiérrez et al., 2016).

Consistent the poor neutralisation from the tested antivenom on *T. gumprechti,* and *T. mcgregori* venoms, and the lack of effect upon *T. hageni*, these same three species were also not inhibited by the known serine protease inhibitor AEBSF, which was effective in neutralising the effects of the other species. Thus, while the actions upon fibrinogen for species such as *T. albolabris* and *T. borneensis* are due to serine proteases, the coagulotoxic effects from *T. gumprechti*, *T. hageni* and *T. mcgregori* are due to the other fibrinogenolytic enzyme class present in the venoms: metalloproteases. Interestingly, these three species are not closely related to each other and therefore this unique activity has been derived on three independent occasions. As antivenom is made using the venom of *T. albolabris* venom, which contains fibrinogenolytic serine proteases, this explains the poor performance against the venoms which are rich in fibrinogenolytic metalloproteases. As these three species were phylogenetically distinct from each other, and therefore convergently derived their venoms away from the plesiotypic serine protease driven actions upon fibrinogen with the consequent impact upon antivenom cross-neutralisation, this underscores the unreliability of organismal phylogeny as a predictor of antivenom efficacy. Similarly, organismal was a poor predictor of venom potency, with the two most potent venoms (*T. albolabris* and *T. hageni*) being distant to each other on the organismal tree.

These findings therefore have real world implications for the

treatment of patients envenomed by *Trimeresurus* species using the widely available Thai Red Cross Green Pit Viper antivenom. An important caveat is that the positive cross-reactivity with *T. flavomaculatus* and *T. fucatus* requires *in vivo* confirmation before clinical recommendations can be made regarding treatment protocols. However, as *T. macrops* was also well neutralised, consistent with clinical reports, the congruent positive effects upon *T. flavomaculatus* and *T. fucatus* in the tests conducted in this study are strongly suggestive of clinical usefulness of the antivenom against these species. In contrast, the poor performance of the antivenom against *T. gumprechti* and *T. mcgregori,* and failure against *T. hageni,* despite the ideal scenario of preincubation of antivenom:venom, is cause for significant concern and the likelihood of clinical usefulness for these species is low (*T. gumprechti* and *T. mcgregori*) to non-existant (*T. hageni*). Another important note to be made is that the antivenom efficacy in this paper is purely from the perspective fibrinogenolysis. Studies into other clinically relevant pathophysiological functions, such as myotoxicity, may produce differential antivenom efficacy patterns and should be the subject of future research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2019.09.003.

References

Alencar, L.R., Martins, M., Greene, H.W., 2018. Evolutionary History of Vipers. eLS. pp. 1–10.

Alencar, L.R., Quental, T.B., Grazziotin, F.G., Alfaro, M.L., Martins, M., Venzon, M., Zaher, H., 2016. Diversification in vipers: phylogenetic relationships, time of divergence and shifts in speciation rates. Mol. Phylogenet. Evol. 105, 50–62.

Alirol, E., Sharma, S.K., Bawaskar, H.S., Kuch, U., Chappuis, F., 2010. Snake bite in South Asia: a review. PLoS Negl. Trop. Dis. 4, e603.

Bell, W.R., 1997. Defibrinogenating enzymes. Drugs 54, 18–31.

Casewell, N.R., Wüster, W., Vonk, F.J., Harrison, R.A., Fry, B.G., 2013. Complex cocktails: the evolutionary novelty of venoms. Trends Ecol. Evol. 28 (4), 219–229.

Chan, T., Chan, J., Tomlinson, B., Critchley, J., 1993. Clinical features and hospital management of bites by the white-lipped green pit viper (*Trimeresurus albolabris*). Southeast Asian J. Trop. Med. Public Health 24, 772–775.

Chippaux, J.P., 1998. Snake-bites: appraisal of the global situation. Bull. World Health Organ. 76, 515.

Chotenimitkhun, R., Rojnuckarin, P., 2008. Systemic antivenom and skin necrosis after green pit viper bites. Clin. Toxicol. 46, 122–125.

Clark, R.F., Davidson, T.M., 1997. Intraarticular envenomation by *Trimeresurus flavomaculatus mcgregori* resulting in joint destruction. Toxicon 35, 837–842.

Cockram, C., Chan, J., Chow, K., 1990. Bites by the white-lipped pit viper (*Trimeresurus albolabris*) and other species in Hong Kong. A survey of 4 years' experience at the Prince of Wales Hospital. J. Trop. Med. Hyg. 93, 79–86.

Coimbra, F.C., Dobson, J., Zdenek, C.N., Op den Brouw, B., Hamilton, B., Debono, J., Masci, P., Frank, N., Ge, L., Kwok, H.F., Fry, B.G., 2018. Does size matter? Venom proteomic and functional comparison between night adder species (Viperidae: *Causus*) with short and long venom glands. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 211, 7–14.

Collet, J., Park, D., Lesty, C., Soria, J., Soria, C., Montalescot, G., Weisel, J., 2000. Influence of fibrin network conformation and fibrin fiber diameter on fibrinolysis speed. Arterioscler. Thromb. Vasc. Biol. 20, 1354–1361.

Dambisya, Y.M., Lee, T.-L., Gopalakrishnakone, P., 1994. Action of *Calloselasma rhodostoma* (Malayan pit viper) venom on human blood coagulation and fibrinolysis using computerized thromboelastography (CTEG). Toxicon 32, 1619–1626.

Debono, J., Bos, M.H., Coimbra, F., Ge, L., Frank, N., Kwok, H.F., Fry, B., 2019a. Basal but divergent: clinical implications of differential coagulotoxicity in a clade of Asian vipers. Toxicol. Vitr. 58, 195–206.

Debono, J., Bos, M.H., Do, M.S., Fry, B., 2019b. Clinical implications of coagulotoxic variations in Mamushi (Viperidae: *Gloydius*) snake venoms. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol., 108567.

Debono, J., Bos, M.H., Nouwens, A., Ge, L., Frank, N., Kwok, H.F., Fry, B., 2018. Habu coagulotoxicity: clinical implications of the functional diversification of *Protobothrops* snake venoms upon blood clotting factors. Toxicol. Vitr. 55, 62–74.

Debono, J., Dobson, J., Casewell, N.R., Romilio, A., Li, B., Kurniawan, N., Mardon, K., Weisbecker, V., Nouwens, A., Kwok, H.F., Fry, B.G., 2017. Coagulating colubrids: evolutionary, pathophysiological and biodiscovery implications of venom variations between boomslang (*Dispholidus typus*) and twig snake (*Thelotornis mossambicanus*). Toxins 9, 171.

Dobson, J., Yang, D.C., Op den Brouw, B., Cochran, C., Huynh, T., Kurrupu, S., Sánchez, E.E., Massey, D.J., Baumann, K., Jackson, T.N., Nouwens, A., Josh, P., Neri-Castro, E., Alagón, A., Hodgson, W.C., Fry, B.G., 2018. Rattling the border wall: pathophysiological implications of functional and proteomic venom variation between Mexican and US subspecies of the desert rattlesnake *Crotalus scutulatus*. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 205, 62–69.

Escalante, T., Rucavado, A., Fox, J.W., Gutiérrez, J.M., 2011. Key events in microvascular damage induced by snake venom hemorrhagic metalloproteinases. J. Proteomics 74 (9), 1781–1794.

Esnouf, M., Tunnah, G., 1967. The isolation and properties of the thrombin-like activity from *Agkistrodon rhodostoma* venom. Br. J. Haematol. 13, 581–590.

Fry, B.G., 2018. Snakebite: when the human touch becomes a bad touch. Toxins 10, 170.

Greene, S., Galdamez, L.A., Tomasheski, R., 2017. White-lipped tree viper (*Cryptelytrops albolabris*) envenomation in an American viper keeper. J. Emerg. Med. 53, e115–e118.

Gutiérrez, J.M., Calvete, J.J., Habib, A.G., Harrison, R.A., Williams, D.J., Warrell, D.A., 2017. Snakebite envenoming. Nat. Rev. Dis. Primers 3, 17063.

Gutiérrez, J.M., Escalante, T., Rucavado, A., Herrera, C., 2016. Hemorrhage caused by snake venom metalloproteinases: a journey of discovery and understanding. Toxins (Basel) 268 (4), 93.

Gutiérrez, J.M., Theakston, R.D.G., Warrell, D.A., 2006. Confronting the neglected problem of snake bite envenoming: the need for a global partnership. PLoS Med. 3, e150.

Huang, T.-F., Chang, M.-C., Peng, H.-C., Teng, C.-M., 1992. A novel α-type fibrinogenase from *Agkistrodon rhodostoma* snake venom. Biochimica et Biophysica Acta (BBA)-Protein Struct. Mol. Enzymol. 1160, 262–268.

Hutton, R., Looareesuwan, S., Ho, M., Silamut, K., Chanthavanich, P., Karbwang, J., Supanaranond, W., Vejcho, S., Viravan, C., Phillips, R., Warrell, D., 1990. Arboreal green pit vipers (genus *Trimeresurus*) of South-East Asia: bites by *T. albolabris* and *T. macrops* in Thailand and a review of the literature. Trans. R. Soc. Trop. Med. Hyg. 84, 866–874.

Jones, B.K., Saviola, A., Reilly, S.B., Stubbs, A.L., Arida, E., Iskandar, D.T., McGuire, J.A., Yates, J.R., Mackessy, S.P., 2019. Venom composition in a phenotypically variable pit viper (*Trimeresurus insularis*) across the Lesser Sunda Archipelago. J. Proteome Res. 18 (5), 2206–2220.

Kasturiratne, A., Wickremasinghe, A.R., de Silva, N., Gunawardena, N.K., Pathmeswaran, A., Premaratna, R., Savioli, L., Lalloo, D.G., de Silva, H.J., 2008. The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. PLoS Med. 5, e218.

Kolev, K., Longstaff, C., 2016. Bleeding related to disturbed fibrinolysis. Br. J. Haematol. 175, 12–23.

Kuniyoshi, A.K., Kodama, R.T., Moraes, L.H.F., Duzzi, B., Iwai, L.K., Lima, I.F., Cajado-Carvalho, D., Portaro, F.V., 2017. *In vitro* cleavage of bioactive peptides by peptidases from *Bothrops jararaca* venom and its neutralization by bothropic antivenom produced by Butantan Institute: Major contribution of serine peptidases. Toxicon 137, 114–119.

Levy, D.E., Del Zoppo, G.J., 2006. Ancrod: a potential treatment for acute, ischemic stroke from snake venom. Toxin Rev. 25, 323–333.

Lin, Y., Yu, X., He, Q., Li, H., Li, D., Song, X., Wang, Y., Wen, H., Deng, H., Deng, J., 2009. Expression and functional characterization of chitribrisin, a thrombin-like enzyme, in the venom of the Chinese green pit viper (*Trimeresurus albolabris*). Protein Expr. Purif. 67, 48–52.

Lister, C., Arbuckle, K., Jackson, T.N.W., Debono, J., Zdenek, C.N., Dashevsky, D., Dunstan, N., Allen, L., Hay, C., Bush, B., Gillett, A., Fry, B.G., 2017. Catch a tiger snake by its tail: differential toxicity, co-factor dependence and antivenom efficacy in a procoagulant clade of Australian venomous snakes. Comp. Biochem. Physiol. Part - C 202, 39–54. https://doi.org/10.1016/j.cbpc.2017.07.005.

Liu, S., Marder, V.J., Levy, D.E., Wang, S.-J., Yang, F., Paganini-Hill, A., Fisher, M.J., 2011. Ancrod and fibrin formation: perspectives on mechanisms of action. Stroke 42, 3277–3280.

Longstaff, C., Kolev, K., 2015. Basic mechanisms and regulation of fibrinolysis. J. Thromb. Haemost. 13.

Malhotra, A., Thorpe, R.S., 2004. A phylogeny of four mitochondrial gene regions suggests a revised taxonomy for Asian pitvipers (*Trimeresurus* and *Ovophis*). Mol. Phylogenet. Evol. 32, 83–100.

Mitrakul, C., 1973. Effects of green pit viper (*Trimeresurus erythrurus* and *Trimeresurus popeorum*) venoms on blood coagulation, platelets and the fibrinolytic enzyme systems: studies *in vivo* and *in vitro*. Am. J. Clin. Pathol. 60, 654–662.

Mosesson, M., 2005. Fibrinogen and fibrin structure and functions. J. Thromb. Haemost. 3, 1894–1904.

Muanpasitporn, C., Rojnuckarin, P., 2007. Expression and characterization of a recombinant fibrinogenolytic serine protease from green pit viper (*Trimeresurus albolabris*) venom. Toxicon 49, 1083–1089.

Nielsen, V.G., 2016. Ancrod revisited: viscoelastic analyses of the effects of *Calloselasma rhodostoma* venom on plasma coagulation and fibrinolysis. J. Thromb. Thrombolysis 42, 288–293.

Nolan, C., Hall, L., Barlow, G., 1976. Ancrod, the coagulating enzyme from Malayan pit viper (*Agkistrodon rhodostoma*) venom. Meth. Enzymol. 205–213 Elsevier.
Orme, D., Freckleton, R., Thomas, G., Petzoldt, T., Fritz, S., Isaac, N., Pearse, W., 2013. Caper: comparative analyses of phylogenetics and evolution in R. R package version 0.5.2. Methods Ecol. Evol. 3, 145–151.
Oulion, B., Dobson, J.S., Zdenek, C.N., Arbuckle, K., Lister, C., Coimbra, F.C., Op den Brouw, B., Debono, J., Rogalski, A., Violette, A., Fourmy, R., Frank, N., Fry, B.G., 2018. Factor X activating *Atractaspis* snake venoms and the relative coagulotoxicity neutralising efficacy of African antivenoms. Toxicol. Lett. 288, 119–128.
Paradis, E., Claude, J., Strimmer, K., 2004. APE: analyses of phylogenetics and evolution in R language APE. Bioinformatics 20, 289–290. https://doi.org/10.1093/bioinformatics/btg412.
Peng, M., Lu, W., Kirby, E.P., 1992. Characterization of three alboaggregins purified from *Trimeresurus albolabris* venom. Thromb. Haemost. 68, 702–707.
Pradniwat, P., Rojnuckarin, P., 2015. The structure–function relationship of thrombin-like enzymes from the green pit viper (*Trimeresurus albolabris*). Toxicon 100, 53–59.
Premawardena, A., Seneviratne, S., Gunatilake, S., De Silva, H., 1998. Excessive fibrinolysis: the coagulopathy following Merrem's hump-nosed viper (*Hypnale hypnale*) bites. Am. J. Trop. Med. Hyg. 58, 821–823.
Revell, L.J., 2012. Phytools: an R package for phylogenetic comparative biology (and other things). Methods Ecol. Evol. 3, 217–223. https://doi.org/10.1111/j.2041-210X.2011.00169.x.
Rogalski, A., Soerensen, C., op den Brouw, B., Lister, C., Dashvesky, D., Arbuckle, K., Gloria, A., Zdenek, C.N., Casewell, N.R., Gutiérrez, J.M., Wüster, W., Ali, S.A., Masci, P., Rowley, P., Frank, N., Fry, B.G., 2017. Differential procoagulant effects of saw-scaled viper (Serpentes: Viperidae: Echis) snake venoms on human plasma and the narrow taxonomic ranges of antivenom efficacies. Toxicol. Lett. 280, 159–170. https://doi.org/10.1016/j.toxlet.2017.08.020.
Rojnuckarin, P., Banjongkit, S., Chantawibun, W., Akkawat, B., Juntiang, J., Noiphrom, J., Pakmanee, N., Intragumtornchai, T., 2007. Green pit viper (*Trimeresurus albolabris* and *T. macrops*) venom antigenaemia and kinetics in humans. Trop. Doct. 37, 207–210.
Rojnuckarin, P., Intragumtornchai, T., Sattapiboon, R., Muanpasitporn, C., Pakmanee, N., Khow, O., Swasdikul, D., 1999. The effects of green pit viper (*Trimeresurus albolabris* and *Trimeresurus macrops*) venom on the fibrinolytic system in human. Toxicon 37, 743–755.
Rojnuckarin, P., Mahasandana, S., Intragumthornchai, T., Sutcharitchan, P., Swasdikul, D., 1998. Prognostic factors of green pit viper bites. Am. J. Trop. Med. Hyg. 58, 22–25.
Ryan, E.A., Mockros, L.F., Weisel, J.W., Lorand, L., 1999. Structural origins of fibrin clot rheology. Biophys. J. 77, 2813–2826.
Symonds, M.R.E., Blomberg, S.P.A., 2014. Primer on phylogenetic generalised least squares. In: Garamszegi, L.Z. (Ed.), Modern Phylogenetic Comparative Methods and Their Application in Evolutionary Biology. Springer-Verlag Berlin Heidelberg, Berlin, pp. 105–130 37.
Tai, H., Wei, Q., Jin, Y., Su, M., Song, J.-X., Zhou, X.-D., Ouyang, H.-M., Wang, W.-Y., Xiong, Y.-L., Zhang, Y., 2004. TMVA, a snake C-type lectin-like protein from *Trimeresurus mucrosquamatus* venom, activates platelet via GPIb. Toxicon 44, 649–656.
Tan, C.H., Liew, J.L., Tan, N.H., Ismail, A.K., Maharani, T., Khomvilai, S., Sitprija, V., 2017. Cross reactivity and lethality neutralization of venoms of Indonesian *Trimeresurus* complex species by Thai Green Pit Viper Antivenom. Toxicon 140, 32–37.
Tan, N.-H., Choy, S.-K., Chin, K.-M., Ponnudurai, G., 1994. Cross-reactivity of monovalent and polyvalent *Trimeresurus* antivenoms with venoms from various species of *Trimeresurus* (lance-headed pit viper) snake. Toxicon 32, 849–853.
Tan, N.H., 2010. Isolation and characterization of the thrombin-like enzyme from *Cryptelytrops purpureomaculatus* venom. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 151, 131–136.
Tan, N.H., Fung, S.Y., Yap, Y.H.Y., 2012. Isolation and characterization of the thrombin-like enzyme from *Cryptelytrops albolabris* (white-lipped tree viper) venom. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 161, 79–85.
Torres-Bonilla, K.A., Andrade-Silva, D., Serrano, S.M., Hyslop, S., 2018. Biochemical characterization of venom from *Pseudoboa neuwiedii* (Neuwied's false boa; *Xenodontinae*; *Pseudoboini*). Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 213, 27–38.
Trookman, N.S., Rizer, R.L., Ford, R., Ho, E., Gotz, V., 2009. Immediate and long-term clinical benefits of a topical treatment for facial lines and wrinkles. J. Clin. Aesthet. Dermatol. 2, 38.
Visudhiphan, S., Tonmukayakul, A., Tumliang, S., Dumavibhat, B., Piankijagum, A., 1989. Dark green pit viper (*Trimeresurus popeorum*) bite: clinical and serial coagulation profiles in 51 cases. Am. J. Trop. Med. Hyg. 41, 570–575.
Witharana, E., Gnanathasan, A., Dissanayake, A., Wijesinghe, S., Kadahetti, S., Rajapaksha, R., 2019. Sri Lankan green pit viper (*Trimeresurus trigonocephalus*) bites in Deniyaya: a clinico-epidemiological study. Toxicon 169, 34–37.
Wolberg, A.S., 2007. Thrombin generation and fibrin clot structure. Blood Rev. 21, 131–142.
Wolberg, A.S., Campbell, R.A., 2008. Thrombin generation, fibrin clot formation and hemostasis. Transfus. Apher. Sci. 38, 15–23.
Wongtongkam, N., Wilde, H., Sitthi-Amorn, C., Ratanabanangkoon, K., 2005. A study of 225 Malayan pit viper bites in Thailand. Mil. Med. 170, 342–348.
Xin, Y., Dong, D., Chen, D., Li, R., 2009. Structural and biological characterization of a novel acutobin-like enzyme isolated from the venom of the sharp-nosed pit viper (*Deinagkistrodon acutus*). Biotechnol. Appl. Biochem. 53, 123–131.
Yamashita, K.M., Alves, A.F., Barbaro, K.C., Santoro, M.L., 2014. *Bothrops jararaca* venom metalloproteinases are essential for coagulopathy and increase plasma tissue factor levels during envenomation. PLoS Negl. Trop. Dis. 8, e2814.
Yang, J.Y., Hui, H., Lee, A.C., 2007. Severe coagulopathy associated with white-lipped green pit viper bite. Hong Kong Med. J. 13, 392.
Youngman, N.J., Zdenek, C.N., Dobson, J.S., Bittenbinder, M.A., Gillett, A., Hamilton, B., Dunstan, N., Allen, L., Veary, A., Veary, E., 2019. Mud in the blood: novel potent anticoagulant coagulotoxicity in the venoms of the Australian elapid snake genus *Denisonia* (mud adders) and relative antivenom efficacy. Toxicol. Lett. 302, 1–6.
Zulys, V.J., Teasdale, S.J., Michel, E.R., Skala, R.A., Keating, S.E., Viger, J.R., Glynn, M., 1989. Ancrod (Arvin) as an alternative to heparin anticoagulation for cardio-pulmonary bypass. Anesthesiology 71, 870–877.