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Venom Composition in a Phenotypically Variable Pit Viper (*Trimeresurus insularis*) across the Lesser Sunda Archipelago

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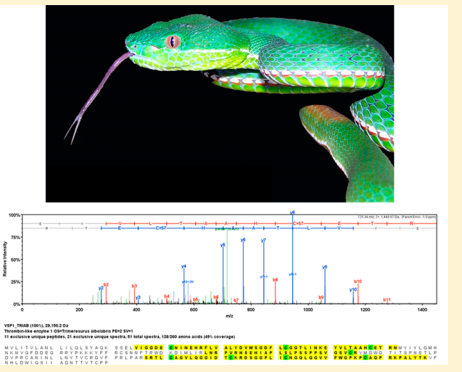
Supporting Information

ABSTRACT: The genus *Trimeresurus* comprises a group of venomous pitvipers endemic to Southeast Asia and the Pacific Islands. Of these, *Trimeresurus insularis*, the White-lipped Island Pitviper, is a nocturnal, arboreal species that occurs on nearly every major island of the Lesser Sunda archipelago. In the current study, venom phenotypic characteristics of *T. insularis* sampled from eight Lesser Sunda Islands (Flores, Lembata, Lombok, Pantar, Sumba, Sumbawa, Timor, and Wetar) were evaluated via SDS-PAGE, enzymatic activity assays, fibrinogenolytic assays, gelatin zymography, and RP-HPLC, and the Sumbawa sample was characterized by venomic analysis. For additional comparative analyses, venoms were also examined from several species in the *Trimeresurus* complex, including *T. borneensis*, *T. gramineus*, *T. puniceus*, *T. purpureomaculatus*, *T. stejnegeri*, and *Protobothrops flavoviridis*. Despite the geographical isolation, *T. insularis* venoms from all eight islands demonstrated remarkable similarities in gel electrophoretic profiles and RP-HPLC patterns, and all populations had protein bands in the mass ranges of phosphodiesterases (PDE), L-amino acid oxidases (LAAO), P−III snake venom metalloproteinases (SVMP), serine proteases, cysteine-rich secretory proteins (CRISP), phospholipases A_2 (PLA_2), and C-type lectins. An exception was observed in the Lombok sample, which lacked protein bands in the mass range of serine protease and CRISP. Venomic analysis of the Sumbawa venom also identified these protein families, in addition to several proteins of lesser abundance (<1%), including glutaminyl cyclase, aminopeptidase, PLA_2 inhibitor, phospholipase B, cobra venom factor, 5′-nucleotidase, vascular endothelial growth factor, and hyaluronidase. All *T. insularis* venoms exhibited similarities in thrombin-like and PDE activities, while significant differences were observed for LAAO, SVMP, and kallikrein-like activities, though these differences were only observed for a few islands. Slight but noticeable differences were also observed with fibrinogen and gelatin digestion activities. *Trimeresurus insularis* venoms exhibited overall similarity to the other *Trimeresurus* complex species examined, with the exception of *P. flavoviridis* venom, which showed the greatest overall differentiation. Western blot analysis revealed that all major *T. insularis* venom proteins were recognized by Green Pitviper (*T. albolabris*) antivenom, and reactivity was also seen with most venom proteins of the other *Trimeresurus* species, but incomplete antivenom−venom recognition was observed against *P. flavoviridis* venom proteins. These results demonstrate significant conservation in the venom composition of *T. insularis* across the Lesser Sunda archipelago relative to the other *Trimeresurus* species examined.



KEYWORDS: *Asia, enzyme, evolution, Indonesia, island, proteomics, toxin, venomics*

INTRODUCTION

Islands, and island-like habitats (such as mountaintops and landlocked lakes), represent remarkable biodiversity hotspots for observing and understanding evolutionary diversification and adaptive radiation.[1] Classical examples of adaptive radiations often acknowledge visible changes in morphological characteristics, such as divergence in body size and limb dimensions in Greater Antillean Anoles,[2−4] and variations in cranial morphology in both Hawaiian honeycreepers[5] and

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Darwin's finches of the Galapagos.[6,7] In addition, physiological divergence has been documented as a driving force of adaptive radiation, as observed with the photosynthetic traits of Hawaiian lobeliads adapting to different light regimes throughout the archipelago.[8] Undoubtedly, these morphological and physiological characteristics are functionally related to resource utilization and food or prey consumption. However, in vertebrates, molecular adaptations that also facilitate optimal utilization of resources and successful prey capture are often overlooked.

Snake venoms are trophic adaptations that have allowed for the transition from a mechanical (constriction) to a chemical (venom) means of subduing prey.[9] Venoms comprise a complex mixture of proteins and peptides[10] that likely evolved from endogenous proteins with normal physiological functions early in the evolution of advanced snakes.[11] Estimates suggest that there are over 700 front-fanged venomous snake species worldwide (reptile-database.org; December 2018), and experimental studies have revealed substantial phenotypic variation in venom composition due to phylogeny (at the family, species, and population levels),[12−15] ontogeny,[16−24] diet,[12,19,20,25−27] geography,[29−31] and perhaps in response to captivity,[32] but see ref 33. Understanding these factors, and how they contribute to venom phenotypic plasticity, can offer clues about the dynamic effects of the local ecology, population interactions, and the potential impact these factors may have on the evolutionary history of a species.

The White-lipped Island Pitviper (*Trimeresurus insularis*) is a nocturnal, arboreal species that occurs on nearly every major island of the Lesser Sunda archipelago.[34] Like many other pitvipers, *T. insularis* appears to be an opportunistic feeder and has a diet consisting of small mammals, birds, frogs, lizards, and other snakes.[35] The taxonomic history of Asian pitvipers, including the genus *Trimeresurus*, has been convoluted and inferred more recently primarily using mitochondrial DNA (Figure 1).[36,37] *Trimeresurus insularis* appears to be most closely related to other Southeast Asian species, such as *T. albolabris*, *T. erythrurus*, *T. fasciatus*, and *T. purpureomaculatus*, and it was once considered a subspecies of *T. albolabris*. In addition, *T. insularis* was formerly placed in the genus *Cryptelytrops*[36] before being returned to *Trimeresurus*.[38]

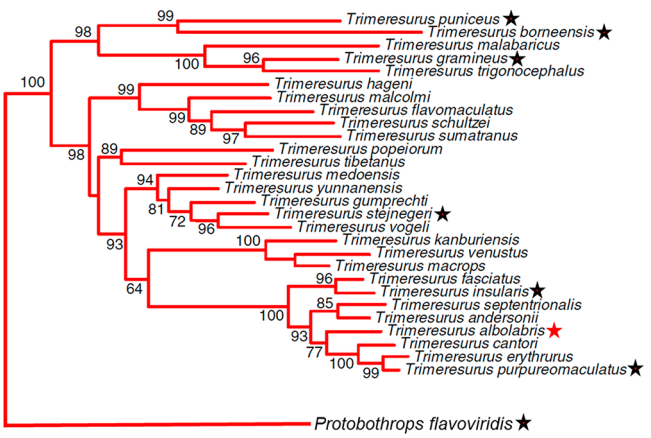

Figure 1. Phylogeny of the Asian Pitviper (*Trimeresurus*) complex. Adapted from Pyron et al.;[37] *Protobothrops* is a member of a clade sister to *Trimeresurus*. Species sampled in this study are indicated with black stars. The Green Pit Viper antivenom used in the study was produced against *T. albolabris* venom (red star).

Interestingly, phenotypic variation in *T. insularis* color patterns have been observed on several islands, and although most populations are green, blue individuals have been found on Komodo Island and yellow individuals on Timor and Wetar Islands; however, these islands also contain green individuals (Figure 2).

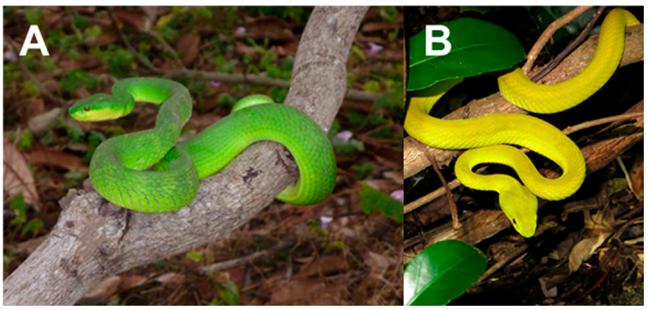

Figure 2. Two color morphs of *T. insularis*. A. Sumbawa Id., B. Wetar Id. (photos: S. Reilly).

As a variable phenotypic characteristic, it can be hypothesized that the venom composition of *T. insularis* will vary significantly between islands throughout the Lesser Sunda archipelago. Variation may be due to isolation and genetic drift but also may result from divergent selection pressures in response to different prey communities. Elucidating the venom compositional patterns of the same species located in geographically isolated regions (e.g., different islands) can provide remarkable insight into the evolutionary relationships among venomous snakes and the possible mechanisms contributing to venom evolution. Therefore, in the current study we utilized RP-HPLC, SDS-PAGE, and enzymatic activity assays to examine and compare the venom composition of *T. insularis* sampled from eight islands throughout the Lesser Sunda archipelago. Furthermore, to estimate relative abundance of venom protein families, and to provide a reference for protein identity in RP-HPLC chromatograms and SDS-PAGE gels, the Sumbawa venom sample was characterized by venom proteomic (venomic) analysis. Lastly, to highlight intraspecific heterogeneity/homogeneity and to evaluate variation within the *Trimeresurus* genus, venoms from six other species of the *Trimeresurus* complex were analyzed and compared to the venoms of *T. insularis*.

EXPERIMENTAL SECTION

Supplies and Reagents

Protein concentration reagents were purchased from BioRad, Inc. (Hercules, CA). NuPage gels, Mark 12 standards, and Western blot materials were obtained from Life Technologies, Inc. (Grand Island, NY). High performance liquid chromatography equipment and materials were obtained from Waters Corporation (Milford, MA), and reversed-phase columns were purchased from Phenomenex, Inc. (Torrance, CA). All other reagents were purchased from Sigma Biochemical Corp. (St. Louis, MO) and were of analytical grade or better.

Venom Samples

Venoms from adult *T. insularis* from the Indonesian islands of Flores ($n = 7$), Lembata ($n = 2$), Lombok ($n = 1$), Pantar ($n = 3$), Sumba ($n = 6$), Sumbawa ($n = 1$), Timor ($n = 3$), and Wetar ($n = 6$) were extracted during fieldwork conducted in 2011, 2012, and 2013 (Figure 3). Fieldwork was performed under local permits issued by the Natural Resources Conservation Agency

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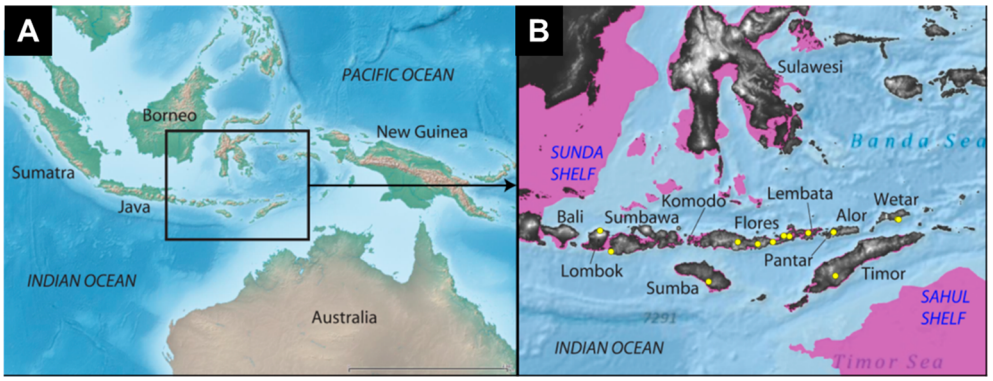

Figure 3. Map of Southeast Asia and Australia (A) with the area in the black box enlarged in (B) to show the Lesser Sunda Islands situated between the Sunda and Sahul shelves. Areas of the ocean in purple (120 m depth) became land positive during glacial maxima, and yellow dots represent venom sample localities.

(BKSDA), and research permits were granted to J.A.M. by the Indonesian Institute of Sciences (LIPI) and the Ministry of Research and Technology (RISTEK). All venoms were dried over silica gel desiccant in a Falcon tube immediately after extraction, and stored at −20 °C until use. Localities and museum accession numbers are provided in Supplemental Table S3. For comparative analyses, venoms of other species in the *Trimeresurus* complex, including *T. borneensis* (Borneo), *T. stejnegeri* (Taiwan), and *T. puniceus* (Java), were extracted from captive snakes and analyzed. Additional samples obtained from a commercial source (Miami Serpentarium) included *T. purpureomaculatus* (Thailand), *T. gramineus* (India), and *Protobothrops flavoviridis* (Okinawa). These species were chosen from venoms available to represent a moderate diversity within the genus *Trimeresurus*. All venoms were reconstituted in 18.2 MΩ Millipore-filtered water at a concentration of 4.0 mg/mL, and protein concentrations were determined using a modified Bio-Rad Inc. method,[39] with bovine gamma globulin (BGG) as a protein standard. Each venom stock solution was adjusted to exactly 4.0 mg/mL for all subsequent experiments.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

Venom proteins were separated by RP-HPLC using a Phenomenex Jupiter C_{18} column on a Waters HPLC System. For venomic analysis, approximately 2.7 mg of crude *T. insularis* venom from the Sumbawa individual was resolubilized in 200 μL of 0.1% trifluoroacetic acid (TFA) in ddH_2O and centrifuged at 10,000*g* for 5 min to remove cellular debris. Sumbawa venom was used due to venom availability as we lacked sufficient venom from the other populations for venomic analysis; additionally, it appeared to contain all venom components present in other samples. For all runs, the column was equilibrated with 95% solution A (0.1% trifluoroacetic acid (TFA) in ddH_2O) with 5% solution B (80% acetonitrile (ACN) and 0.1% TFA in ddH_2O). Protein separation was carried out under the following conditions: 5% solution B for 5 min, 5−15% B from 5 to 10 min, 15−22% B from 10 to 30 min, 22−35% B from 30 to 40 min, 35−45% B from 40 to 50 min, 45−65% B from 50 to 100 min, 65−100% B from 100 to 105 min, 100% B from 105 to 110 min, 100% to 5% B from 110 to 112 min, and 5% B from 112 to 120 min. Protein detection was carried out at 220 and 280 nm using a Waters 2487 Dual λ absorbance detector operating under Empower software. RP-HPLC fractions were collected using a Waters Fraction Collector II at a flow rate of 1 mL/min and dried in a vacuum centrifuge (Savant, Thermofisher Scientific) for subsequent characterization.

Characterization of RP-HPLC Fractions

Fractions obtained from RP-HPLC were further separated by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D SDS-PAGE) as previously described.[40] Briefly, dithiothreitol (DTT) reduced fractions or Dalton Mark 12 protein standards (5 μL/lane) were loaded onto precast NuPage bis-tris 12% acrylamide gels (Life Technologies). Gels were run at 200 V (100 to 125 mA) for 40−45 min, stained overnight with 0.1% Coomassie brilliant blue R-250, destained with 30% methanol/7% acetic acid, and stored in 7% acetic acid. Destained gels were scanned with a HP Scanjet 4570c. Estimated molecular weights were based on protein standards.

Electrophoretic protein bands were excised from Coomassie brilliant blue-stained gels, destained, and subjected to in-gel reduction (10 mM dithiothreitol) and alkylation (50 mM 2-chloro-2-iodacetamide) at room temperature. Gel bands were washed with 25 mM ammonium bicarbonate and dehydrated with ACN. Bands were rehydrated in 20 μL of sequencing-grade trypsin (6.66 ng/μL in 25 mM ammonium bicarbonate) for 60 min on ice. Excess trypsin was removed, an additional 20 μL of 25 mM ammonium bicarbonate was added to gel pieces, and samples were digested overnight at 37 °C. Following the overnight digestion, the samples were vortexed for 1 h at room temperature and sonicated in an ultrasonic bath for 3 min, and the tryptic peptides were extracted in 20 μL of 50% ACN/0.1% TFA. Digests were dried in a vacuum centrifuge and resuspended in 12 μL of 5% ACN containing 0.1% formic acid (FA) and submitted to liquid chromatography mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS experiments were performed with an Easy nLC 1000 instrument coupled to a LTQ Orbitrap Velos mass spectrometer (both from ThermoFisher Scientific). Tryptic peptides were loaded on a C_{18} column (75 μM inner diameter x 15 cm) packed in-house with Aqua 3 μm C_{18} 125 Å resin. The flow rate was set to 0.6 μL/min and the column was developed with a linear gradient of 5% ACN, in 0.1% formic acid (FA) in ddH_2O (solution A) and 0.1% FA in ACN (solution B) at 1% B for 1 min, followed by 1−5% B for 5 min, 5−80% B for 50 min, isocratic at 80% B for 5 min, and from 80−0% B for 3 min. MS/MS was performed using a data-dependent acquisition (DDA) top 10 method, with the instrument operating in positive nanoelectrospray with a spray voltage of +2.3 kV. Precursor ion scans were executed in the Orbitrap mass analyzer at 60 K

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resolving power, and monoisotopic precursor selection (MIPS) was enabled for charge states ≥2 (+1 charge state was enabled for RP-HPLC peak 26 which contained small peptides). Fragment ion scans were performed in the linear ion trap mass analyzer using a collision-induced dissociation at 35% normalized collision energy. Dynamic exclusion was set to 60s at 10 ppm tolerance. Fragmentation spectra were interpreted using Integrated Proteomics Pipeline (IP2, version 6.0.2; www.integratedproteomics.com) against the NCBI nonredundant database downloaded on 30 September 2018. MS/MS mass tolerance was set to ±0.6 Da and carbamidomethyl cysteine and oxidation of methionine were selected as fixed and variable modifications, respectively.

The relative abundances (expressed as percentage of the total venom proteins) of the different protein families and subfamilies were calculated as the ratio of the sum of the areas of the RP-HPLC chromatographic peaks (at 220 nm) containing proteins from the same family to the total area of the protein peaks in the RP-HPLC chromatogram.[41,42] In the case of more than one protein band being present in a single RP-HPLC peak, their proportions were estimated by densitometry of the Coomassie-stained SDS-PAGE gels using ImageJ (version 1.51; National Institute of Health, Bethesda, MD). When more than one protein was identified in a single SDS-PAGE protein band, their relative abundances were estimated based on the relative ion intensities of the three most abundant peptide ions associated with each protein by MS/MS analysis. Protein family and subfamily abundances were estimated as the percentages of the total venom proteome.

To examine for potential population-level variation in *T. insularis* venom composition, venom from one individual *T. insularis* representing each island (except for Lombok due to lack of venom) were analyzed under identical RP-HPLC conditions mentioned above. Furthermore, individual *T. insularis* venoms from Flores ($n = 7$), Lembata ($n = 2$), Lombok ($n = 1$), Pantar ($n = 3$), Sumba ($n = 6$), Timor ($n = 3$), and Wetar ($n = 6$), were assessed for the relative number and molecular masses of protein components by 1D SDS-PAGE as described above. For a comparison of *T. insularis* venom compositional patterns with other species in the *Trimeresurus* genus, *T. borneensis*, *T. stejnegeri*, *T. puniceus*, *T. purpureomaculatus*, *T. gramineus*, and *Protobothrops flavoviridis* venoms were also subjected to RP-HPLC and SDS-PAGE analyses. Characterization of these additional *T. insularis* and *Trimeresurus* complex samples was based on the detailed venomic characterization of the Sumbawa sample. RP-HPLC peak identifications were determined by comparison of elution times and visual inspection of RP-HPLC chromatograms with the Sumbawa *T. insularis* sample. Tentative SDS-PAGE protein band identity was based on characteristic migration patterns of venom proteins as observed in previous analyses[10,13] as well as the LC-MS/MS analysis of the electrophoretic protein bands in the Sumbawa venom.

Enzyme Assays

Venoms from each individual *T. insularis* were assayed for snake venom metalloproteinase (SVMP), L-amino acid oxidase (LAAO), phosphodiesterase (PDE), and thrombin-like (TLE) and kallikrein-like (KLE) serine proteinase activities as previously described.[43] All assays were performed in triplicate and results reported as product formed/min/mg venom protein (or ΔA/min/mg).

Fibrinogenolytic Activity Assay

Venom of one *T. insularis* from each island population, in addition to the representative venoms from the *Trimeresurus* complex, were assayed for fibrinogenolytic activity as described previously;[44] modified by ref 21. A single venom representative for each island population was used instead of all venom samples due to the high conservation between samples (see Results). Assays were conducted by incubating 5 μg of crude venom with 100 μL of human fibrinogen (2 mg/mL) in 100 mM Tris-HCl buffer (pH 8.0) at 37 °C. At 0, 1, 5, 10, 30, and 60 min intervals, 15 μL of reaction was removed and mixed with 15 μL termination solution (4% SDS, 10% β mercaptoethanol, 20% glycerol), boiled for 5 min, and then allowed to cool to room temperature. Samples were loaded onto a NuPage bis-tris 12% acrylamide gel and run in SDS running buffer for 50 min at 200 V. The gels were stained, destained, and imaged as described above.

Metalloproteinase Zymography Assays

Gelatin hydrolysis activity of *T. insularis*, *T. borneensis*, *T. gramineus*, *T. puniceus*, *T. purpureomaculatus*, *T. stejnegeri*, and *P. flavoviridis* venoms were visualized by zymography. Individual venom samples were diluted to 1 μg/μL, and further diluted 1:10 with ddH_2O and mixed with an equal volume of 2X Laemmli sample buffer. Samples (5 μL, 0.5 μg) were loaded onto 10% acrylamide gelatin zymogram gels (Life Technologies), and run at 125 V (30−40 mA) for approximately 90 min in ice-cold running buffer. The gel was then rinsed several times with ddH_2O and incubated with Novex Zymogram Renaturing Buffer (1X) on a shaker for 1 h at room temperature. The renaturing buffer was decanted and the gel was incubated with Novex Zymogram Developing Buffer in a shaker water bath overnight at 37 °C. The developing buffer was then decanted and the gel was stained with 0.1% Coomassie Brilliant Blue R-250, lightly destained with rapid destain, imaged, and stored in 7% acetic acid as described above. Bands of (metallo)proteolytic activity appear as clear bands on a darker background.

Western Blot-Green Pit Viper Antivenom Binding Assay

Venoms (8 μg/lane) from one *T. insularis* representing each island population (except Pantar, due to lack of venom), and from other individuals of the *Trimeresurus* complex were subjected to Western blot analysis following reducing SDS-PAGE on 12% acrylamide NuPAGE Bis-Tris precast gels.[22,40] Proteins were transferred to a nitrocellulose membrane for 1 h at 150 mA, washed with PBS (×3), and blocked in 3% bovine serum albumin in PBS for 2.5 h with gentle shaking at room temperature. The membrane was rinsed in PBS (x3) and incubated overnight at room temperature with primary antibody (Green Pit Viper (*Trimeresurus albolabris*) antivenom, Queen Saovabha Memorial Institute/Thai Red Cross, expiration date 7/17/2017; 30 mg of antivenom in 15 mL of PBS) and gentle shaking. The membrane was then rinsed with 15 mL of Tris-buffered saline (TBS; ×4) and incubated with the secondary antibody (10 μL of antihorse IgG labeled with alkaline phosphatase in 15 mL of TBS) for 1 h at room temperature with gentle shaking. Unbound secondary was removed by washing with TBS (×3) prior to the addition of 10 mL of alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablet). The reaction was quenched with 15 mL of 20 mM EDTA in PBS and visualized as described above.

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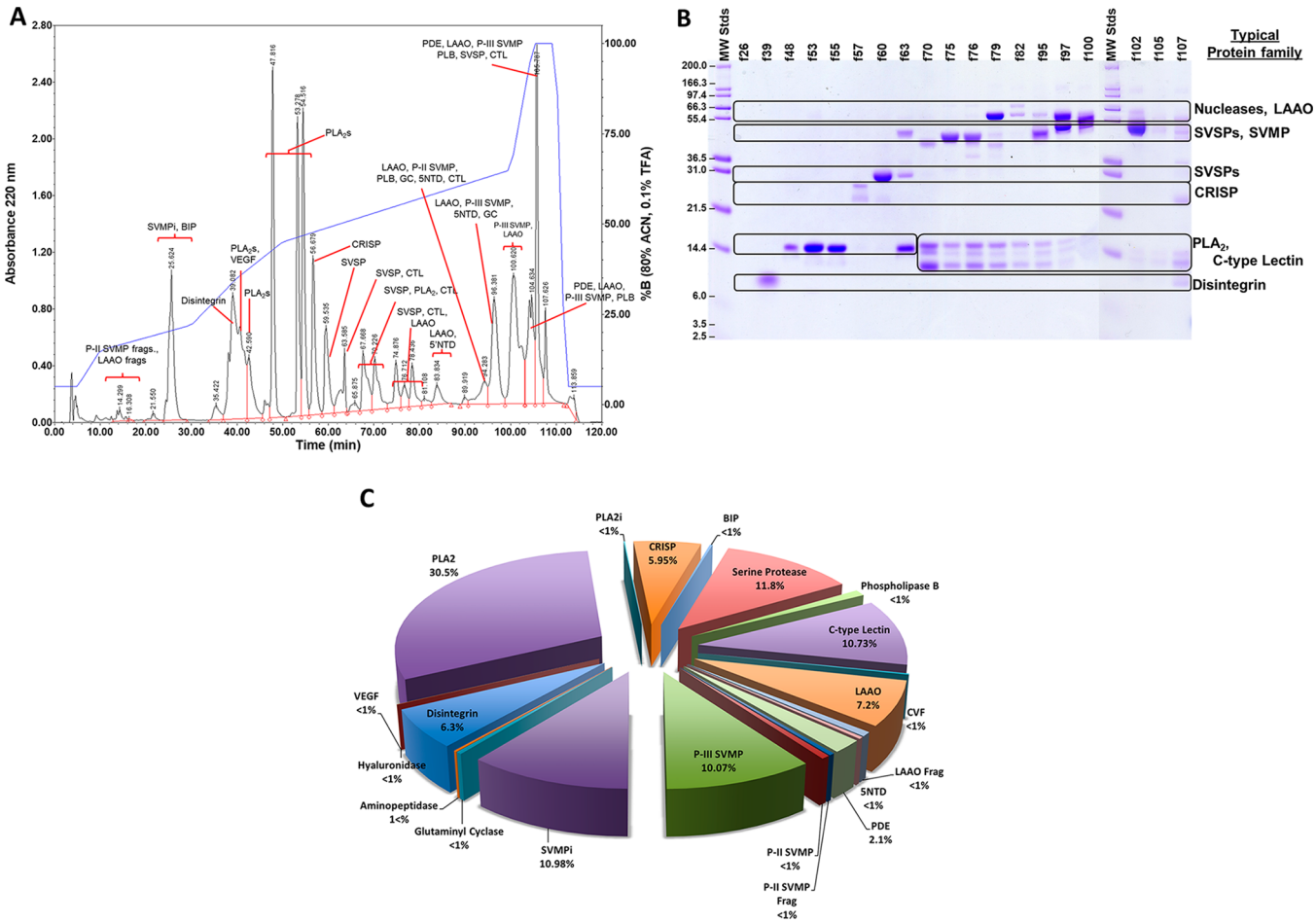

Figure 4. RP-HPLC fractionation of venom proteins from *T. insularis* from Sumbawa (panel A). Fractions were collected and subjected to reducing SDS-PAGE (B), and the protein bands were excised, in-gel digested with trypsin, and identified by bottom-up LC-MS/MS (Table S1). The pie chart in panel C displays the relative occurrence (in percentage of total venom proteins) of toxins from the different protein families in the venom of *T. insularis* from Sumbawa. 5NTD, 5′-nucleotidase; BIP, Bradykinin-inhibitory peptide; CRISP, cysteine-rich secretory protein; CVF, cobra venom factor; LAAO, L-amino acid oxidase; PDE, phosphodiesterase; PLA_2, phospholipase A_2; PLA_2i, PLA_2 inhibitor; PLB, phospholipase B; SVMP, snake venom metalloproteinase; SVMPi, SVMP inhibitor; VEGF, vascular endothelial growth factor.

Statistical Analyses of Assays

Enzymatic assays were analyzed using a One-Way Analysis of Variance (ANOVA) followed by a Tukey's posthoc analysis, with $p < 0.05$ considered statistically significant. Samples from Lombok and Sumbawa Islands were not considered in this analysis since they both consisted of one individual each, which would violate the assumptions of the tests.

RESULTS

Venom Proteome of Trimeresurus insularis from Sumbawa

To provide a reference for protein identity in the RP-HPLC chromatograms and SDS-PAGE gels, the venom toxin composition of Sumbawa *T. insularis* was characterized through a bottom-up protocol. Although we were unable to complete venomic experiments on the other island populations, the high conservation in venom composition as observed by RP-HPLC and SDS-PAGE (see below) leads us to believe that the Sumbawa venom proteome is highly representative of the venom composition of the additional *T. insularis* examined here. Venom was fractionated by RP-HPLC (Figure 4A), the individual peaks quantified spectrophotometrically, the chromatographic fractions analyzed by LC-MS/MS and DTT-reducing SDS-PAGE (Figure 4B), and the protein bands identified through a tryptic-peptide-centric MS/MS method (Table S1). In total, our venomic analysis identified 32 different proteins and 6 different peptides belonging to 18 protein/peptide families (Figure 4C and Table S2). Characterization of RP-HPLC fractions 41, 43, 47, 53, and 55 (Figure 4A) yielded five proteins in the PLA_2 family comprising 30.5% of the venom proteome (Figure 4C and Tables S1 and S2). Three of these PLA_2s show sequence similarity to *T. albolabris* A0A0H3U1W4 (8.1% of total PLA_2s), *T. albolabris* A0A0H3U206 (5.2%), and *T. erythrurus* A0A0H3U239 (2.7%) and belong to the Asp-49 subfamily (Table S1). In addition, two PLA_2 homologues similar to *T. erythrurus* A0A0H3U270 (10.9%) and *T. cardamomensis* A0A0H3U1Y7 (3.6%) were identified, with the former exhibiting an Asn in position 49, corresponding to Asn-49 PLA_2. Threonine is in position 49 of A0A0H3U1Y7; however, we were unable to identify peptides confirming Thr-49 in our LC-MS/MS analysis (Table S1). Serine proteinases, which eluted primarily in RP-HPLC fractions 59−78, comprise 11.8% of the venom proteome, and consisted of an α-fibrinogenase (3.8%) [~*T. albolabris* P0CJ41], a plasminogen activator (3.4%) [~ *T. albolabris* P0DJF5], a thrombin-like enzyme (1.8%) [~*T. albolabris* A7LAC6], and a kallikrein-like enzyme (1.5%) [~*Crotalus oreganus helleri* T1E6T7] (Tables S1 and S2).

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Sequences showing similarity to two other serine proteases, ~*T. gramineus* O13061 and ~*T. stejnegeri* Q71QH9, comprising 1.3% of the venom proteome were also identified; however, the specific activity of these two enzymes is currently unknown.

SVMPs were predominately the P−III subclass comprising 10.1% of the venom proteome, and our LC-MS/MS analysis identified proteins similar to *T. stejnegeri* Q2LD49 (8.0%), Q3HTN1 (1.8%), and Q3HTN2 (0.27%) eluting in RP-HPLC peaks 91−107 (Table S1 and Figure 4B). LC-MS/MS of the 50 kDa SDS-PAGE protein band of RP-HPLC peak 95 (Figures 4A and 4B) identified peptides similar to the P−II SVMPs P0DM87 from *T. stejnegeri* (0.3%) and P0C6B6 from *T. albolabris* (0.26%), representing <1% of the Sumbawa *T. insularis* venom proteome. P−II SVMP fragments were also identified in RP-HPLC peaks 15, 102, and 107 (representing <1% of the venom proteome); P−I SVMPs were not identified in our analyses (Figures 4C and 5A and Tables S1 and S2). Additional enzymes

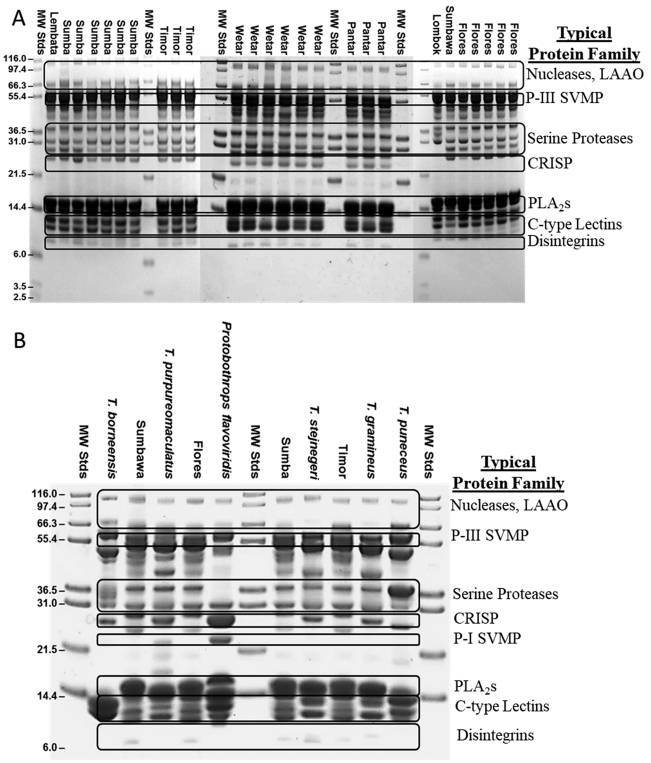

Figure 5. Comparison of SDS-PAGE patterns of *T. insularis* from the different Lesser Sunda islands (A) and of several species from the *Trimeresurus* complex (B) on NuPage gels (20 μg/lane). Typical protein families of bands of specific masses are indicated on the right; mass standards (MW Stds) are given in kilodaltons. Abbreviations: LAAO, L-amino acid oxidase; SVMP, snake venom metalloproteinase (P−III class); CRISP, cysteine rich secretory proteins; PLA_2, phospholipase A_2.

identified in moderate abundance include two LAAOs similar to *T. stejnegeri* Q6WP39 (1.1%) and *Protobothrops flavoviridis* T2HRS5 (6.1%), which eluted in the range of 79−97 and 100−107 min, and a PDE [~*P. flavoviridis* T2HQA0] (2.1%) was characterized from RP-HPLC fractions 91 and 102−107. LAAO fragments were also identified in RP-HPLC peak 15.

Five tripeptide SVMP inhibitors detected at *m*/*z* 444.22 (pEKW, 10.59%), 430.17 (pENW, 0.12%), 472.2 (pERW, 0.012%), 444.187 (pEQW, 0.24%), and 427.16 (pEQ[-17.03]W, 0.02%)[45−48] were identified in RP-HPLC peak 26, in addition to a bradykinin inhibitor peptide [*Agkistrodon bilineatus* P85025] comprising 0.24% of the venom proteome. The very similar mass SVMP inhibitors were unequivocally distinguished from one another by individually examining mass spectra and differentiating peptides by the mass differences of the b_2 ions. A recent study has identified many of these same peptide inhibitors in the venom of *P. flavoviridis*.[48]

C-type lectins were broadly distributed among RP-HPLC peaks 63−97 and comprise 10.9% of the Sumbawa *T. insularis* venom proteome. We identified peptide sequences matching the homodimeric TSL[49] (~ Q9YGP1) from *T. stejnegeri* (1.9%), the alpha [~ P0DJL2] and beta [~ P0DJL3] subunits of the heterodimeric purpureotin (6.73%) from *T. purpureomaculatus*, and the alpha [~ P81113] and beta [~ P81115] subunits of alboaggregin (2.1%) from *T. albolabris*. RP-HPLC peak 39 yielded a single SDS-PAGE band of ~7 kDa that showed sequence similarity to the disintegrin flavoridin [P18619] from *Protobothrops flavoviridis* (6.3%). Furthermore, our LC-MS/MS analysis identified peptides showing sequence similarity to the CRISP F2Q6F8 from *T. stejnegeri* (5.9%) in both the 23 and 25 kDa SDS-PAGE bands of RP-HPLC peak 57.

Additional proteins identified in low abundance, each representing <1% of the overall venom proteome (Figure 4C and Table S2), consisted of a glutaminyl cyclase [~*Daboia russelii* M9NCG3], aminopeptidase [~*Protobothrops flavoviridis* T2HQ95], PLA_2 inhibitor [~*Gloydius brevicaudus siniticus* O93233], phospholipase B [~*Protobothrops elegans* A0A077L7E7], cobra venom factor [~*Naja kaouthia* Q91132], 5′-nucleotidase [~*Protobothrops flavoviridis* A0A077L7M9], vascular endothelial growth factor [~*Protobothrops flavoviridis* U3TAE4], and a hyaluronidase [~*Ovophis okinavensis* U3TDI3].

1D SDS-PAGE

The 1D SDS-PAGE profiles of individual *T. insularis* venoms showed similar banding patterns between the different island populations, with prominent bands representing at least 8−9 protein families typically found in many viper venoms (Figure 5A). All individuals examined had protein bands in the mass range of nucleases (PDEs), LAAOs, P−III SVMPs, serine proteases, CRISPs, PLA_2s, and C-type lectins. Individuals sampled from the islands of Lembata, Sumba, Timor, and Pantar also had very faint bands in the 7 kDaA range, likely representing disintegrins. Surprisingly, following RP-HPLC separation, the Sumbawa sample also showed a moderate band at 7 kDa that was identified as a disintegrin (Figure 4B and Table S1), demonstrating that sample decomplexing by RP-HPLC prior to SDS-PAGE analysis provides significantly better visualization of low to moderately abundant proteins. Furthermore, a clear difference is seen in the single *T. insularis* sample from Lombok, which is missing two bands at approximately 25 and 29 kDa, typical masses of CRISPs and serine proteases, respectively.[10] In fact, LC-MS/MS of the 23 and 25 kDa SDS-PAGE bands of HPLC fraction 57 (Figure 4A,B) showed sequence similarity to the CRISP F2Q6F8 from *T. stejnegeri* (Table S1), and the 29 kDa SDS-PAGE protein band of HPLC Fractions 60 and 63 (Figure 4A, B) showed sequence similarity to the serine protease α-fibrinogenase P0CJ41 from *T. albolabris* (Table S1). All banding patterns of venoms from species in the *Trimeresurus* complex, though distinct, were qualitatively similar to *T. insularis* venoms, although *P. flavoviridis* venom showed a very different pattern,

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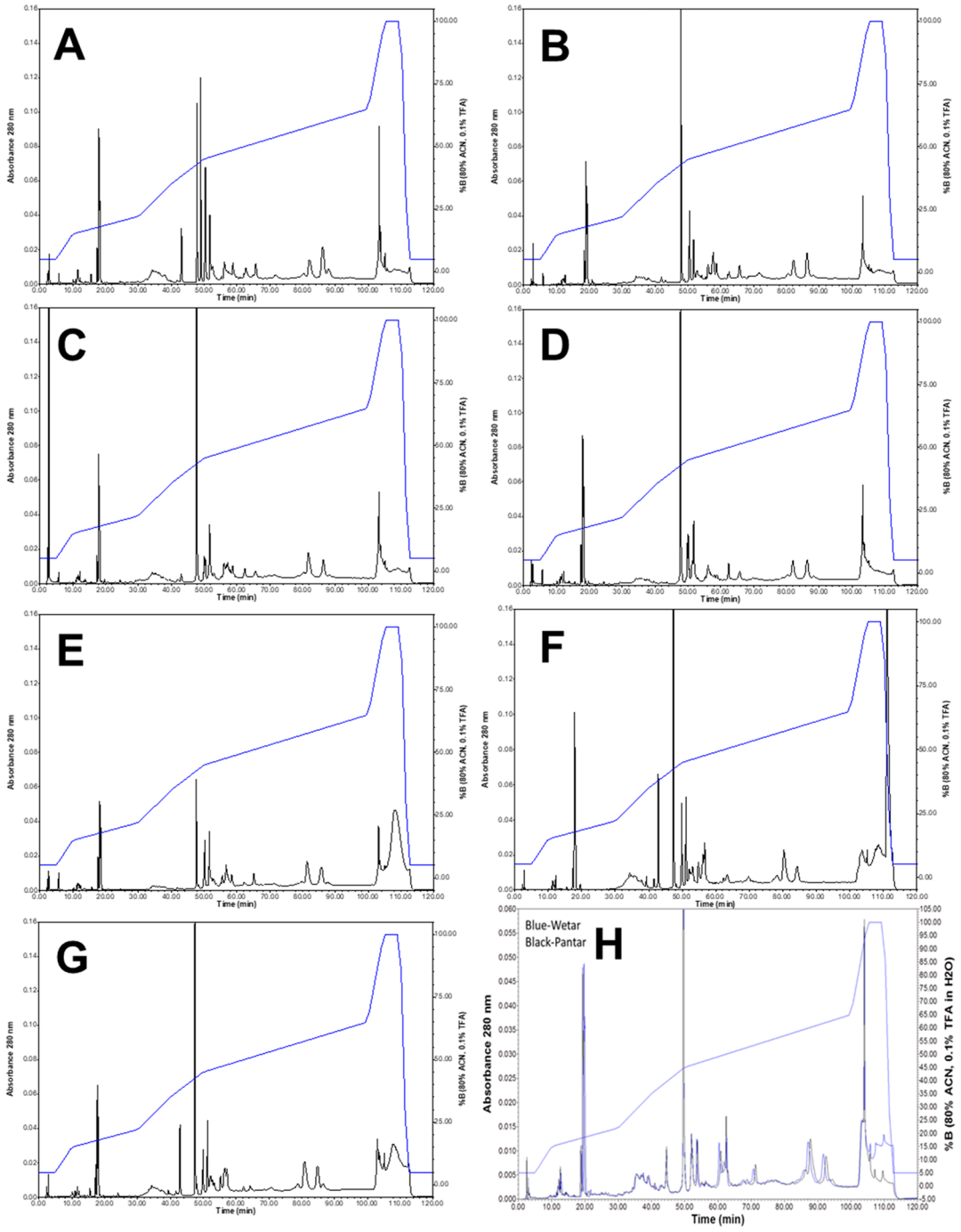

Figure 6. RP-HPLC chromatograms of *T. insularis* venom samples: (A) Sumbawa; (B) Lembata; (C) Pantar; (D) Wetar; (E) Timor; (F) Flores; (G) Sumba; and (H) overlay of chromatograms of venoms from Wetar (blue) and Pantar (black); note that these chromatograms are very similar.

particularly in the region of serine proteases and SVMPs (Figure 5B).

Reversed-Phase High-Performance Liquid Chromatography

To evaluate any similarities or differences among protein/peptide peak composition, one *T. insularis* venom sample from each island population (except Lombok, due to lack of venom) and venoms of the *Trimeresurus* complex were subjected to RP-HPLC fractionation (Figure 6). The chromatograms showed similarities in regards to peak presence, retention time, and (in general) peak height; chromatograms of venoms from Wetar and Pantar overlain in Figure 6H illustrate the high degree of similarity. However, there were some differences between peak presence and height for several venom samples. Most notable is a moderate peak at approximately 43−45 min (identified as two PLA_2s in our venomics analysis) in Sumbawa, Flores, and Sumba venoms that is barely discernible, to completely absent, in Lembata, Pantar, Wetar, and Timor samples. This peak also appears to be present in *T. puniceus* and *T. stejnegeri* but absent from the other *Trimeresurus* venoms examined (Figure 7).

There are noticeable differences in peak height in the 48−52 min range, also identified as PLA_2s (Table S1), when comparing Timor and Sumbawa to all other islands. The Sumbawa Island venom sample had an extra protein peak at 59 min (identified as

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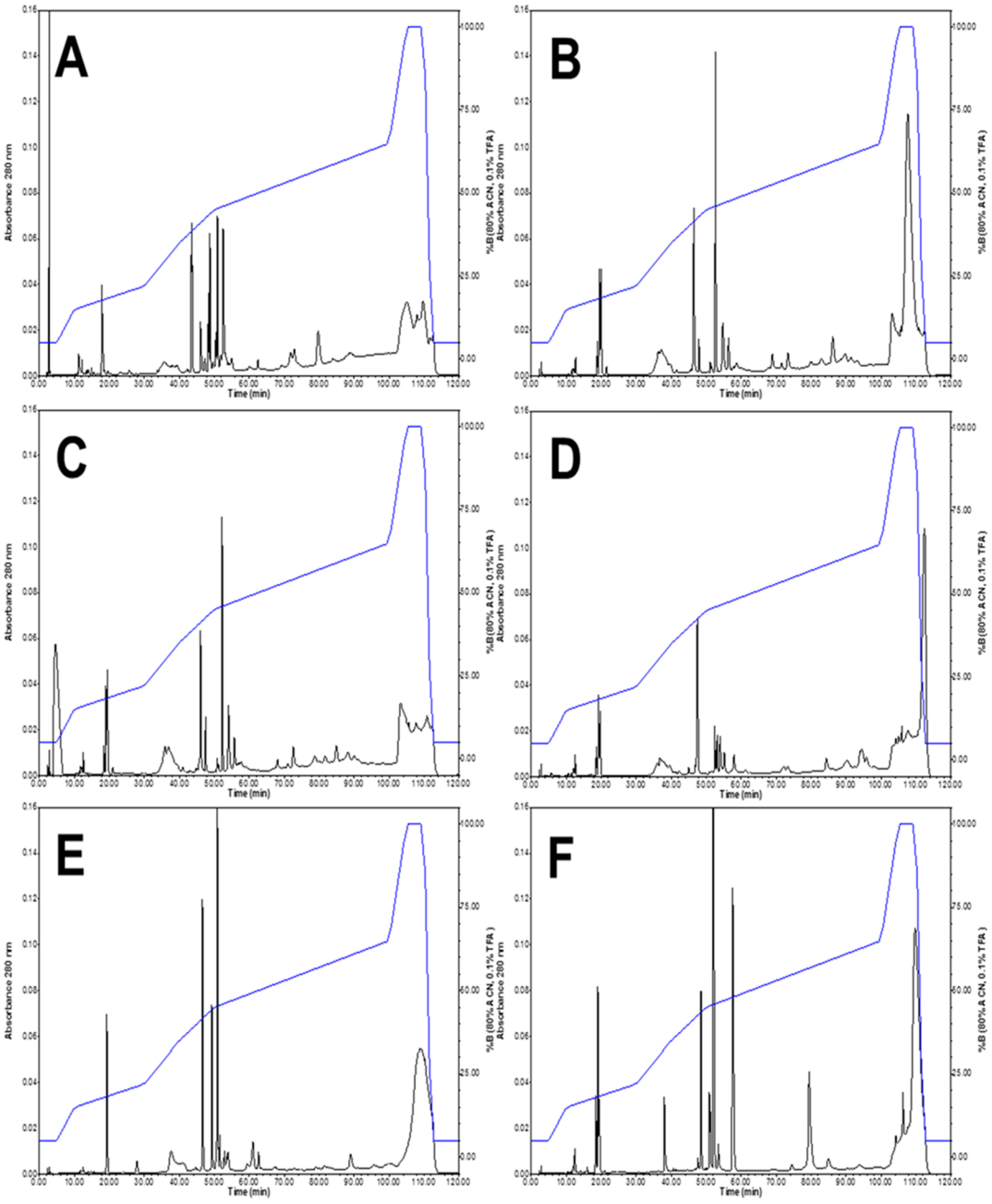

Figure 7. RP-HPLC chromatograms for several species of Asian vipers using the same protocol optimized for *Trimeresurus insularis*. (A) *T. puniceus*; (B) *T. gramineus*; (C) *T. stejnegeri*; (D) *T. purpureomaculatus*; (E) *T. borneensis*; and (F) *Protobothrops flavoviridis* venom.

an α-fibrinogenase, Figure 4A and Table S1) that was not seen in the other *T. insularis* samples. All *T. insularis* venoms showed similar HPLC peaks in the 80−90 min range (Figure 6) that are primarily large molecular mass enzymes (Figure 4A); however, this region seemed to vary in appearance when compared to the other venoms of the *Trimeresurus* complex (Figure 7). Peak height differences were also observed among most samples in the 100 to 115 min range, also primarily high molecular weight enzymes. These differences in protein-specific concentrations in the venom were consistent with the enzyme assay variation noted below.

Enzymatic Assays

All *T. insularis* venoms demonstrated activity for five enzymes commonly found in viper venoms (Figure 8). SVMP activity showed a statistically significant difference between islands ($F_{5,21}$ = 16.16, $p < 0.001$). Tukey's posthoc analysis revealed that both Pantar and Wetar populations had significantly less SVMP activity compared to the other three islands (Flores, Sumba, and Timor; all p's < 0.05), and there was also a statistically significant difference between Timor and Lembata populations ($p < 0.05$). No other comparisons of SVMP activity were statistically significant (all p's > 0.05). LAAO activity also exhibited a significant difference ($F_{5,21} = 2.792$, $p < 0.05$); however, this was only observed when comparing Wetar and Timor islands ($p <$ 0.05). All other comparisons were not significant (all p's > 0.05). KLE activity differences were also statistically significant ($F_{5,21}$ = 5.709, $p < 0.01$), with the Flores samples having significantly more activity compared to both Wetar and Pantar islands (both p's < 0.01); no other comparisons were significant (all p's > 0.05). TLE and PDE activities were not significantly different

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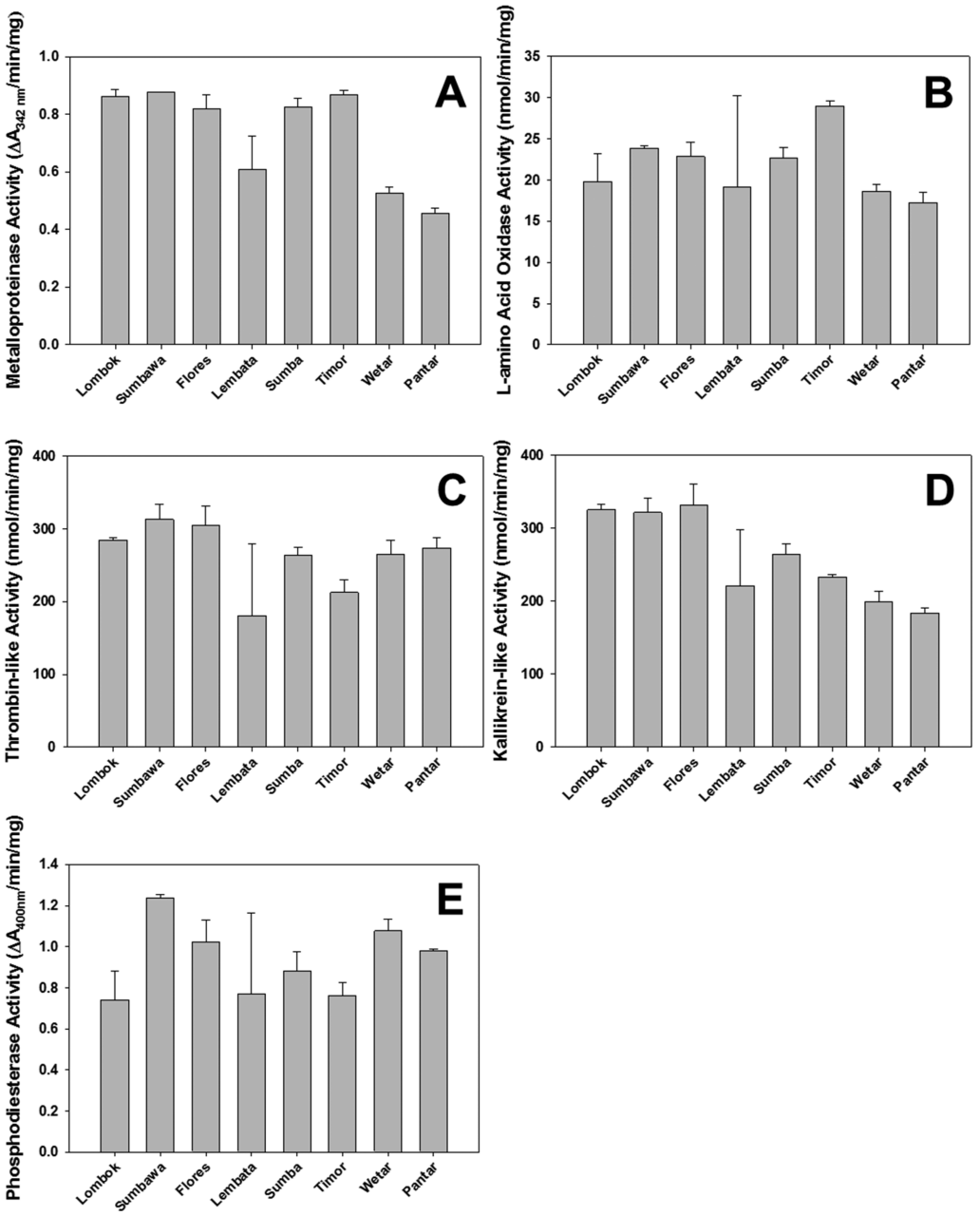

Figure 8. Enzyme activity assays of venoms from *T. insularis* from the islands of Lombok (n = 1), Sumbawa (n = 1), Flores (n = 7), Lembata (n = 2), Sumba (n = 6), Timor (n = 3), Wetar (n = 6), and Pantar (n = 3): metalloproteinase (SVMP) (A), L-amino acid oxidase (B), thrombin-like serine protease (C), kallikrein-like serine protease (D), and phosphodiesterase (E). Activities are expressed as units/min/mg venom protein.

between the populations following an ANOVA ($p > 0.05$). As mentioned above, due to only one sample each, Lombok and Sumbawa islands were not included in the ANOVA.

Fibrinogen Digest Assays

One representative venom sample from each island, as well as venoms of the representative species in the *Trimeresurus* complex, were assayed for their ability to digest human fibrinogen. Qualitative analysis showed slight differences in the time required for digestion of intact α-, β-, and γ-subunits between the different venoms (Figure 9). Lembata, Sumba, and Timor *T. insularis* venom samples completely digested the α- and β-subunits and most of the γ-subunit of fibrinogen by 5 min. A comparable digestion pattern was observed for *T. purpureomaculatus* and *T. borneensis* venoms. Similarly, *T. puniceus* venom degraded the α-subunit of fibrinogen by 5 min, while the β- and γ- subunits were completely digested by 10 min. Venom from Pantar and Wetar individuals showed identical fibrinogenolytic patterns, completely digesting the α- and β-subunits by 5 min, while the γ-subunit remained partially intact following 60 min of incubation. *Trimeresurus gramineus* and *T. stejnegeri* venoms exhibited a similar digestion pattern as that seen for the Pantar and Wetar individuals. Lombok and Sumbawa venom samples also digested the α-subunit by 5 min but required longer time to digest the β-subunit completely (30 and 60 min, respectively), while the γ-subunit was incompletely digested at 60 min. The fibrinogenolytic pattern produced by the Flores venom sample showed that the α-, β-, and γ-subunits remained largely intact until 10 min. *Protobothrops flavoviridis* venom

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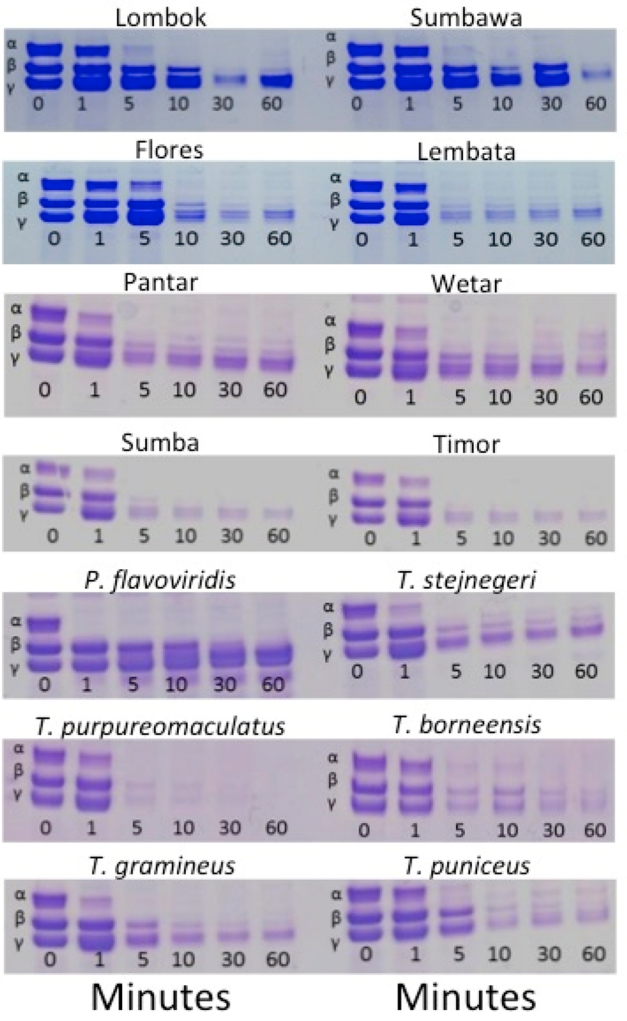

Figure 9. Fibrinogenolytic assays showing the degradation of fibrinogen subunits after incubation with venom from the different *T. insularis* populations or from the representative *Trimeresurus* species during specific time intervals (1, 5, 10, 30, and 60 min). Note that all venoms appear to rapidly catalyze the hydrolysis of the α-subunit by 5 min, but require more time to digest the β- and γ-subunits. *Protobothrops flavoviridis* failed to digest the γ-subunit even after 60 min of venom incubation.

digested the α-subunit within 1 min, but the β-subunit was not digested until 30 min, and the γ-subunit was not digested at all.

Zymogram Gel

All *T. insularis* samples (one from each island population) exhibited similar patterns of gelatin digestion, though band intensity varied between several islands (Figure 10). Venoms sampled from Sumbawa, Lembata, Wetar and Pantar individuals exhibited several prominent gelatin-digesting bands, likely corresponding to mature forms of P−II and P−III SVMPs. These bands are also present, although significantly fainter, in the Lombok, Flores, Sumba and Timor samples. Venoms from most other members of the *Trimeresurus* complex (*T. borneensis*, *T. stejnegeri*, *T. gramineus*, and *T. puniceus*) also digested gelatin and showed two to four bands; *T. borneensis* showed the highest activity, with two prominent bands and three fainter bands. *Trimeresurus purpureomaculatus* showed several very faint bands, while *P. flavoviridis* venom did not appear to digest gelatin. *Trimeresurus stejnegeri* and *T. gramineus* had very similar patterns, and *T. puniceus* and *T. borneensis* also had similar

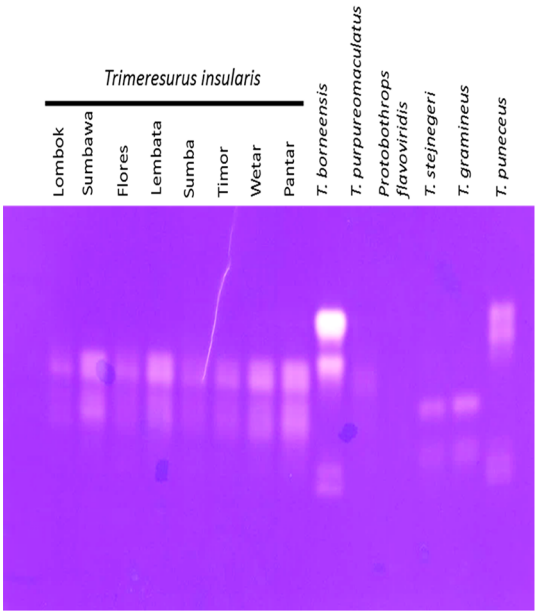

Figure 10. Venoms from each *T. insularis* population and from the representative *Trimeresurus* species were analyzed by gelatin zymography. All *T. insularis* venoms exhibited identical banding patterns, although digestion intensities appear to vary between several populations. Species in the *Trimeresurus* complex appear to have drastically different gelatinolytic compared to *T. insularis*. *Trimeresurus purpureomaculatus* showed weak gelatinolytic activity similar to *T. insularis*, and *P. flavoviridis* had no apparent activity.

patterns, though the bands of *T. puniceus* were much fainter. Interestingly, *T. puniceus* and *T. borneensis* are closely related, while *T. stejnegeri* and *T. gramineus* are not (see Figure 1). None of the other species in the *Trimeresurus* complex had banding patterns similar to those of *T. insularis*.

Western Blot of *Trimeresurus* Venoms

One venom sample from each island population of *T. insularis* (except Pantar, due to lack of venom) and all individuals from the *Trimeresurus* complex were tested for recognition by Green Pit Viper (*T. albolabris*) antivenom (Figure 11). Western blot analysis showed good reactivity of Green Pit Viper antivenom to all *T. insularis* samples, and it appears that all venom components (as visualized by 1D SDS-PAGE) were recognized, indicating that extensive shared epitopes exist between *T. insularis* and *T. albolabris* venoms. Venoms of all individuals in the *Trimeresurus* complex also showed strong binding with the exception of *P. flavoviridis* venom, for which only a few bands were recognized by antivenom.

DISCUSSION

In the current study, venom phenotypic characteristics of *T. insularis* from eight of the major Lesser Sunda Islands, representing most of this species' range, were examined. Though overall venom phenotypes were conserved in this species, significant differences in enzymatic activity levels were detected between several island populations for azocasein SVMP, kallikrein-like, and LAAO activities. Most prominent is the significant difference in SVMP activity, and venoms of individuals from Pantar and Wetar had significantly lower (>40%) activity compared to the Flores, Sumba, and Timor samples; Lombok and Sumbawa samples, represented by a single individual each, had levels similar to Flores, Sumba, and

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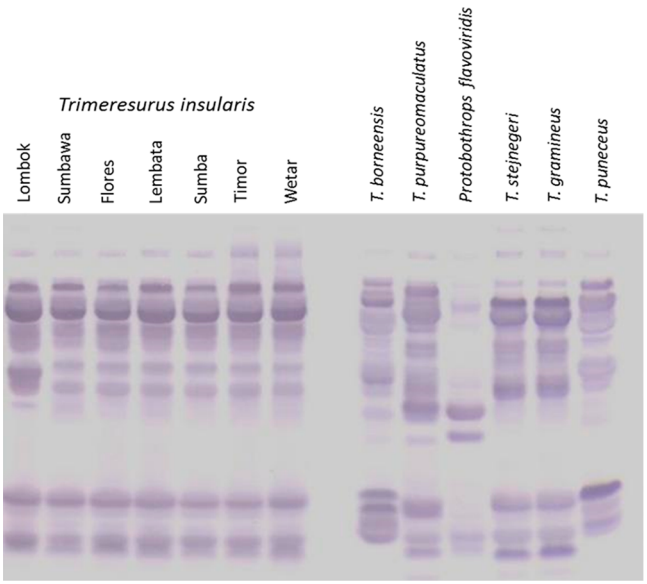

Figure 11. Western blot of *Trimeresurus* venoms using Green Pit Viper (*T. albolabris*) antivenom as the primary antibody. Each island (except Pantar) is represented for *T. insularis* venoms, as well as each species of the *Trimeresurus* complex assayed. Note that all samples of *T. insularis* (except Lombok) are essentially identical, and like 1D SDS-PAGE, the other *Trimeresurus* venoms all differ from *T. insularis*, though many shared epitopes are evident.

Timor. There was also a significant difference in SVMP activity between Timor and Lembata venoms. These differences in SVMP activity likely occur through differential expression of the P−II and P−III classes of SVMPs, which were both identified in the proteomic analysis of Sumbawa *T. insularis* venom. Further, gelatin zymography, which allows for the visualization of SVMP gelatinolytic activity,[50] revealed slight differences in band intensity between several *T. insularis* populations. Most apparent is the increased level of gelatin digestion by venoms from Sumbawa, Lembata, Wetar, and Pantar islands, also likely corresponding to mature forms of P−II and P−III SVMPs. Surprisingly, Lembata, Wetar, and Pantar had the lowest azocasein SVMP activity, suggesting substrate-specific differences exist between *T. insularis* SVMPs. More discrete differences in gelatinolytic activity were observed when examining venoms of other species in the *Trimeresurus* complex, with *T. borneensis* exhibiting the most distinct pattern and the highest gelatinolytic activity. SVMPs represent one of the most studied components of snake venoms, as they contribute to the often extreme levels of systemic hemorrhage and tissue degradation frequently observed following viperid envenomation.[51] The biological relevance of these substrate-specific differences is currently unclear, but it is likely related to differential degradation of specific components of vascular basement membranes and extracellular matrix by different SVMPs.[52]

Like most viper venoms, all *T. insularis* venoms exhibited TLE and KLE serine protease activities, and for KLE activity, both Wetar and Pantar populations had significantly lower activity compared to Flores. These two serine proteases affect the blood coagulation cascade at several points: KLEs liberate bradykinin from kininogen precursor, producing vascular relaxation and hypotension, and TLEs and some SVMPs are known to digest fibrinogen and deplete many of the major coagulation factors involved in clot formation, particularly fibrinogen.[28,53−55] From a trophic standpoint, these compounds inhibiting clot formation can facilitate the dissemination of venom compounds throughout the prey or bite victim. Slight but noticeable differences were also observed with fibrinogen digestion times between the different island populations of *T. insularis* and various species of the *Trimeresurus* complex. Venoms from *T. purpureomaculatus* and *T. borneensis*, and Lembata, Sumba, and Timor *T. insularis*, rapidly degraded the α- and β-subunits, and the majority of the γ-subunit of fibrinogen after 5 min of incubation; *T. insularis* from these islands also had moderate to high azocasein SVMP activity, moderate KLE activity, and low to moderate TLE activity. The venoms sampled from *T. gramineus* and *T. stejnegeri*, and Pantar and Wetar *T. insularis*, also exhibited a similar pattern of fibrinogenolytic activity, and all venoms degraded the α- and β-subunits of fibrinogen by 5 min, with minimal effect on the γ-subunit after 60 min of incubation. Wetar and Pantar samples exhibited the lowest SVMP and KLE activities, but had moderate TLE activity, suggesting that TLEs may be responsible for the rapid degradation of fibrinogen observed in these samples (though SVMPs are also known to be fibrinogenolytic). Lombok and Sumbawa venoms both showed similar fibrinogenolytic patterns and venoms from both islands also had very similar KLE, TLE, and azocasein SVMP activities. Venom from the Flores individual exhibited the most distinct fibrinogen digestion pattern of all *T. insularis*, requiring the longest time to digest the α subunit completely (10 min), and while the β and γ- subunits were heavily digested after 10 min, faint intact bands remained even at 60 min. Surprisingly, Flores samples exhibited high levels of SVMP, TLE, and KLE activities. Of all species examined, *P. flavoviridis* exhibited the most distinct fibrinogenolytic pattern, as the γ-subunit of fibrinogen remained completely intact following 60 min of venom incubation, suggesting the presence of an α/β-fibrinogenase in this species' venom.[53] This action is also likely due to the presence in *P. flavoviridis* venom of a metalloproteinase, triflamp,[56] and a thrombin-like enzyme, flavoxobin,[57] both of which are reported to catalyze hydrolysis of only the α-subunit of fibrinogen. Further, a recent venomic analysis indicated that serine proteases represent only 1.4% of *P. flavoviridis* venom.[48] Our venomic analysis confirmed the presence in *T. insularis* venom of thrombin-like, kallikrein-like, α-fibrinogenase, and plasminogen activator serine proteases, which collectively comprise 11.8% of the Sumbawa *T. insularis* venom proteome.

PDE and LAAO activities were also observed in all *T. insularis* venoms, and for the latter enzyme, a statistically significant difference was observed between Wetar and Timor islands. PDEs, which make up approximately 2.1% of the Sumbawa *T. insularis* venom proteome, likely facilitate induction of hypotension and assist with prey immobilization through the generation of purine nucleosides.[58,59] LAAOs have a broad range of pharmacological actions, and they may contribute to overall venom toxicity through the generation of hydrogen peroxide via the enzymatic deamidation of amino acids.[60] Some LAAOs have also been shown to induce cellular apoptosis[61−63] and hemorrhage,[64] and affect platelet aggregation.[61,65] Our venomic analysis indicates that LAAOs comprise 7.3% of the Sumbawa *T. insularis* venom proteome.

Despite the variation in enzymatic activities discussed above, RP-HPLC revealed only slight differences in venom compositional patterns between the different islands. Venoms sampled from Wetar and Pantar snakes again showed remarkable similarity in RP-HPLC patterns. More noticeable differences

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are observed when comparing the *T. insularis* populations with other species in the *Trimeresurus* complex, and especially with *P. flavoviridis,* which had the most unique HPLC pattern of all species examined. In addition, 1D SDS-PAGE analysis indicates that all *T. insularis* have the same gel electrophoretic patterns at approximately the same density, and all populations appear to have venoms containing PDEs, LAAOs, P−III SVMPs, serine proteases, CRISPs, C-type lectins, and PLA_2s. An exception is observed in the single Lombok venom sample that lacks two protein bands at approximately 25 and 29 kDa, which appear to be a CRISP and a serine protease, respectively[10,13] (Figure. 4B and Table S1). It is unknown if these missing bands are unique to just this individual or if it is representative of the entire Lombok population. *Protobothrops flavoviridis* had the most distinct 1D SDS-PAGE pattern of all of the species examined and completely lacked a protein band at approximately 36.5 kDaA, potentially a serine protease, but has a CRISP band at 21 kDaA[48] which is not present in any of the other samples examined. SVMPs, PLA_2s, serine proteases, and LAAOs were recognized as major constituents following transcriptomic analyses of the *P. flavoviridis* venom gland.[66,67] However, a recent quantitative proteomic analysis of *P. flavoviridis* venom revealed significant differences in protein family abundances when compared to Sumbawa *T. insularis* venom. *Protobothrops flavoviridis* venom is dominated by high PLA_2 levels (55.1%), in addition to SVMPs and disintegrins (31.3%), and contains low amounts of C-type lectins (2.8%), CRISP (1.8%), serine proteases (1.4%), LAAO (0.7%), PDE (0.07%), 5′-nucleotidase (0.02%) and low molecular weight peptides (6.4%).[48] Our results generally indicate that the venom phenotype for *T. insularis* appears to be fairly conservative across the Lesser Sunda archipelago, and it is also rather conserved in members of the *Trimeresurus* complex analyzed here. The differences in enzymatic activity between island populations but similarity of RP-HPLC and 1D SDS-PAGE profiles may be explained by the presence/absence of enzyme isoforms that exhibit varying levels of activity and substrate specificity not detected by these analyses.

The high conservation in venom composition is further supported by Western blot analysis with Green Pit Viper antivenom, which showed strong reactivity across the full range of *T. insularis* venom proteins, as well as many of the proteins in *T. borneensis, T. gramineus, T. puniceus, T. purpureomaculatus,* and *T. stejnegeri* venoms. These results indicate high conservation of the immunoreactive epitopes of *T. albolabris* venom with numerous toxin families in *T. insularis* venom. Although the clinical efficacy of an antivenom must be evaluated in animal protection models to ensure cross-reactivity, as a first approximation, these results suggest that Green Pit Viper antivenom may be effective for bites by *T. insularis*, for which a specific antivenom is not currently available. A similar result was obtained in a recent study using ELISAs and in vivo neutralization assays,[68] suggesting that this antivenom will likely be effective in treating human envenomations by *T. insularis*, which was formerly considered a subspecies of *T. albolabris*.[69] Again, *P. flavoviridis* venom appears to have a distinct composition compared to the *Trimeresurus* species, as only a handful of venom proteins were recognized by Green Pit Viper antivenom.

Venomic analysis of Sumbawa *T. insularis* venom confirmed the presence of the protein families identified by 1D-SDS PAGE and enzyme assays. PLA_2s represent the most abundant toxin family at 30.5%, and they are often some of the most toxic components of snake venoms, exhibiting a multitude of pharmacological activities.[70] Disintegrins and C-type lectins, which interact with platelet receptors and affect hemostasis,[71,72] are moderately abundant components, comprising a respective 6.3 and 10.73% of the entire *T. insularis* venom proteome. During predatory episodes, disintegrins in rattlesnake venoms also appear to play a role in relocation of envenomated prey released following a predatory strike,[73] and they may have a similar role for *T. insularis*. Numerous protein families of lesser abundance (<1%) were also identified in the *T. insularis* venom proteome (Figure 4C and Table S2), and while many of these compounds commonly occur in viperid venoms, they have somewhat elusive trophic roles during envenomation. A surprising result was the identification of numerous peptides for cobra venom factor (CVF; <1% of the total venom proteome), a toxin previously thought to be restricted to cobra species of the family Elapidae.[74] Although the exact biological role of CVF is not fully understood, upon introduction into the bloodstream it activates complement and generates anaphylatoxins, causing hemolysis; however, continuous complement activation by CVF results in complement depletion.[74,75]

Overall, the venom characteristics of *T. insularis* from the eight islands sampled showed a high degree of similarity, suggesting that *T. insularis* may have colonized the archipelago recently; a very recent phylogeographical study estimates colonization of the Lesser Sundas between 0.2 and 2.5 million years ago (Ma).[76] Land bridges may have facilitated movement of ancestral populations of *T. insularis* between certain islands (Lombok + Sumbawa and Pantar + Alor are separated by channels ∼100 m in depth, whereas Flores + Adonara + Lembata are connected by shallower channels), followed by periods of isolation as the northern ice sheets retreated and water levels rose. However, the islands of Wetar, Timor, and Sumba were never connected to the other islands by a land bridge, so it is likely that *T. insularis* arrived to these islands by rafting. Further, *T. insularis* was once considered a subspecies of *T. albolabris*. However, recent molecular analyses have determined that they diverged long ago (see Figure 1 above) and that *T. insularis* is the sister taxon to *T. fasciatus*, which occurs on a small island north of Flores;[77,78] estimated time of divergence of *T. fasciatus* and *T. insularis* is approximately 2.5 Ma.[76] Due to the extensive cross-reactivity of *T. insularis* venom with *T. albolabris* (Green Pit Viper) monovalent antivenom (see above), it is quite possible that the other species nested between them phylogenetically also have very similar venom profiles. In fact, with the exception of *P. flavoviridis* venom, which showed the greatest overall differentiation of venom phenotype, all *Trimeresurus* venoms analyzed exhibited rather similar phenotypes, demonstrated by similar epitope recognition by *T. albolabris* (Green Pit Viper) antivenom and by SDS-PAGE. As *P. flavoviridis* is the most distantly related species and inhabits islands of Japan while the other species inhabit various parts of India and Southeast Asia, the closer similarity of these *Trimeresurus* venoms is not unexpected.

The lack of island-specific natural history data for *T. insularis* makes it difficult to predict local effects on venom phenotypic evolution. However, based on the dietary information available, and the general diversity of venom proteins (similar to that of many other vipers), *T. insularis* can be considered to possess a "generalized" venom with efficacy at subduing diverse prey taxa. Given that each island contains a distinct community of prey species, this generalized venom composition may be partly

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responsible for the successful establishment of *T. insularis* upon colonization of a new island in the Lesser Sunda chain.

CONCLUSIONS

This study provides a comprehensive comparative analysis of *T. insularis* venoms sampled from eight Lesser Sunda Islands and of several other species of the genus *Trimeresurus*.

Venomic analysis of the Sumbawa individual shows that the venom is overwhelmingly dominated by PLA_2s, followed by serine proteases, SVMPs, SVMP inhibitors, C-type lectins, LAAOs, disintegrin, CRISP, and PDE. Numerous proteins of lower abundance (<1%) were also detected including CVF, which, to the best of our knowledge, is the first time this protein has been identified in a viperid venom. Comparisons between the different *T. insularis* populations revealed similarities in 1D SDS-PAGE, RP-HPLC chromatograms, and TLE and PDE activities. However, both Pantar and Wetar populations had significantly less SVMP activity compared to Flores, Sumba, and Timor islands, LAAO activity was significantly different between Wetar and Timor islands, and KLE activity was significantly higher in Flores individuals compared to both Wetar and Pantar individuals. Slight but noticeable differences were also observed with fibrinogenolytic and gelatinolytic activities. Overall, venom composition of *T. insularis* appears to be rather conservative across the archipelago, and high similarities in intraspecific venom composition are also apparent when compared to *T. borneensis, T. gramineus, T. puniceus, T. purpureomaculatus,* and *T. stejnegeri* venoms. This lack of significant intraisland variation in venom composition is also reflected in a lack of phylogeographical structure, as indicated by a very recent mtDNA-based analysis.[76] This study provides a robust analysis of the venom phenotype of *T. insularis* that can be extended into future studies of this and other species of Southeast Asian viperid snakes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.9b00077.

Supplemental Table S1: A full list of LC-MS/MS derived tryptic peptide sequences (XLS)

Supplemental Table S2: Relative occurrence of the different protein families and subfamilies present in the venom of Sumbawa *Trimeresurus insularis*; abbreviations as in Figure 1 (PDF)

Supplemental Table S3: Localities and museum accession numbers for all *Trimeresurus insularis* sampled (XLS)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

1D SDS-PAGE, one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis; 5NTD, 5′-nucleotidase; ACN, acetonitrile; BIP, bradykinin-inhibitory peptide; CRISP, cysteine-rich secretory protein; CVF, cobra venom factor; DTT, dithiothreitol; LAAO, L-amino acid oxidase; PDE, phosphodiesterase; PLA_2, phospholipase A_2; PLA_2i, PLA_2 inhibitor; PLB, phospholipase B; RP-HPLC, reversed-phase high-performance liquid chromatography; SVMP, snake venom metalloproteinase; SVMPi, SVMP inhibitor; TFA, trifluoroacetic acid; VEGF, vascular endothelial growth factor.

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