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Analysis of snake venom metalloproteinases from Myanmar Russell's viper transcriptome

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ABSTRACT

Snake venom metalloproteinases (SVMPs) are the key enzymes in Russell's viper (RV) venom which target all important components of haemostasis, such as clotting factors, platelets, endothelial cells and basement membrane. The structural diversity of SVMPs contributes to the broad spectrum of biological activities. The aim of the study was to investigate the SVMP transcript profile to gain better insights into the characteristic clinical manifestations of the Myanmar Russell's viper (MRV) bites that distinguish it from the RVs of other habitats. Next generation sequencing (RNA-Seq) of mRNA from MRV venom glands (2 males and 1 female) was performed on an Illumina HiSeq2000 platform and then *de novo* assembled using Trinity software. A total of 59 SVMP contigs were annotated through a Blastn search against the serpent nucleotide database from NCBI. Among them, disintegrins were the most abundant transcripts (75%) followed by the P-III class SVMPs (25%). The P-II SVMPs were scarce (0.002%), while no P-I SVMPs were detectable in the transcriptome. For detailed structural analysis, contigs were conceptually translated and compared with amino acid sequences from other RVs and other vipers using Clustal Omega. The RTS-disintegrin (jerdostatin homolog) was the most abundant among transcripts corresponding to 5 disintegrin isoforms. From 10 isoforms of SVMPs, RVV-X, and *Vipera lebetina* apoptosis-inducing protease (VLAIP) homolog, hereby termed *Daboia siamensis* AIP (DSAIP), were found to be highly expressed. Venom protein analysis using SDS-PAGE followed by mass spectrometry revealed that the disintegrin was scarce, while the latter two SVMPs were abundant. These two proteins can contribute to severe clinical manifestations caused by MRV envenomation.

1. Introduction

Russell's viper is a highly venomous snake found throughout Southeast Asian countries. According to molecular phylogeographic investigations (Thorpe et al., 2007), there are currently two full species, *Daboia russelii* (South Asia) and *Daboia siamensis* (Southeast Asia and China). Previously, subspecies were defined by morphology (Wuster, 1998; Wuster et al., 1992). *Vipera russelii nordius* (Northern India) and *Daboia russelii pulchella* (Sri Lanka) have now been incorporated into *D. russelii*. Similarly, *V. r. limitis* (Indonesia) and *V. r. formosensis* (Taiwan) are considered as synonym species with *D. siamensis* (http://reptile-database.reptarium.cz/species?genus=Daboia&species=russelii) (http://reptile-database.reptarium.cz/species?genus=Daboia&species=siamensis).

The clinical profile of Russell's viper bites varies in different countries. Haemostatic abnormalities are seen in all countries but the presentation of the more severe coagulopathy, renal failure and pituitary haemorrhage are reported more often in Myanmar than in any other countries. Vascular leakage resulting in oedema and

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hypotension is the unique manifestation in Myanmar (Antonypillai et al., 2011; Belt et al., 1997; Hung et al., 2002; Kularatne et al., 2014; Kularatne, 2003; Warrell, 1989). However, the toxin components causing increased vascular permeability have not been identified.

Such variable clinical manifestations of envenoming in different countries indicate geographical variations in venom composition and suggest that there are differences in the sequences and/or quantities of the active components of the venoms. Transcriptomic profiling of venom gland tissue is a comprehensive approach to understand the composition and complexity of the snake venom, although it cannot investigate post-translational modifications. Toxin profiling from the transcriptome is useful for both targeted searches for individual toxins or isoforms and discovery of all toxins attributed to a species (Brahma et al., 2015).

Snake venom metalloproteinases (SVMPs) are amongst the key enzymes contributing to local and systemic haemorrhage in viper bites and constitute the major component (11- over 65%) of total protein in Viperidae venoms (Calvete et al., 2007). SVMPs show a wide spectrum of biological activities, such as haemorrhage, fibrin (ogen)olysis, prothrombin activation, factor X activation, apoptosis, platelet aggregation inhibition, pro-inflammation and inactivation of blood serine proteinase inhibitors (Markland and Swenson, 2013). This diversity in biological functions is a consequence of the diverse structural domains of the SVMPs.

Transcriptome analysis using Expressed Sequence Tags (ESTs) derived from a cDNA library has some limitations due to low coverage and is generally not very quantitative. Since Next-generation sequencing (NGS) provides a more comprehensive coverage for transcriptomic characterisations of venom glands of snakes than low-throughput sequencing approaches, such as cloning and EST generation, RNA-Seq of Myanmar Russell's viper (MRV) was performed to obtain a more comprehensive qualitative and quantitative analysis of SVMP transcripts. Analysis of the expression profile of SVMPs from MRV venom glands could provide information on expression level of different SVMP isoforms and their sequences. In addition, comparison with SVMP sequences from various Russell's vipers could give insights into the reasons for different signs and symptoms among Russell's viper bites from different countries.

2. Materials and methods

2.1. Sample collection and RNA extraction

Venom gland transcriptomes of 3 Russell's vipers (2 adult male and 1 female *Daboia russelii siamensis*) from the Snake Farm, Myanmar Pharmaceutical Factory, Myanmar, were sequenced. The habitat of the snakes was Kyun Gyan Gon Township, Yangon, Myanmar. The experimental plan was approved by the Animal Care and Use Committee, Chulalongkorn University (CU-ACUC) (No. 17/2558). The venom was milked 3 days before dissection of the venom glands to stimulate the production of messenger RNAs (mRNAs) in the venom glands. The venom glands were dissected while the snake was anaesthetised using chloroform. The tissues were kept in RNAlater solution (Ambion Inc., Canada) to stabilise and protect the cellular RNA for storage without endangering the quality and quantity of RNA. The tissues samples were stored at −80 °C until further processed.

Total RNA from male gland tissues was extracted using Trizol reagent (Life Technologies, Carlsbad, California) and that from female was extracted using a Total RNA Purification Kit (Jena Bioscience GmbH, Jena, Germany). The concentration of total RNA was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The sample was stored at −80 °C. Subsequently, mRNA from the 2 male samples was isolated using PolyAT Tract mRNA Isolation System (Promega, Madison, USA). For the female sample, mRNA was isolated using a FastTrack MAG mRNA Isolation Kit (Invitrogen, Carlsbad, California). The concentration of mRNA was then determined using a NanoDrop spectrophotometer. Subsequently, mRNA was precipitated, concentrated with 3 M sodium acetate/isopropanol and stored at −80 °C.

2.2. Next-generation sequencing (NGS) and contig assembly

The mRNA was resuspended in RNase-free water before sending to Macrogen Inc. Geumchun-gu, Seoul, Korea for RNA sequencing. The mRNA sequencing libraries were prepared using the TruSeq RNA sample preparation kit (Illumina) with the selected insert sizes of 200–400 bp. Libraries were then sequenced on the Illumina HiSeq2000 platform using (2 × 100bp) paired-end reads.

NGS reads were assessed for quality using the FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The raw data from the Illumina platform was then processed by Trimmomatric (0.32) (http://www.usadellab.org/cms/?page=trimmomatic) to remove adaptor sequences, error nucleotides and low quality sequences. The resulting data were assembled using Trinity (r20140717) (http://trinityrnaseq. sourceforge. net/).

2.3. Annotation of SVMP transcripts

De novo transcripts were annotated using Blastn and Blastx searches. Firstly, transcript annotation was archived through Blastn searches against the collected NCBI nucleotide sequences database with search words “venom” and “serpent”. Annotations with high scores (the e-value cutoff $<10^{-5}$) at the top of the Blastn hit list were considered after filtering out genome sequences, transcribed sequences and duplicated contigs.

The toxins were then grouped manually in a Microsoft Excel work sheet according to key words of known toxin names. From different toxin groups, metalloproteinase contigs were fetched from whole data set using Python programming language. Lastly, using Blastx with the cut-off e-value of 10^{-5}, analysis of homologs was carried out against the NCBI SwissProt database. The best annotation was taken after Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) alignment of the translated sequences.

2.4. Transcript abundance analysis

Transcript quantification of the *de novo* assembly was carried out with RSEM (1.2.15), which estimated transcript abundance based on the metrics standardised the number of RNA-seq reads mapped to a particular exon by the total number of mapped reads and the size of the exon. Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values were used for analysis of relative transcript expression levels.

2.5. Prediction of open reading frames (ORF), coding DNA sequences (CDS) and conserved domain searches

The CDS were selected based on Blastn and Blastx results. The open reading frame of each sequence was predicted by ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) and their conserved domains were identified against NCBI's conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

2.6. Availability of the sequences

All nucleic acid sequences and translated amino acid sequences in this study were deposited to the NCBI GenBank database

(accession numbers MF589235-MF589243) (GenBank, http://www.ncbi.nlm.nih.gov/genbank/).

2.7. SDS-PAGE analysis of the venom

Crude venom extracted from the snakes of which venom glands were used for NGS were analysed by SDS-PAGE (Laemmli, 1970) on a 15% resolution gel and 4% stacking gel. Samples were loaded in either reduced or non-reduced form. Gels were run at 200 V, 30 mA per gel, for 60 min. Proteins were visualised with Coomassie Brilliant Blue R250 followed by destaining with methanol: water: acetic acid (10: 80: 10). The gels were read with Molecular Imager Gel DocTM XR+using Image Lab Version 3.0 in UV Trans illumination with Commassie Fluor Orange.

2.8. Mass spectrometry (MS) analysis of the protein bands

The protein bands of interest were excised from Coomassie Brilliant Blue-stained SDS-PAGE gels and subjected to in-gel digestion using the following steps: reduction (10 mM dithiothreitol, 45 min at 56 °C) and alkylation (10 mM iodacetamide, 30 min in the dark at room temperature), then overnight digestion with sequencing-grade trypsin (12.5 ng/μL in 25 mM ammonium bicarbonate, 0.63 μg trypsin/gel band) at 37 °C. The supernatant was transferred into a 1.5 mL Protein LoBind Tube (Eppendrof, Hamburg, Germany). The peptides were then extracted for 3 times from the gel piece with 0.1% formic acid/50% acetonitrile by vortexing for 15 min. These peptide extracts were combined with the original supernatant and the combined peptide extracts then dried in a vacuum centrifuge, and redissolved in 25 μL of 0.1% formic acid. A 12 μL of this sample was submitted to LC-ESI-MS/MS.

The tryptic peptides were separated by nano-liquid chromatography (EASY-nLC 1000, Thermo Fisher Scientific) coupled to a mass spectrometer (Q-Exactive Plus Hybrid Quadrupole-Orbitrap, Thermo Fisher Scientific) through an EASY-Spray nano-electrospray ion source (Thermo Fisher Scientific). The flow rate was set to 300 nL/min and column was developed with a linear gradient of 0.1% formic acid in water (Solution A) and 0.1% formic acid in acetonitrile (Solution B), 4–40% B for 50 min, followed by 40–95% B for 10 min. The analysis included a full MS scan at a resolution of 70,000 followed by 10 data-dependent MS2 scans at a resolution of 17,500. The full MS scan range of 200–2000 m/z was selected, and precursor ions with the charge states of +1 or greater than +8 were excluded. Normalised collision energy of HCD fragmentation was set at 28%. Raw LC-MS/MS files were searched by Proteome Discoverer 2.1 against *Daboia russelii* databases from Uniprot plus RVV-X, DSAIP and Disintegrin sequences from transcriptome and common contaminants concatenated with their reversed sequences. A target-decoy approach was used to limit a false discovery rate (FDR) of the identified peptides to less than 1%. Parent and fragment monoisotopic mass errors were set at 10 ppm. Carbamidomethyl cysteine was used as a fixed modification mass. Variable modifications were oxidation at methionine. A maximum of 1 missed cleavage site was allowed.

3. Results and discussion

3.1. Next-generation sequencing (NGS) and de novo assembly of contigs

The purity of mRNA of male and female samples was measured using a NanoDrop spectrophotometer. The 260/280 ratios of samples were 1.96 and 2.17 respectively (reference ratio: 2.0). The TruSeq RNA library QC result showed 105 ng/μL (570 nM) concentration and 281 base pair (bp) size for the male library and 99 ng/μL (540 nM) concentration and 284 bp size for the female library.

From the Illumina HiSeq2000 platform, 82 million reads from the male library and 79 million reads from the female library were generated. After removal of low-quality reads and adaptors using Trimmomatric, 78 million reads with 98.9% Q20 bases (the base quality more than 20 and an error rate of less than 0.01) from the male library and 74 million reads with 98.6% Q20 bases from the female library remained for *de novo* assembly. The largest contigs assembled by Trinity were 31,000 bp and 17,000 bp for male and female libraries respectively.

3.2. Annotation of SVMP transcripts

Contig gene name annotation was archived through a Blastn search against the collected serpent nucleotides database (142,183 sequences). The Blastn search showed that 23,525 out of 88,325 transcripts (26.6%) from the male sample and 20,355 out of 50,858 transcripts (40.0%) from the female sample have a best hit after filtering.

The contigs with a metalloproteinase annotation (N = 109; male = 69 and female = 40) were grouped according to their gi numbers obtained from the Blastn result. Subsequently, 59 contigs (male = 37 and female = 22) with SVMP annotations were clustered into 4 disintegrins, 7 P-III class SVMPs and 2 P-II class SVMPs (Table 1). The contigs from each cluster, according to a strong homology between their overlapping regions, are likely to have originated from the same gene. The presence of only 3′-untranslated regions of toxin mRNA points to incompletely sequenced transcripts. Some transcripts contained parts of both exons and introns indicating that they were still in the pre-mRNA stage at the time of venom gland extraction or that there were potentially new splice variants for the transcripts.

For detailed structural analysis, contigs containing full-length and partial length CDS were then conceptually translated and compared to the translated amino acid sequences from the Blastn annotation results. The translated sequences that were not matched with Blastn annotation result were also compared to the Blastx annotation results. After Blastx hits against UniProtKB/Swiss-Prot database, 2 contigs from the P-III 2 cluster from the female sample matched to 2 different reference contigs c11797_g1_i1_F (MF589241) (disintegrin, full-length CDS) and c11797_g1_i4_F (MF589243) (VLAIP-A, partial length CDS). Similarly, out of 6 contigs containing partial length CDS from the P-III 7 cluster from the male sample showed 2 different reference transcript matches: c9453_g1_i1_M, c66080_g1_i1_M (homologous to *Protobothrops mucrosquamatus* zinc metalloproteinase-disintegrin-like NaMP) and; c22734_g1_i1_M, c34552_g1_i1_M, c41009_g1_i1_M, c71069_g1_i1_M (homologous to *Echis coloratus* svmp-u mRNA) (Table 2).

3.3. Transcript abundance

The toxins were grouped manually based on the key words of individual toxin names. The FPKM values of contigs within each toxin group were combined to represent the individual toxin group. The most common toxin transcripts in both male and female samples were annotated as metalloproteinases (approximately 34.8%), more than any other toxin annotated transcripts.

As shown in Table 3, the abundance distribution of SVMP toxin transcripts: disintegrin (75%), P-III class SVMPs (25%) and P-II class SVMPs (approximately 0.002%), were the same for male and female samples indicating that the relative proportions of toxin transcripts are not related to the size of the cDNA library. In another way, it could be observed that the highly expressed contigs from both viper sexes shared the same identity: P-III class SVMPs were found to

Table 1
The list of snake venom metalloproteinase (SVMP) transcripts with their expression level in FPKM values and portions of mRNA.

Classes	Annotation	Male		Female	
		FPKM	Portion of mRNA	FPKM	Portion of mRNA
Dis 1	gi|66390955|gb|AY987816.1| *Macrovipera lebetina lebetase* isoform Le-4 mRNA partial cds	62514	Full CDS	18456	Full CDS
Dis 2	gi|95007580|emb|AM261811.1| *Macrovipera lebetina transmediterranea* ml-G1 gene for VGD-containing dimeric disintegrin subunit ML-G1 precursor exons 1-2	82.5	Intron/Intron + Exon	10.8	Intron/Intron + Exon
Dis 3	gi|110346543|emb|AM286800.1| *Echis ocellatus* gene for MLD-containing dimeric disintegrin subunit exons 1-2	62.4	Exon +3′UTR	6.93	3′UTR
Dis 4	gi|95007582|emb|AM261812.1|*Macrovipera lebetina transmediterranea* ml-G2 gene for MLD-containing dimeric disintegrin subunit ML-G2 precursor, exons 1-2	2.46	Intron	–	–
P-III 1	gi|300079899|gb|GQ420354.1| *Daboia russellii russelii* factor X activator heavy chain mRNA complete cds	9225	Full CDS	3829	Full
P-III 2	gi|61104774|gb|AY835996.1| *Macrovipera lebetina* VLAIP-A mRNA complete cds	5794	Partial CDS	7846	Full, Partial CDS
P-III 3	gi|727360728|gb|GBUG01000035.1| TSA: *Echis coloratus* svmp-n mRNA sequence	5490	3′UTR	2012	3′UTR
P-III 4	gi|83523625|emb|AM039691.1| *Echis ocellatus* mRNA for Group III snake venom metalloproteinase (Svmp3-Eoc1 gene) clone Eo_venom_04E07	139	Partial CDS, intron, 3′UTR, SINE	25.7	Partial CDS, Intron, 3′UTR, SINE
P-III 5	gi|387014235|gb|JU173711.1| TSA: *Crotalus adamanteus* Cadam_SVMPIII-2e mRNA sequence	5.66	3′UTR	3.34	3′UTR
P-III 6	gi|387014223|gb|JU173705.1|TSA: *Crotalus adamanteus* Cadam_SVMPIII-1a mRNA sequence	14.51	3′UTR	–	–
P-III 7	gi|727360714|gb|GBUG01000042.1| TSA: *Echis coloratus* svmp-u mRNA sequence	6.43	Partial CDS, 3′UTR	–	–
P-II 1	gi|320579332|gb|GU594194.1| *Echis ocellatus* clone 04C07 group II snake venom metalloproteinase (Eoc00006) mRNA partial cds	1.41	Partial CDS	–	–
P-II 2	gi|31322300|gb|AY204244.1| *Gloydius saxatilis* metalloproteinase/disintegrin saxin precursor mRNA partial cds	–	–	8.44	Partial CDS

CDS = Coding DNA Sequence.

be far more highly expressed than P-II in Myanmar Russell's viper (MRV) venom glands. No P-I class SVMP transcripts were detected by our analysis. There are no other transcriptomic data for *Daboia* genus to compare with our findings. However, the relative abundance of different classes of in our study is similar to that of Urutu (*Bothrops alternatus*) (Cardoso et al., 2010). SVMPs from the same family of *Viperidae*, *i.e.* the most abundant SVMP transcripts being P-III; low number transcripts of P-II SVMPs; and no detectable P-I SVMP transcript. The abundance of P-III class SVMP transcripts (assuming that transcripts are proportionally translated) might explain the intense pro-coagulant activity of *Viperidae* snake venom.

The comparison of the content of contigs encoding toxins in adult male and female venom glands showed some gender-related differences. A disintegrin transcript isoform (Dis 1b) was highly expressed only in the female venom gland. Some P-III SVMP isoforms (P-III 6, 7a and 7b) were only expressed in the male venom glands but at low expression levels. The P-II SVMP transcripts expressed in male and female were found to be different isoforms. However, the small sample size used in the current study did not allow us to conclude if a gender-related or individual variability was reflected in the discovered differential abundance of SVMP transcripts.

Four contigs (MRV1-4) of Snake Venom Metalloproteinases Inhibitor (SVMPI) were also identified in these transcriptomes, and detailed analysis of those sequences was reported in our previous study (Yee et al., 2016).

3.4. Sequence analysis of disintegrins

Snake venom disintegrins are generated by proteolytic processing of P-II SVMP precursors (Kini and Evans, 1992) or are

Table 2
Annotations of snake venom metalloproteinase (SVMP) contigs containing full-length and partial length coding DNA sequences (CDS) against Swiss-Prot NCBI database.

Classes	Sub-classes	Contigs	Annotation	Coverage	% identity	e-value
Dis 1	Dis 1a	c13890_g2_i1_M	gi|48428040|sp|Q7ZZM2.1|Disintegrin jerdostatin (*Protobothrops jerdonii*)	100%	100%	5e-81
		c11797_g1_i2_F	gi|48428040|sp|Q7ZZM2.1|Disintegrin jerdostatin (*Protobothrops jerdonii*)	100%	100%	5e-81
P-III 1		c20464_g1_i1_M	ADJ67475.1 factor X activator heavy chain [*Daboia russellii russelii*]	100%	97%	0.0
		c11797_g1_i3_F	ADJ67475.1 factor X activator heavy chain [*Daboia russellii russelii*]	100%	97%	0.0
P-III 2		c20464_g1_i2_M	gi|82228619|sp|Q4VM08.1|VM3VA_MACLB| Zinc metalloproteinase-disintegrin-like VLAIP-A (*Macrovipera lebetina*)	82%	89%	0.0
		c11797_g1_i4_F	gi|82228619|sp|Q4VM08.1|VM3VA_MACLB| Zinc metalloproteinase-disintegrin-like VLAIP-A (*Macrovipera lebetina*)	100%	90%	0.0
	Dis 1b	c11797_g1_i1_F	gi|48428040|sp|Q7ZZM2.1|Disintegrin jerdostatin (*Protobothrops jerdonii*)	100%	87%	3e-71
P-III 7	P-III 7a	c9453_g1_i1_M	XP_015683143.1 PREDICTED: zinc metalloproteinase-disintegrin-like NaMP (*Protobothrops mucrosquamatus*)	59%	97%	2e-13
		c66080_g1_i1_M	XP_015683143.1 PREDICTED: zinc metalloproteinase-disintegrin-like NaMP (*Protobothrops mucrosquamatus*)	100%	93%	6e-43
	P-III 7b	c22734_g1_i1_M	JAC96590.1 Snake venom metalloproteinase U (*Echis coloratus*)	100%	95%	2e-50
		c34552_g1_i1_M	JAC96590.1 Snake venom metalloproteinase U (*Echis coloratus*)	100%	92%	3e-49
		c41009_g1_i1_M	JAC96590.1 Snake venom metalloproteinase U (*Echis coloratus*)	100%	93%	2e-52
		c71069_g1_i1_M	JAC96590.1 Snake venom metalloproteinase U (*Echis coloratus*)	100%	98%	3e-54
P-II 1		c19938_g2_i1_M	sp|U5PZ28.1|VM2H1_BOTLA|Zinc metalloproteinase-disintegrin BlatH1 (*Bothriechis lateralis*)	55%	58%	2e-11
P-II 2		c11787_g3_i1_F	AAP20639.1 metalloproteinase/disintegrin saxin precursor (*Gloydius saxatilis*)	92%	78%	2e-25

Table 3
Summary expression level of disintegrin and snake venom metalloproteinase (SVMP) isoforms in male and female transcriptomes.

Classes	Annotation	Male		Female	
		FPKM	FPKM per class	FPKM	FPKM per class
Dis 1a	Disintegrin jerdostatin (*Protobothrops jerdonii*)	62514	62661	18456	24205
Dis 1b	Disintegrin jerdostatin (*Protobothrops jerdonii*)	0	(75%)	5731	(75%)
Dis 2	VGD-containing dimeric disintegrin subunit ML-G1 (*Macrovipera lebetina transmediterranea*)	82.5		10.8	
Dis 3	MLD-containing dimeric disintegrin (*Echis ocellatus*)	62.4		6.93	
Dis 4	MLD-containing dimeric disintegrin subunit ML-G2 (*Macrovipera lebetina transmediterranea*)	2.46		0	
P-III 1	factor X activator heavy chain (*Daboia russelii russellii*)	9225	20678	3829	7986
P-III 2	Zinc metalloproteinase-disintegrin-like VLAIP-A (*Macrovipera lebetina*)	5794	(25%)	2115	(25%)
P-III 3	Snake venom metalloproteinase N (*Echis coloratus*)	5490		2012	
P-III 4	Group III snake venom metalloproteinase (Svmp3-Eoc1 gene) (*Echis ocellatus*)	139		25.76	
P-III 5	Cadam_SVMPIII-2e (*Crotalus adamanteus*)	5.66		3.34	
P-III 6	Cadam_SVMPIII-1a (*Crotalus adamanteus*)	14.5		0	
P-III 7a	Zinc metalloproteinase-disintegrin-like NaMP (*Protobothrops mucrosquamatus*)	2.37		0	
P-III 7b	Snake venom metalloproteinase U (*Echis coloratus*)	4.06		0	
P-II 1	Zinc metalloproteinase-disintegrin BlatH1 (*Bothriechis lateralis*)	1.41	1.41	0	8.44
P-II 2	Metalloproteinase/disintegrin saxin precursor (*Gloydius saxatilis*)	0		8.44	

synthesised from short coding mRNA without the metalloproteinase-coding region (Okuda et al., 2002). One disintegrin isoform (Dis 1a) was highly expressed in both male and female venom glands and another isoform (Dis 1b) was selectively expressed in the female glands. The Dis 1a and Dis 1b proteins were found to possess amino acid variations in the signal peptide and N-terminus of the coding region with conserved functional disintegrin motifs (Fig. 1). The Dis 1a sequence was found to be identical to the russellistatin from *D. russelii,* as well as to jerdostatin from *Protobothrops jerdonii*. The disintegrin domains of all 3 sequences contain an RTS motif.

The contigs classified in Dis 2, Dis 3 and Dis 4 classes are of various lengths. Some are likely to be in pre-mRNA stage containing both intron and exon portions, while some have only intron or 3′ untranslated region (UTR) of the subject sequences (Supplementary Table T1). These contigs have the annotation of VLD-containing and MLD-containing dimeric disintegrins from other vipers.

MRV venom appears to contain several forms of disintegrins with different kinds of functional motifs (Table 3). RTS-disintegrins (*Disintegrin jerdostatin*) are more highly expressed than non-RGD disintegrins (MLD- & VGD-containing disintegrins). The proteomic studies of Pakistan RVV (0.4%) (Mukherjee et al., 2016), Southern Indian RVV (3.2%) (Sharma et al., 2015) and Western Indian RVV (4.9%) (Kalita et al., 2017) showed that disintegrins contribute only a minor portion of the toxin pool in these species. Disintegrins were not identified in Sri Lankan RV (Tan et al., 2015) or even in the same species, *D. r. siamensis* from Myanmar (Risch et al., 2009; Yee et al., 2016).

3.5. Sequence analysis of Russell's viper venom factor X activator (RVV-X)

The high abundance of P-III SVMP transcripts in the venom gland transcriptome is in agreement with a detection of RVV-X heavy chain in venomic analysis of Siamese Russell's viper (Risch et al., 2009; Yee et al., 2016). The Clustal alignment of translated sequences of Myanmar Russell's viper species [c20464_g1_i1_M(MF589235) and c11797_g1_i3_F (MF589236)] with those from other species showed 99% sequence identity to Indian species (ADJ67475.1); 97% identity to Indonesian species (Q7LZ61.2) (Chen et al., 2008a) and Thailand species (Sai-Ngam, 2007). The N-terminal portion of the latter sequence is not completely available (Fig. 2).

Since the heavy chain of RVV-X functions cooperatively with light chains, RVV-X light chain transcripts [c20336_g1_i7_M (MF589237) and c20336_g1_i9_M (MF58938)] from the MRV transcriptome have also been retrieved and aligned with the light chains of different species. RVV-X light chains are C-type lectin-like proteins which are linked to the heavy chain through disulfide bonds (Takeda et al., 2007). The two light chains recognise the Ca^{2+}-bound conformation of the Gla domain of factor X helping the protease action of the heavy chain on Factor X (Takeya et al., 1992).

The alignment of light chain 1 showed that light chain 1 transcript of MRV has a 97% similarity to those of Thai (Sai-Ngam, 2007), Indian (ADJ67474.1), Myanmar (ADK22820.1) species and Eastern Russell's viper [Q4PRD1 (SLLC1_DABSI)]; and 98% identity to that of Indonesian (Chen et al., 2008a) species. It is noticeable that the glutamate residues next to the Ca^{2+} binding site and in the C-terminal region of protein sequences from Thailand, Indonesia and

```
                    Signal peptide
c13890_g2_i1_M      MIQVLLVTICLAVFPYQVSSKTLKSGSVNEYEVVNPGTVTGLPKGAVKQPEKKHEPMKGN 60
jerdostatin         MIQVLLVTICLAVFPYQVSSKTLKSGSVNEYEVVNPGTVTGLPKGAVKQPEKKHEPMKGN 60
c11797_g1_i2_F      MIQVLLVTICLAVFPYQVSSKTLKSGSVNEYEVVNPGTVTGLPKGAVKQPEKKHEPMKGN 60
c11797_g1_i1_F      MMQVLLVTISLAVFPYQGSSIILEAGNVNDYEVVYPQKVTAMPKGAVKQPEKKHEPMKGN 60
                    *:*******.******* **   *::*.**:**** * .**.:******************
                                 Disintegrin domain
c13890_g2_i1_M      TLQKLPLCTTGPCCRQCKLKPAGTTCWRTSVSSHYCTGRSCECPSYPGNG 110 (100%)
jerdostatin         TLQKLPLCTTGPCCRQCKLKPAGTTCWRTSVSSHYCTGRSCECPSYPGNG 110
c11797_g1_i2_F      TLQKLPLCTTGPCCRQCKLKPAGTTCWRTSVSSHYCTGRSCECPSYPGNG 110 (100%)
c11797_g1_i1_F      TLQKLPLCTTGPCCRQCKLKPAGTTCWRTSVSSHYCTGRSCECPSYPGNG 110 (87%)
                    **************************************************
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Fig. 1. Clustal alignment of conceptually translated disintegrin transcripts from MRV with that of jerdostatin (disintegrin from *Trimeresurus jerdonii*) with conserved integrin binding motif RTS. Isoform 1 (Dis 1a) [c13890_g2_i1_M (MF589239) and c11797_g1_i2_F (MF589240)] protein sequence has 100% identity and isoform 2 (Dis 1b) (c11797_g1_i1_F) (MF589241) sequence has 87% identity to jerdostatin sequence. (*) Identical residues; (:) strongly similar residues; (.) weakly similar residues.

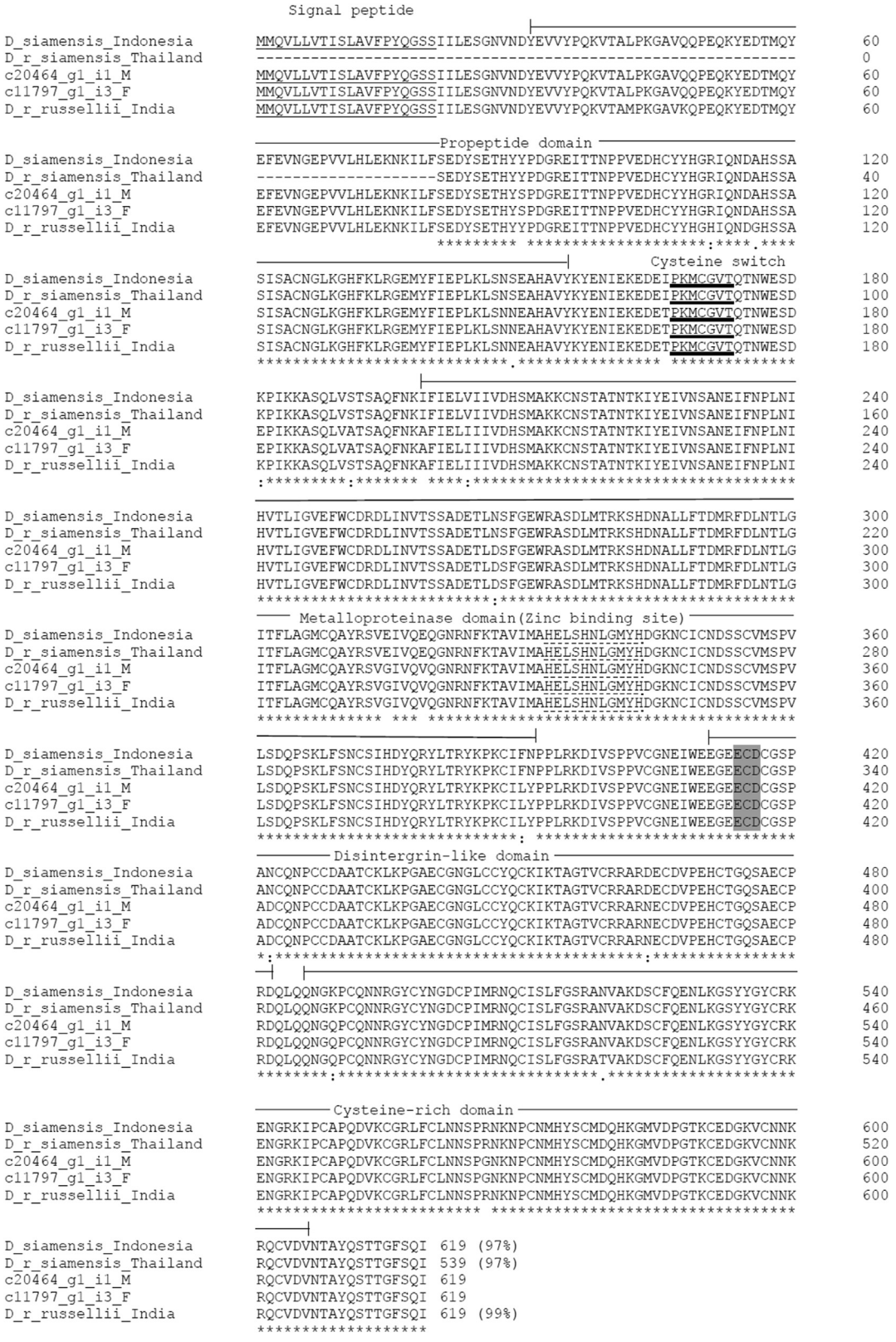

Fig. 2. Clustal alignment of RVV-X heavy chains from different viper species. The putative signal peptide, cysteine switch motif, zinc binding site and ECD sequence are indicated by single underline, bold underline, dotted underline, and shadow, respectively. (*) Identical residues; (:) strongly similar residues; (.) weakly similar residues.

Fig. 3. Sequence alignment of RVV-X light chain 1 proteins from different viper species. The conserved cysteine residues are highlighted in gray. The putative signal peptide, N-glycosylation and Gla-domain binding sites are indicated by dotted underline, single underline, and double underline, respectively. The Ca^{2+} binding sites are marked with closed circle (•).

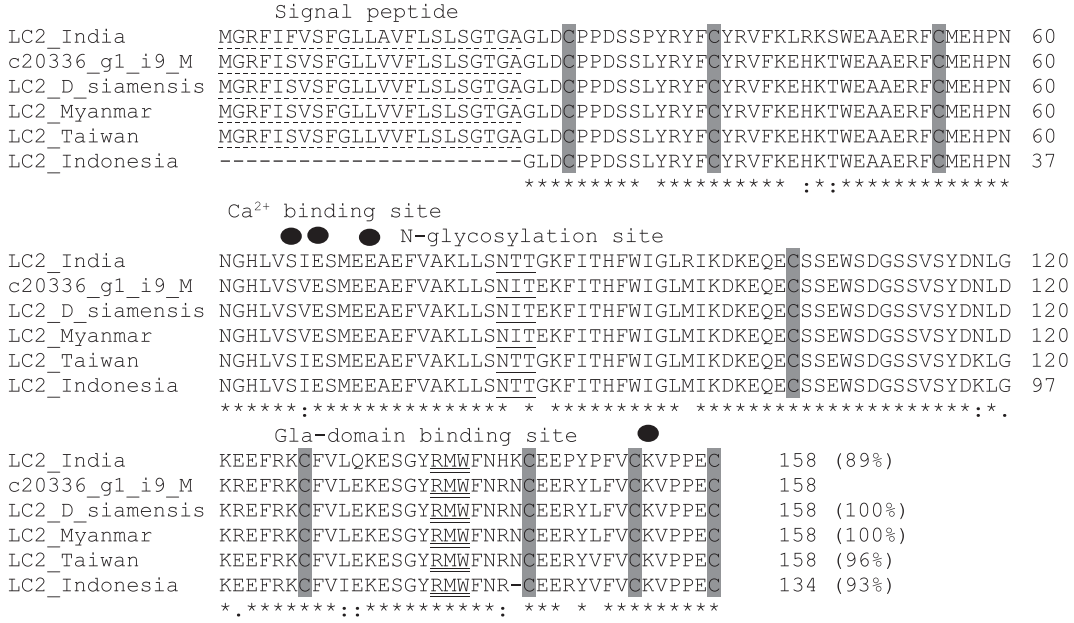

Fig. 4. Sequence alignment of RVV-X light chain 2 proteins from different viper species. The conserved cysteine residues are highlighted in gray. The putative signal peptide, N-glycosylation and Gla-domain binding sites are indicated by dotted underline, single underline, and double underline, respectively. The Ca^{2+} binding sites are marked with closed circle (•).

Eastern Russell's viper species are substituted by lysine and valine in those of Myanmar and Indian species, respectively (Fig. 3).

The translated amino acid sequence of the light chain 2 transcript of MRV has 100% identity to those of Eastern Russell's viper [Q4PRD2 (SLLC2_DABSI)] and Myanmar (ADK22819.1) species, 96% to Taiwan (AFE61611.1) species, 93% to Indonesian (Chen et al., 2008a) species, and 89% to that of Indian (ADJ67473.1) species (Fig. 4). The protein alignment of light chains shows that species variation in amino acid residues in light chain 2 is more than that in light chain 1. In the future, detailed interactions of those active residues should be further studied by constructing a model with suitable computational software.

3.6. Sequence analysis of Vipera lebetina apoptosis-inducing protease-A (VLAIP-A) homolog

The second most highly expressed P-III SVMP transcripts, c20464_g1_i2_M (MF589242) and c11797_g1_i4_F (MF589243), in both male and female transcriptomes are homologous to zinc metalloproteinase-disintegrin-like VLAIP-A from *Macrovipera*

Fig. 5. Clustal alignment of translated transcripts (partial-length CDS) of VLAIP-A homolog from MRV with that of zinc metalloproteinase-disintegrin-like VLAIP-A (*V. lebetina* apoptosis-inducing protease from *Macrovipera lebetina*). The shorter translated sequence from the male sample, c20464_g1_i2_M(MF589242) has 486 amino acid residues. The longer translated sequence from the female sample, c11797_g1_i4_F (MF589243) has 619 residues and 90% identity to VLAIP-A sequence. The putative signal peptide, cysteine switch motif, zinc binding site and GECD sequence are indicated by single underline, bold underline, dotted underline, and shadow, respectively. (*) Identical residues; (:) strongly similar residues; (.) weakly similar residues.

lebetina (Fig. 5). These transcripts are partially sequenced but contain regions encoding signal peptide, pro-domain, metalloproteinase, disintegrin-like and cysteine-rich domains. Our deduced amino acid sequences have the critical functional motifs that belong to VLAIP-A as follows: the cysteine switch motif (PKMCGV) in the prodomain and the Zn^{2+}-chelating sequence HEXXHXXGXXHD in the metalloproteinase domain. The exact sequence of the Zn-binding site, **HE**MG**H**NL**G**ME**HD,** differs from that of RVV-X (**HE**LS**H**NL**G**MY**HD)**. The sequence also possesses the conserved intra-chain disulfide bond pattern of 6-Cys: Cys314-Cys394, Cys354-Cys378 and Cys356-Cys361. The typical Cys369 residue for vascular apoptosis inducing proteases was also present. The disintegrin-like domain was found to contain the ECD motif for binding to integrin receptor. Since the VLAIP homolog is a new finding in regards to the Myanmar Russell's viper, it will be named as *Daboia siamensis* apoptosis-inducing protease (DSAIP) from hereon.

3.7. Identification of RVV-X, DSAIP and disintegrin in MRV venom

The crude venoms from the snakes in this study were subjected to SDS-PAGE (Fig. 6). The visible bands, reflecting the most abundant venom proteins, appear to be similar in all 3 snakes under both reduced and non-reduced conditions. The 67-kDa band of

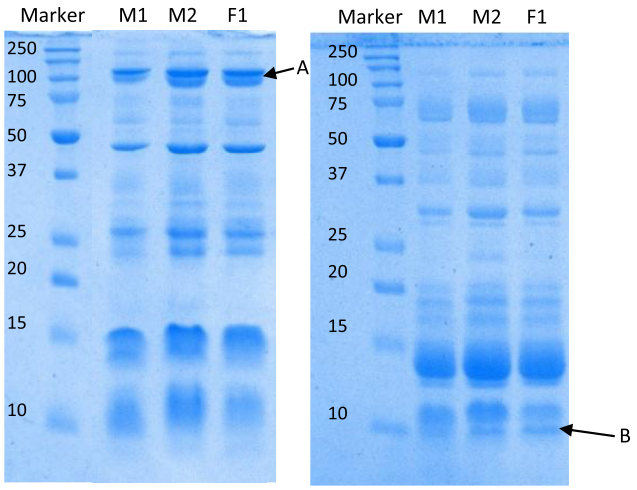

Fig. 6. SDS-PAGE, under non-reduced (left) and reduced (right) conditions, of the crude venoms from MRVs from which venom glands were extracted. The lanes are labeled for venoms from 2 males (M1 and M2), venom from a female (F1) and the molecular weight marker in kDa. Each lane contains 14 μg of protein.

Daborhagin identified as the major component of the MRV venom used in a previous study (Yee et al., 2016) was not observed, supporting the finding that it was not found in the transcriptome of the snakes in this study. This is because of a regional difference between 2 lots of snake venoms used. A proteomic study showed that venom composition of MRV was found to be varied according to the geographical locations (Thomas et al., 2017).

The main proteins bands observed on SDS-PAGE were identified using a proteomics approach. Of direct relevance to this SVMP study were the two protein bands, A and B (Fig. 6), cut from non-reducing and reducing gels respectively. The trypsin digested products from band A showed 40.2% coverage (Table 4) to the RVV-X heavy chain (c11797_g1_i3_F, MF589236) (29 unique peptides out of 31 total matched peptides, Supplementary Table T2) and 51.9% coverage (Table 4) to DSAIP (c11797_g1_i4_F, MF589243) (25 unique peptides out of 27 total matched peptides, Supplementary Table T3). Band B contained the tryptic peptides from Kunitz-type serine protease inhibitor 1 (VKT1_DABRR, Q2ES50) (2 unique peptides out of 4 total matched peptides) and one unique peptide from Disintegrin (c11797_g1_i1_F, MF589241) (Table 4 & Supplementary Table T4). The finding of only one tryptic digested peptide from disintegrin might probably due to incomplete digestion of the protein by the enzyme during in-gel digestion.

Depending on the intensity of the identified protein bands on SDS-PAGE, the relative abundance of disintegrin in the venom compared with RVV-X and DSAIP is not as high as would be expected from the transcript levels. The reasons for such discrepancies between the transcriptomic and proteomic abundance of toxins remain elusive. A study on six viperid species suggested that translational and/or post-translational regulations affect final protein abundances (Casewell et al., 2014). However, the transcriptomic and proteomic quantities of RVV-X and DSAIP are matched. The RVV-X was slightly more abundant than DSAIP in both venom glands (mRNA) and venoms (proteins).

3.8. Potential clinical implications

Haemotoxic manifestations in humans, such as circulatory shock, spontaneous bleeding, and acute renal failure, often lead to death following viper bite envenoming. The venom toxins act on components of the haemostatic system to disturb the normal haemostatic mechanisms, resulting in intravascular coagulation, increased vascular permeability and impaired platelet aggregation (Fatima and Fatah, 2014; Hutton and Warrell, 1993; Kini, 2011; Meier and Stocker, 1991; Rojnuckarin, 2008).

The geographical, age-dependent, and even individual difference in venom composition and isoforms of each toxin may influence the outcome of snakebite envenomation. In the current transcriptomic study, 5 isoforms of disintegrin transcripts and 10 isoforms of SVMP transcripts were expressed. Among them, RTS-containing disintegrin, RVV-X heavy chain, and DSAIP, in decreasing order, are found to be the most highly expressed.

Analysis of the MRV proteins showed that SVMPs comprise a higher percentage (20%) in this venom (Yee et al., 2016) than in those of Russell's viper from other countries, such as Southern India (9.5%) (Sharma et al., 2015) and Sri Lanka (6.9%) (Tan et al., 2015). On the other hand, the venoms of Russell's viper from Pakistan (Mukherjee et al., 2016) and Western India (Kalita et al., 2017) have a higher content of SVMPs than that of Myanmar venom. Differences in venom composition of Russell's viper from India and Sri Lanka was shown by their complete venomic studies, while the venomics of Myanmar, Taiwan and Thailand species have only been partially studied.

The proteomes of Russell's viper venoms from Thailand and Taiwan have not been studied in detail, particularly the composition and identification of toxins contained within it, but 2D gel analysis of these venoms showed similar protein patterns in both *V. russelli formosensis* and *V. russelli siamensis* (Nawarak et al., 2003). However, the differences in venom compositions between Myanmar and Thailand species have not been determined.

The high phospholipase activity in the venom of Thailand Russell's viper is compatible with the finding of the high abundance of PLA_2 toxin transcripts in a cDNA library of Thai RV venom gland (Sai-Ngam, 2007). A further study (Suwansrinon et al., 2007) showed that the renal haemodynamic changes in dogs induced by Thai Russell's viper venom appeared to correlate better with proteolytic enzyme activity than PLA_2 activity. Suntravat et al. (2011) demonstrated that RVV-X from Thai Russell's viper is the key component of renal dysfunction and coagulopathy *in vivo*. They suggested that RVV-X is a major cause of renal failure through intra-glomerular clotting (Suntravat et al., 2011). However, the significant haemolytic feature (Warrell, 1989) in Thai Russell's viper bite might be mainly contributed by PLA_2. Signs and symptoms, such as oedema and shock, are unlikely to occur in Russell's viper bites in Thailand, but are prominent in the Myanmar Russell's viper bite (Warrell, 1989).

High expression of RVV-X heavy chain (P-IIId SVMP) in the MRV transcriptome indicates that RVV-X is one of the principal

Table 4
Proteins identified from the selected bands from SDS-PAGE of Myanmar Russell's viper venom.

Band	Description	Coverage	MW (kDa)
A	RVV-X (c11797_g1_i3_F) (MF589236)	40.2	67.2
	DSAIP (c11797_g1_i4_F, partial CDS) (MF589243)	51.9	66.9
B	Kunitz-type serine protease inhibitor 1 (VKT1_DABRR) (Q2ES50)	36.9	9.3
	Disintegrin (c11797_g1_i1_F) (MF589241)	12.2	9.8

components of SVMPs in MRV venom, and this is supported by protein studies (Risch et al., 2009; Yee et al., 2016). One of the strong bands at 100 kDa was seen on the non-reducing SDS-PAGE gel (Fig. 6). RVV-X affects the coagulation cascade, as well as platelet aggregation, leading to severe haemorrhage, renal failure, shock and pituitary infarction via activation of factor X, formation of thrombin and fibrin clots (Mizuno et al., 2001), as well as potent inhibition to collagen- and ADP-stimulated platelet aggregation via its disintegrin-like domain (Takeya et al., 1992).

Daborhagin, geographically specific component of MRV, causes haemorrhage, oedema and shows fibrin (ogen)olytic activities (Chen et al., 2008b; Khin-Than Yee et al., 2014; Yee et al., 2016). The resultant truncated fibrinogen hinders blood coagulation. A brief thrombotic event occurs in early stages of procoagulant envenoming, just before the activation of fibrinolysis that could explain cerebral infarction following a Myanmar Russell's viper bite. Either inhibition or activation of platelet aggregation by SVMPs (especially via the functional motif of the disintegrin-like domain) and disintegrins increase the risk of bleeding in snake bite-patients (Berling and Isbister, 2015; Kini and Koh, 2016; White, 2005).

Another highly expressed P-III SVMP, DSAIP, was newly detected in the transcriptome of MRV in the present study. This VLAIP homolog is also identified in the venom of Russell's viper from Sri Lanka (Tan et al., 2015), and Pakistan (Mukherjee et al., 2016). However, no such homolog was identified in RVV of Western Indian (Kalita et al., 2017) and Southern Indian (Sharma et al., 2015). The reason for such variation is not clear.

The VLAIP from *M. lebetina* showed proteolytic activity on fibrinogen. The VLAIP inhibits endothelial cell adhesion to extracellular matrix proteins: fibrinogen, fibronectin, vitronectin, collagen I and collagen IV. Moreover, it induces apoptosis of human endothelial cells (Trummal et al., 2005). The biological function of DSAIP should be characterised further.

The most highly expressed disintegrin transcripts in MRV venom glands were found to be RTS-disintegrins. Disintegrins with an RTS motif are found in venom of the Southern (Sharma et al., 2015) and Western Indian species (Kalita et al., 2017). In the study of Sanz-Soler et al. (2012), the RTS-disintegrin, russellistatin, was shown to comprise 2% of the total venom proteins of *Daboia russelii* (Sanz-Soler et al., 2012, 2016). Although RTS-disintegrins are highly expressed in Myanmar Russell's viper venom glands, there is no report on purification and characterisation of disintegrins from MRV venom. The RTS-Disintegrin from the Chinese Jerdons pit viper is reported to inhibit the selective binding of integrins of endothelial cells to basement membrane collagen (Sanz et al., 2005). Nevertheless, protein analysis showed only a small quantity of this disintegrin in the studied venom. The discovered discrepancy between transcript and protein abundancies is striking and deserves more detailed analysis and comprehension.

4. Conclusions

Our transcriptomic profile of SVMPs is the first analysis of this kind for Russell's viper species. It gives general information of the abundantly expressed SVMP genes, together with sequence analysis, drawing a picture of their particular abundance and potentially distinctive role in MRV venom. The most represented component of the SVMP pool, RVV-X which acts as pro-coagulant resulting in disseminated intravascular coagulation and renal failure. DSAIP and RTS-disintegrin are newly identified as components of the MRV in the current study and their functions in MRV venom are yet to be established. Further study on their potential effect on endothelial cells is recommended for understanding of pathophysiology of increased vascular permeability in MRV snake bite.

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Transparency document

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Appendix A. Supplementary data

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