



Deciphering the main venom components of the ectoparasitic ant-like bethylid wasp, *Scleroderma guani*



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ABSTRACT

Similar to venom found in most venomous animals, parasitoid venoms contain a complex cocktail of proteins with potential agrichemical and pharmaceutical use. Even though parasitoids are one of the largest group of venomous animals, little is known about their venom composition. Recent few studies revealed high varied venom composition existing not only in different species but also between closely related strains, implying that increasing information on the venom proteins from more greater diversity of species of different taxa is key to comprehensively uncover the complete picture of parasitoid venom. Here, we explored the major protein components of the venom of ectoparasitic ant-like bethylid wasp, *Scleroderma guani* by an integrative transcriptomic-proteomic approach. Illumina deep sequencing of venom apparatus cDNA produced 49,873 transcripts. By mapping the peptide spectral data derived from venom reservoir against these transcripts, mass spectrometry analysis revealed ten main venom proteins, including serine proteinase, metalloprotease, dipeptidyl peptidase IV, esterase, antithrombin-III, acid phosphatase, neural/ectodermal development factor IMP-L2 like protein, venom allergen 3, and unknown protein. Interestingly, one serine proteinase was firstly identified with rarely high molecular weight about 200 kDa in parasitoid venom. The occurrence of abundant acid phosphatase, antithrombin-III and venom allergen 3 demonstrated that *S. guani* venom composition is similar to that of social wasp venoms. All identified venom genes showed abundantly biased expression in venom apparatus, indicating their virulent functions involved in parasitization. This study shed light on the more better understanding of parasitoid venom evolution across species and will facilitate the further elucidation of function and toxicity of these venom proteins.

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1. Introduction

As many as 10%–20% of all insects may be parasitoid wasps classified under the order Hymenoptera, which comprises approximately 130,000 insect species and includes more parasitoids than any other order of insects (Pennacchio and Strand, 2006). The large evolutionary grade of parasitoids with thousands of species diversity in over 40 families is matched by their own favored life stages of particular host species in many other orders of insects. In order to successfully oviposit and develop inside or on top of hosts, they need to evade or counteract the different types of host physiological or immunological defenses according to their endoparasitic or ectoparasitic nature (Godfray, 1994). During the antagonistic coevolution between parasitoids and hosts, parasitoids have evolved a newly virulent mechanism by injecting inside the

host virulent factors such as venom, polydnavirus, virus-like particle or ovarian calyx fluid that alter the host immunity and/or manipulate its development and physiology to cope with a wide spectrum of developmental interactions with their hosts (Beckage and Gelman, 2004; Burke and Strand, 2014). Parasitoid venoms consist of a complex cocktail of proteinaceous components (proteins and peptides, commonly referred to as toxins) and non-proteinaceous (salts and organic) compounds. Generally, the proteinaceous components are the most abundant. Due to its unique and diverse physiological functions, they demonstrate its appealing agrichemical as well as pharmaceutical application as biological control and therapeutic agents in pest management and human diseases (Danneels et al., 2015).

With more than 50 years of a continued interest, over 50 venom proteins have been identified from the venoms of 17 species belonging to Ichneumonidae, Braconidae, Pteromalidae, Figitidae or Eucilidae (Moreau and Guillot, 2005; Colinet et al., 2013). Physiological functions of only a small number of wasp venoms

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(about 20) are unraveled (Moreau and Asgari, 2015). Many of them did not share significant homology with known proteins. Interestingly, preliminary data demonstrate the occurrence of unexpectedly distinguished action of modes and physiological functions of parasitoid venom proteins, which are strongly different from those of their high similar counterparts which have known functions. For example, the abundant phenoloxidas in venom of *Pimpla hypochondriaca* are notably active without the addition of exogenous proteases because of the absence of sequences which specify the site of proteolytic activation in insect pro-phenoloxidas and unprecedented occurrence of signal peptide sequences (Parkinson et al., 2001). A venom defensin-like peptide characterized in *Nasonia vitripennis* interfered with the host phenoloxidase cascade (Tian et al., 2010). In contrast to venom composition of bees, spiders, fishes, frogs, snakes, scorpions or centipedes, as well as in secretions from salivary glands, being streamlined and highly conserved, available studies conducted in select species indicated that parasitoid venom components are more complex and often highly variable with a high degree of functional redundancy (Casewell et al., 2013; Poirié et al., 2014). Additionally, its components are heterogeneous within species but also differ from one phylogenetically distant species to another, even between the closely related species, such as *Leptopilina boulardi* and *Leptopilina heterotoma*, *Microctonus aethiopoulos* and *M. hyperodae*, and *Chelonus insularis* and *C. near curvamaculatus* (Crawford et al., 2008; Vincent et al., 2010; Colinet et al., 2013; Goecks et al., 2013; Heavner et al., 2013). It is remarkable that the complex nature and mixtures of parasitoid venom is far from completely understood. Knowledge of the diverse venom components of multiple parasitoid species is a key need to provide a springboard to clarify the evolution and elucidate the functional role of them for contributing to an improved utilization.

The ectoparasitic ant-like bethylid wasp, *Scleroderma guani* (Hymenoptera: Bethyridae), firstly discovered in Guangdong province of China in 1973, is a polyphagous parasitoid that can attack larval or pupal stages of more than 50 insect species across 22 families among three orders of Coleoptera, Lepidoptera and Hymenoptera (Zhang, 1980; Chen and Cheng, 2000). It has been widely used as an efficiently biological control agent in China to control wood-boring insects, including the most important vector of the pinewood nematode, *Monochamus alternatus* (Zhang et al., 1995). Within hours of being parasitized by *S. guani*, its host is paralyzed by injecting venom. Before laying eggs on the host, female adults feed the hemolymph releasing from the hosts piercing surface to facilitate ovary development. Then eggs are deposited and glued upon the host externally. The hosts are eaten alive by larvae until mature. During this process, the larvae will be protected by the adult wasps. When the larvae are mature, they will be removed by female wasps from the hosts body then spin congested cocoon and pupate. The wide spectrum of host range and interesting parasitic features make *S. guani* a valuable model for investigating the venom proteins. However, to date, only several studies have aimed at understanding the roles of *S. guani* venom as a whole functions involved in regulating hosts genes during parasitism (Zhu et al., 2013, 2014a,b,c).

The next generation sequencing technology has emerged as a powerful new approach to efficiently high-throughput gene discovery in non-model organisms. This method can cost-effectively generate huge amount of sequence to facilitate the construction a reference protein database for proteomic analysis. The proteomic approach has the advantage to allow identification of protein from different cellular locations by providing information from the regulatory steps between transcription and the final protein product. Take advantages of next generation sequencing and proteomics, we here took an integrative transcriptomic and proteomic approach to

identify the main venom proteins from this ectoparasitic wasp. The present study is the first in-depth venom analysis of a bethylid parasitoid, as well as a parasitoid of Coleoptera and Hymenoptera. In addition to a number of conserved or novel venom proteins, toxins of antithrombin-III and venom allergen 3 typically reported in venom of social wasps were abundantly revealed. This dataset will contribute to further investigate insight on the evolution and functional diversity of parasitoid venoms.

2. Materials and methods

2.1. Insects

Tenebrio molitor larvae were reared on the wheat bran under room temperature. Their pupae (3 h post-pupation) were used for maintaining the laboratory colony of *S. guani* as described by Zhu et al. (2013). Adult parasitoid wasps were provided with a cotton pad soaked in 20% honey solution.

2.2. Venom apparatus collection

Female *S. guani* of mixed age were used to collect the venom apparatus including venom reservoir, venom gland and Dufour gland (Fig. 1). Firstly, female adults were anaesthetized by cooling in a refrigerator. Then, venom apparatus were removed out from wasp abdomen by pulling the ovipositor with pins and forceps in phosphate buffered saline (PBS) on a slide glass under a stereomicroscope with cold light illuminator. After thoroughly wash using the PBS to wipe off the debris, venom apparatus were frozen in liquid nitrogen prior to use.

2.3. Transcriptome library preparation and sequencing

Total RNA was extracted from about 500 venom apparatus using Trizol reagent (Invitrogen) according to the user manual. After DNase (Takara) digestion to remove any remaining DNA, total RNA was subjected to prepare the mRNA with an oligo (dT) magnetic beads (Invitrogen). The quality and quantity of mRNA were estimated on a 1% agarose gel plus with an Agilent 2100 Bioanalyzer (Agilent Technologies). The mRNA (5 µg) was used to prepare a cDNA library using Illumina's kit following manufacturer's protocol. The library was enriched by PCR. Sequencing was performed on Illumina HiSeq™ 2000 platform at the Novogene (Beijing, China).



Fig. 1. Morphology of venom apparatus. Vr, Venom reservoir; Vg, venom gland; Dg, Dufour gland.

2.4. Assembly and bioinformatic analysis of transcriptome

Raw data was deposited to Sequence Read Archive (SRA) database of DNA Data Bank of Japan (DDBJ) (<http://www.ddbj.nig.ac.jp/>) under the accession number DRA001255. Raw reads with adapters, more than 10% unknown bases, and low quality were filtered to get clean reads, which were assembled into transcripts by the Trinity program (Grabherr et al., 2011). All assembled transcripts were used for BlastX search and annotation with a significant cut-off E-value of 10^{-5} against the databases including Nr, Nt, Swiss-Prot, Pfam, GO (Gene Ontology), COG (Clusters of Orthologous Groups of proteins), and KEGG (Kyoto Encyclopedia of Genes and Genomes). The best matches were used to identify coding regions and to determine the sequence direction. When different databases conflicted, the results were prioritized in the following order: Nr, Nt, Swiss-Prot, Pfam, COG and KEGG. When a transcript was not covered in BlastX, it was predicted by ESTScan. Functional annotation by GO terms, COG categories and metabolic pathways were performed using the Blast2GO program (www.blast2go.org). The expression levels of transcripts were calculated and normalized using a variation of the RPKM (reads/kb/million) method (Mortazavi et al., 2008).

2.5. LC–MS/MS

Twenty venom reservoirs of *S. guani* were dissected from the female wasps and transferred into a 1.5 mL tube containing ddH₂O₂. The crude venom was obtained by centrifuging at 15,000 g at 4 °C for 30 min to remove debris. Protein quantification was determined by the method reported by Bradford (1976) using bovine serum albumin (BSA) as standard. In total, 20 µg protein aliquots were separated by molecular weight on 12% SDS-PAGE gel. Staining was done with Coomassie Brilliant Blue R-250. The main gel bands were excised out and cut into small blocks. After ring with water followed by acetonitrile, they were subjected to reduction (10 mM dithiothreitol) at 56 °C for 1 h, alkylation (55 mM iodoacetamide) at room temperature for 45 min, and overnight digestion with trypsin at 37 °C. The resulting peptides were extracted once by 50% acetonitrile and 0.1% formic acid, then twice by 100% acetonitrile and 0.1% formic acid. After vacuum drying, they were reconstituted in solvent A (2% acetonitrile, 0.1% formic acid) and determined by a Thermo Scientific Orbitrap Velos Pro mass spectrometer coupled to Shimadzu LC-20AD nanoHPLC system packed with C18 analytical column at BGI (Shenzhen, China). Peptides were eluted with a flow rate 400 nL/min using a linear gradient at 2–35% solvent B (98% acetonitrile, 0.1% formic acid) over 44 min, then from 35% to 80% over 2 min, followed by 80% solvent B for 4 min, and finally solvent A for 1 min. The spray voltage of the Orbitrap was set at 2.5 kV. The spray temperature was set at 150 °C for peptides. Collision energy was set at 35 ± 5 eV. Mass spectra were acquired from proteins at a resolution of 30,000 and the 20 most-abundant ions per precursor spectrum via collision were subjected to MS/MS by CID fragmentation in the ion trap.

2.6. Database search and protein identification

Searches using Mascot 2.3.024 were against NCBI insecta database plus with the protein database (35,970 predicted proteins) derived from the above sequenced venom apparatus transcriptome with trypsin specificity. Search parameters were as follows: a maximum of one missed cleavage, fragment mass tolerance of ± 0.1 Da, peptide mass tolerance of ± 0.05 Da, mass value of mono-isotopic, variable modifications of deamidated (NQ), fixed modifications of carbamidomethyl (C) and no restriction on protein mass. All identifications were filtered in order to achieve a protein

false discovery rate (FDR) of 1%. Only candidate proteins with a significant Mascot score were taken into consideration.

2.7. Quantitative real-time PCR (qPCR)

Total RNAs were extracted from 100 venom apparatus, 10 female adults and 10 male adults using Trizol reagent (Invitrogen), following the manufacturer's protocol. After analyzing for RNA quality on 1% agarose gel and assessing RNA purity and quantity by spectrophotometer, RNA samples were treated with DNase (Takara) to remove the genomic DNA contamination. Then, 1 µg RNA for each sample was used for synthesizing the single strand cDNA using PrimeScript RT Master Mix (Takara). qPCR was performed in a Qiagen real time PCR system (Qiagen) using SYBR Premix EX Taq™ (Takara) in three biological replicates. Specific primers for selected venom genes were designed by Primer Premier 5.0 software based on the sequences derived from the above assembled transcripts (Table S1). qPCR protocol used was as follows: 2 min at 94 °C, followed by 39 cycles of 1 min at 94 °C, 30 s at 60 °C and 30 s at 72 °C. The 5.8S ribosomal RNA gene was used as a reference. The relative quantification of gene expression was analyzed using the comparative $2^{-\Delta\Delta CT}$ method with male adult as a reference (Livak and Schmittgen, 2001). Data were analyzed using a one-way nested analysis of variance (ANOVA), followed by least significant difference (LSD) comparison using Data Processing System (DPS) software (Tang and Zhang, 2013).

3. Results and discussion

3.1. Transcripts of venom apparatus

Illumina deep sequencing generated about 4.42 Gb of data (Table 1). *De novo* assembly produced 49,873 transcripts with an average length of 1460 bp. Of these, more than 99.88% transcripts were completely covered by at least one read, representing the completeness of assembly. The length distribution of transcripts revealed that 29,535 transcripts (59.22%) were longer than 500 bp, 21,301 transcripts (42.71%) were longer than 1 kp, and 12,615 transcripts (25.29%) were longer than 2 kb (Fig. S1). In previous studies using whole insect body as sample for sequencing by the same technology, the length of transcripts were abundantly shorter than 1 kb (Zhu et al., 2013; Salvemini et al., 2014; Xu et al., 2015).

Table 1
Summary of sequencing, assembly and annotations for transcriptome.

Reads	
Raw reads	53,526,912
Clean reads	43,833,148
Total bases (Gb)	4.42
Q20 percentage (%)	96.76
GC content (%)	45.87
Sequence duplication level (%)	89.3
Transcripts (≥ 200 bp)	
Total Number	49,873
N50 length (bp)	2838
N90 length (bp)	580
Minimum length (bp)	201
Maximum length (bp)	21,754
Mean length (bp)	1460
Transcripts annotated in databases	
Nr	29,948
Nr	13,561
SwissProt	24,363
Pfam	25,984
GO	23,610
COG	20,818
KEGG	29,526

Here, much better assembly results obtained might be due to the specific organ, venom apparatus, used for sequencing resulting in more in-depth sequencing of genes present in this tissue.

For annotation, 29,948 (60.05%), 13,561 (27.19%), 24,363 (48.85%), 25,984 (52.10%), 23,610 (47.34%), 20,818 (41.74%), 29,526 (59.20%) transcripts showed significant similarity to known proteins in Nr, Nt, SwissProt, Pfam, GO, COG and KEGG databases, respectively (Table 1 and Table S2). The remaining transcripts unannotated in the databases might be novel transcripts and genes specific to *S. guani*. After Nr database annotation, 44.33% of the transcripts had a significant similarity with a stringent threshold of less than $1E^{-45}$, and 38.37% of the similar sequences ranged from $1E^{-45}$ to $1E^{-100}$ (Fig. 2A). Transcripts with e-value = 0 were corresponded to 17.30% of the total number of annotated transcripts. As expected, 97.42% of the transcripts had best matches and first hit against sequences of hymenopteran species including *Apis mellifera*, *A. florea*, *Acromyrmex echinator*, *Bombus impatiens*, *B. terrestris*, *Camponotus floridanus*, *Harpegnathos saltator*, *Megachile rotundata*, *N. vitripennis*, and *Solenopsis invicta* (Fig. 2B). Among these genome available species, *M. rotundata* genes had the greatest number of matches (5757, 19.22%), implying the more closely genetic relationship between *S. guani* and *M. rotundata* than the other species.

To further reveal the functions of transcripts, *S. guani* transcriptome was GO categorized, COG classified, and KEGG pathway mapped. Under three GO ontologies, transcripts were respectively assigned into biological process with dominant terms of cellular process (14,649, 22.99%) and metabolic process (13,648, 21.42%), cellular component with dominant terms of cell (13,569, 28.72%) and cell part (13,569, 28.72%), and molecular function with dominant terms of binding (15,254, 42.08%) and catalytic activity (12,192, 33.63%) (Fig. 3A). COG was classified into 25 categories (Fig. 3B). Among these, the highest represented were general functional prediction only (3359, 14.18%) and signal transduction (2962, 12.50%). To understand which biological pathways are active in the venom apparatus, a comparison of the transcripts against the KEGG database yielded a significant match to 291 pathways in total,

most represented by signal transduction (1522, 6.79%) and carbohydrate metabolism (1263, 5.63%) at the second level (Fig. 3C). There were 71 pathways with below 20 transcripts assigned. Functional annotation results revealed that metabolism and transport might be active in the venom apparatus, similar to the results indicated in the secretory organs including salivary gland and testis (Su et al., 2012; Wei et al., 2015). Additionally, transcripts assigned into the COG categories and pathways mainly involved transport and biosynthesis of RNA and protein such as RNA processing and modification, post-translational modification and protein turnover, and transcription and translation were abundant, suggesting venom apparatus is mainly involved in the processes of protein biosynthesis (Zhang et al., 2014). Although GO, COG and KEGG analysis is an effective method to categorize gene functions based on sequence homology, it is unable to obviously discover the potential venous genes because of no toxin group been produced in these database. However, this approach will assist in the further discovery and annotation of novel genes that play key roles in venom reproduction.

3.2. Identification of venom proteins

Venom proteins were fractionated using SDS-PAGE, which consistently produced eight major protein bands (Fig. 4). All of them were excised and typically digested. After elution of tryptically digested proteins from the SDS-PAGE gel slices, tryptic peptides were identified by LC-MS/MS analyses using the above transcriptomic sequences as reference (Table S3). In total, 10 venom proteins were identified (Table 2). They were serine proteinase, metalloprotease, dipeptidyl peptidase IV, esterase, antithrombin-III, acid phosphatase, neural/ectodermal development factor IMP-L2 like, venom allergen 3, and unknown protein. Based on the transcriptions of genes encoding these venom proteins generated in the transcriptomic analysis using RPKM method, parallel mRNA levels and protein abundance in the SDS-PAGE gel were recorded except for acid phosphatase (Table 2). In terms of the bands of Vn2 and Vn3, they were composed by two venom proteins. According to the RPKM values of their compositions, dipeptidyl peptidase IV occupied main proportion in the band of Vn2, while esterase was dominant in the band of Vn3.

Serine proteinases in insects encode a large family of proteins that play important roles in various physiological processes, including digestion and immunity (Cao et al., 2015). They are generally synthesized as zymogens featured a high specificity catalytic triad in their catalytic domain composed of histidine (His), aspartic acid (Asp) and serine (Ser) for the requirement of proteolytic activation (Bao et al., 2014; Rawlings et al., 2014). In the digestive system of many vertebrates, they hydrolyze proteins to their component peptides and amino acids. With respect of digestive enzyme, this venom protein might be involved in liquefying the host body mass for parasitoid feeding. A trypsin-like enzyme in the saliva of *Euplectrus separatae* larvae is responsible for digesting the host tissues following migration and lethal to the host (Nakamatsu and Tanaka, 2004). Especially, they function as cellular and humoral defense factor by incorporating prophenoloxidase activation pathway to interfere with melanization (Lu et al., 2014). They are common in the animal venoms. Serine proteinase from venom of the endoparasitoid *Cotesia rubecula* inhibits melanization of the host hemolymph by interfering with the prophenoloxidase activation cascade (Asgari et al., 2003; Zhang et al., 2004). There are two serine proteinases identified from the venom of *S. guani*. One of them was at about 30 kDa, which is similar to the reports of other parasitoid species. But it is rarely found that another one was at about 200 kDa. This is the first report of such high molecular weight of serine proteinase in the parasitoid venom.

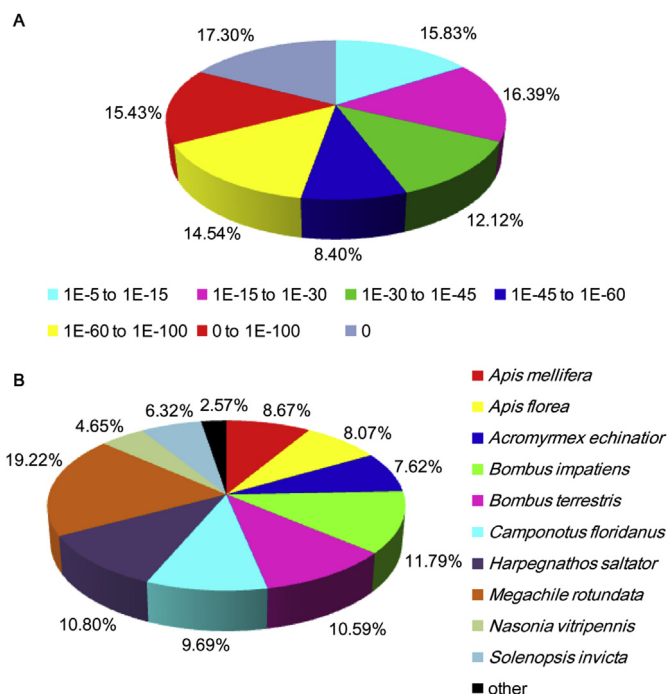


Fig. 2. E-value and species distributions of the top BlastX hits against Nr database. A. E-value distribution. B. Species distribution.

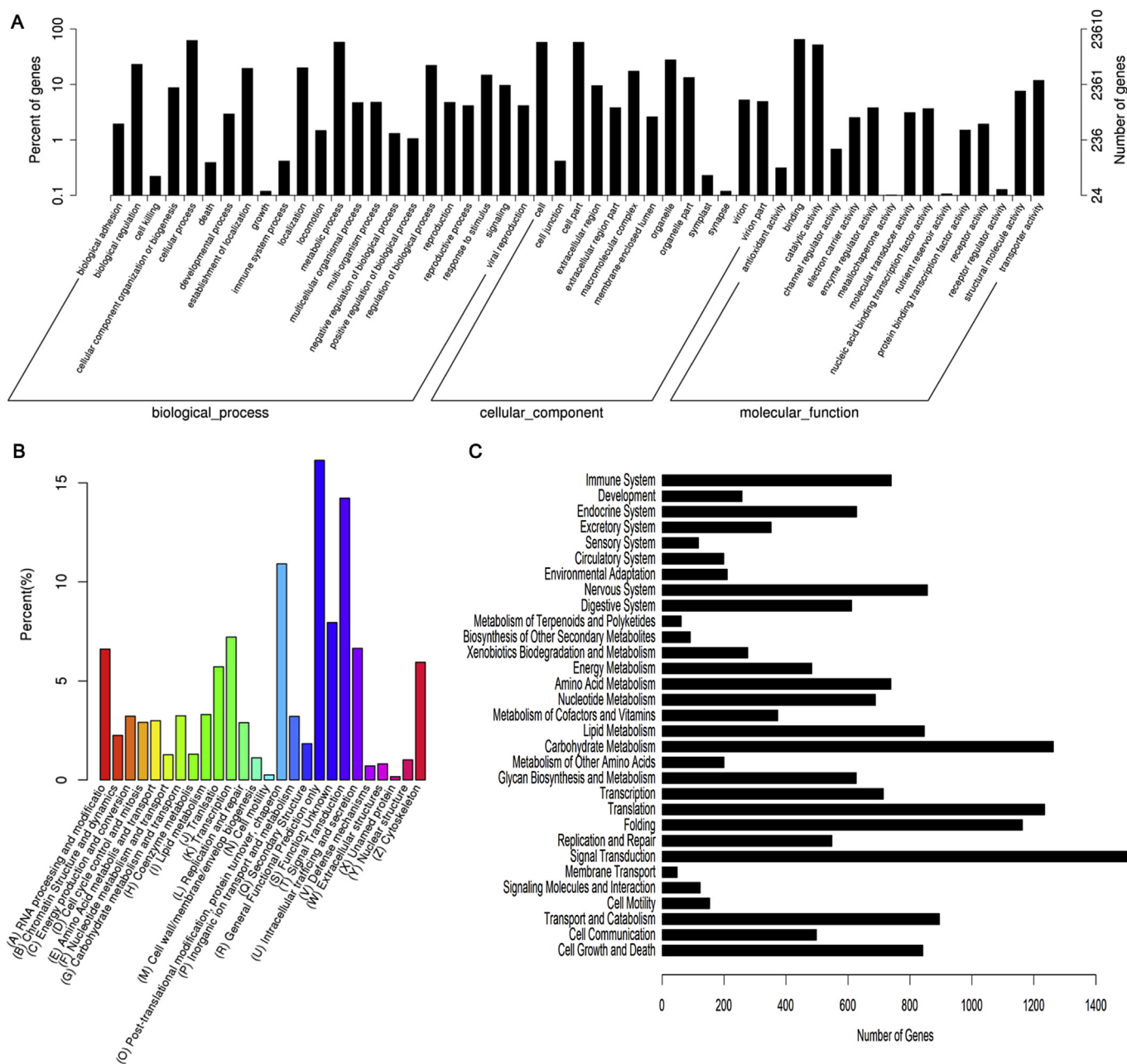


Fig. 3. Functional transcript annotations. A. GO classification assigned at the second level to three ontologies (biological process, cellular component, and molecular function). B. COG classification functionally grouped into 25 classes. C. Pathway assignment based on KEGG.

Metalloproteases are noted major component in most snake venoms or social stinging wasps with well-known anti-hemostatic activity implicated in the hemostatic disorders associated with envenomation (Limam et al., 2010). In addition to hemorrhagic activity, they also have fibrin(ogen)olytic activity acting as pro-thrombin activators, possess apoptotic activity, inhibit platelet aggregation and contribute to other pathways that lead to local tissue damage (Markland and Swenson, 2013). Similarly, more recent investigations have evidenced that metalloprotease is a common enzyme occurring in many of the parasitoid venoms (Poiré et al., 2014). Due to well associate with a disintegrin-like domain, it is likely to be a good candidate as an effector of host immune suppression by interacting with immune related proteins circulating in the hemolymph (Parkinson et al., 2002). Although metalloprotease

is found to be among the main cytotoxic compounds in *N. vitripennis* venom, which may have tissue specific functions (Formesyn et al., 2013), its suppression of the host immune is required to be experimentally demonstrated. However, metalloprotease from venom of the ectoparasitic wasp *Eulophus pennicornis* with gelatinase activity is able to manipulate host development (Price et al., 2009).

Dipeptidyl peptidase IV is a membrane-anchored glycosylated serine protease that selectively cleaves dipeptides from the N-terminus of oligopeptides with proline or alanine in the penultimate position (De Meester et al., 2000). This protein as the main constituent stored in the venom reservoir of *A. mellifera* has a role in activating pro-melittin into highly toxic melittin (Kreil et al., 1980). The maturation of major toxin peptide, mastoparan B, in venom of

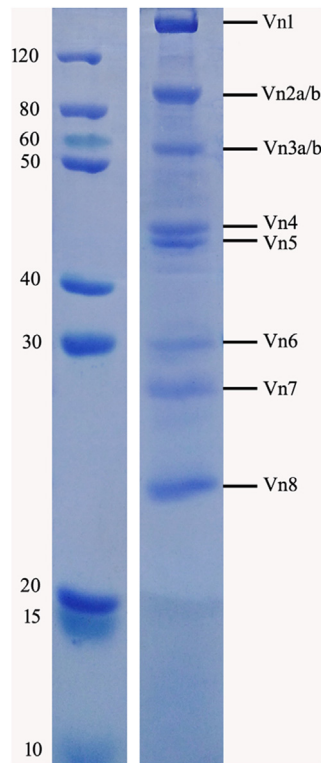


Fig. 4. Electrophoretic fractionation of venom proteins. Sample was separated on a 12% Tris-glycine-SDS-PAGE gel. Gel was stained with Coomassie Brilliant Blue R-250. Positions of molecular size markers (kDa) were indicated on the side of gel. Major protein bands excised for identification by LC–MS/MS were marked with solid lines and were numbered. Identified proteins were listed in Table 2.

Table 2
Venom proteins identified by LC–MS/MS.

Name	Identification	Protein score	Protein mass (kDa)	Isoelectric point	Coverage (%)	Unique peptide hits	Transcripts ID	RPKM of transcripts
Vn1	Serine proteinase 1	12,394.85	193.42	6.89	23.68	36	comp8207_c0_seq1	6217.12
Vn2a	Metalloprotease	4511.52	85.07	6.68	72.36	45	comp7401_c0_seq2	163.41
Vn2b	Dipeptidyl peptidase IV	3970.81	87.76	6.51	66.23	43	comp7931_c0_seq1	633.61
Vn3a	Esterase	4559.63	62.52	7.66	74.01	41	comp5176_c0_seq1	400.22
Vn3b	Antithrombin-III	2238.26	50.15	5.54	52.24	18	comp8440_c0_seq72	32.81
Vn4	Acid phosphatase	2670.28	46.99	5.61	62.56	23	comp3477_c0_seq1	29.43
Vn5	Unknown protein	2131.99	34.09	10.85	68.20	20	comp5504_c0_seq1	3081.18
Vn6	Neural/ectodermal development factor IMP-L2 like	2465.85	30.33	5.47	73.48	22	comp8214_c18_seq1	371.76
Vn7	Serine proteinase 2	2099.12	30.46	8.61	72.56	19	comp5246_c0_seq1	386.96
Vn8	Venom allergen 3	1585.08	26.62	8.54	73.39	16	comp8539_c0_seq1	499.96

the black-bellied hornet, *Vespa basalis*, requires dipeptidyl peptidase IV of its prosequence via sequential liberation of dipeptides (Lee et al., 2007; Hsieh et al., 2011). It is also present in the venom of *N. vitripennis* and *Hyposoter didymator*, suggesting that its function may be mainly limited to activation or inactivation of biomolecules within the venom reservoir (de Graaf et al., 2010; Dorémus et al., 2013). As one of the neuropeptidase families, dipeptidyl peptidase IV have been implicated in the metabolic inactivation of neuropeptides in the central nervous system of insects (Isaac et al., 2009). Independent of proteolytic activity, cell-adhesion by binding to collagen and fibronectin as well as regulation of immune response have been assigned to dipeptidyl peptidase IV in human (Tansi et al., 2010). Additional such roles except for processing of prosequence may thus be speculated for further unraveling of this enzyme in parasitoid venom involved in manipulating host

physiology.

Esterases and lipases are hydrolase enzymes that act on carboxylic esters. They have four invariant important catalytic residues Ser, Gly, Asn, and His (Chahinian and Sarda, 2009). Esterases differ from lipases by typically showing highest activity towards the soluble state of its substrate, whereas lipases display high activity towards the aggregated state of its substrate (Fojan et al., 2002). Esterases are shared between venoms of many parasitoid species while their functions are unclear (Poirié et al., 2014).

Antithrombin-III is an anticoagulant for the prevention of clots and has been shown to have anti-inflammatory properties. It acts by inhibiting the serine proteases (known as serpin) in both intrinsic and extrinsic pathways, which dramatically enhanced by heparin (Yang et al., 2004). Previously, peptides from *H. didymator* venom displaying similarities with antithrombin-III sequence of the *C. floridanus* has been observed (Dorémus et al., 2013). The content of antithrombin-III is very low in *H. didymator* venom, while this protein is one of the main venom components of *S. guani*, as indicated by the corresponding protein bands in the gel and number of peptides. This toxin was well represented in snake venom known as blood coagulation inhibitor (Morais-Zani et al., 2015), so *S. guani* venom antithrombin-III may cause a significant effect on inhibiting hosts haemocyte aggregation. Taken its action mode as serpin into consideration, it may also interfere with host hemolymph melanization cascade, thus altering the host immune response.

Acid phosphatase, a major lysosomal enzyme generally associated with degradation and cell death, is abundant in venom of *P. hypochondriaca* and *Pteromalus puparum* (Dani et al., 2005; Zhu et al., 2008). It functions as a major allergen (Api m 3) in honey bees venom (Grunwald et al., 2006). In venom apparatus of *P. puparum*, its mRNA was expressed quickly after female adult

emergence followed by a fast increase to the high level at 2 days after emergence, suggesting that it would be a precise and complex regulation mechanism linked with the oviposition process (Zhu et al., 2008). In *P. hypochondriaca* venom, this enzyme plays no role in the antihemocytic activity (Dani et al., 2005). It supposed that it may be involved in providing nutrients for parasitoid development by releasing carbohydrates or hampering host immunity by dephosphorylating immune related proteins, as those of fungus (Dani et al., 2005; Zhu et al., 2008). It was confirmed that the proper pH range values for *Pimpla* and *Pteromalus* venom acid phosphatase were very narrow with the optimal activity at 4.8 (Dani et al., 2005; Zhu et al., 2008), raising the question about this enzyme displaying functional role in near to neutral or prior to alkaline hosts hemolymph.

Neural/ectodermal development factor IMP-L2 is an apparent

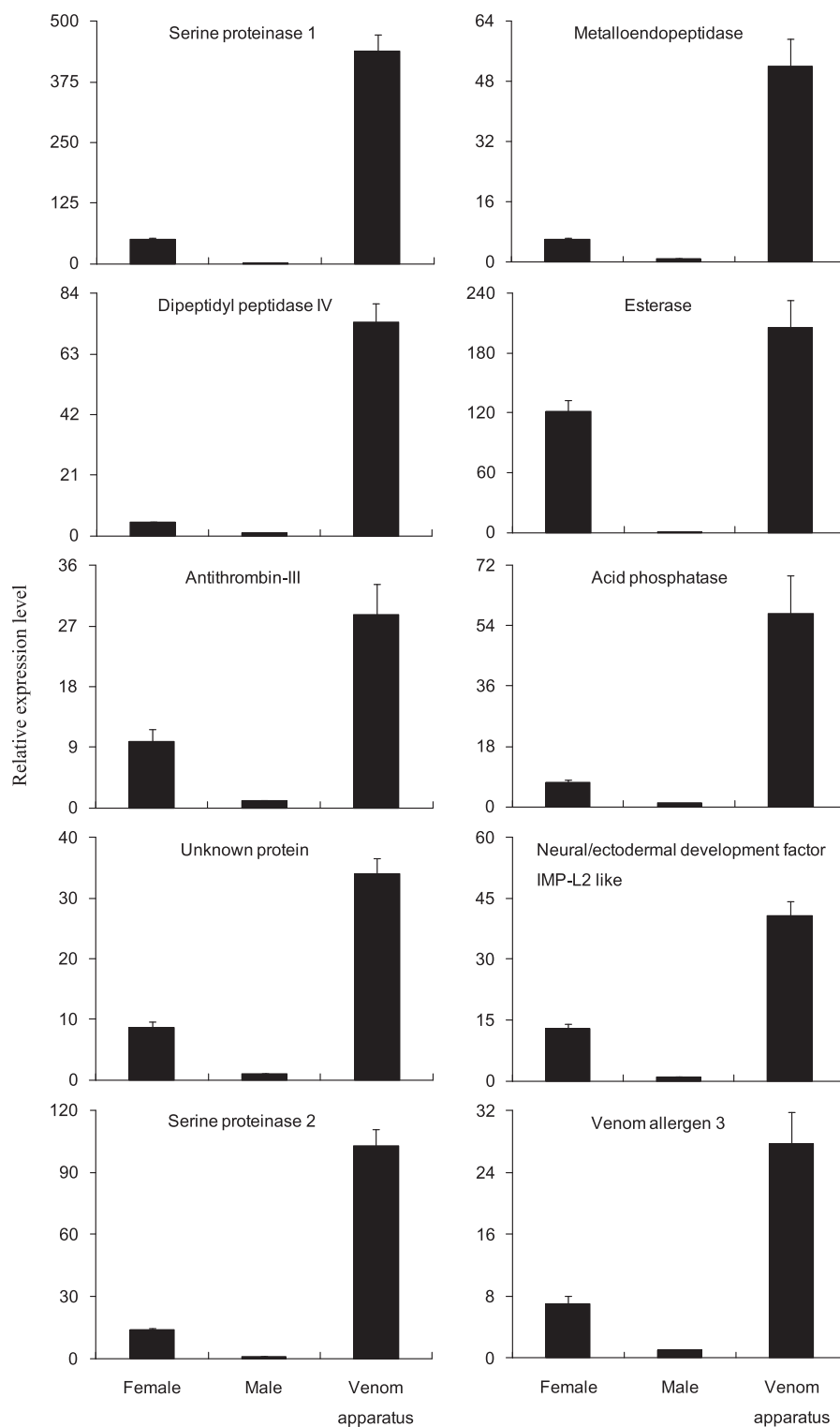


Fig. 5. qPCR analysis of expression profiles of venom genes. Data were indicated as mean $\pm$ SD. Transcriptional levels of the genes were calculated relative to the expression of the control gene, and normalized transcriptional levels to that of male wasp.

secreted member of the immunoglobulin superfamily involved in development, in particular the normal development of the nervous system (Garbe et al., 1993). It has recently been shown to bind to DILP2 and DILP5 to impair insulin signaling via inhibiting the activity of insulin-like peptides, resulting in nutritional regulation (Sarraf-Zadeh et al., 2013). A protein with similarities to it was

found in venom of *N. vitripennis*, which could be an excellent candidate responsible for the developmental arrest of host (de Graaf et al., 2010).

Venom allergen 3 represents one of the major allergenic proteins of the venom from Hymenopteran Apoidea, Vespidae and Formicidae with a cysteine-rich secretory belonging to the antigen

5 family (Hoffman, 2006). Recently, it has been reported in the venom of several parasitic wasps (Dorémus et al., 2013). To date, even progress on identification and molecular characterization of this venom allergen enables the resolution of crystal structure (Henriksen et al., 2001), its biological function is still unknown. In addition to venom allergen 3, another allergen, icarapin-like peptide, has been found in *M. hyperodae*, *M. aethiopoides* and *H. didymator* venom (Crawford et al., 2008; Dorémus et al., 2013).

3.3. Specific expression of venom genes

Most venom genes would show specific expression in venom apparatus and might be expressed higher in female than in male wasps. In order to verify this hypothesis, transcriptional levels of all identified venom proteins were estimated in venom apparatus, female and male by qPCR (Fig. 5). As expected, all of them were abundantly expressed in venom apparatus compared to female and male, indicating that they might be virulent factors. Besides acid phosphatase gene, this is similar to their RPKM results (Table 2). But qPCR results validated the high abundance of acid phosphatase in the venom, of which is not evidenced by RPKM method. The different expression levels of this gene in qPCR and RPKM analysis might be caused by a sensitivity of biases occurred between these two methods. Also, they expressed significantly higher expression in female than male. It suggests that the genes likely to be involved in parasitization are most highly expressed in the female. In addition to venom apparatus, they may express in other tissues of female or male. With respect of the venom genes associated with global expression, they can't be excluded from having a virulence function, as suggested by Goecks et al. (2013). Such kind genes expressed in venom apparatus might serve unique virulent functions but play different roles when appeared in other tissues. For instance, calreticulin known with widespread expression were identified from the venom of other parasitoid wasps (Poirié et al., 2014). Calreticulin present on the surface of haemocytes is involved in non-self recognition in insect cellular defense reactions of phagocytosis and encapsulation (Choi et al., 2002; Asgari and Schmidt, 2003; Wang et al., 2012), while this protein from parasitoid venom plays a role in inhibiting haemocyte spreading and encapsulation of the host (Zhang et al., 2006; Wang et al., 2013). Serine proteases and its inhibitors (serpins) in the hemolymph are essential for regulating phenoloxidase cascade leading to humoral immune response, but they from parasitoid venom inhibit melanization of the host hemolymph (Asgari et al., 2003; Colinet et al., 2009).

4. Conclusion

The integrative transcriptomic and proteomic analysis led to the identification of ten main venom proteins from the ant-like bethylid wasp, *S. guani*. Most of them such as serine proteinase, metalloprotease, dipeptidyl peptidase or esterase were shared between many parasitoids, demonstrating the presence of conserved venom proteins across species. However, acid phosphatase, antithrombin-III and venom allergen 3 that are generally main venom proteins of social wasps were abundant in *S. guani* venom, which is a big difference from previously reported venom compositions of other parasitoid wasps. It indicates that the diversity and variation in venom composition between different parasitoid species is adopted by phylogenetic evolution. Being a complex cocktail of *S. guani* venom, additional venom proteins epically with low abundance are expected to be unraveled. This study provides the data from Bethyilidae for comparative investigation of the nature of venom evolution in parasitic wasps and will facilitate future functional study of the deciphered venom proteins.

Ethics statement

The parasitoid wasp *S. guani* used for this study is not involved in endangered or protected species.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.toxicon.2016.02.003>.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.toxicon.2016.02.003>.

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