A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Identification of Venom Components from Aculeate Hymenoptera and Characterization of Their Properties

Aculeata 벌류의 독성분 동정 및 특성분석

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ABSTRACT

To identify the venom components and their expression patterns of some Aculeata bees/wasps, venom gland-specific transcriptome analysis was conducted. FPKM values were normalized with the average of the transcription level of reference gene (α-tubulin). Common components in both solitary and social wasp venoms include hyaluronidase, phospholipase A2, metalloendopeptidase, etc. Although it has been expected that more diverse bioactive components with the functions of prey inactivation and physiology manipulation are present in solitary wasps, the information on venom compositions of solitary wasps obtained in this study was not sufficient to generalize this notion. Nevertheless, some neurotoxic peptides (e.g., pompilidotoxin and dendrotoxin-like peptide) and proteins (e.g., insulin-like peptide binding protein) appear to be specific to solitary wasp venom.

In contrast, several proteins, such as venom allergen 5 protein, venom acid phosphatase, and various phospholipases, appear to be relatively more abdundant in social wasp venom. In the venom gland transcriptome of bumblebees, major allergens or pain producing factors were barely identified, implying that bumblebee venoms are relatively less toxic than those of social or solitary wasps. Finally, putative functions of main venom components and their application are also discussed.

To investigate the differences in venom properties and toxicities between *Vespa crabro* and *V. analis*, the transcriptomic profiles of venom glands, along with the venom components, were analyzed and compared. A total of 35 venom-specific genes were identified in both venom gland transcriptomes, but their transcriptional profiles were different between *V. crabro* and *V. analis*. In addition, the major venom components were identified and confirmed by mass spectroscopy. In general, most major venom genes were more abundantly expressed in *V. crabro*, whereas some minor venom genes exhibited higher transcription rates in *V. analis*, including muscle LIM protein, troponin, paramyosin, calponin, etc. Our findings reveal that the overall venom components of *V. crabro* and *V. analis* are similar, but that their expression profiles and levels are considerably different. The comparison of venom gland transcriptomes suggests that *V. crabro* likely produces venom with more highly enriched major

venom components, which has potentially higher toxicity compared with *V. analis* venom.

To examine the differences in the potential toxicity and bioactivity of mastoparans, vespid chemotactic peptides, vespakinins and bombolitins of *V. crabro*, *V. analis*, *Parapolybia varia*, *V. mandarina*, *Bombus ardens*, *B. consobrinus*, *B. terrestris* and *B. ussurensis*, differential toxicological and pharmacological activities of synthesized venom peptides were investigated.

The hemolytic, antimicrobial, and antitumor activities of synthesized *V. analis* mastoparan were higher than those of *V. crabro* mastoparan. These differential bioactivities are likely due to the amino acid sequence differences in the mature peptides.

*Pv*VCP, *Pv*Vespk, and *Vm*Vespk showed little to low hemolytic activities. Only *Vm*VCP showed hemolytic activity at a high concentration. Among the four peptides tested, *Vm*VCP showed both anti-microbial and anti-fungal activities, whereas *Pv*VCP showed only anti-fungal activity to *Candida albicans*. Interestingly, *Pv*VCP showed significantly stronger anti-tumor activities to two ovarian cancer cell lines compared with *Vm*VCP. Vespks only showed anti-tumor activity to SK-OV-3 cells but not to NIH-OVCAR-3 cells.

Among the four bombolitins tested, bombolitin T showed the highest hemolytic and anti-tumor activities. All bombolitins exhibited strong anti-

microbial and anti-fungal activities, and bombolitin A specifically possessed the highest anti-microbial activity against the Gram-negative bacteria *Escherichia coli*.

To design selective anti-tumor peptides with reduced hemolytic activity, bombolitin T was chosen as a model to be modified by amino acid substitution. The lowest hemolytic activity was observed with the addition of a hydrophobic amino acid (Ile^{14}) to the C-terminal end as well as the substitution of Ser^{10} and Leu^{12} with a hydrophobic amino acid (Leu) and positively charged amino acid (Lys), respectively. This finding implies that the balanced substitution of hydrophobic and positively charged amino acids could affect the cytotoxicity. This study provides new information on the properties of anti-tumor peptides in the venoms of bumblebees and the basic approaches for peptide design tools for the reduction of cytotoxicity.

Key words: venom gland, venom peptide, transcriptional profiles, RNA sequencing, circular dichroism, biological acitivity, principal component analysis, peptide substitution

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ABBREVIATIONS

AMP	anti-microbial peptide	ORF	open reading frame
BHI	brain heart infusion	PBS	phosphate-buffered saline
CAP	cysteine-rich antimicrobial peptide	PCA	principal component analysis
CD	circular dichroism	PD	potato-dextrose
DMSO	dimethyl sulfoxide	PFT	pore-forming toxin
EC_{50}	half maximal effective concentration	pI	isoelectric pont
ECM	extracellular matrix	qPCR	quantitative real-time PCR
FPKM	fragments per kilobase million	RBC	red blood cell
GO	gene ontology	RIN	RNA integrity number
IC_{50}	half maximal inhibitory concentration	SCP	sterol carrier protein
LB	luria-bertani	SDS	sodium dodecyl sulfate
LPS	lipopolysaccharide	TFE	trifluoroethanol
MIC	minimal inhibitory concentration	VCP	vespid chemotactic peptide
OD	optical density	Vespk	vespakinin

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LITERATURE REVIEW

1. Comparative Aspects of Venom Components between Solitary vs. Social Wasp

Contents of venom components can be directly estimated from their proportion in the proteomic/peptidomic profiles of venom. Alternatively, they can also be indirectly predicted from the proportions of their transcripts in the transcriptome or cDNA library of venom gland. Previous comparisons of the relative contents of venom components suggest that venom peptides (mastoparan-like and chemotactic peptide-like), metalloendopeptidase, hyaluronidase, and arginine kinase are predominantly found in solitary wasps (Baek and Lee, 2010a, b; Baek et al., 2013) while mastoparan, vespid chemotactic peptide (VCP), venom allergen 5, serine protease, and hyaluronidase were generally ranked as top five components in social wasps (Baek et al., 2013; Yoon et al., 2015b).

Both solitary and social wasp venoms contain several common components, including hyaluronidase. Hyaluronidase is hydrolase that can hydrolyze the viscous hyaluronic acid polymer, an important constituent of the extracellular matrix of all vertebrates (Kreil, 1995). Hyaluronidase is also commonly found in the venoms of various venomous organisms, including bees and wasps. Due to the degradation of hyaluronic acid in the extracellular matrix, venom hyaluronidase

functions as a diffusion factor that facilitates the diffusion of injected venom from the site of sting into circulation, thereby potentiating the action of other venom ingredients (Kemparaju and Girish, 2006; Nagaraju et al., 2007). Besides, hydrolyzed hyaluronan fragments are known to stimulate inflammation, angiogenesis, and immune response, thereby resulting in a quicker systemic envenomation (Girish and Kemparaju, 2007). Owing to its role in venom spreading, hyaluronidase is common not only in social wasp venoms, but also in solitary wasp venoms as an essential venom ingredient (Table 1 and Table 2). In addition to hyaluronidase, other venom proteins that are commonly found in both wasps include phospholipase A2, metalloendopeptidase, etc., which are also well-known allergens.

Table 1. Venom peptides and proteins from solitary wasps and their putative functions

Protein/peptide	Putative function [a]	Species [b]
Neurotoxins		
α-pompilidiotoxin	Paralysis (Na^+ channel blocking)	As
β-pompilidiotoxin	Paralysis (Na^+ channel blocking)	Bm
Dendrotoxin-like	Paralysis (K^+ channel blocking)	Ep
Kinins		
Wasp kinin	Pain production	Ca, Cd, Ci, Cm, Mf, Mp
Mast cell degranulating peptides		
Mastoparan-like	Allergic inflamation (Mast cell degranulation), Antimicrobial activity	Af, As, Ep, Er, Od1, Od2
Chemotactic peptides		
Wasp chemotactic peptide	Inflammatory activity Antimicrobial activity	Cf, Ep, Od1, Rb
Enzymes		
Acetyl-CoA synthase	Involvement in metabolism of acetate	Rb
Alcohol dehydrogenase	Oxidation of ethanol to acetaldehyde	Ep
Amidophosphoribosyltransferase	Regulation of cell growth	Rb
Arginine kinase	Paralysis	Cd, Od1, Ep, Rb
ATP synthase	ATP synthesis	Od1, Ep, Rb
Carboxylesterase	Lipid metabolism	Rb
Citrate synthase	Catalyzing the citric acid cycle	Rb
Cytochrome P450 monooxygenase	Metabolism of toxic compounds	Od1, Rb
DNA-directed RNA polymerase	Synthesis of mRNA precursor	Rb
Farnesoic acid o-methyltransferase	Regulation of biosynthetic pathway of juvenile hormone	Rb
Glutamate decarboxylase	Involvement in beta-cell-specific autoimmunity	Ep
Glyceraldehyde-3-phosphate dehydrogenase	Direct hemolytic factor	Rb
Glycogenin	Synthesis of glycogen	Rb
HECT E3 ubiquitin ligase	Regulation of cell trafficking	Ep
Hyaluronidase	Venom dissemination	Ep, Rb
Myo inositol monophosphatase	Regulation of inositol homeostasis	Rb
Phospholipase A2	Hydolysis of lecithins	Ep, Rb
Protein tyrosin phosphatase	Regulation of cellular processes	Rb
Serine/threonine protein phosphatase	Regulation of biochemical pathways	Rb
Tyrosine 3-monooxygenase	Regulation of dopamine synthesis	Od1, Ep

Hemostasis affecting proteins		
Metalloendopeptidase	Inhibition of platelet aggregation	Od1, Ep, Rb
Neprilysin	Inhibition of platelet aggregation	Rb
Serine protease/Chymotrypsin/ Thrombin-like	Fibrinolytic activity Kinin releasing activity Melanization	Od1, Ep
Muscle-related proteins		
Actin	Regulation of hemocyte cytoskeleton gene expression	Od1, Ep, Rb
Ankyrin	Attachment of membrane proteins to membrane cytoskeleton	Rb
Bmkettin	Development of flight muscles	Od1, Rb
Calponin	Regulation of myogenesis	Od1, Ep, Rb
Muscle LIM protein	Regulation of myogenesis	Od1, Ep, Rb
Muscle protein 20	Regulation of muscle cotraciton	Od1, Ep
Myomesin	Anchoring the thick filaments	Rb
Myosin heavy chain	Regulation of muscle functions	Od1, Ep, Rb
Myosin light chain	Modulation of the affinity of myosin for actin	Od1, Ep, Rb
Paramyosin	Regulation of thick filament in muscles	Od1, Ep, Rb
Titin	Assembly of contractile machinery in muscle cells	Od1, Rb
Tropomyosin	Muscle contraction	Od1, Rb
Troponin	Muscle contraction	Od1, Ep, Rb
Tubulin	Regulation of hemocyte skeleton genes expression	Od1, Ep, Rb
Other proteins/peptides		
Chemosensory protein	Transferring metabolism-related small molecules	Rb
Cytochrom C	Protein wire	Od1, Rb
Heat shock proteins	Prevention of protein misfolding	Od1, Ep, Rb
Insulin-like peptide binding protein	Developmental arrest (Inhibition of insulin signaling)	Ep
Sialin	Nitrate transporter	Rb
Sugar transporter	Maintenance of glucose homeostasis	Rb

[a] The functional categories are either molecular, cellular or biological functions. When biological functions in venom are known, they were underlined.

[b] Wasp species abbreviations: Af, *Anterhynchium flavomarginatum*; As, *Anoplius samariensis*; Bm, *Batozonellus maculifrons*; Ca, *Campsomeriella annulata*; Cd, *Cyphononyx dorsalis*; Cf, *Cyphononyx fulvognathus*; Ci, *Colpa interrupta*; Cm, *Carinoscolia melanosome*; Ep, *Eumenes pomiformis*; Er, *Eumenes rubronotatus*; Mf, *Megascolia flavifrons*; Mp, *Megacampsomeris prismatica*; Od1, *Orancistrocerus drewseni*; Od2, *Oreumenes decorates*; Pt, *Philanthus Triangulum*; Rb, *Rhynchium brunneum*.

Table 2. Venom peptides and proteins from social wasps and their putative functions

Protein/peptide	Putative function [a]	Species [b]
Neurotoxins		
AvTx-7,8	Paralysis (K^+ channel blocking)	Av
Agatoxin-like	Paralysis (Ca^{2+} channel blocking)	Vv1
Analgesic polypeptide	Paralysis (Na^+ channel blocking)	Vv1
Calsyntenin	Paralysis (Ca^{2+} channel blocking)	Vc
Conophysin-R	Paralysis (Ca^{2+} channel blocking)	Vv1
Latrotoxin-like	Channel formation	Vv1
Leucine rich repeat domain-containing protein	Paralysis (Involvement in synaptic vescle trafficking)	Va2, Vc
Orientotoxin-like	Paralysis (Presynaptic effect, lysophospholipase activity)	Vo, Vv1
Kinins		
Wasp kinin	Pain production	Pa, Pc, Pe1, Pe2, Pf, Pi, Pj, Pm1, Pm2, Pp, Pr, Va2, Vc, Vm2, Vt, Vx
Mast cell degranulating peptides		
Mastoparan	Allergic inflamation (Mast cell degranulation)	Ap, Pe2, Pi, Pj, Pm2, Pp, Ps, Rs, Va1, Va2, Vb1, Vb2, Vc, Vd, Vl, Vm1, Vm2, Vo, Vt, Vv1, Vx
Chemotactic peptides		
Wasp chemotactic peptide	Inflammatory activity Antimicrobial activity	Ap, Pl, Pm2, Pp, Ps, Va2, Vb2, Vc, Vm1, Vm2, Vo, Vt, Vx
Enzymes		
Acetylcholinesterase	Pain processing (Hydrolysis of neurotransmitter)	Va2, Vc, Vv1
Acetyltransferase	Synthesis of acetylcholine	Vt
Acid phosphatase	Female reproduction	Va2, Vc
Acyl-CoA delta-9 desaturase	Insertion of double bond in fatty acids	Vt
AMP dependent coa ligase	Production of fatty acyl-CoA esters	Vt
Arginine kinase	Paralysis	Va2, Vc
Argininosuccinate synthase	Arginine synthesis	Vt
ATP-dependent protease	Mediation of protein quality	Vt
Carboxylesterase	Lipid metabolism	Va2, Vc
Chitinase	Chitinolysis	Va2, Vt
Core alpha 1,3-fructosyltransferase A	Glycoprotein production	Vt

Cytochrome P450 monooxygenase	Metabolism of toxic compounds	Vt
Dipeptidyl peptidase IV	Liberation of bioactive peptides	Va2, Vb1
Esterase FE4	Sequestration	Vc
Fatty acid synthase	Biosynthesis of hormones	Vt
Fibrinogenase brevinase	Fibrinolysis	Vv1
Glyceraldehyde-3-phosphate dehydrogenase	Direct hemolytic factor	Va2, Vc
Glycerol-3-phosphate acyltransferase	Synthesis of triacylglycerol	Vt
Glycogenin	Synthesis of glycogen	Va2, Vc
GTP cyclohydrolase I isoform A	Production of neurotransmitter	Vt
Hyaluronidase	Venom dissemination	Dm, Pa, Pp, Va2, Vc, Vm1, Vt, Vv3
Laccase	Oxidation, cuticle sclerotization	Vc
Myosin light chain kinase	Muscle contraction	Va2, Vc
O-linked n-acetylglucosamine transferase	Insulin signaling reduction	Vt
Peptidyl-prolyl cis-trans isomerase	Immune mediator	Vt
Phospholipase A1	Production of lipid mediator	Dm, Pa, Va1, Va2, Vc, Vv3
Phospholipase A2	Hydrolysis of lecithins	Va2, Vc, Vv1
Phospholipase B1	Hydrolysis of lysolecithins	Va2
Phospholipase D	Induction of inflammatory responses	Va2, Vc
Phospholipase DDHD	Synaptic organization	Va2, Vc
Purine nucleoside phosphorylase	Apoptosis of lymphocytes	Vt
Reverse transcriptase	Production of high venom yield	Vt
Thrombin-like enzyme	Coagulation factor	Va2, Vv1
γ-glutamyl transpeptidase	Apoptosis of ovariole cells	Va2, Vc
CAP superfamily		
Defensin	Antimicrobial activity	Va2, Vc
Venom allergen 5	Allergenic activity	Dm, Pa, Pe1, Pf, Va2, Vc, Vf, Vg, Vm1, Vm3, Vp, Vs, Vt, Vv2, Vv3
Hemostasis affecting proteins		
Blarina toxin	Production of kinins	Vv1
Coagulation factor	Platelet aggregation	Vv1
Disintegrin	Platelet aggregation	Va2
Factor V activator	Coagulation factor	Vv1
Lectoxin-Enh4	Anticoagulant factor	Vv1
Metalloendopeptidase	Inhibition of platelet aggregation	Va2, Vc, Vv1
Nematocyte expressed protein-6	Inhibition of platelet aggregation	Vv1
Neprilysin	Inhibition of platelet aggregation	Va2, Vc
Oscutarin-C	Fibrinolysis	Vv1
Ryncolin-3/4	Platelet aggregation	Vv1

Serine protease/Chymotrypsin/ Thrombin-like	Fibrinolytic activity Kinin releasing activity Melanization	Va2, Vc, Vm1, Vt, Vv1
Snaclec	Platelet aggregation	Vv1
Vescular endothelial growth factor	Coagulation factor	Vv1
Veficolin	Platelet aggregation	Vv1
Venom plasminogen activator	Fibrinolysis	Vv1
Venom prothrombin activator	Fibrinolysis	Vv1
Muscle-related proteins		
Actin	Expression of hemocyte cytoskeleton	Va2, Vc
Calponin	Binding with actin	Va2, Vc
Muscle LIM protein	Regulation of myogenesis	Va2, Vc
Myosin heavy chain	Regulation of muscle functions	Va2, Vc
Paramyosin	Regulation of thick filament in muscle	Va2, Vc
Tropomyosin	Muscle contraction	Va2, Vc
Troponin	Muscle contraction	Va2, Vc
Vespin	Smooth muscle contraction	Vm1
Protease inhibitor		
Leukocyte elastase inhibitor isoform	Reduction of tissue damage	Vc
Serpin	Immune suppression (Inhibition of melanization)	Va2, Vc
Other proteins/peptides		
Anaphase-promoting complex subunit 13	Protein degradation	Vt
Apolipophorin-III	Lipid transport	Vt
Bhlh factor math 6	Regulation of developmental process	Vt
Bombolitin	Antimicrobial activity	Vc
CRAL/TRIO domain-containing protein	Regulation of cell growth	Vt
Cytochrome b	Transferring electrons	Vt
Doublesex isoform 1	Sex determination factor	Vt
Ejaculatory bulb-specific protein 3	Odorant binding protein	Va2, Vc
Elongation factor 2	Protein synthesis	Va2, Vc
Endopeptidase inhibitor	Inhibition of atrial natriuretic peptides	Vt
Endoplasmin	Protein folding	Va2, Vc
ETR-3 like factor 2	Pre-mRNA alternative splicing	Vt
Gigantoxin-1	Hemolytic activity	Vv1
Growth hormone inducible transmembrane protein	Apoptosis	Vt
GTPase-activating protein	Regulation of G protein signaling	Va2
Heat shock proteins	Prevention of protein misfolding	Vt
Insulin binding protein	Inhibition of insulin signaling	Va2, Vc
NADH-ubiquinone oxidoreductase	Involvement in respiratory chain	Vt

chain 4		
Natterin-4	Kininogenase activity	Vv1
Peptidoglycan-recognition protein 1	Antimicrobial activity	Vt
Phd finger protein	Protein-protein interaction	Vt
Plancitoxin	DNase activity	Vv1
Polyubiquitin	Proteolysis	Vt
SE-cephalotoxin	Paralysis	Vv1

[a] The functional categories are either molecular, cellular or biological functions. When biological functions in venom are known, they were underlined.

[b] Wasp spicies abbreviations: Ap, *Agelaia pallipes*; Av, *Agelaia vicina*; Dm, *Dolichovespula maculate*; Pa, *Polistes annularis*; Pc, *Polistes chinensis*; Pe1, *Polistes exclamans*; Pe2, *Protopolybia exigua*; Pf, *Polistes fuscatus*; Pi, *Parapolybia indica*; Pj, *Polistes jadwigae*; Pl, *Paravespula lewisii*; Pm1, *Paravespula maculifrons*; Pm2, *Polistes major*; Pp, *Polybia paulista*; Pr, *Polistes rothneyi*; Ps, *Protonectarina sylveirae*; Rs, *Ropalidia* sp.; Va1, *Vespa affinis*; Va2, *Vespa analis*; Vb1, *Vespa basalis*; Vb2, *Vespa bicolor*; Vc, *Vespa crabro*; Vd, *Vespa ducalis*; Vf, *Vespa flavopilosa*; Vg, *Vespula germanica*; Vl, *Vespula lewisii*; Vm1, *Vespa magnifica*; Vm2, *Vespa mandarina*; Vm3, *Vespula maculifrons*; Vo, *Vespa orientalis*; Vp, *Vespula pensylvanica*; Vs, *Vespula squamosa*; Vt, *Vespa tropica*; Vv1, *Vespa velutina*; Vv2, *Vespula vidua*; Vv3, *Vespula vulgaris*; Vx, *Vespa xanthoptera.*

2. Solitary Wasp Venom-Specific Features

Many peptides, which constitute a sizeable portion in solitary wasp venom, are not exactly categorized in the well-defined groups of social wasp venom peptides, such as kinin, mastoparan, or chemotactic peptides. Although it has been expected that more diverse bioactive components with the functions of prey inactivation and physiology manipulation are present in solitary wasps, available studies on venom compositions of solitary wasps are simply too scarce to generalize this notion. Nevertheless, several neurotoxic peptides or proteins appear to be specific to solitary wasps and are involved in prey paralysis.

Non-kinin neurotoxic peptides isolated from Pompilidae solitary wasps [α-

pompilidotoxin (α-PMTX) from *Anoplius samariensis* and β-PMTX from *Batozonellus maculifrons*] (Konno et al., 1998; Konno et al., 1997) are known to affect both vertebrate and invertebrate nervous systems by slowing or blocking sodium channel inactivation (Sahara et al., 2000), thereby paralyzing cockroach prey.

A novel dendrotoxin-like peptide containing the Kunitz/BPTI domain was identified from in *A. samariensis* (As-fr-19), *E. pomiformis* (EpDTX), and *R. brunneum* venoms (Baek and Lee, 2010a, b; Baek et al., 2013; Hisada et al., 2005). Since dendrotoxin is known to block K^+ channel, the presence of dendrotoxin-like venom peptides in solitary wasp venoms suggests its involvement in the paralysis of prey.

More extensive research on the components of solitary wasp venom is likely to identify other neuroactive peptides with similar functions. However, it is also worth mentioning that low molecular mass substances functioning as neurotoxin or neuromodulator, such as philanthotoxins found in the Egyptian solitary wasp *Philanthus Triangulum* venom (Piek, 1982; Piek et al., 1984; Vanmarle et al., 1984) and biogenic amines, [e.g., γ-aminobutyric acid (GABA), taurine, and β-alanine] found in the jewel wasp Ampulex compressa venom (Moore et al., 2006), can play a more decisive role than neuroactive venom peptides in solitary wasps.

In particular, IBP was found to be a major component (22.4% of total venom

protein) in the venom of a solitary wasp *E. pomiformi* (Baek and Lee, 2010b). To elucidate biological and molecular functions of EpIBP, EpIBP and its homologous protein of *Spodoptera exigua* (SeIBP) were in vitro expressed using an *E. coli* expression system (Baek et al., 2016). *S. exigua* larvae injected with EpIBP exhibited an increased survivorship and a reduced loss of body weight under a starvation condition. Both EpIBP and SeIBP were found to interact with apolipophorin III (apoLp III), implying that EpIBP might control the apoLp III-mediated metabolism, thereby regulating the growth of prey (Baek et al., 2016). Similarly, an IBP was also identified in the venom of a parasitoid wasp *Nasonia vitripennis* and suggested to inhibit the growth of the host (Danneels et al., 2010). Although IBP was found in the venom gland transcriptomes of *V. crabro* and *V. analis*, its transcription level, as judged by the FPKM value, was very low (Yoon et al., 2015b). Considering that the expression level of IBP in a solitary wasp *E. pomiformi* is much higher (Baek and Lee, 2010b), IBP in social wasp venom with a negligible level of expression is likely to be non-functional.

Vitellogenin-like protein, which is known to be involved in immune stimulation by enhancing melanin synthesis (Lee et al., 2000), was identified from both *E. pomiformis* and *O. drewseni* venoms, suggesting that they are likely involved in the protection of prey from microbial invasion (Baek and Lee, 2010b).

3. Social Wasp Venom-Specific Features

Venom allergen 5 proteins, venom acid phosphatase, and various phospholipases (A1, B1, D, and DDHD) appear to be relatively more specific to social wasp venom (see Table 2). Together with hyaluronidase and acid phosphatase, venom allergen 5 protein is one of the major allergenic Vespid venom proteins (Fang et al., 1988). It is reported that venom allergen 5 is a major venom component in social wasps and has been identified in all social wasps examined in the present review (see Table A1). Although the gene encoding venom allergen 5 is found from a solitary wasp R. brunneum (Vincent et al., 2010), it was not identified at all in the venom gland/sac transcriptome and proteome libraries of solitary wasps, including *R. brunneum* (Baek and Lee, 2010a, b; Baek et al., 2013; Baek et al., 2009), suggesting that it is not common in solitary wasps. Phylogenetic analysis of venom allergen 5 proteins of several Hymenopterans suggests that the gene can date back to the common ancestor of the Ichneumonoidea and the Aculeata, indicating its ancient origin (Vincent et al., 2010). Since all of the Vespid venom allergen 5 proteins show >57% of sequence identity, the tertiary conformations and allergenic capacities may not significantly differ (Hoffman, 1993; Yoon et al., 2015b). Thus, a high degree of cross-reactivity in serological testing was observed among the venom allergen 5 proteins of the common group of yellow jackets and among those of the two common North

American subgenera of paper wasps (Hoffman, 1993).

Venom acid phosphatase belongs to the enzyme group which hydrolyzes phosphomonoesters at acidic pH. It has been characterized as a glycoprotein causing histamine release from sensitized human basophils, as well as an acute swelling and flare reaction after intradermal injection into the skin of allergenic patients (Barboni et al., 1987; Hoffman, 1977; Hoffman, 2006; Kim and Jin, 2014). Venom acid phosphatase is a high-molecular-weight protein composed of 404–411 amino acid residues in the order Hymenoptera (de Graaf et al., 2010; Grunwald et al., 2006; Kim and Jin, 2014). Venom acid phosphatase is not common in solitary wasp venoms (Table 1), whereas its gene was found in the venom gland transcriptomes of *V. crabro* and *V. analis* (Table 2), suggesting its relatively wider distribution in social wasp venoms and its importance as a major allergen.

Acetylcholine, a neurotransmitter and neuromodulator, is commonly found in social wasp venom and is likely to be involved in pain processing. Acetylcholinesterase, which hydrolyzes the acetylcholine, was specifically identified in social wasps (Yang et al., 2012), suggesting that it may play a role in the regulation of pain sensation in envenomed vertebrates.

A number of venom proteins tentatively associated with hemostasis have been identified by the venom gland transcriptome analysis of Vespa social wasps (Table

2, (Liu et al., 2015; Yoon et al., 2015b)). A relatively fewer numbers of hemostasis-related proteins have been identified from solitary wasps (Table 1), suggesting that this group of proteins are likely to be associated with social defense by disrupting hemostasis of vertebrate predators.

4. Venom Peptides

Among a variety of venom components, peptides with a molecular weight range of 1.4–7 kDa are predominant in wasp venoms, comprising up to 70% of the dried venoms (Baek and Lee, 2010a, c; Baek et al., 2013; Palma, 2006; Yoon et al., 2015b). In addition to neurotoxic peptides including wasp kinins, a series of amphipathic α-helical peptides (mastoparans and chemotactic peptides) are also major peptidergic components. These venom peptides commonly exist in both solitary and social wasp venoms. However, no kinins have been identified yet in Vespidae solitary wasps. Only three chemotactic peptide-like peptides have been reported in Vespidae solitary wasps so far, implying that kinin-like peptides are not likely a major component in Vespidae solitary wasp venoms (Table A1).

Kinins, mastoparans, and chemotactic peptides share a common secondary structure: an N-terminal signal sequence, a prosequence, a mature peptide, and/or an appendix G or GKK at the C-terminus. They are post-translationally processed via the sequential liberation of dipeptides (A/P-X-A/P-X) in the prosequence by

dipeptidyl peptidase IV (DPP-IV) (Gorrell, 2005; Lee et al., 2007). Additionally, some of mastoparans and chemotactic peptides are further processed at C-terminal G or GKK residues via the C-terminal amidation catalyzed by peptidylglycan α-hydroxylating monooxygenase and peptidyl-α-hydroxyglycine α-amidating lysase (Eipper et al., 1993; Kreil and Kreil-Kiss, 1967). Although many venom peptides pass through a similar posttranslational processing, the matured peptides have distinguishable structures and bioactivities. Moreover, several venom peptides matured via the same processing are not even categorized into kinin, mastoparan, or chemotactic peptides in solitary wasps (e.g., 6 venom peptides from *O. drewseni* and *E. pomiformis* in "Uncategorized Peptides" in Table A1). Since peptides are major components of both solitary and social wasp venoms, their kinds, properties, and putative functions are reviewed in detail in this section.

Table 3. Venom peptides of hunting wasps

	Name	Sequence	Length (a.a)	Species
	Neurotoxins			
Solitary	α-PMTX	RIKIGLFQDLSKL	13	*Anoplius samariensis*
	β-PMTX	RIKIGLFQDLSRL	13	*Batozonellus maculifrons*
Social	AvTx-7	1210 Da (α-PMTX 1530 Da)		*Agelaia vicina*
	AvTx-8	1567 Da		*Agelaia vicina*
	Kinins	-PPGF(T/S)P(F/L)-		
	Bradykinin (BK)	RPPGFSPFR		mammal
Solitary	Megascoliakinin	RPPGFTPFRKA	11	*Megascolia flavifrons*
	Bradykinin	RPPGFSPFR	9	*Megacampsomeris prismatica*
	Thr^6-BK	RPPGFTPFR	9	*Megacampsomeris prismatica* *Campsomeriella annulata annulata* *Carinoscolia melanosoma fascinate* *Cyphononyx dorsalis* *Megascolia flavifrons* *Colpa interrupta*
Social	RA-Thr^6-Bradykinin	RARPPGFTPFR	11	*Polybia paulista*
	RA-Thr^6-Bradykinin-DT	RARPPGFTPFRDT	13	*Polybia paulista*
	Vespakinin-M	GRPHypGFSPFRID	14	*Vespa mandarinia*
	Vespakinin-X	ARPPGFSPFRIV	12	*Vespa xanthoptera*
	Vespakinin-A	GRPPGFSPFRVI	12	*Vespa analis*
	Vespakinin-AP **	ELPPGFTPFRII	12	*Vespa analis parallela*
	Vespakinin-T	GRPHypGFSPFRVI	12	*Vespa tropica*
	Vespakinin-C	KLPPGFTPFRII	12	*Vespa crabro flavogasciata*
	Vespulakinin	TAT(carbhy)T(carbhy)RRRGRPPG FSPFR	17	*(Para)Vespula maculifrons*
	Vespulakinin-L	TAR(NAcGal-	17	*Vespula lewisii*

		Gal)TKRRGRPPGFSPFR		
	Polisteskinin 3	PyrTNKKKLRGRPPGFSPFR	18	*Polistes exclamans, Polistes annularis Polistes fuscramatus*
	Polisteskinin-R	ARRPPGFTPFR	11	*Polistes rothneyi*
	Polisteskinin-J	RRRPPGFT(S)PFR	11	*Polistes jadwigae*
	Polisteskinin-C	SKRPPGFSPFR	11	*Polistes chnensis*
	PMM1	KRRPPGFTPFR	11	*Polistes major major*
	Protopolybiakinin-I	DKNKKPIRVGGRRPPGFTR	19	*Protopolybia exigua*
	Protopolybiakinin-II	DKNKKPIWMAGFPGFTPIR	19	*Protopolybia exigua*
	Mastoparan-like Peptides			
Solitary	EMP-AF	INLLKIAKGIIKSL-NH2	14	*Anterhynchium flavormarginatum micado*
	Eumenitin	LNLKGIFKKVASLLT	15	*Eumenes rubronotatus*
	EMP-OD (OdVP1)	GRILSFIKGLAEHL-NH2	14	*Orancistrocerus drewseni*
	OdVP3 [a]	KDLHTVVSAILQAL-NH2	14	*Orancistrocerus drewseni*
	EpVP1 [a]	INLKGLIKKVASLLT	15	*Eumenes pomiformis*
	EpVP2a [a]	FDLLGLVKKVASAL-NH2	14	*Eumenes pomiformis*
	EpVP2b [a]	FDLLGLVKSVVSAL-NH2	14	*Eumenes pomiformis*
Social	Mastoparan (MP)	INLKALAALAKKIL-NH2	14	*Vespula lewisii*
	Mastoparan-X	INWKGIAAMAKKLL-NH2	14	*Vespa xanthoptera*
	Mastoparan-A	IKWKAILDAVKKVL(I)-NH2	14	*Vespa analis*
	Mastoparan-B	LKLKSIVSWAKKVL-NH2	14	*Vespa basalis*
	Mastoparan-C	INW(L)KALLAVAKKIL-NH2	14	*Vespa crabro*
	Mastoparan-II	INLKALAALVKKVL-NH2	14	*Vespa orientalis*
	HR1	INLKAIAALVKKVL-NH2	14	*Vespa orientalis*
	Mastoparan-T1*	INLKVFAALVKKFL-NH2	14	*Vespa tropica*
	Mastoparan-T2*	INLKVFAALVKKLL-NH2	14	*Vespa tropica*
	Mastoparan-T3*	INLRGFAALVKKFL-NH2	14	*Vespa tropica*
	Mastoparan-T4*	INLFGFAALVKKFL-NH2	14	*Vespa tropica*

	protopolybia-MP I	INWLKLGKKVSAIL-NH2	14	*Protopolybia exigua*
	protopolybia-MP II	INWKAIIEAAKQAL-NH2	14	*Protopolybia exigua*
	protopolybia-MP III	INWLKLGKAVIDAL-NH2	14	*Protopolybia exigua*
	P-8	INWKALLDAAKKVL-NH2	14	*Protonectarina sylveirae*
	polybia-MP I	IDWKKLLDAAKQIL-NH2	14	*Polybia paulista*
	polybia-MP II	INWLKLGKMVIDAL-NH2	14	*Polybia paulista*
	polybia-MP III	IDWLKLGKMVMDVL-NH2	14	*Polybia paulista*
	polybia-MP IV	IDWLKLRVISVIDL-NH2	14	*Polybia paulista*
	polybia-MP V	INWHDIAIKNIDAL-NH2	14	*Polybia paulista*
	polybia-MP VI	IDWLKLGKMVM	11	*Polybia paulista*
	parapolybia-MP	INWKKMAATALKMI-NH2	14	*Parapolybia indica*
	parapolybia-MP	INWAKLGKLALEVI-NH2	14	*Parapolybia indica*
	Ropalidia-MP	INWAKLGKLALQAL-NH2	14	*Ropalidia*
	polistes-MP	VDWKKIGQHILSVL-NH2	14	*Polistes jadwigae*
	PMM2	INTKKIASIGKEVLKAL-NH2	17	*Polistes major major*
	Agelaia MP-I	INWLKLGKAIIDAL-NH2	14	*Agelaia pallipes pallipes*
	Chemotactic Peptides			
Solitary	OdVP2 (Orancis-protonectin)	ILGIITSLLKSL-NH2	12	*Orancistrocerus drewseni*
	EpVP6[b]	FGPVIGLLSGILKSLL	16	*Eumenes pomiformis*
	RbVP1*[b]	FLGGLIKGLVKAL-NH2	13	*Rhynchium brunneum*
Social	Protonectin	ILGTILGLLKGL-NH2	12	*Protonectarina sylveirae* *Agelaia pallipes pallipes*
	Protonectin(1-6)	ILGTIL-NH2	6	*Agelaia pallipes pallipes*
	Paulista-CP (polybia-CP)	ILGTILGLLKSL-NH2	12	*Polybia paulista*
	Polybia-CP 2	ILGTILGKIL	10	*Polybia paulista*
	Polybia-CP 3	ILGTILGTFKSL-NH2	12	*Polybia paulista*
	Crabrolin	FLPLILRKIVTAL-NH2	13	*Vespa crabro*
	Ves-CP-T	FLPILGKILGGLL-NH2	13	*Vespa tropica*

	Ves-CP-T2*	FLPIIGKLLSGLL-NH2	13	*Vespa tropica*
	Ves-CP-M	FLPIIGKLLSGLL-NH2	13	*Vespa mandarina*
	Ves-CP-A	FLPMIAKLLGGLL-NH2	13	*Vespa analis* *Vespa analis parallela*
	Ves-CP-X	FLPIIAKLLGGLL-NH2	13	*Vespa xanthoptera*
	Ves-CP-L	FLPIIAKLVSGLL-NH2	13	*Vespula lewisi*
	VCP-5e	FLPIIAKLLGGLL-NH2	13	*Vespa magnifica*
	VCP-5f	FLPIPRPILLGLL-NH2	13	*Vespa magnifica*
	VCP-5g	FLIIRRPIVLGLL-NH2	13	*Vespa magnifica*
	VCP-5h	FLPIIGKLLSGLL-NH2	13	*Vespa magnifica*
	HP-1	LFRLIAKTLGSLM	13	*Vespa basalis*
	HP-2	LFRLLANTLGKIL	13	*Vespa basalis*
	HP-3	IFGLLAKTLGNLF	13	*Vespa basalis*
	HR2	FLPLILGKLVKGLL-NH2	14	*Vespa orientalis*
	PMM3	FLSALLGMLKNL-NH2	12	*Polistes major major*
	Uncategorized Peptides			
Solitary	Anoplin	GLLKRIKTLL-NH2	10	*Anoplius samariensis*
	Decoralin	SLLSLIRKLIT-NH2	11	*Oreumenes decoratus*
	OdVP4	LDPKVVQSLL-NH2	10	*Orancistrocerus drewseni*
	EpVP3	AINPKSVQSLL-NH2	11	*Eumenes pomiformis*
	EpVP3S	INPKSVQSLL-NH2	10	*Eumenes pomiformis*
	EpVP4a	LSPAVMASLA-NH2	10	*Eumenes pomiformis*
	EpVP4b	LSPAAMASLA-NH2	10	*Eumenes pomiformis*
	EpVP5	VHVPPICSHRECRK	14	*Eumenes pomiformis*
	As-peptide126	QDPPVVKMK-NH2	9	*Anoplius samariensis*
	Cd-125	DTARLKWH	8	*Cyphononyx dorsalis*
	Cd-146	SETGNTVTVKGFSPLR	16	*Cyphononyx dorsalis*
	EpDTX	IATICNLPIVSGNGQEEHIRWAYSIITH VCVSFRYTGKGGNRNNFFTERECRSYCY F	57	*Eumenes pomiformis*

	As-fr-19	VSFCLLPIVPGPCTQYVIRYAFQPSISA CRRFTFGGCEGNDNNFMTRRDCEHYCEE LL	58	*Anoplius samariensis*
Social	Vespin	CYQRRVAITAGGLKHRLMSSLIIIIIIR INYLRDNSVIILESSY	44	*Vespa magnifica*

* No name in the reference. Named in this review.
** Named the same with a previously known, different peptide. Renamed in this review.
[a] Categorized as mastoparan-like peptides based on the sequence similarity with the previously reported venom peptides, without a mast cell degranulation activity test.
[b] Categorized as chemotactic peptide-like peptides based on the sequence similarity with the previously reported venom peptides, without a chemotactic activity test.

4.1. Neurotoxic peptides

Neurotoxic peptides modulating ion channel and receptor functions have been described in wasp venoms. The first neurotoxin component that was isolated from wasp venom was a nicotinic acetylcholine receptor (nAChR) inhibitor, kinin. In 1954, the first wasp kinin was isolated from a social wasp, Vespa vulgaris (Schachter and Thain, 1954). Afterwards, many kinins of the Vespoidea wasp venoms were found to be responsible for the pain and paralysis after a wasp sting (Konno et al., 2002; Nakajima, 1986). Until now, most of the neurotoxic peptides of hunting wasps are kinins. Wasp kinins will be discussed further in Section 4.2.

There are 2 non-kinin neurotoxic peptides isolated from Pompilidae solitary wasps: α-PMTX from A. samariensis and β-PMTX from B. maculifrons (Konno et al., 1998; Konno et al., 1997). PMTXs, 13-amino acid venom peptides, affect both vertebrate and invertebrate nervous systems by slowing or blocking sodium channel inactivation (Sahara et al., 2000). α-PMTX greatly potentiates synaptic transmission of lobster leg neuromuscular junction by acting primarily on the presynaptic membrane (Konno et al., 1997). Interestingly, β-PMTX, in which the lysine at 12 position of α-PMTX was replaced with arginine, is 5 times more potent than α-PMTX (Konno et al., 1998).

Recently, novel venom peptides, AvTx-7 and AvTx-8, were also reported as neurotoxins of the social wasp Agelaia vicina (de Oliveira et al., 2005; Pizzo et al.,

2004). Although their primary structures have not been elucidated so far, they seem to be new types of venom peptides, different from kinins as judged by their distinct neural activity. AvTx-7 stimulates glutamate release through K^+ channel and AvTx-8 inhibits GABAergic neurotransmission, whereas wasp kinins block nAChR.

4.2. Kinins

Bradykinin was first reported in 1949 as a mammalian blood serum substance that triggers a slow contraction of the guinea-pig ileum (Silva et al., 1949). This nonapeptide acts on smooth muscles with contractions or relaxations. In the neuronal cells of vertebrates, bradykinin evokes a release of neuropeptides (galanin, neuropeptide Y, and vasoactive intestinal peptide) and catecholamines (dopamine, norepinephrine, and epinephrine) by depolarizing nerve terminals (Podvin et al., 2015; Rochat and Martin-Eauclaire, 2000).

Bradykinin-like peptide was found in wasp venoms as the first neurotoxic and pain-producing peptide (Schachter and Thain, 1954). It irreversibly blocks the synaptic transmission of nAChR in the insect central nervous system (CNS) (Piek et al., 1990; Piek et al., 1987). While wasp kinins have remarkable sequence similarities to the main structure of mammalian bradykinin [-PPGF(T/S)P(F/L)-], most of them are longer than bradykinin or differ at position 3 or 6, where proline

is replaced by hydroxyproline or serine is replaced by threonine (Thr6-bradykinin), which has a single extra hydroxyl or methyl group, respectively. By the single amino acid substitution, Thr6-bradykinin displayed 3-fold higher anti-nociceptive effects on the rat CNS and remained active longer than bradykinin (Mortari et al., 2007). Considering their action on nAChR, bradykinins in solitary wasp venoms may play a crucial role in paralyzing prey during hunting (Konno et al., 2002).

Almost all social wasp venoms may have kinin or kinin-like activities, while, among solitary wasps, Cyphononyx dorsalis (Pompilidae) and several Scoliidae wasps have Thr6-bradykinin in their venom, and bradykinin was found only in the Megacampsomeris prismatica (Scoliidae) venom (Konno et al., 2001b; Konno et al., 2002). Since one of major pharmacological effects of kinins is pain sensation in vertebrates (Eisner et al., 1978), the ubiquitous distribution of kinins in social wasp venoms suggests that kinins may function as a major defense and alarm device by generating pain in the envenomed vertebrate predators. The presence of kinins in the venom of Vespidae and Scoliidae, as well as that of Formicidae (ants), and no kinin-like activities in bees (Apidae) and bee-related solitary wasps (Crabronidae and Sphecidae) was supposed to support the suggestion that these three families are associated by synapomorphies (Brothers, 1999; Piek, 2000). Later on, however, kinin-like activity was also found in Ampulicidae (*A. compressa*) that is closely located to Apidae (Piek et al., 1989), and no kinins have

been isolated from solitary wasps in Vespidae so far. Thus, the relationship between the venom kinins and evolution remains obscure, requiring a more extensive identification and characterization of Hymenopteran venom kinins.

4.3. Mastoparans

The most abundant peptide component of hunting wasp venoms, both in solitary and social wasps, is mastoparan. Of note, however, mastoparans have been thus far isolated only in Vespidae (both social and solitary), not in other solitary hunting wasp families. Mastoparans (mostly tetradecapeptides) act on mast cells to liberate granules and release histamine (mast cell degranulation, MCD), resulting in inflammatory response (Hirai et al., 1979). Their structural properties, net positive charge, and amphipathic α-helical structure, in which all side chains of the hydrophobic amino acids are located on one side of the axis, and those of the basic or the hydrophilic amino acid residues are on the opposite side, allow them to attach to biomembranes and form pores via barrel-stave, carpet, or toroidal-pore mechanisms, resulting in an increase of cell membrane permeability (Brogden, 2005). Mastoparans are often highly active against the cell membranes of bacteria, fungi, and erythrocytes, as well as mast cells, resulting in antimicrobial, hemolytic, and MCD activities. Meanwhile, MCD by mastoparan may occur also through the exocytosis of granules, triggered by mastoparan

modulating G-protein activity without receptor interaction (Higashijima et al., 1988). The net effect of MCD depends on the cell types: the secretion of histamine from mast cells, serotonin from the platelets, catecholamines from chromaffin cells, prolactin from the anterior pituitary, and even insulin from pancreatic β-cells (Baptista-Saidemberg et al., 2012; Hirai et al., 1979; Kurihara et al., 1986). Cell lytic activity also varies depending on the cell types. Generally, antimicrobial activity of mastoparans is higher against fungi than Gram-negative bacteria, *E. coli* (Baek et al., 2011; Xu et al., 2006). In addition, probably due to the cell lytic activity against insect cells, antimicrobial mastoparans also caused feeding disorder in caterpillars, although they are not active against human erythrocytes (Baek et al., 2011).

Cell lytic activity of mastoparans also leads to mitochondrial permeability transition that affects cell viability and triggers tumor cell cytotoxicity (reviewed in (Moreno and Giralt, 2015)). Besides MCD and cell lytic activity, mastoparans also stimulate phospholipases A, C and D, mobilization of Ca2+ from mitochondria and sarcoplasmic reticulum, and necrosis and/or apoptosis (Moreno and Giralt, 2015; Rocha et al., 2007). A variety of biological functions of mastoparans have attracted attention to them as components for potential therapeutic and biotechnological applications in biomedicine (reviewed in (Moreno and Giralt, 2015; Silva et al., 2015)). Due to the lack of cell specificity,

however, mastoparans could not be used as they are. That is, they would damage not only tumor cells, but would also negatively affect healthy cells. Accordingly, researchers are developing a delivery system for venom peptides targeting tumor cells and a selective release system inside tumor cells that would make venom peptides accumulate in a specific and controlled manner (Moreno et al., 2014).

4.4. Chemotactic peptides

The second major peptide group in hunting wasp venom is chemotactic peptides. Similarly to mastoparans, venom chemotactic peptides have also been isolated only from social and solitary wasps in Vespidae, not from other solitary wasp families. Like mastoparans, venom chemotactic peptides are generally tridecapeptides with an amphipathic, α-helical, linear, cationic, and C-terminal amidated secondary structure. Their primary activity is described as inducing cellular chemotactic response in polymorphonuclear leukocytes and macrophages (Jensen and Moody, 2006) and, due to the structural homology, chemotactic peptides often reveal mastoparan-like MCD, antimicrobial, and hemolytic activities. Chemotactic activity results in a mild edema, accompanied by an inflammatory exudate around the stinging site, where polymorphonuclear leukocytes are mainly concentrated. In other words, chemotactic peptides do not directly trigger pain, but enhance the inflammatory response by wasp stings

(Pieck, 1986); therefore, they are likely to be involved in defense. Their widespread distribution in most social wasp venoms supports this prediction.

Although chemotactic peptides are a major venom component, only three of them have been reported in the venom of solitary hunting wasps: Orancis-protonectin (OdVP2) (Baek and Lee, 2010c; Murata et al., 2009), EpVP6 (Baek and Lee, 2010a), and the one found in *R. brunneum* (named RbVP1 hereafter) (Baek et al., 2013). These solitary wasp venom peptides were categorized into chemotactic peptides based on the amino acid sequence homology with the previously known peptides, without a chemotaxis analysis. Thus, they indeed should be further evaluated to be referred to chemotactic peptides.

There is no known conserved main structure for recognition of venom chemotactic peptides. In Table A1, venom chemotactic peptides reported in solitary and social hunting wasps are summarized and compared, revealing representative motives XX(G/R)XX, XX(G/A/S/R/K/T)(G/T/K/S)XX or, sometimes, an overlapped form of the two motives, where X is a hydrophobic amino acid, most frequently, Ile or Leu. While only some mastoparanshave these motives, all chemotactic peptides have them. In addition, chemotactic peptides possess no or only one Lys residue, rarely 2 (RbVP1 and HR2), while most of mastoparans have 2 or 3 Lys residues. Mastoparans with 3 Lys residues generally have a single Lys (-K-) and separately double Lys residues (-KK-). These

characteristics were inferred from the sequences collected during the preparation of the present manuscript, thereby the two motives suggested above are not fully confirmed yet.

4.5. Other venom peptides

Many peptides in solitary wasp venom are not exactly categorized in kinin, mastoparan, or chemotactic peptides. Most of them are not functionally analyzed.

Amphipathic linear cationic α-helical peptides anoplin and decoralin, found in a Pompilidae wasp *A. samariensis* and an Eumeninae wasp *Oreumenes decoratus*, respectively, commonly have MCD and antimicrobial activities (Jindrichova et al., 2014; Konno et al., 2001a; Konno et al., 2007). Anoplin has hemolytic activity as well (Ifrah et al., 2005).

Non-helical coil venom peptides OdVP4, EpVP3, EpVP3S, EpVP4a, EpVP4b, and EpVP5 of Eumeninae wasps have neither antimicrobial and hemolytic, nor insect cell lytic activities (Baek et al., 2011; Baek and Lee, 2010a, c), which implies that those peptides might have novel properties other than cell lytic activity.

Bioactivities of As-peptide126, Cd-125 and Cd-146 (Konno et al., 2001b), isolated from Pompilidae wasps, have not been evaluated so far. Another *A. samariensis* venom peptide As-fr-19, as well as its homologue EpDTX of *E.*

pomiformis, has a sequence similarity to potassium or calcium channel blocker, dendrotoxins from snakes, cone snails, and sea anemones (Baek and Lee, 2010a; Hisada et al., 2005). The precise biochemical functions of As-fr-19 and EpDTX have not been clarified so far, but they are likely to function as neurotoxins.

Vespin of *Vespa magnifica*, a 44 amino-acid peptide, exerts contractile effects on isolated guinea pig ileum smooth muscle by interacting with bradykinin receptors (Chen et al., 2010). However, vespin does not share the conserved motif of kinins [-PPGF(T/S)P(F/L)-], suggesting that vespin is a novel kind of venom peptide with kinin-like activity.

Recently, genes encoding putative neurotoxic peptides (i.e., agatoxin-like, conophysin-R-like, latrotoxin-like and orientotoxin-like) have been identified from the venom transcriptome of *V. velutina*, though their transcription levels were very low (Liu et al., 2015). Neurotoxic effects of these tentative venom peptides remain to be addressed in further research.

5. Useful Wasp Venom Components for Pharmacological, Medical, and Agricultural Applications

Considering the huge diversity of wasp venom components, wasp venoms can be employed as a rich source of novel bioactive substances for pharmacological, therapeutic, and agricultural applications (Pieck, 1986). Some venom proteins and

peptides have been exploited as candidates for the discovery of novel therapeutic agents. Furthermore, studies on social wasp venoms have provided crucial information on the main allergenic molecules that are responsible for the hypersensitivity reaction in humans and enabled for the development of immunotherapy for preventing venom-induced anaphylaxis (Boyle et al., 2012; Spillner et al., 2014). Similarly to the venom of parasitoid wasps, venoms of solitary hunting wasps are also known to contain various substances that can manipulate the physiology of prey (Baek and Lee, 2010b; Baek et al., 2016; Beckage and Gelman, 2004). Such regulatory molecules produced by wasps would serve as innovative leads for developing novel, environmentally safe insect control agents (Beckage and Gelman, 2004).

5.1. Anti-microbial agents

Anti-microbial peptides (AMPs), which are relatively small (<10 kDa), cationic, and amphipathic peptides, are a basic humoral immune component of most organisms, including wasps, against invading microbial pathogens (Bulet et al., 2004; Rydlo et al., 2006). These AMPs exhibit a broad-spectrum antimicrobial activity against various microorganisms, including Gram-positive and Gram-negative bacteria, protozoa, yeast, and fungi (Reddy et al., 2004). Over the last several decades, a number of AMPs, mostly belonging to the groups of

mastoparans, VCPs, and kinins, have been isolated from a wide variety of wasp species (Yang et al., 2013). Most AMPs with the origin of wasp venom belong to the peptides forming alpha-helical structures, or coils rich in cysteine residues (Baek et al., 2011; Brogden, 2005) and are suggested to act by perforating the plasma membrane, thus resulting in the cell lysis and death (Brogden, 2005).

Mastoparan or mastoparan-like peptides are the alpha-helical peptides and have been identified in a wide range of wasps, including both solitary and social wasps (Baek and Lee, 2010a, b; Baek et al., 2013; Baptista-Saidemberg et al., 2012; Cabrera et al., 2004; Cerovsky et al., 2007; Chen et al., 2008; de Souza et al., 2009; Ho and Hwang, 1991; Konno et al., 2001a; Konno et al., 2006; Konno et al., 2007; Lee et al., 2007; Lin et al., 2011; Mendes et al., 2004; Mendes et al., 2005; Murata et al., 2009; Piek and Spanjer, 1986; Sforca et al., 2004; Souza et al., 2005; Xu et al., 2006; Yang et al., 2013; Yoon et al., 2015b). The AMP from the Brazilian wasp *P. paulista* venom (MP1) has a broad-spectrum antibiotic activity against Gram-negative and Gram-positive bacteria without showing apparent hemolytic and cytotoxic activities (de Souza et al., 2009). The applicability of mastoparans for therapeutic and biotechnological use has been also reviewed elsewhere (Moreno and Giralt, 2015).

Three venom peptides (OdVP1, OdVP2 and OdVP3) isolated from the venom of the solitary wasp *O. drewseni* showed the typical features of amidated C-

termini proteins and had a high content of hydrophobic and positively charged amino acids, resembling the amphipathic α-helical secondary structure of mastoparans (Baek and Lee, 2010c). Despite the distinctive sequences context in mature peptide, the overall transcript structure of the OdVPs showed a high similarity to that of *Vespa basalis* mastoparan-B by containing a signal sequence, a prosequence, a mature peptide, and a C-terminal glycine (Baek and Lee, 2010c). The OdVPs exhibited strong activities against fungi, but weak antibacterial activities. OdVP2L, having additional Glu–Pro residues, showed a high antifungal activity against the gray mold Botrytis cinerea, but did not show antimicrobial activity against bacteria or Gram-positive yeast (Baek and Lee, 2010c). Venom peptides of a-helical structure from a solitary wasp *E. pomiformis* (EpVP1, EpVP2a, EpVP2b, and EpVP6) also exhibited varying degrees of anti-microbial activities against Gram-negative *E. coli*, Gram-positive *Staphylococcus aureus*, Gram-positive yeast *C. albicans*, and the gray mold *B. cinerea* (Baek et al., 2011).

Since microbial infection mediated by biofilms has been a major problem in the use of implantable devices, several approaches, including the covalent immobilization of AMPs, have been attempted to tackle this problem (Vasilev et al., 2009). To this end, the immobilization of MP1, a broad-spectrum AMP from a social wasp P. paulista venom, onto silicon surfaces has been attempted via the “allyl glycidyl ether brush”-based polymerization chemistry (Basu et al., 2013).

The antibacterial activity of the MP1-immobilized surfaces was retained after 3 days of incubation in artificial urine without causing any significant cytotoxicity against human red blood cells, suggesting the stability and safety of the AMP coating in physiological environments (Basu et al., 2013). Based on this finding, a general approach to exploit and immobilize other AMPs as novel surface-sterilizing agents can be attempted.

5.2. Anti-tumor agents

Mitoparan, a synthetic mastoparan analog, can form pores in the cancer cell plasma membrane and eventually lead to its death either by necrosis or by triggering apoptosis (Jones and Howl, 2004; Jones et al., 2008). Due to their non-specific cytolytic activity and instability when injected in blood, however, the use of cytosolic peptides, such as mitoparan, is limited (Moreno et al., 2014). To overcome this limitation, Moreno et al., (2014) have devised a pro-cytotoxic system based on mitoparan conjugated to poly (l-glutamic acid) PGA polymer through specific cleavage sequences that are cleaved by overexpressed tumor proteases, such as the metalloproteinase-2 or cathepsin B, in which the conjugated mitoparan becomes active only when it reaches cancer cells, then is cleaved and released by the tumor proteases (Moreno et al., 2014).

The MP1 AMP, a mastoparan-like pore-forming peptide, has been determined

to have highly selective antitumor activities against several types of cancer cells, including bladder and prostate cancer cells (Wang et al., 2008) and multidrug-resistant leukemic cells (Wang et al., 2009). Recently, the highly specific antitumor activity of MP1 was determined to be due to its selective affinity to phosphatidylserine (PS) and phosphatidylethanolamine (PE), thereby enhancing the MP1-driven poration of cancer cell membrane, in which the outer lipid bilayer has an enriched PS and PE composition (Leite et al., 2015). When combined with other anticancer drugs, the selectivity of MP1 peptide to disturb the cancer cell membrane may provide synergistic potentials, which can dramatically improve the therapeutic efficacy (Leite et al., 2015). The mastoparans from social wasps *V. crabro* and *V. analis* also exhibited antitumor activities against ovarian tumor cells, with *V. analis* mastoparan showing a greater antitumor activity (Yoon et al., 2015a). Taken together, mastoparan-like peptides from wasps can serve as good candidates for lead compounds of novel anticancer drugs (Leite et al., 2015).

5.3. Venom Allergy Diagnosis and Immunotherapy

Hymenoptera venom allergy (HVA) is an anaphylactic reaction of human to stings of social Hymenopteran insects, including honey bees, yellow jackets, hornets, bumble bees, and paper wasps (Spillner et al., 2014). A small but significant portion (0.3%–3.4%) of general human population is known to show

systemic allergic reactions to Hymenoptera stings (Spillner et al., 2014). While whole venoms have been usually used for both diagnosis and immunotherapy, their diagnostic precision, when based on whole venom preparations, has been often impaired by immunoglobulin E (IgE) cross-reactivity between different venoms, which might be due to highly conserved venom allergens present in venom of different families or due to the presence of common cross-reactive carbohydrate determinants on venom allergens (Aalberse et al., 2001; Spillner et al., 2014). Nevertheless, the information on single venom allergens for diagnostic and therapeutic purposes has been limited, impeding an in-depth understanding of molecular basis underlying HVA. Recent employment of omics technologies for venom study has enabled a rapid discovery of novel venom components of medical importance and, thus allowed for a better molecular understanding of the entire "venome" as a system of unique and characteristic components (Spillner et al., 2014). Recombinant allergens, such as phospholipase A1, hyaluronidase, and venom allergen 5, have been generated from four important genera in Vespidae (i.e., Vespula, Dolichovespula, Vespa, and Polistes) and used for diagnosis (reviewed in (Müller, 2002)). When the IgE-binding capacity of recombinant and purified natural venom allergens was compared, recombinant allergens exhibited higher specific responses without cross-reactivity and false positive results, indicating that they are better than highly purified natural preparations in terms of

the clinical relevance of an individual allergen (Kreil, 1995; Muller et al., 1997). The potential of recombinant allergens for diagnostic and therapeutic applications has been well reviewed by Muller (2002) (Müller, 2002). The use of cocktails with recombinant allergens for diagnosis can significantly increase the specificity of conventional diagnostic tests, such as immediate-type skin tests and the assays for serum-specific IgE antibodies (Müller, 2002). The hypoallergenic mutants or modified variants of major venom allergens or the T-cell epitope peptides generated by recombinant technologies can be used as vaccines for immunotherapy to treat HVA (King and Spangfort, 2000; Müller, 2002).

5.4. Biopesticides

Manipulation of host by parasitoid wasp venom can be achieved via a variety of means, such as transient paralysis, immune suppression, endocrine dysfunction, metabolic alteration, and developmental arrest (reviewed in (Beckage and Gelman, 2004)). Several peptide/protein neurotoxins, including AvTx, pompilidotoxin, agatoxin-like, latrotoxin-like, orientotoxin-like, dendrotoxin-like peptides, among others, have been identified in solitary and social wasp venoms, of which those from solitary wasps (i.e., pompilidotoxin and dendrotoxin-like peptide) are known to be involved in prey paralysis (Table 1 and Table 2; (Baek and Lee, 2010a, b; Konno et al., 1998; Konno et al., 1997; Magloire et al., 2011; Quistad et al., 1994;

Sahara et al., 2000)). However, only one venom component that can regulate prey physiology has been identified and characterized in solitary wasps (Baek and Lee, 2010a, b; Baek et al., 2016; Baptista-Saidemberg et al., 2012). Nevertheless, given that solitary hunting wasps are also in need of the long-term metabolic alteration and developmental arrest of prey to provide fresh provisions to their progeny, a more versatile array of physiology-manipulating components is likely to be present in the venom of solitary wasps. Once these protein/peptide components with insecticidal or growth-regulating activity are identified, they can be exploited as alternative insect control agents, provided proper delivery protocols are established (Smith et al., 2013). A spider venom neurotoxin (*Segestria florentina* toxin 1, SFI1) fused to the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) exhibited insecticidal activity against two homopteran sucking pests, the peach-potato aphid *Myzus persicae* and the rice brown planthopper *Nilaparvata lugens* (Down et al., 2006), where the fusion protein gene can be employed for developing sucking pest-resistant transgenic crops. More recently, the *ω*-hexatoxin-Hv1a peptide (Hv1a), a neurotoxin from the Australian funnel web spider Hadronyche versuta acting on voltage-sensitive calcium channels, was fused to the carrier protein GNA to make Hv1a traverse the insect gut epithelium and access the central nervous system, thereby enhancing its oral toxicity (Fitches et al., 2012; Nakasu et al., 2014). In addition, recombinant baculoviruses

expressing insect-selective toxins, hormones, or enzymes could enhance their insecticidal properties (Mccutchen et al., 1991). Once such neurotoxins or host/prey-regulatory molecules are identified and characterized from wasp venoms, similar biotechnical approaches can be attempted. Therefore, further research is needed for searching and characterizing wasp venom components with insecticidal and growth-regulating potential.

The main contents of this literature review were published as Lee et al., 2016.

CHAPTER I

Characterization of Venom Evolution via Venom Component Phylogenetic Analysis in Aculeate Hymenoptera

Abstract

To identify and compare the venom components and expression patterns of some bees/wasps, venom gland-specific transcriptome analyses were conducted for 14 Aculeata bees/wasps. FPKM (Fragments Per Kilobase Million) values were normalized using the average transcription level of a reference housekeeping gene (α-tubulin). Most of the allergens and pain-producing factors (arginine kinase, hyaluronidase, mastoparan, phospholipase A1, and venom allergen 5) showed extremely high expression levels in social wasps, implying that social wasps have evolved to use venom to defend the colony against intruders. Acid phosphatase and tachykinin, which are known as allergens and neurotoxic peptides, were found with higher frequencies in the venom glands of solitary wasps compared to social wasps. This suggests that solitary wasps might use their venom for catching and preserving prey, and by changing the prey's physiology and behavioral state. In the venom glands of bumblebees, little or no transcripts of major allergens or pain producing factors were identified, implying that bumblebee venoms are relatively less toxic than those of social or solitary wasps. Taken together, the differential expression patterns of venom genes in some Aculeata bees/wasps implies that bees/wasps have unique groups of highly expressed venom components, which appear to have evolved in response to both ecological and behavioral influences.

1. Introduction

Wasps present an extremely diverse group in the suborder Apocrita (Hymenoptera), which is conventionally divided into two groups: Parasitica and Aculeata (Brothers, 1999; Pieck, 1986). The clade Parasitica comprises the majority of parasitoid wasps, whereas the clade Aculeata contains most parasitic and predatory wasps for which the ovipositor is completely modified into a stinger for injecting venom (Pieck, 1986). These stinging Aculeata wasps are further divided into two subgroups (solitary vs. social) depending on their lifestyle in the context of sociality (Dowton, 2001).

Approximately 95% of 15,000 species of Aculeata wasps are solitary and are widely distributed across various families in the Aculeata (O'Neill, 2001). The lifestyle of solitary wasps is unsocial; they do not form colonies (O'Neill, 2001). After mating, the female solitary wasp builds one or more nests, hunts prey, and stores prey in the cell(s) of the nest as provisions for the young.

Most solitary wasps sting their prey to paralyze and preserve it. It can then be used as food for the hatched wasp larvae. Thus, solitary wasp venom primarily comprises various bioactive molecules that function in paralysis, antimicrobial activity, developmental arrest, etc.

Carnivorous social wasps represent only a small portion of the Aculeata.

Social wasps form colonies and some species, such as hornets and yellow jackets, build very large nests. Unlike solitary wasps, social wasps usually sting to defend themselves and their colonies from vertebrate predators (Schmidt, 1990). Once disturbed, the entire colony is mobilized via an attack pheromone to sting the intruder, resulting in mass envenomation which can be fatal (Spradbery, 1973). Most social wasps generally butcher their prey (mostly insects and spiders) without stinging, and bring the more nourishing parts of the prey back to the colony to feed the larvae. Therefore, social wasps do not need to paralyze or preserve hunted prey with their venom. Social wasp venom appears to have evolved to maximize the defense potential. This is achieved by intensifying the venom-induced pain and/or augmenting the allergic and immune responses of humans and animals (Schmidt, 1990). Since social wasp venom contains molecules that cause hypersensitivity reactions such as anaphylaxis, it has been of great medical and clinical importance.

Bumblebees, which belong to the family Apidae, are increasingly used for pollination of greenhouse plants because of their naive characteristics and lifestyle fit for pollinating. They are not aggressive and do not sting unless disturbed near their nests or intentionally touched (Bucher et al., 2001). Although more frequent stinging incidents are expected with the increased use of bumblebees in agriculture, little information on the comparative toxicities of bumblebee venoms

has been available to date.

In addition, the venoms of bumblebees are considered a potential source of novel bioactive substances for pharmacological, therapeutic, and agricultural uses (Pieck, 1986). Until now, the key components that differentiate the venoms of solitary and social wasps had not been accurately identified.

To this end, we conducted a comparative transcriptomic characterization of the venom glands from 14 Aculeata bee/wasp species. We investigated the evolutionary aspects of the venom components in accordance with ecological and behavioral features.

2. Materials and Methods

2.1. Bees/wasps collection and total RNA purification

Female solitary hunting wasps (*Eumenes decorates*, *Sphecidae* spp., *Anterhynchium flavomarginatum*, and *Sceliphron deforme*), social wasps (*Vespa analis*, *V. crabro*, *V. dybowskii*, *V. simillima*, *Parapolybia varia*, *Polistes snelleni*, and *P. rothneyi*), and bumble bees (*Bombus ardens*, *B. consobrinus*, and *B. ussurensis*) were collected from several southern regions in Korea. Bees/wasps were anesthetized using low-pressure carbon dioxide. Twenty venom glands were dissected, and total RNA was extracted using 200 μL TRI reagent according to the

manufacturer's protocol.

2.2. Construction of RNA-Seq library

RNA purity and total RNA integrity were evaluated using a NanoDrop 8000 spectrophotometer and Agilent Technologies 2100 Bioanalyzer. Next, mRNA sequencing libraries were prepared as described previously (I-1).

A reference gene (α-tubulin) (Gao et al., 2017) was selected for the verification of transcriptional abundance, and total reads were normalized using average FPKM values for the reference gene in 14 Aculeata bees/wasps.

2.3. Comparison of amino acid sequences

To investigate structural differences in venom proteins, deduced amino acid sequences obtained from the transcriptome data were aligned with full-length sequences using CLC Main Workbench 7 (CLC Bio, Waltham, MA, USA).

2.4. Construction of phylogenetic tree

Based on the amino acid alignment of the cellulase genes, a phylogenetic tree was constructed. We used CLC Main Workbench 7 (CLC Bio) with the neighbor-joining method and 1000 bootstrap replications. Acid phosphatase, defensin 1, defensin 2, hyaluronidase, and phospholipase A2 of *Homo sapiens* were used as

outgroups. The following were also used as outgroups: arginine kinase and phospholipase A1 of *Hadrurus spadix*; mastoparan of uncultured bacteria (Moore et al., 2015); venom allergen 5 of *Varroa destructor*; and tachykinin of *Blattella germanica*.

3. Results and Discussion

3.1. Differential transcriptional profiles in the venom gland of some Aculeata Bees/wasps

Total reads of 5.89, 6.39, 7.13, 5.74, 6.22, 6.16, 11.3, 11.4, 8.2, 8.07, 8.1, 7.66, 10.9, and 10.6 Gb were obtained from RNA sequencing the venom glands of *E. decorates*, *Sphecidae* spp., *A. flavomarginatum*, *S. deforme*, *V. crabro*, *V. analis*, *V. dybowskii*, *V. simillima*, *P. varia*, *P. snelleni*, *P. rothneyi*, *B. ardens*, *B. consobrinus*, and *B. ussurensis*. *De novo* assembly of the trimmed sequence data resulted in 30,744, 54,171, 51,224, 34,333, 49,536, 72,510, 54,823, 55,668, 57,822, 48,513, 58,857, 71,954, 71,152, and 84,228 non-duplicate contigs, respectively. A total of 17,441, 24,662, 23,250, 18,274, 27,281, 33,104, 22,461, 20,999, 20,951, 18,567, 20,988, 27,176, 28,652, and 24,996 genes were identified by BLAST search, respectively (Table 1).

In general, social wasp venoms (of which the most studied is the venom of the

social Vespidae) induce local edema and erythema. This is caused by an increased permeability of the blood vessels in the skin, which is a net effect of active peptides such as bradykinin-like peptides, mastoparans, and chemotactic peptides (Lee et al., 2016). These local reactions to venom peptides produce prolonged pain that often continues for several hours, and itching that can last for days. In addition to the direct effects of wasp stings, immunological reactions caused by venom allergens are also frequently observed. Venom allergens include phospholipase A (A1 and A2), hyaluronidase, cysteine-rich secretory proteins, antigen 5, pathogenesis-related proteins (*e.g.* CAP), and serine proteases. Allergic reactions can lead to anaphylaxis. With a large dose of venom, systemic toxic reactions including hemolysis, coagulopathy, rhabdomyolysis, acute renal failure, hepatotoxicity, aortic thrombosis, or cerebral infarction are possible (Lai and Liu, 2010; Palma, 2006). As expected, most of the allergens and pain-producing factors (arginine kinase, hyaluronidase, mastoparan, phospholipase A1, and venom allergen 5) showed significantly higher expression in the social wasps compared to the solitary wasps or social bumblebees. This implies that social wasps have evolved to use these components as their main defensive tools against intruding species (Table 2).

In contrast, the venoms of most solitary wasps are not lethal to their prey. Instead, the venoms induce paralysis and regulate development and metabolism to

maintain the life of the prey while feeding the wasp larvae (Schmidt, 1990). Acid phosphatase and tachykinin were found with much higher frequencies in the venom glands of solitary wasps. These wasps also showed lower expression levels of allergens and pain-producing factors compared to social wasps and bumblebees (Table 2). The venom acid phosphatase of the endoparasitoid wasp *Pteromalus puparum* has been cloned and characterized. Acid phosphatase possibly affects a host's immune response and physiology (Zhu et al., 2008). However, that of the endoparasitoid wasp *Pimpla hypochondriaca* plays no known role in hemocyte immunology (Dani et al., 2005). Further studies are needed to elucidate the biological activity of acid phosphatase. Tachykinin is a neurotoxic peptide in wasp venom that is known to induce hypokinesia. Hypokinesia, a "zombie-like" state, is a venom-induced behavioral state caused by direct envenomation into the central nervous system. Synthetic AcVTK mimics the venom tachykinins of the parasitoid jewel wasp (*Ampulex compressa*). Injection of synthetic AcVTK into the subesophageal ganglion of the American cockroach (the jewel wasp host) showed behavioral changes, including suppression of the escape response, reduced spontaneous walking, and an increased escape response threshold (Arvidson et al., 2016). Thus, solitary wasps might use their venom to change a prey's physiology and behavior, ensuring the availability of fresh prey for larvae. Solitary and social wasps have distinctly different venom properties. Considerable

differences in the main components of their venoms are expected, given their different primary functions. Since many solitary wasps are specific in selecting a prey species, it would be intriguing to investigate the differences in venom composition according to the prey species, and the factors that influence the evolution of different venom substances.

In the venom glands of bumblebees, few transcripts for major allergens or pain-producing factors were found. This implies that bumblebee venoms are less toxic than those of social or solitary wasps (Table 2). Interestingly, defensin 2 was not detected in the venom glands of three bumblebees, but defensin 1 (an antimicrobial peptide) showed extremely high expression levels in *B. ardens* and *B. ussurensis*. The bumblebee is host to a variety of gut parasites, such as the trypanosomatid *Crithidia bombi*. Reducing the expression of defensins with an RNAi knock-down system led to an increase of *C. bombi* parasitemia in bumblebees, implying that defensins are strong anti-trypanosome agents (Deshwal and Mallon, 2014). Defensin 1 found in the royal jelly of honey bees contributes to cutaneous wound closure by enhancing and stimulating keratinocyte migration and metalloproteinase-9 secretion (Bucekova et al., 2017). Bumblebees might use their venom more in self-healing than as a defense from other species. The lack of defensin 1 transcripts in *B. ussurensis* suggests that this species is more susceptible to infection than other bumblebees examined in this

study.

Table 1. Summary of the venom gland transcriptome cDNA libraries of some Aculeata bees/wasps.

Species	Total number of reads	Total base pairs	Trimmed reads	Total number of assembled transcripts	Total number of annotated transcripts
E. decorates	58,988,410	5,957,829,410	3,640,686	30,744	17,441
Sphecidae sp.	63,954,192	6,459,373,392	3,657,286	54,171	24,662
A. flavomarginatum	71,380,878	7,209,468,678	3,990,196	51,224	23,250
S. deforme	57,404,468	5,797,851,268	5,232,438	34,333	18,274
V. crabro	62,246,264	5,756,774,997	1,663,615	49,536	27,281
V. analis	61,648,078	5,686,981,519	1,425,630	72,510	33,104
V. dybowskii	113,343,134	11,447,656,534	7,054,698	54,823	22,461
V. simillima	114,592,946	11,573,887,546	17,508,908	55,668	20,999
P. varia	82,025,386	8,284,563,986	4,539,352	57,822	20,951
P. snelleni	80,798,216	8,160,649,816	3,799,964	48,513	18,567
P. rothneyi	81,060,754	8,187,136,154	3,252,562	58,857	20,988
B. ardens	76,622,404	7,738,862,804	3,681,916	71,954	27,176
B. consobrinus	109,962,550	11,106,217,550	5,303,738	71,152	28,652
B. ussurensis	106,973,794	10,804,353,194	5,402,528	84,228	24,996

Table 2. Annotation of differentially transcribed major venom genes in the venom gland of Aculeata bees/wasps.

Species[a] / Gene	Social wasps							Solitary wasps				Social bumblebees		
	Va	Vc	Vd	Vs	Pv	Pr	Ps	Ed	Ss	Af	Sd	Ba	Bc	Bu
Acid phosphatase	35.01	38.19	68.5	11.3	96.4	11.33	11.72	2.919	32.35	66.26	70833	41.98	81.59	73.84
Arginine kinase	256.9	136.2	337	73.15	1599	5.332	278.7	6.820	31.67	57.64	19.08	10.14	550.5	7.225
Defensin 1	177.5	90.64	318.3		153.3	4.655			69.92	5.674	2.002	1046	25.55	387.6
Defensin 2					17.76	2.567	1.385	5.908	27.35	237.6				
Hyaluronidase	36.11	4476.48	4535	893.4	470.3	788.9	1.074		92.77	11.49	755.3	9.145	2.042	8.275
Mastoparan	22176	2782136					16.54			3.14				54.02
Phospholipase A1	291.1	21874	96336	19807	1252	1326	0.744	34.58	2.195			5.703		8.476
Phospholipase A2	50.6	28.02	6.804	11.88	4.529	2.783	0.923	5.125	2.609	18.96		25.49	2.29	11.76
Venom allergen 5	61.38	62297.5		35483	34.98	126	16.7			3.03	1.709		74	81.79
Tachykinin	1.334		1.572		1.422	3.497	0.881	2580	24.72			3.806	2.616	2.456
Vespakinin	1363	3732.2												

[a] Bees/wasps species abbreviations: Va, *Vespa analis*; Vc, *Vespa crabro*; Vd, *Vespa dybowskii*; Vs, *Vespa simillima*; Pv, *Parapolybia varia*; Pr, *Polistes rothneyi*; Ps, *Polistes snelleni*; Ed, *Eumenes decorates*; Ss, *Sphecidae* spp.; Af, *Anterhynchium flavomarginatum*; Sd, *Sceliphron deforme*; Ba, *Bombus ardens*; Bc, *Bombus consobrinus*; Bu, *Bombus ussurensis*. FPKM values were marked with different colors: Red > 1,000, Orange > 500, Yellow > 100.

3.2. Acid phosphatase

All of the Aculeata bees/wasps showed different transcription levels of venom acid phosphatase. Among all species tested, the solitary hunting wasp *S. deforme* exhibited an extremely high expression level. The overall FPKM values of acid phosphatase in other bees/wasps were less than 100, suggesting that acid phosphatase might have a significant role in the venom of solitary hunting wasps (Table 2).

The amino acid alignments showed several highly conserved regions. Based on amino acid sequences, the phylogenetic tree indicated that the acid phosphatase proteins of social wasps, solitary hunting wasps, and bumblebees were separately clustered into their own groups (with the exception of *B. consobrinus*, *E. decorates*, and *A. flavomarginatum*) (Fig. 1). *Vespa*, *Parapolybia*, and *Polistes* species were grouped with *E. decoratus,* which has a close relationship with the social wasps in one clade. Since the social wasps *P. snelleni* and *V. dybowskii* belong to another clade, the phylogenetic relationship of acid phosphatase is not well correlated with that of the Aculeata in general. This indicates that the venom acid phosphatase in each group of bees/wasps may have evolved to acquire its own unique features. Nevertheless, a functional characterization of each group's acid phosphatase would be required to confirm this notion.

3.3. Arginine kinase

Both acid phosphatase and arginine kinase were identified in the venom glands of all Aculeata bees/wasps, suggesting their ancient origins. The arginine kinase of *P. varia* had the highest FPKM value among the 14 species, and most of the social wasps showed values over 100 (except *V. simillima* and *P. rothneyi*). Solitary hunting wasps and bumblebees (except *B. consobrinus*) showed considerable expression of arginine kinase, although expression levels were lower than those of the social wasps (*i.e.* FPKM values < 100). This finding suggests that arginine kinase, which is known to cause an allergic reaction in invertebrates (Binder et al., 2001), has likely been employed as an allergen for a very long time (*i.e.* before the divergence of bees and wasps).

A phylogenetic analysis revealed that the arginine kinase proteins of the bees/wasps tested were not categorized according to ecological and behavioral characteristics. For example, social wasps were grouped with *Sphecidae* spp., which belongs to another clade in the Aculeate phylogenetic tree. This suggests that the molecular evolution of arginine kinase does not necessarily align with the evolution of bees/wasps (Fig. 2).

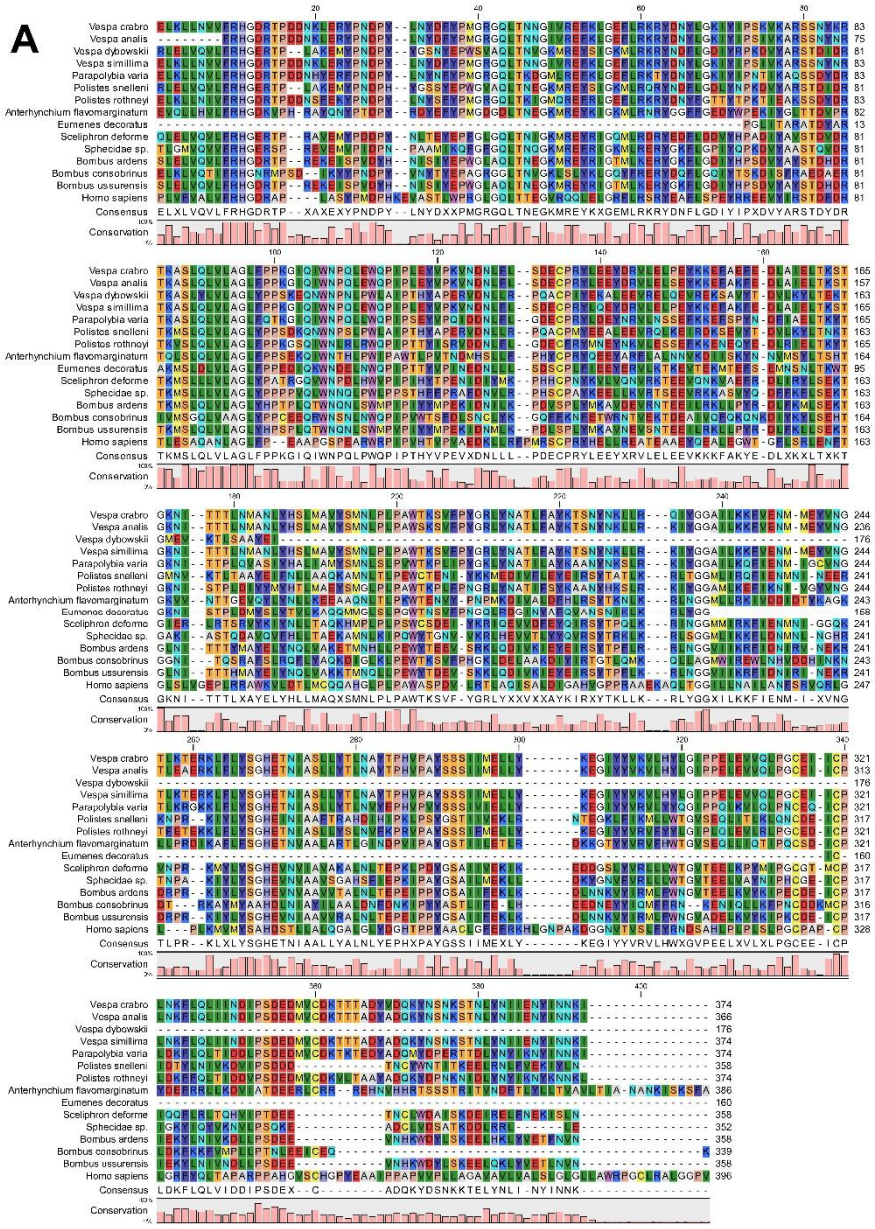

Figure 1. Amino acid alignments of acid phosphatase. A) Alignment of amino acid sequences from *V. crabro*, *V. analis*, *V. dybowskii*, *V. simillima*, *P. varia*, *P. snelleni*, *P. rothneyi*, *A. flavomarginatum*, *S. deforme*, *Sphecidae* spp., *E. decoratus*, *B. ardens*, *B. consobrinus*, and *B. ussurensis*. B) Phylogenetic analysis of acid phosphatase.

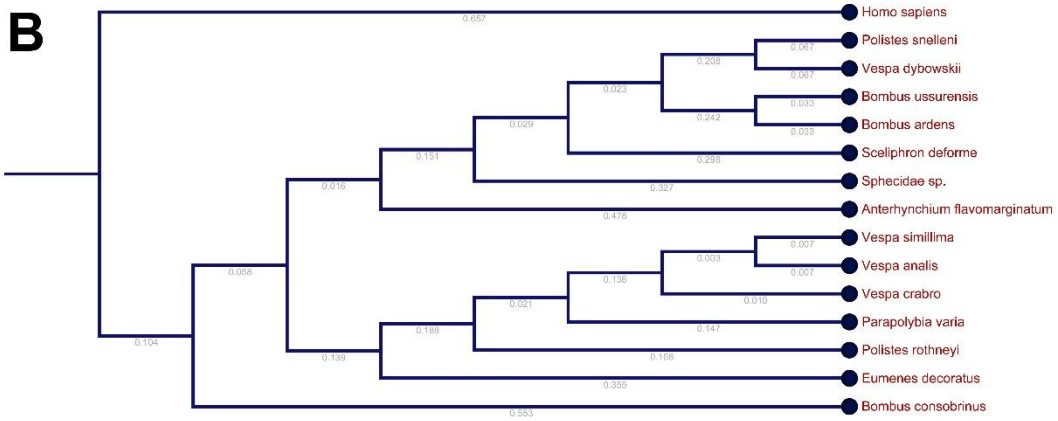

Figure 1. Continued

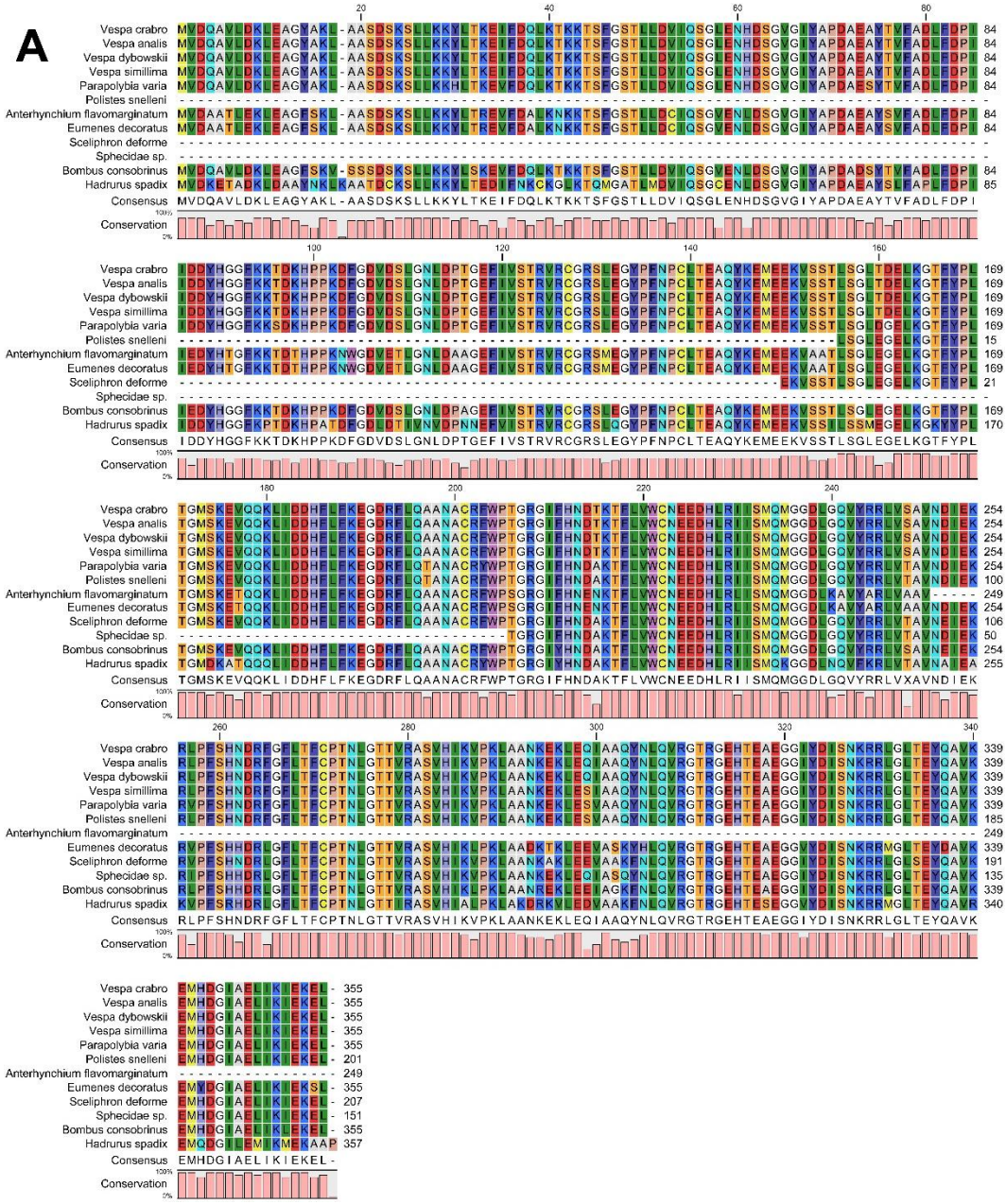

Figure 2. Amino acid alignments of arginine kinase. A) Alignment of amino acid sequences from *V. crabro*, *V. analis*, *V. dybowskii*, *V. simillima*, *P. varia*, *P. snelleni*, *A. flavomarginatum*, *S. deforme*, *Sphecidae* spp., *E. decoratus*, and *B. consobrinus*. B) Phylogenetic analysis of arginine kinase.

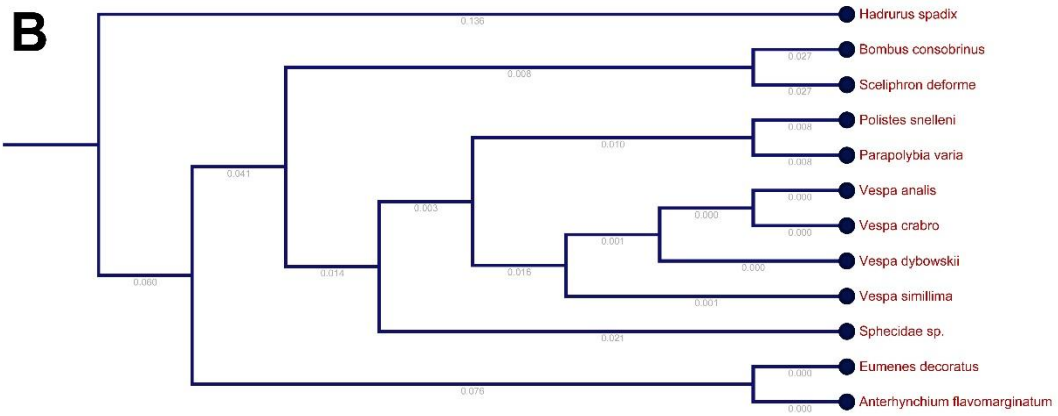

Figure 2. Continued

3.4. Defensins

Bumblebees (except for *B. consobrinus*) had the highest expression of defensin 1 compared to other species. Only the social wasps *V. analis*, *V. dybowskii*, and *P. varia* showed FPKM values > 100; other wasps exhibited little to no defensin 1 transcription. The high bumblebee-specific expression of defensin 1, which is well known as an antimicrobial peptide, suggests that bumblebees may have exploited defensin for a special purpose. It is possible they use it for sanitizing their nests, which are built underground (Table 2).

Vespa and *Bombus* species were grouped with closely related species, whereas the defensin 1 of *P. rothneyi* did not cluster with any of the groups in the phylogenetic tree (Fig. 3).

Defensin 2 was mainly identified in the venom glands of solitary hunting wasps (*E. decoratus*, *Sphecidae* spp., and *A. flavomarginatum*), with only slight expression in some of the social wasps (*P. varia* and *P. rothneyi*). This finding suggests that defensin 2 may be specialized for preserving the prey of solitary wasps. Our phylogenetic analysis revealed that defensin 2 clearly separated into two groups depending on behavioral properties (solitary vs. social) (Fig. 4). These results imply that the evolution of defensin, and particularly defensin 2, closely reflects Aculeate phylogenetic relationships.

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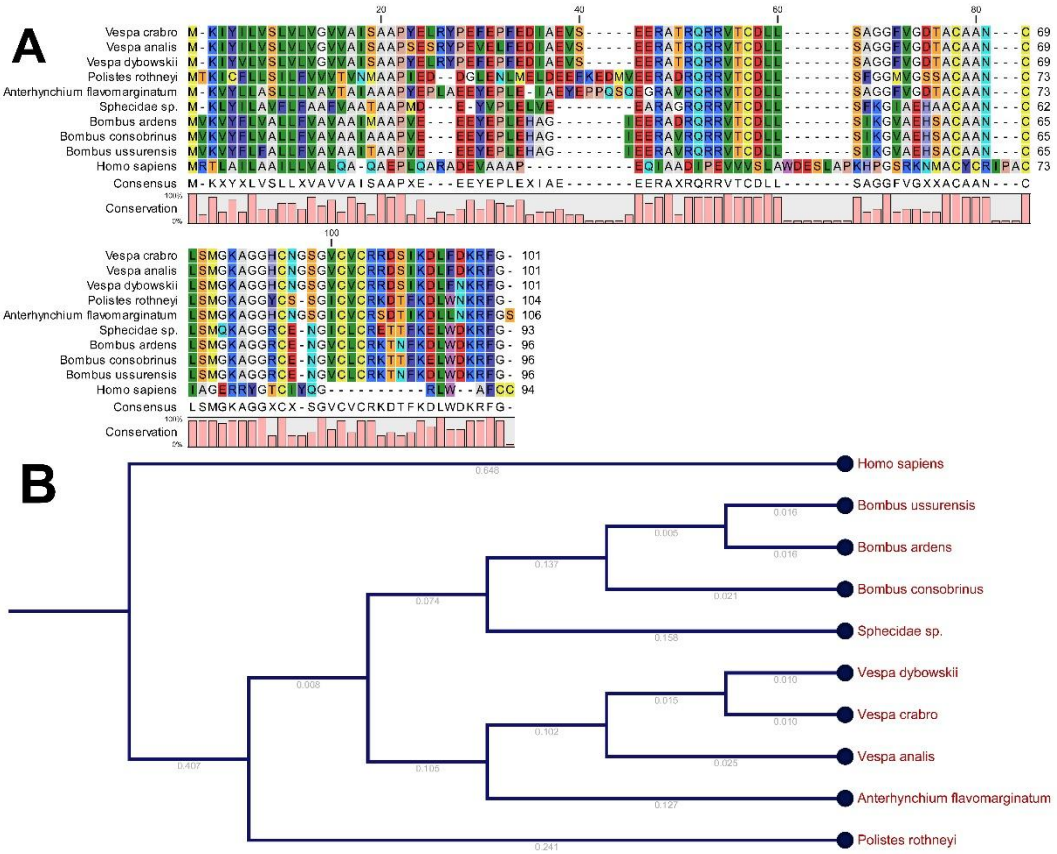


Figure 3. Amino acid alignments of defensin 1. A) Alignment of amino acid sequences from *V. crabro*, *V. analis*, *V. dybowskii*, *P. rothneyi*, *A. flavomarginatum*, *Sphecidae* spp., *B. ardens*, *B. consobrinus*, and *B. ussurensis*. B) Phylogenetic analysis of defensin 1.

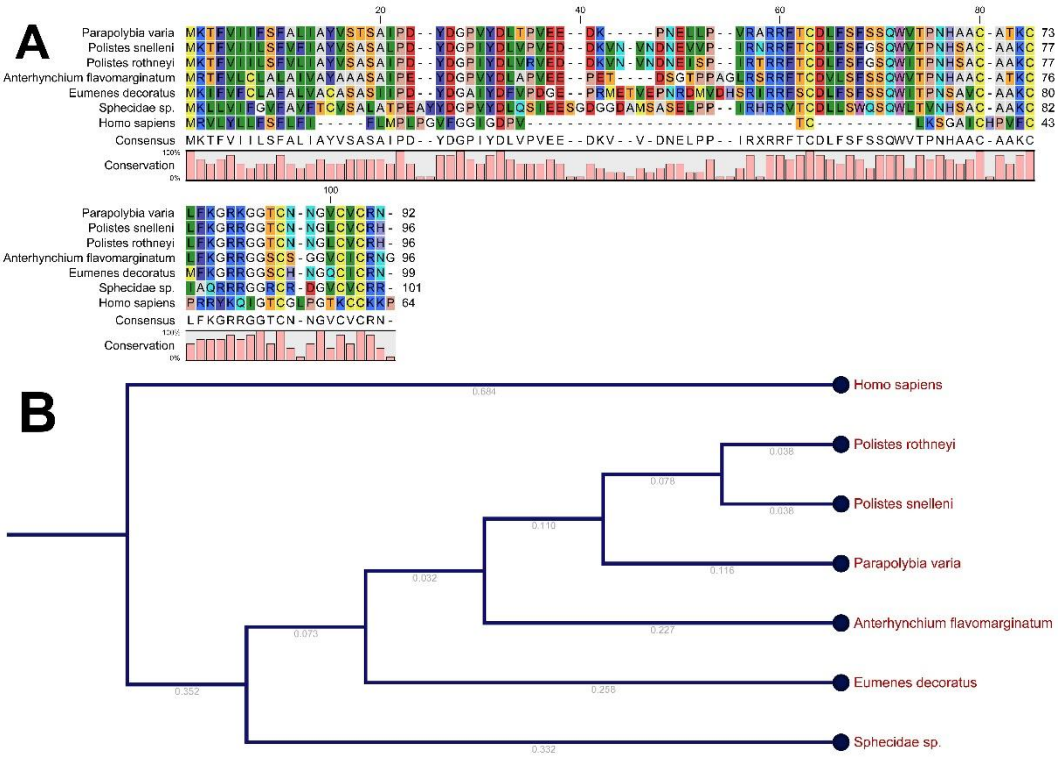


Figure 4. Amino acid alignments of defensin 2. A) Alignment of amino acid sequences from *P. varia*, *P. snelleni*, *P. rothneyi*, *A. flavomarginatum*, *Sphecidae* spp., and *E. decoratus*. B) Phylogenetic analysis of defensin 2.

3.5. Hyaluronidase

Most of the social wasps, and especially *V. analis* and *V. dybowskii,* showed extremely high expression levels of hyaluronidase (FPKM values > 4,000). One solitary hunting wasp (*S. deforme*) exhibited a moderate level of expression (FPKM = 500). This result indicates that hyaluronidase, which is a venom spreading factor, plays a major role in the venom of social wasps (Lee et al., 2016) (Table 2).

The hyaluronidase proteins of the solitary hunting wasps, social wasps, and bumblebees clearly separated into distinct groups in the phylogenetic tree, matching the pattern of the Aculeate phylogenetic relationships. This implies that hyaluronidase may have diverged according to ecological and behavioral pressures during the evolution of bees/wasps (Fig. 5).

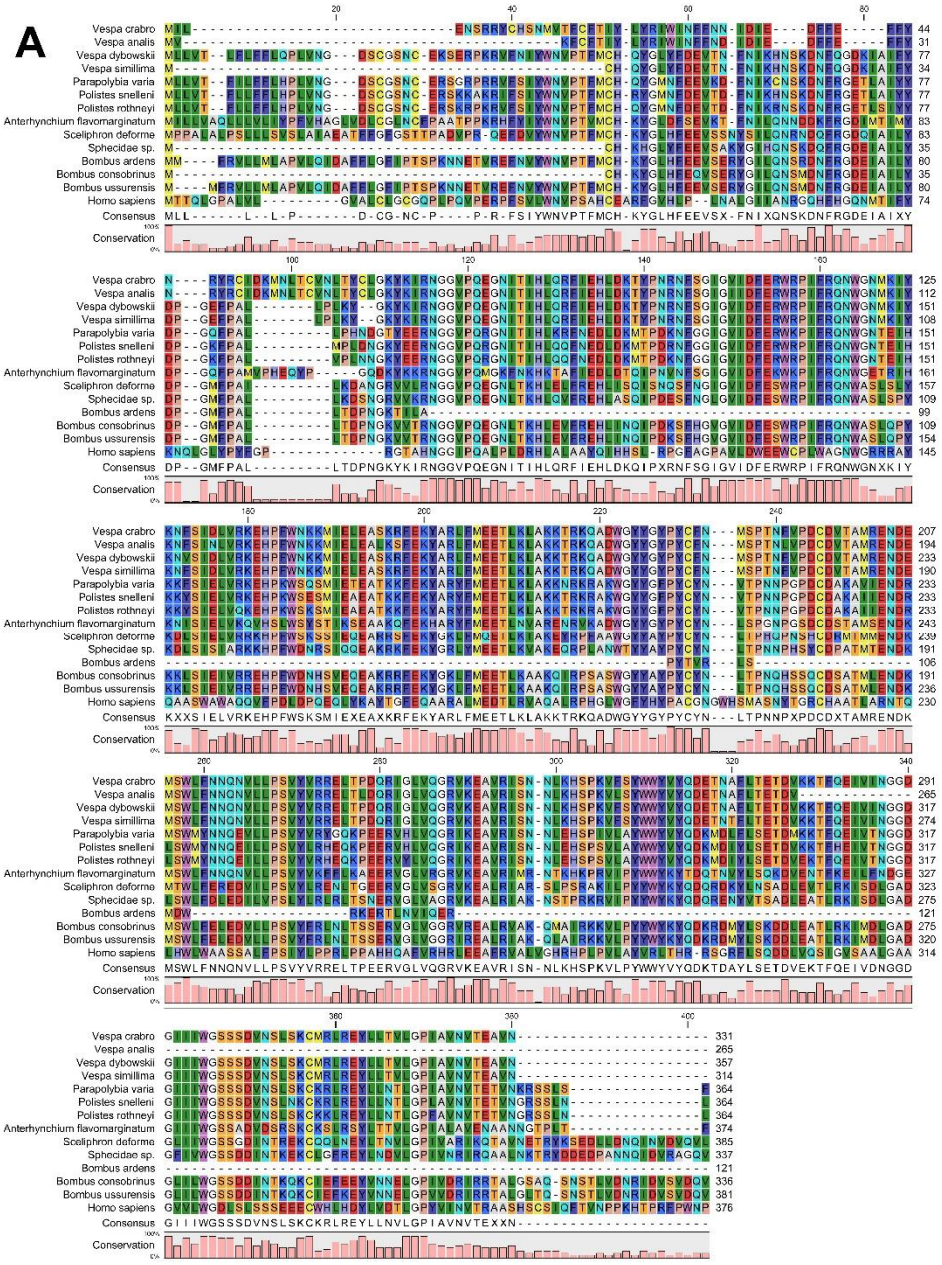


Figure 5. Amino acid alignments of hyaluronidase. A) Alignment of amino acid sequences from *V. crabro*, *V. analis*, *V. dybowskii*, *V. simillima*, *P. varia*, *P. snelleni*, *P. rothneyi*, *A. flavomarginatum*, *S. deforme*, *Sphecidae* spp., *B. ardens*, *B. consobrinus*, and *B. ussurensis*. B) Phylogenetic analysis of hyaluronidase.

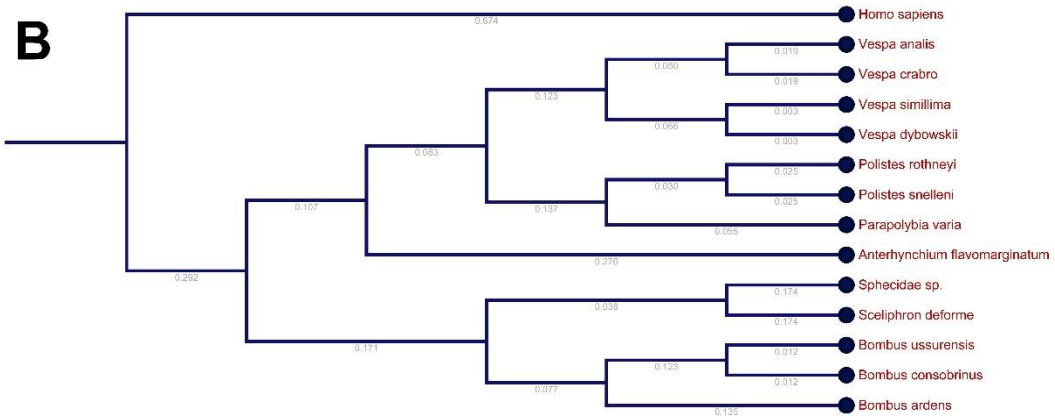


Figure 5. Continued

3.6. Mastoparan

Mastoparan was uniquely identified in most social wasps (*V. analis*, *V. crabro*, and *P. snelleni*), a solitary hunting wasp (*A. flavomarginatum*), and a bumblebee (*B. ussurensis*). *V. crabro* mastoparan showed an extremely high FPKM value, which was the highest among all of the venom genes. Mastoparan causes allergic inflammation and mast cell degranulation in vertebrates (Yoon et al., 2015a). Therefore, social wasp venom is likely to be very toxic to vertebrates due to its extremely high level of mastoparan (Table 2).

Prepromastoparans showed amino acid sequence similarities of 55.4 to 86.7%, and the mature peptide regions showed 33.3 to 60% similarity. The phylogenetic tree of mature mastoparans showed different patterns than that of the Aculeata. This indicates that mastoparans have different amino acid structures in the mature peptide domain, which is positioned at the C-terminus. Even if only a few amino acids differ, this can cause significant changes in a small peptide. Similarly, small amino acid changes may be sufficient to modify the activity of mastoparans (Fig. 6).

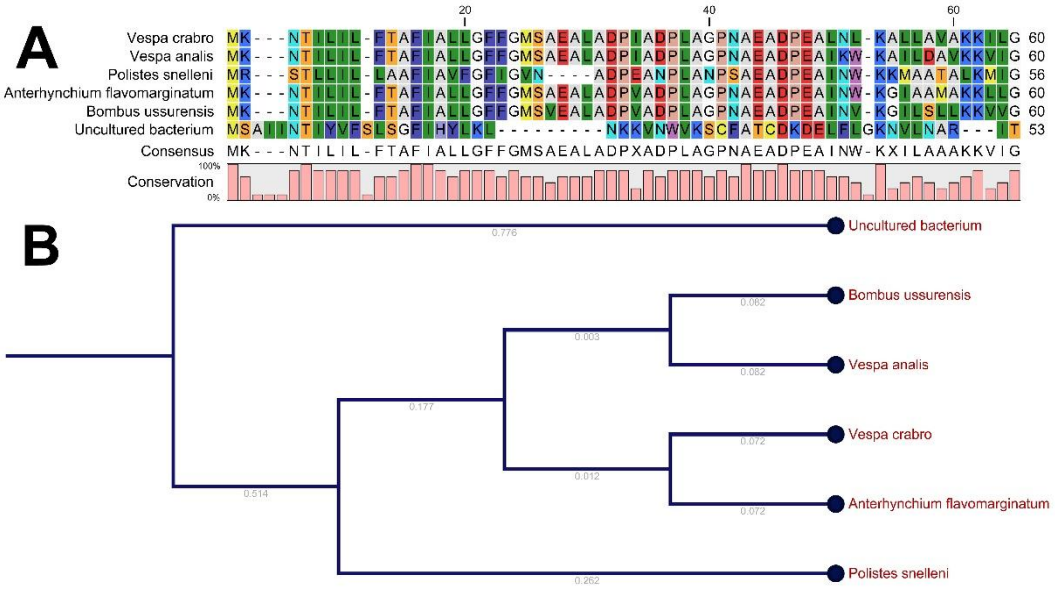


Figure 6. Amino acid alignments of mastoparan. A) Alignment of amino acid sequences from *V. crabro*, *V. analis*, *P. snelleni*, *A. flavomarginatum*, and *B. ussurensis*. B) Phylogenetic analysis of mastoparan.

3.7. Phospholipase A

Phospholipase A1 and A2 are major bee venom allergens for humans (Borodina et al., 2011; Corthesy et al., 2001). Phospholipase A1 was identified in the venom glands of all the social wasps, and some of the solitary hunting wasps and bumblebees (Table 2). Most of the social wasps exhibited extremely high expression of phospholipase A1 (with the exception of *P. snelleni*), implying that phospholipase A1 plays a significant role as an allergen in the venom of social wasps.

The result of an amino acid sequence alignment showed several conserved regions in all bee/wasp species. *B. ussurensis* clustered into a group of social wasps, whereas *V. analis* and *E. decoratus* were separated into another group. This implies that the phospholipase A1 of each species has a unique sequence, structure, and function (Fig. 7).

Phospholipase A2 was identified in all species except *S. deforme*, and exhibited FPKM values < 100 (Table 2). Most of the social wasp and bumblebee species tested grouped together, respectively. *A. flavomarginatum,* which has semi-social characteristics, showed a close relationship with the social wasps (Fig. 8).

The result of our phylogenetic analysis revealed that phospholipase A1 has a unique evolutionary pattern, whereas phospholipase A2 reflects the Aculeate

phylogenetic relationship.

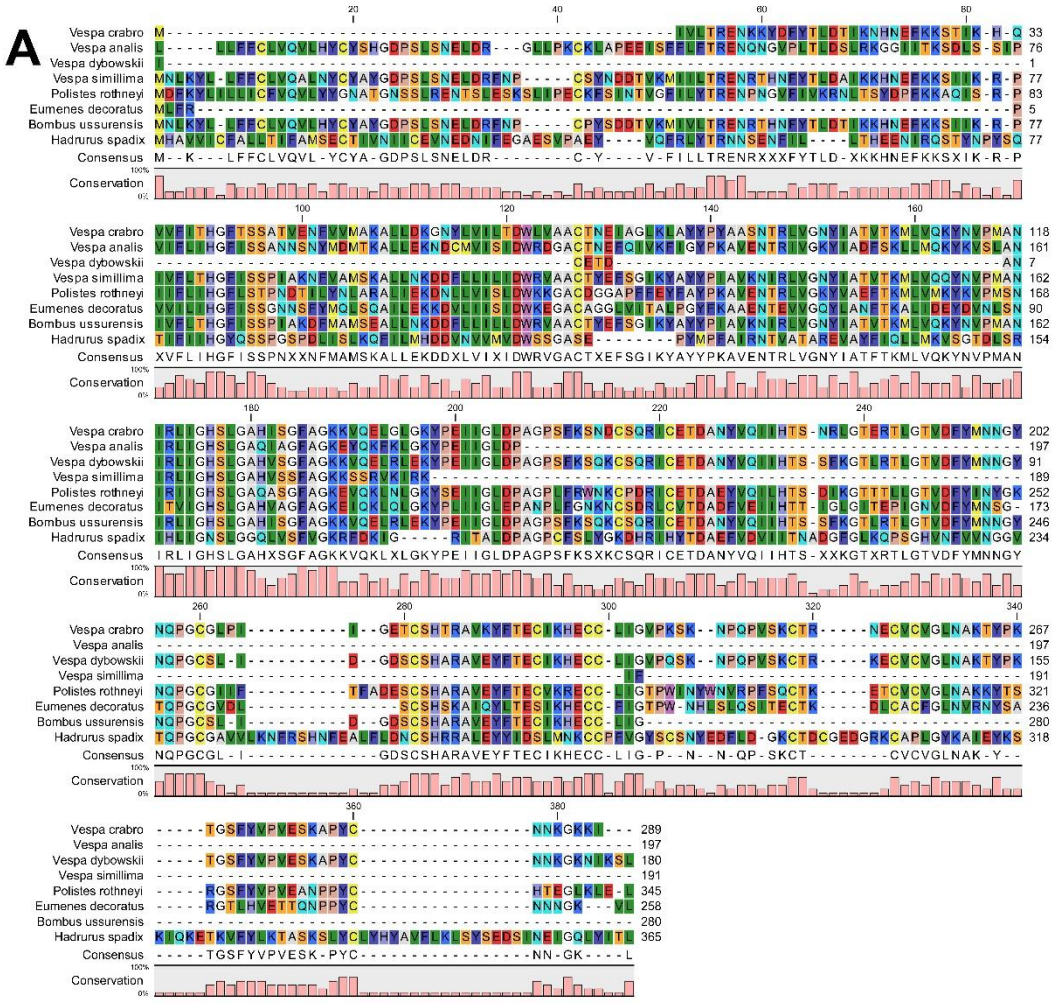
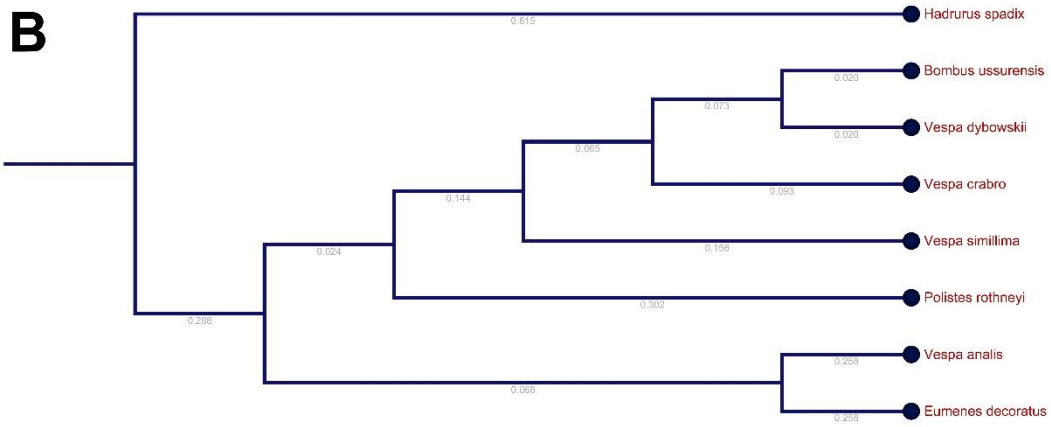


Figure 7. Amino acid alignments of phospholipase A1. A) Alignment of amino acid sequences from *V. crabro*, *V. analis*, *V. dybowskii*, *V. simillima*, *P. rothneyi*, *E. decoratus*, and *B. ussurensis*. B) Phylogenetic analysis of phospholipase A1.



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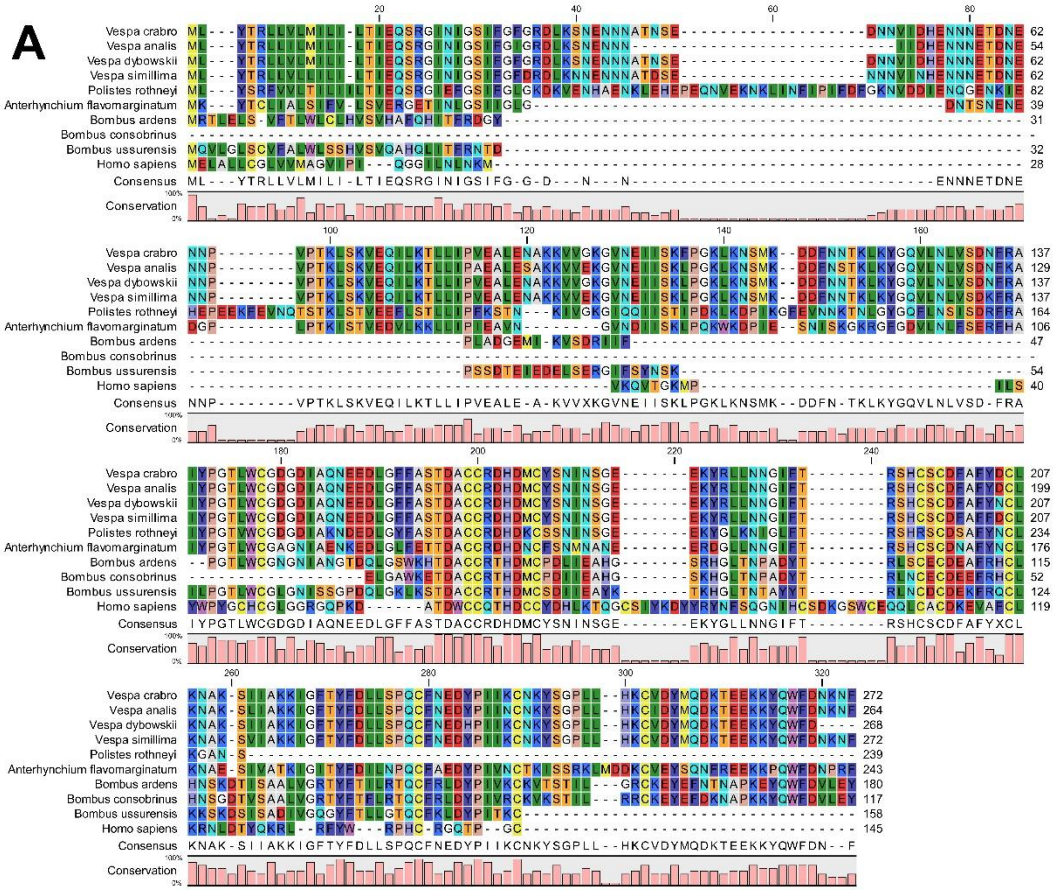


Figure 8. Amino acid alignments of phospholipase A2. A) Alignment of amino acid sequences from *V. crabro*, *V. analis*, *V. dybowskii*, *V. simillima*, *P. rothneyi*, *A. flavomarginatum*, *B. ardens*, *B. consobrinus*, and *B. ussurensis*. B) Phylogenetic analysis of phospholipase A2.

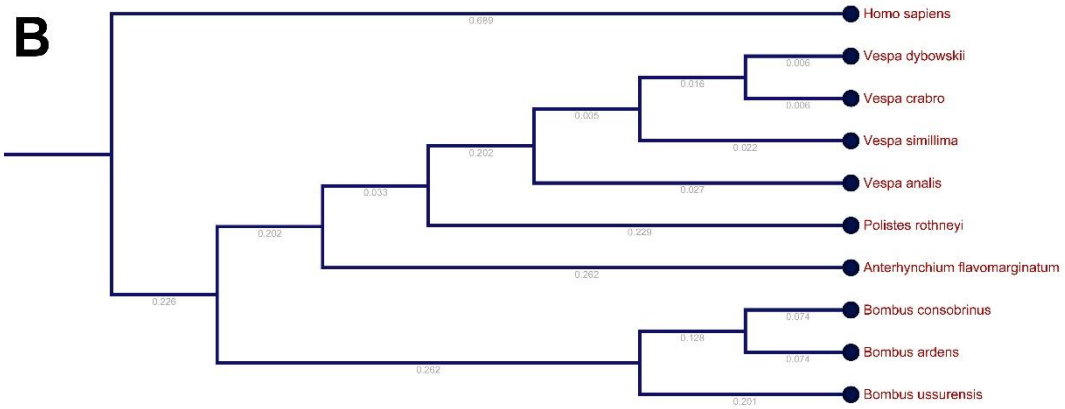


Figure 8. Continued

3.8. Venom allergen 5

The social wasps *V. crabro*, *V. simillima*, and *P. rothneyi* had significantly high expression levels of venom allergen 5, which is one of the major allergens identified in many insect venoms (King and Spangfort, 2000).

An amino acid sequence alignment showed a high degree of sequence conservancy, but the phylogeny of venom allergen 5 did not clearly match the species phylogeny (Fig. 9). Two solitary hunting wasps, which belong to different clades in the Aculeate phylogeny, were grouped together. Likewise, two paper wasps and two bumblebees were grouped together. These findings imply that venom allergen 5 is a major allergen in the venom of social wasps, but has a unique evolutionary history.

3.9. Tachykinin

Tachykinin was highly expressed in the solitary hunting wasp *E. decorates*, whereas other bees/wasps showed FPKM values < 50 (Table 2). The solitary hunting wasp *Sphecidae* spp. fell into a clade with social wasps, which did not match the Aculeate phylogeny. The tachykinin of some hunting wasps is known to induce hypokinesia. *E. decoratus* tachykinin may have a different activity (*i.e.* as a neurotoxic peptide for feeding fresh prey to larvae) than the tachykinin of bumblebees, although they were categorized into the same group (Fig. 10).

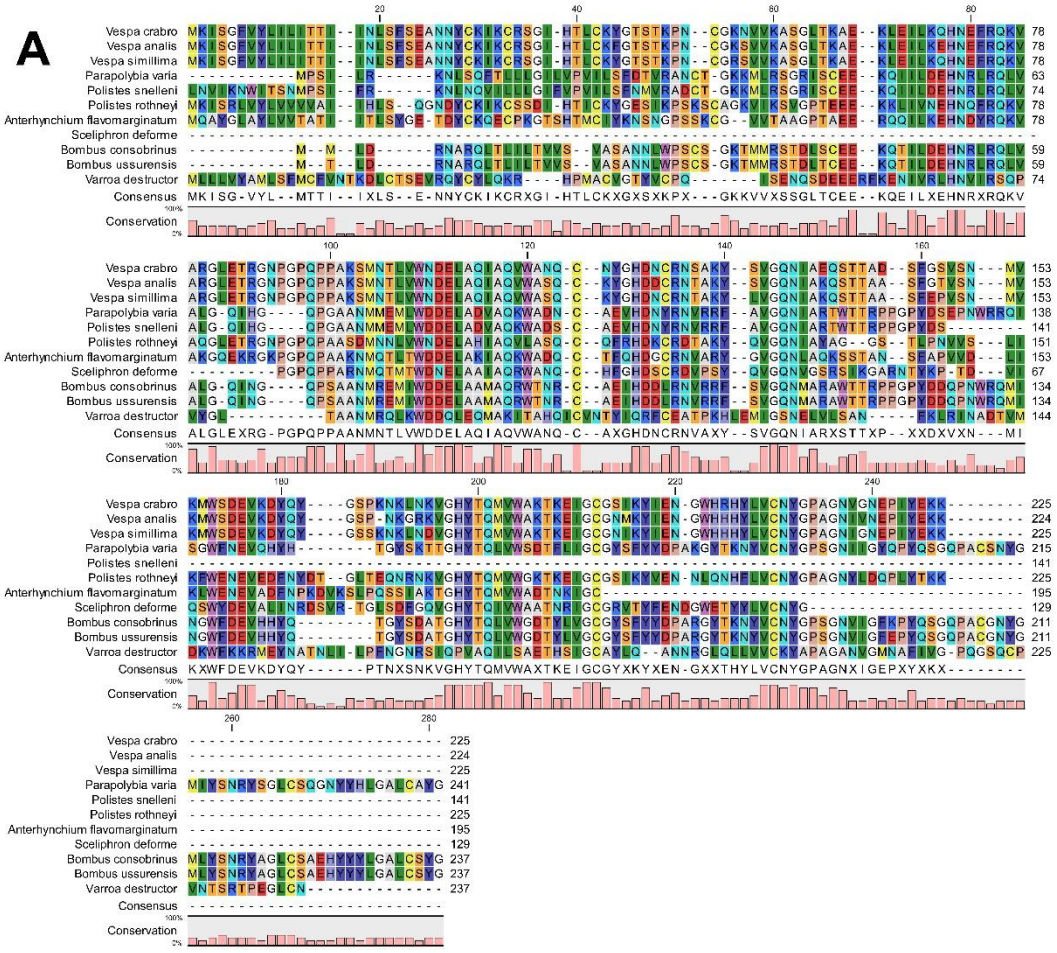


Figure 9. Amino acid alignments of venom allergen 5. A) Alignment of amino acid sequences from *V. crabro*, *V. analis*, *V. simillima*, *P. varia*, *P. snelleni*, *P. rothneyi*, *A. flavomarginatum*, *S. deforme*, *B. consobrinus*, and *B. ussurensis*. B) Phylogenetic analysis of venom allergen 5.

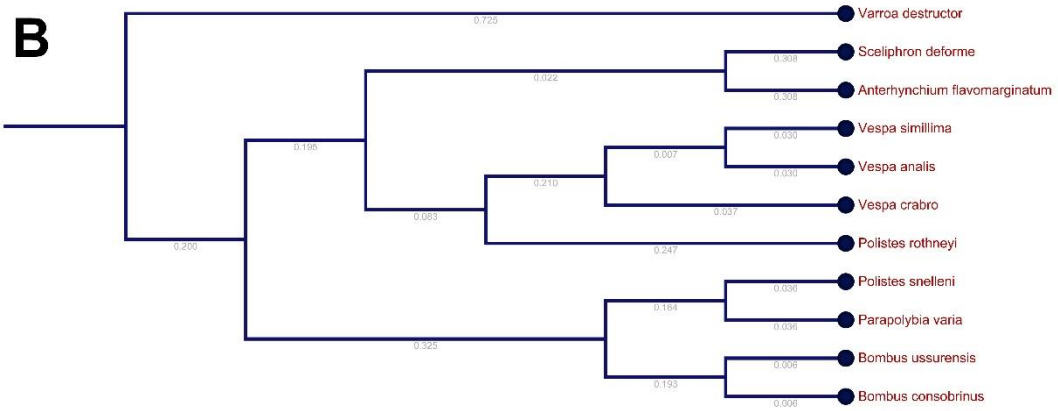


Figure 9. Continued

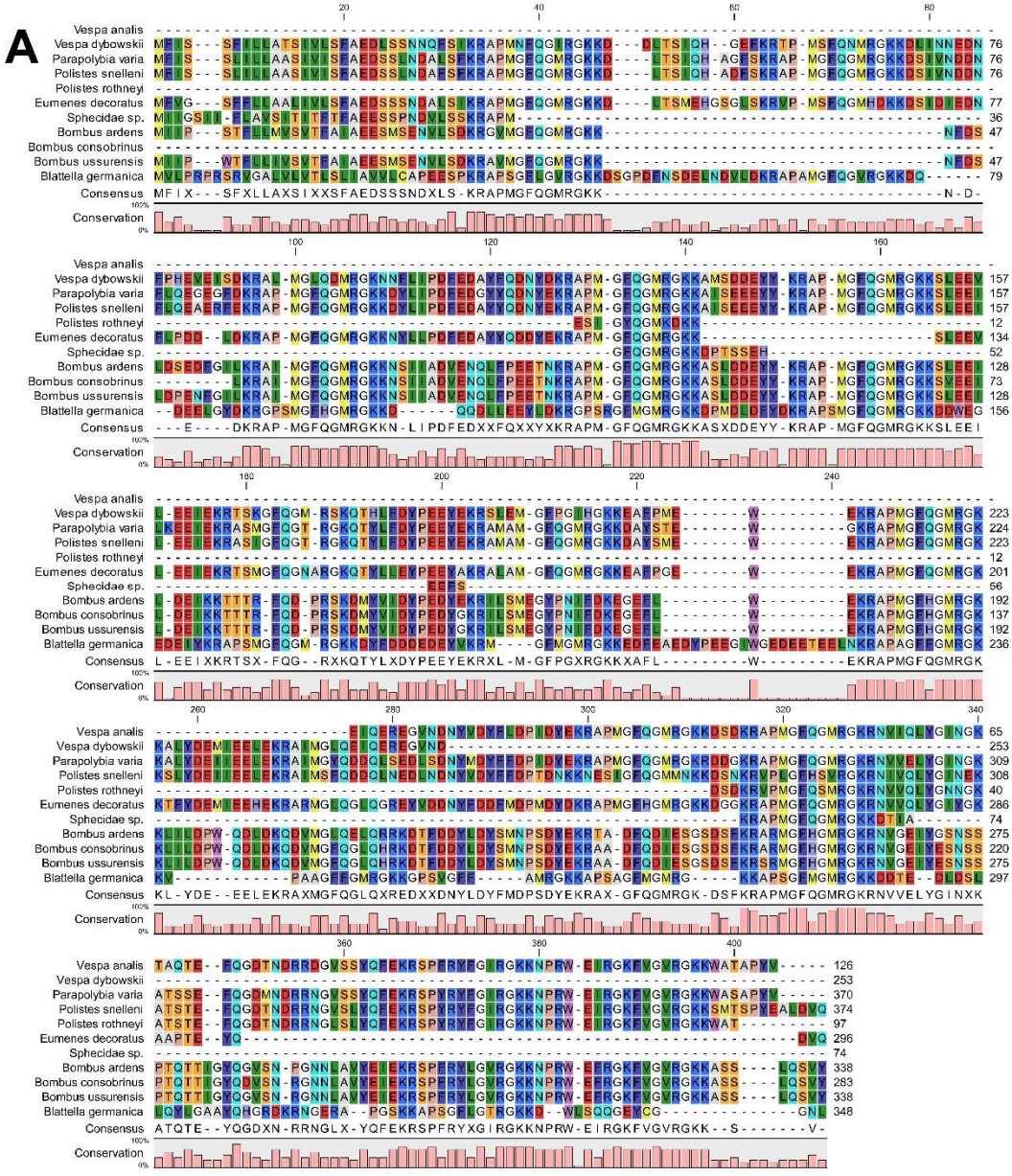


Figure 10. Amino acid alignments of tachykinin. A) Alignment of amino acid sequences from *V. analis*, *V. dybowskii*, *P. varia*, *P. snelleni*, *P. rothneyi*, *Sphecidae* spp., *E. decoratus*, *B. ardens*, *B. consobrinus*, and *B. ussurensis*. B) Phylogenetic analysis of tachykinin.

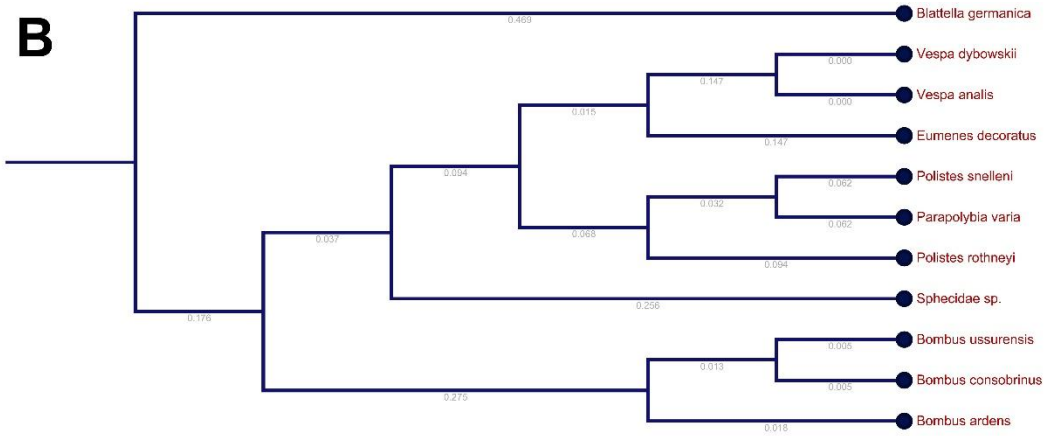


Figure 10. Continued

4. Conclusions

Some venoms (including defensins, hyaluronidase, and phospholipase A2) reflected the Aculeate species phylogeny, but other venoms (including acid phosphatase, arginine kinase, mastoparan, phospholipase A1, venom allergen 5, and tachykinin) did not. This unique evolution of some venom components might have been shaped by specific ecological behaviors. To the best of our knowledge, this is the first systematic approach based on venom gland transcriptomic data to compare major venom components across various species of Aculeate bees and wasps. A broader scale of comparison for venom components (including minor components across diverse bee and wasp species) would elucidate how venoms evolved according to ecological and behavioral pressures.

The recent introduction of cost-effective, high-throughput, deep-sequencing technologies has enabled the rapid identification of genes encoding various venom peptides/proteins from venom gland transcriptomes. In addition, the availability of highly sensitive mass spectrometry techniques has allowed for the efficient proteomic/peptidomic analysis of a limited number of solitary wasp venoms. A systematic and comparative analysis of venoms from solitary wasps, social wasps, and bumblebees would provide further insights into venom evolution and phylogeny. The accumulation of functional data on the bioactivity of various

venom components would facilitate the application of wasp and bumblebee venoms for pharmacological, medical, and agricultural purposes.

CHAPTER II

Comparative Functional Venomics of Social Hornets

Vespa crabro and *V. analis*

Abstract

The hornets *Vespa crabro* and *V. analis* are widely distributed in Asia and are known to be aggressive when disturbed, resulting in frequent stinging accidents. To investigate the differences in venom properties and toxicities between these two hornets, the transcriptomic profiles of venom glands, in conjunction with the venom components, were analyzed and compared. A total of 35 venom-specific genes were identified in both venom gland transcriptomes, but their transcriptional profiles were different between *V. crabro* and *V. analis*. In addition, the major venom components were identified and confirmed by mass spectroscopy. Prepromastoparan, vespid chemotactic peptide precursor and vespakinin were the top three genes most prevalently transcribed in the venom gland of *V. crabro*, and their transcription rates were 112-, 16- and 161-fold higher, respectively, compared with those in V. analis, as judged by fragments per kilobase million (FPKM) values. In the venom gland of *V. analis*, however, vespid chemotactic peptide precursor was the most abundantly transcribed gene, followed by premastoparan and vespakinin. In general, most major venom genes were more abundantly expressed in *V. crabro*, whereas some minor venom genes exhibited higher transcription rates in *V. analis*, including muscle LIM protein, troponin, paramyosin, calponin, etc. Our findings reveal that the overall venom

components of *V. crabro* and *V. analis* are similar, but that their expression profiles and levels are considerably different. The comparison of venom gland transcriptomes suggests that *V. crabro* likely produces venom with more highly enriched major venom components, which has potentially higher toxicity compared with *V. analis* venom.

1. Introduction

Hornets are a group of the largest social wasps belonging to the genus Vespa. Vespa hornets are mostly distributed in the Northern hemisphere, including Europe, Russia, and Asia, with the European hornet, *Vespa crabro* Cameron, having been introduced into the northeastern United States (Spradbery, 1973). Along with *V. crabro*, *V. analis* Andre is also commonly found in Asia. Both species are widely distributed in Korea and known to be aggressive when disturbed, resulting in frequent stinging incidents (Jung et al., 2007; Jung et al., 2014). Unlike the giant Asian hornet *V. mandarinia*, which usually inhabits forests, the habitats of both *V. crabro* and *V. analis* have been rapidly expanding into human residential areas and an increased number of stinging incidents have been reported (Jung et al., 2014).

Hornets sting to hunt prey or to defend their colonies (Piek and Spanjer, 1986). Unlike honey bees, hornets can sting multiple times due to the barbless structure of their stinger. The typical sting event occurs when a single hornet is accidentally disturbed by humans during outdoor activities. However, when the nest is disturbed, the entire colony of hornets, as is the case with many other social wasps, can be mobilized via an attack pheromone to sting the human intruder, resulting in mass envenomation, which is extremely dangerous (Spradbery, 1973).

Single hornet stings are not usually fatal, but can cause severe anaphylactic shock (Vetter et al., 1999). Multiple stings may result in death because of the various toxic components present in the venom (Schmidt et al., 1986). Despite differences in venom composition depending on the hornet species, multiple hornet stings typically result in systemic damage, involving hemolysis, rhabdomyolysis and acute renal failure, and death usually occurs due to renal failure or cardiac problems (Vetter et al., 1999).

The toxicity of hornet stings is species-specific and varies depending on the venom components (Schmidt et al., 1986). Although the major venom components of some Vespa hornets have been reported, no comparative transcriptomic analysis of venom glands of *V. crabro* and *V. analis* has been conducted to date. The goals of this study were to identify the complete set of transcripts in the venom glands, to investigate the differences in venom components and, thereby compare the potential toxicity between these two hornet species. To this end, the transcriptome of the venom gland from each species was analyzed by RNA-sequencing and the venom-related genes were annotated, characterized and compared. In addition, the major venom components were identified and confirmed by mass spectroscopy.

2. Materials and Methods

2.1. Wasp collection, venom purification and total RNA extraction

V. crabro and *V. analis* females were collected from an apiary located in Taehwa Mountain and Suri Mountain in Korea, respectively. Wasps were anesthetized using low pressure carbon dioxide, and the abdominal organs were removed with forceps and placed in 1 × phosphate-buffered saline (PBS) on a slide glass. Twenty venom glands were dissected, and total RNA was extracted using 200 μL TRI reagent following the manufacturer's protocol.

For the collection of venom, 20 venom sacs were dissected from abdomens, punctured using forceps and centrifuged at 12,000 × g for 5 min at 4°C, followed by transfer into a centrifugal filter device with a 0.45-μm pore size (Millipore, Bedford, MA, USA). Peptides were identified using 5 μL of purified venom using a peptide data base acquired from the NCBI and transcriptomic data from this study.

2.2. Construction of RNA-Seq library

RNA purity was determined by assaying 1 μL of total RNA using a NanoDrop8000 spectrophotometer. Total RNA integrity was evaluated using an Agilent Technologies 2100 Bioanalyzer and samples with an RNA Integrity

Number (RIN) value greater than 8 were used. mRNA sequencing libraries were prepared following the manufacturer's protocol (Illumina TruSeq RNA Prep kit v2). mRNA was purified from total RNA (1 μg) using poly-T oligo-attached magnetic beads using two rounds of purification and then fragmented. Cleaved RNA fragments primed with random hexamers were reverse transcribed into first strand cDNA using reverse transcriptase. RNA templates were removed and a replacement strand was synthesized to generate double-strand (ds) cDNA. End repair, A-tailing, adaptor ligation, cDNA template purification and enrichment of the purified cDNA templates using PCR were then performed. The quality of the amplified libraries was verified by capillary electrophoresis (Agilent, Santa Clara, California).

After quantitative real-time PCR (qPCR) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California), index tagged libraries in equimolar amounts were combined into a pool. Cluster generation occurred in the flow cell on the cBot automated cluster generation system (Illumina, San Diego, California). The flow cell was loaded on an HISEQ 2000 sequencing system (Illumina) and sequencing was performed with 2 × 100 bp read lengths. The SOAPdenovo-Trans (Beijing Genomic Center, Beijing, China) and Trans-ABySS programs (Canada's Michael Smith Genome Sciences Centre, Vancouver, Canada) were used to perform de novo assembly of trimmed transcripts. Annotation was performed

using BLASTp searches and FPKM values were calculated using Cufflinks (Trapnell Lab, Seattle, Washington).

2.3. Quantitative real-time PCR (qPCR)

Total RNA was extracted from 20 venom glands and DNase I (Ambion, Austin, Texas) was used to eliminate DNA contamination. Double-stranded cDNA was synthesized from total RNA using SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, California). A total of 9 venom-specific genes (prepromastoparan, venom allergen 5, insulin binding protein, tropomyosin, hyaluronidase, muscle LIM protein, paramyosin, vespid chemotactic peptide precursor and vespakinin) and a reference gene (acidic ribosomal protein P2) (Hornakova et al., 2010) were selected for verification of their transcriptional abundance. Primers specific to the selected venom proteins were designed to have a similar lengths, % GC content and PCR product sizes (Table 1). The following components were used in the PCR reaction mix (20 μL): 25 ng of cDNA, 10 μL of DyNAmo SYBR Green master mix (Finnzymes, Espoo, Finland), and 5 pM of sense and antisense primers. The thermal cycling program started with pre-incubation at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s, 58°C for 10 s and 72°C for 10 s. A melting curve analysis was conducted to confirm the homogeneity of the PCR products. The relative transcription levels of the venom-specific genes were

calculated according to an equation (Pfaffl, 2001) and then plotted against their

Name	Sequence	PCR product length
Mastoparan-F	CACCGCTTTCATCGCACTTT	107 bp
Mastoparan-R	CTTCTGGATCAGCTTCAGCA	

FPKM values.

Table 1. Quantitative real-time PCR primer list

Venom allergen 5-F	TAGACAAAAGGTTGCACGGG	118 bp
Venom allergen 5-R	CCATACTTGGGCGATTTGAG	
Insulin binding protein-F	GATATAGCTCGCGTGGAACA	116 bp
Insulin binding protein-R	GAGGAAGGTAGTTTCCTGTG	
Tropomyosin-F	GGATGAGGAAAGGATGGATC	117 bp
Tropomyosin-R	TCGTCTTCAACGAAGGCCAG	
Hyaluronidase-F	GGGGTTATTATGGATATCCC	114 bp
Hyaluronidase-R	GATTATTGAACAGCCACGAC	
Muscle LIM protein-F	GAGCACGAGGGTGAGCTTTT	115 bp
Muscle LIM protein-R	TCAGATGTTCGCCTTGGTCC	
Paramyosin-F	AGATGTTCATCTCGAGAGCG	112 bp
Paramyosin-R	CCTGGCACGAGCTTTTGAAA	
Vespid chemotactic peptide-F	GCCTCATTGGCATGTTTGCA	119 bp
Vespid chemotactic peptide-R	GGGAGAAAGGGATCAGCCAA	
Vespakinin-F	GCTATAGCAGCAATTTTGGC	123 bp
Vespakinin-R	TGGTGAAAATCCAGGCGGAC	
Reference gene-F	ATGCGTTACGTGGCCGCTTAT	111 bp
Reference gene-F	TTCCCCATCAGCTTCAATGC	

2.4. Venom peptide analysis

Lyophilized venom (5 μL) from each of the two hornet species was used as a template for peptide analysis. The solid phase extraction using a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) was conducted for each venom. Prior to loading, 5 μL of lyophilized venom was diluted with 500 μL aqueous 0.1% trifluoroacetic acid. These venom solutions were fractionated in Sep-Pak C18 cartridges that had been previously washed and conditioned with methanol and 0.1 M NH_4HCO_3 (pH 8.0). After the venom solution was loaded, the cartridge was washed with diluted venom solution, and fractions were eluted with 0.1% TFA in H2O/methanol (5%, 20%, 50% and 100%). The eluted peptides were dried in a SpeedVac concentrator (ThermoSavant, Holbrook, New York) and stored at − 80 °C. The dried samples were suspended in 20 μL of aqueous 0.1% formic acid (FA) for mass spectrometer analysis. Venom peptide profiling and identification were performed on a nanoESI-Q-TOF tandem MS with a nanoAcquilty UPLC system (Waters, Milford, Connecticut). The samples were loading on a 75 μm × 250 mm, nanoAcquity UPLC BEH 130 C18 column (1.7 μm particle size, Waters). The separation was performed using a linear gradient of 3–90% B in 90 min (phase A: aqueous 0.1% FA; phase B: 0.1% FA in ACN) at 250 nL/min. MS and MS/MS spectra by MSE analysis were acquired for 1 s from m/z of 50–1950. MSE data searches were performed using ProteinLynx 2.5 (Waters) against the

hymenopteran related protein data base or 6 frame translated protein data base. Peptide candidates were de novo sequenced by MALDI TOF/TOF MS. MALDI TOF/TOF analysis of the pre-treated venom peptide sample was carried out on a 5800 TOF/TOF mass spectrometer operated in positive reflector mode (AB SCIEX, Framingham, Massachusetts). An aliquot (1 μL) of venom peptide sample was mixed with 1 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, Missouri) in 0.1% TFA in H2O/CAN (50:50) and spotted onto the MALDI sample plate. The dried sample spots were used to perform MS and MS/MS analyses. External calibration was carried out with a mixture of 9 peptides in the 700–3500 Da mass range. The MS analyses were followed by MS/MS experiments on the time ion selected precursor ions. The collision energy was manually adjusted for proper fragmentation. De novo peptide sequencing was carried out using a^+, b^+ and y^+-type fragments and immonium ions derived from the MS/MS analysis. Data interpretation was performed with DeNovo Explorer 4.1 (AB SCIEX).

3. Results and discussion

3.1. Differential transcriptional profiles in the venom gland of V. crabro and V. analis

Total reads of 6.22 and 6.16 Gb were obtained from RNA sequencing and further processed through adaptor and quality trimming to 5.75 and 5.7 Gb for V. crabro and V. analis, respectively. De novo assembly of the trimmed sequence data resulted in 49,536 and 72,510 non-duplicate contigs, from which a total of 27,281 and 33,104 genes were identified by BLAST search in the venom gland transcriptomes of *V. crabro* and *V. analis*, respectively (Table 2).

Gene ontology (GO) functional categorization was conducted from the annotated transcripts of venom glands in *V. crabro* and *V. analis* (Figs. 1-2). Analysis of the molecular function category revealed that the most abundant transcripts were related to ion binding, followed by organic cyclic compound binding, heterocyclic compound binding, small molecule binding, hydrolase activity and transferase activity (Figs. 1A, 2A). Analysis of the biological process category revealed several dominant gene categories related to organic substance metabolic process, followed by primary metabolic process, cellular metabolic process, nitrogen compound metabolic process, single-organism cellular process and single-organism metabolic process (Figs. 1B, 2B). GO analysis of the cellular

components category revealed one dominant category related to cell part. The following gene categories were related to membrane part, membrane-bound organelle, protein complex, organelle part and non-membrane-bound organelle (Figs. 1C, 2C). The overall patterns of functional categories were similar to venom gland of both social wasps, but the number of transcripts was relatively high in *V. analis*. The most abundant GO terms in the venom gland transcriptomes of *V. crabro* and *V. analis* were similar to those of *Urodacus yaschenkoi* scorpion (Luna-Ramirez et al., 2015), suggesting a high degree of commonality in the venom gland genes, even between these distantly related arthropod species.

Thirty-five abundantly expressed genes were identified in both venom gland transcriptomes, but their expression profiles were different between *V. crabro* and *V. analis*. The calculated FPKM values revealed that most major venom genes were more abundantly expressed in *V. crabro* than in *V. analis* (Tables 3, 4). The three most abundantly transcribed genes in *V. crabro* were premastoparan A, vespid chemotactic peptide precursor and vespakinin-T, and their transcription rates were 112-, 16- and 161-fold higher, respectively, compared with *V. analis*. In the venom gland of *V. analis*, the most abundant transcripts were vespid chemotactic peptide precursor, followed by prepromastoparan A, vespakinin-T and calponin. Although a total of 19 genes showed higher transcription levels in *V. analis*, most of them were minor venom genes in terms of expression level (Tables

3, 4). The abundant transcription of premastoparan, Vespid chemotactic peptide precursor and venom allergen 5 was also reported in the venom gland of *V. tropica* (Baek et al., 2013).

To verify the transcriptional abundance of the venom genes, the relative transcription levels of 9 venom-specific genes were investigated by quantitative PCR (qPCR) and compared with their FPKM values (Fig. 4). The correlation coefficient between the relative transcription levels and the FPKM values was 0.9638, indicating that the relative abundance of venom-specific genes estimated from their FPKM values are highly reliable (Fig. 3).

Table 2. Summary of the venom gland transcriptome cDNA libraries of *V. crabro* and *V. analis*

Species	*V. crabro*	*V. analis*
Total number of reads	62,246,264	61,648,078
Trimmed reads	1,663,615	1,425,630
Total base pairs	5,759,774,997	5,686,981,519
Trimmed base pairs	7,684,407	5,717,617
Total number of assembled transcript	49,536	72,510
Total number of ORF	27,311	35,453
Total number of annotated transcript	27,281	

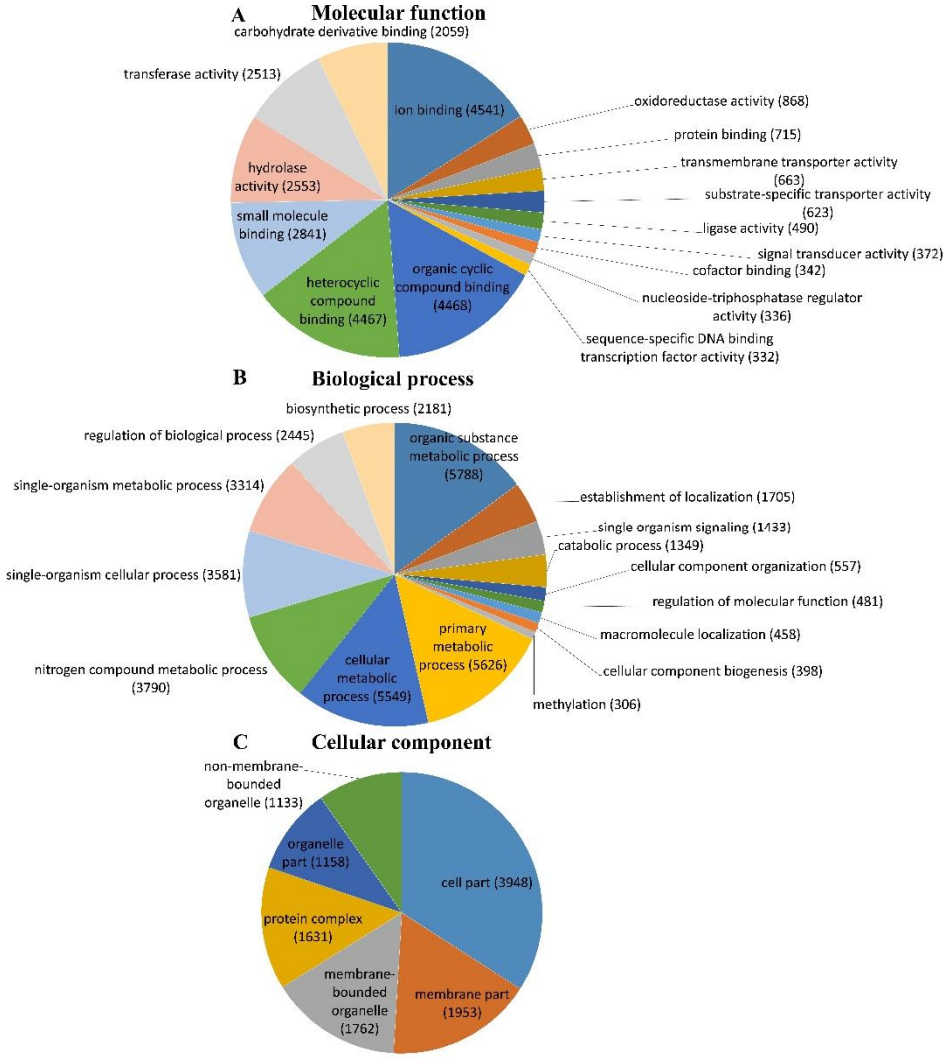


Figure 1. Gene ontology (GO) functional categorization of the contigs from the venom gland of *V. crabro*. A: Category related to molecular function, B: Category related to biological process, C: Category related to cellular component.

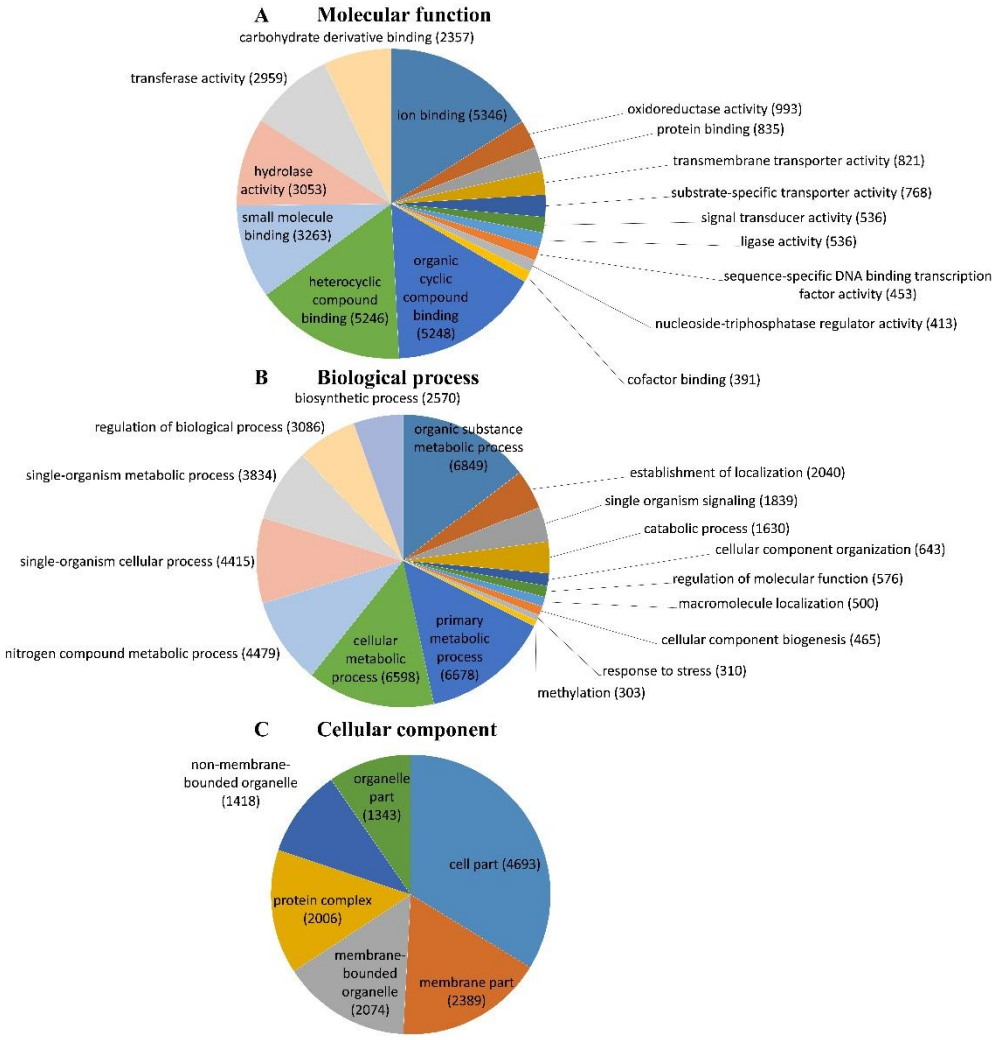


Figure 2. Gene ontology (GO) functional categorization of the contigs from the venom gland of *V. analis*. A: Category related to molecular function, B: Category related to biological process, C: Category related to cellular component.

Table 3. Annotation of differentially transcribed genes in the venom gland of *V. crabro*

Putative venom protein gene[a]	Species	E-Value	FPKM
Prepromastoparan A	*Vespa analis*	4.00E-05	2075944
Vespid chemotactic precursor	*Vespa magnifica*	0	909882.66
Vespakinin-T	*Vespa tropica*	0	184443.81
Venom allergen 5	*Vespa magnifica*	4.00E-117	46484.45
Phospholipase A1	*Vespula vulgaris*	1.00E-159	16322.3
Vespakinin-M	*Vespa magnifica*	6.00E-11	2784.85
Elongation factor 2	*Harpegnathos saltator*	0	980.83
Serine protease	*Vespa magnifica*	4.00E-79	324.54
Glycogenin	*Camponotus floridanus*	2.00E-163	113.77
Arginin kinase	*Cyphononyx dorsalis*	0	101.7
Endoplasmin	*Bombus impatiens*	5.00E-81	79.32
Defensin 1	*Apis mellifera*	4.00E-27	67.64
Phospholipase DDHD1	*Harpegnathos saltator*	0	47.26
Phospholipase A1 2	*Vespa magnifica*	8.00E-93	33.22
Calponin	*Apis mellifera*	1.00E-63	30.17
Venom acid phosphatase	*Cerapachys biroi*	3.00E-29	28.5
Insulin binding protein	*Acromyrmex echinatior*	0	25.11
Phospholipase A2	*Cerapachys biroi*	5.00E-23	20.91
Neprilysin	*Bombus terrestris*	5.00E-20	17.58
Phospholipase D3	*Acromyrmex echinatior*	0	15.9
Actin	*Nasonia vitripennis*	0	10.21
Hyaluronidase	*Vespa magnifica*	2.00E-28	9.83
Phospholipase D2	*Apis dorsata*	0	5.32
Metalloendopeptidase	*Camponotus floridanus*	2.00E-56	4.84
Serpine	*Harpegnathos saltator*	4.00E-177	3.34
Tropomyosin	*Apis dorsata*	6.00E-99	2.41
Troponin	*Harpegnathos saltator*	9.00E-16	2.32
γ-glutamyl transpeptidase	*Acromyrmex echinatior*	0	2.07
Paramyosin	*Megachile rotundata*	0	2.06
Muscle LIM protein	*Bombus terrestris*	2.00E-92	1.39
Glyceraldehyde-3-phosphate dehydrogenase	*Apis mellifera*	4.00E-18	1
Myosin light chain kinase	*Camponotus floridanus*	1.00E-25	0.7
Carboxylesterase	*Apis mellifera*	2.00E-48	0.68
Leucine rich repeat domain-containing protein	*Acromyrmex echinatior*	3.00E-64	0.64
Myosin heavy chain	*Megachile rotundata*	4.00E-14	0.62

[a] Underlined genes were more transcribed in the *V. crabro* venom gland as judged by FPKM values.

Putative venom protein gene[a]	Species	E-Value	FPKM
Vespid chemotactic precursor	*Vespa magnifica*	0	57295.01
Prepromastoparan A	*Vespa analis*	4.00E-11	18615.9
Vespakinin-T	*Vespa tropica*	5.00E-09	1144.35
Calponin	*Apis mellifera*	1.00E-63	382.86
Vespakinin-M	*Vespa magnifica*	0.011	375.03
Elongation factor 2	*Harpegnathos saltator*	0	334.88
Phospholipase A1	*Vespula vulgaris*	0	244.37
Arginin kinase	*Cyphononyx dorsalis*	0	215.66
Actin	*Apis cerana*	0	193.95
Troponin	*Bombus terrestris*	5.00E-54	188.1
Muscle LIM protein	*Bombus terrestris*	0	162.19
Paramyosin	*Megachile rotundata*	0	153.59
Defensin 1	*Apis mellifera*	5.00E-27	149.05
Serpine	*Harpegnathos saltator*	0	111.6
Tropomyosin	*Nasonia vitripennis*	6.00E-62	98.19
Venom allergen 5	*Vespa magnifica*	1.00E-114	51.53
Endoplasmin	*Megachile rotundata*	0	43.04
Phospholipase DDHD1	*Harpegnathos saltator*	0	43.01
Insulin binding protein	*Acromyrmex echinatior*	0	27.2
Hyaluronidase	*Vespa magnifica*	2.00E-163	25.5
Neprilysin	*Bombus terrestris*	7.00E-20	23.63
γ-glutamyl transpeptidase	*Acromyrmex echinatior*	3.00E-22	11.31
Phospholipase D1	*Megachile rotundata*	4.00E-15	8.92
Phospholipase D2	*Apis mellifera*	1.00E-67	8.92
Metalloendopeptidase	*Camponotus floridanus*	2.00E-56	7.81
Phospholipase A2	*Cerapachys biroi*	5.00E-23	7.68
Serine protease	*Vespa magnifica*	1.00E-27	6.27
Phospholipase A1 2	*Vespa analis*	7.00E-34	4.68
Myosin heavy chain	*Cerapachys biroi*	7.00E-22	3.97
Venom acid phosphatase	*Cerapachys biroi*	8.00E-45	2.76
Carboxylesterase	*Apis mellifera*	9.00E-176	2.45
Myosin light chain kinase	*Cerapachys biroi*	0	2.13
Glyceraldehyde-3-phosphate dehydrogenase	*Acromyrmex echinatior*	4.00E-64	1.31
Glycogenin	*Camponotus floridanus*	0.37	0.79

Leucine rich repeat domain-containing protein	*Bombus terrestris*	3.00E-84	0.59

Table 4. Annotation of differentially transcribed genes in the venom gland of *V. analis*

[a] Underlined genes were more transcribed in the *V. analis* venom gland as judged by FPKM values.

3.2. Identification of venom peptides and proteins from V. crabro and V. analis

A total of 17 active venom peptides were directly identified from the venoms of *V. crabro* and *V. analis* by mass spectrometry (Tables 5, 6). In the venom of *V. crabro*, venom peptides revealed as highly transcribed genes in venom gland-specific transcriptomic analysis were identified, including prepromastoparan A, vespid chemotactic peptide precursor and serine protease. In the venom sac of *V. analis*, vespid chemotactic peptide precursor, prepromastoparan A and vespakinin-T, which were the most abundant transcripts in venom gland, were identified as active venom peptides. The peptide sequence information of prepromastoparan derived through peptide analysis was used for peptide synthesis.

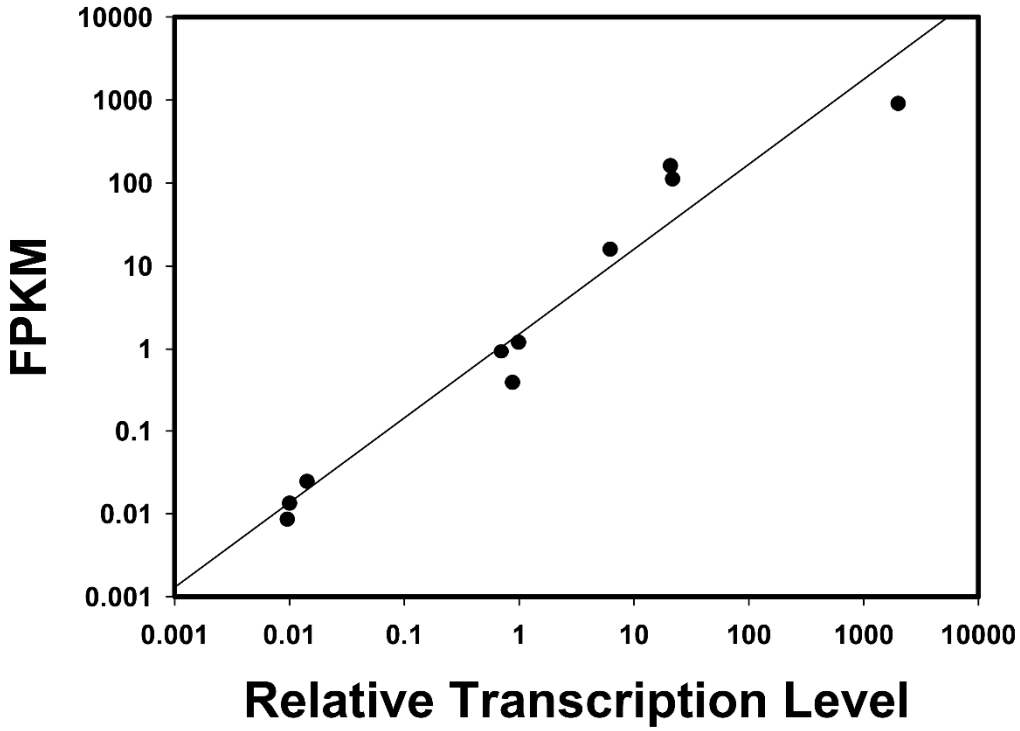


Figure 3. Comparison of the relative transcription levels and FPKM values between the two hornet species. Result of a comparative analysis of the relative transcription levels and FPKM values of 10 genes revealed high similarity ($r^2 = 0.9638$).

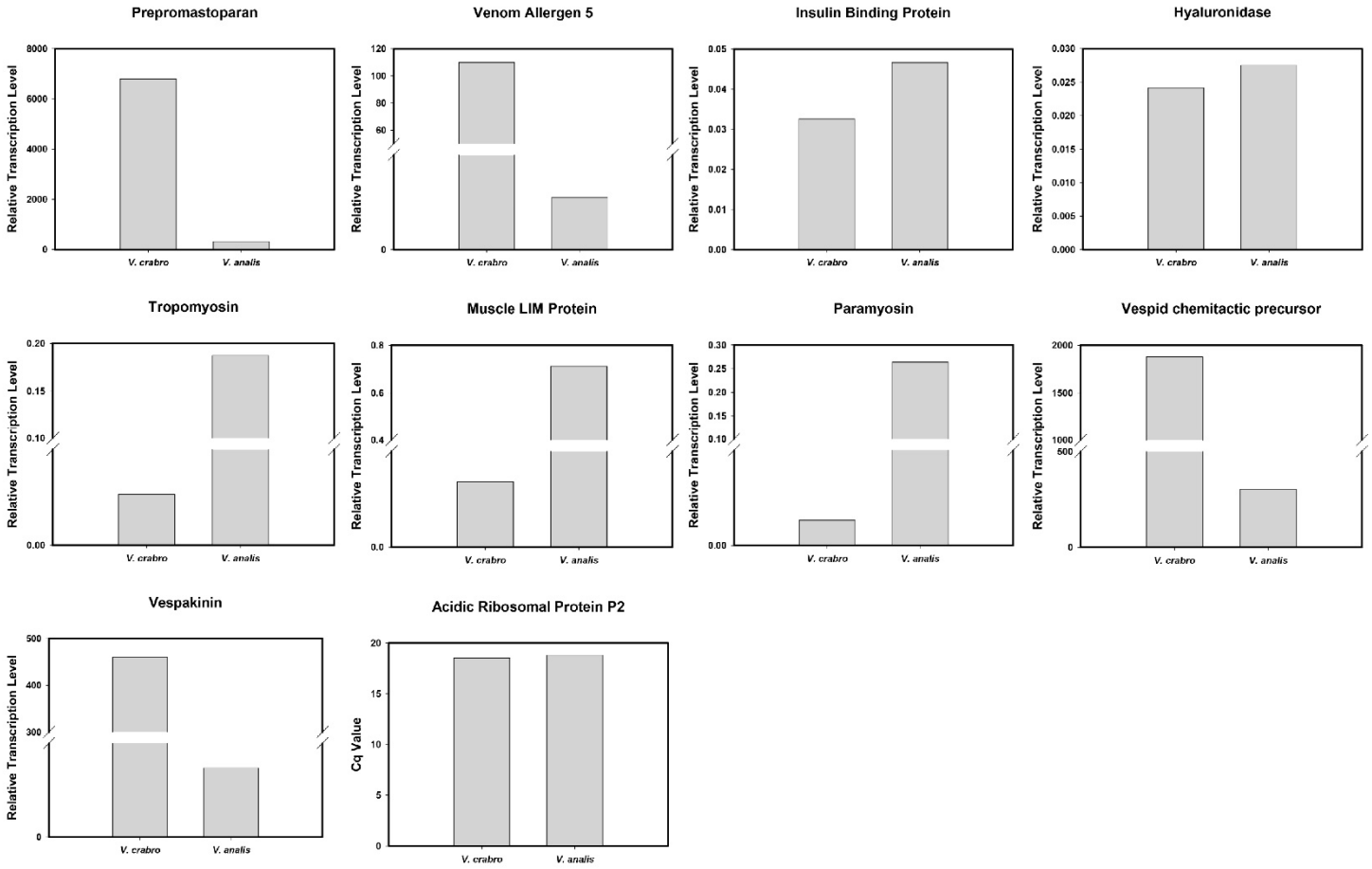


Figure 4. Quantitative real-time PCR analysis of 10 genes from the two social hornet species.

Table 5. Peptides identified from the venom of the social wasps *V. crabro*

Protein name/ID	MW (Da)/PI (pH)	PLGS score/probability (%)	No. of peptide/coverage (%)	Sequence
Group 3 secretory phospholipase A2/EZA56014.1	25,644/7.9277	8.4047/100	1/5.1948	(D)KYVPLIRLNMNL(−)
Mastoparan/Mast_VESCR	1507/10.8457	8.4029/99.82	1/1.4862	(S)RFAALLLLLAAVA(R)
Calsyntenin-1/XP_008206192.1	104,827/5.6089	8.4029/99.82	1/1.4862	(S)RFAALLLLLAAVA(R)
Leukocyte elastase inhibitor isoform X5/XP-003425279.1	42,596/5.7437	6.8023/20.14	1/4.7493	(E)VVALPYENEDLALVIIVP(K)
Crabrolin/CRBL_VESCR	1495/11.4888	8.4047/100	1/100	(−)FLPLILRKIVTAL(−)
Serine proteinase stubble/EFN63998.1	60,953/8.3394	8.4047/100	1/2.139	(V)VQIIATIVPLAV(G)
Calsyntenin-1/XP_008206192.1	104,827/5.6089	804.29/99.82	1/1.4862	(S)RFAALLLLLAAVA(R)
Venom bombolitin 2/ADY75782.1	6073/5.4946	8.4044/99.97	1/35.7143	(P)EADPEALNILGLLGKIGKIL(S)
Prepromastoparan/AIK26613.1	6153/4.1616	8.4044/99.97	1/36.6667	(P)NAEADPEAINLKAIAALVKKVL(G)
Hypothetical protein/BAL70302.1	32,863/9.6533	8.4044/99.97	3/4.8276	(P)AKFSPILLRRQPSR(Y) (A)KFSPILLRRQPSR(Y)(S)PILLR(R)
Laccase/CAD20461.1	76,014/5.7759	8.4044/99.97	1/68.1974	(G)PLIIR(E)
Crabrolin/CRBL_VESCR	1495/11.4888	8.4044/99.97	6/100	(−)FLPLILRKIVT(A)(−)FLPLILRKIVTA(L) (−)FLPLILRKIVTAL(−)(L)PLILR(K) (L)PLILRKIVT(A)(L)PLILRKIVTA(L)
Vespid chemotactic peptide/CRBL_VESTR	1352/10.0957	8.4044/99.97	1/92.3077	(F)LPILGKILGGLL(−)
Serine proteinase stubble/EFN63998.1	60,953/8.3394	8.4044/99.97	1/2.139	(V)VQIIATIVPLAV(G)
Ejaculatory bulb-specific protein 3/EFN65878.1	17,235/6.8965	8.4044/99.97	1/18.3007	(I)IPVAALCALCSSLKYSGYHVLALLLIAV(A)
Esterase FE4/EFN66413.1	63,069/8.1372	8.4044/99.97	1/1.9643	(N)KIPLPVLFWLH(G)
Serpin B10/EGI61105.1	42,542/5.4258	8.4044/99.97	1/1.3055	(E)PLLLR(V)

Table 6. Peptides identified from the venom of the social wasps *V. analis*

Protein name/ID	MW (Da)/PI (pH)	PLGS score/probability (%)	No. of peptide/coverage (%)	Sequence
Premastoparan/AEM43049.1	6309/4.4458	8.4047/100	1/23.3333	(A)IKWKAILDAVKKVI(G)
Mastoparan-A/MASTA_VESAN	1624/10.5527	8.4047/100	1/100	(−)IKWKAILDAVKKVL(−)
Mastoparan-A/CRBL-VESAN	1642/10.5527	8.4047/100	1/100	(−)IKWKAILDAVKKVL(−)
Venom protein D precursor/NP_001155171.1	44,317/7.5293	8.4047/100	1/100	(N)IIFDILLASIVK(Q)
Contactin/XP_008213762.1	105,589/7.8823	8.4047/100	1/2.7957	(L)IESLPPTIRTAVNLNQTLRCMAYTDE(M)
Disintegrin and metalloproteinase domain containing protein 10-like/XP_003692198.1	91,487/6.0542	8.4047/99.97	1/1.451	(L)VLLLILAFLACF(L)
Venom dipeptidyl peptidase 4-like/XP_003399213.1	88,774/5.9678	7.7111/49.98	1/3.2134	(Y)QQSMALNKALVDKDIMFEQQSYTDE(A)
Dipeptidyl peptidase IV/ABG57265.1	88,438/5.6411	8.4047/100	1/2.0645	(A)LEKADIMFEQITYTDE(A)
Probable chitinase 2/EFN82567.1	48,109/6.2695	8.4047/100	1/2.0833	(N)PIGLPALIT(G)
Ejaculatory bulb-specific protein 3/EGI60804.1	21,683/6.813	8.4047/100	1/12.3711	(K)PETWELLKKKYDPTGEYSIKYTDE(A)
GTPase-activating protein and VPS9 domain-containing protein 1/EGI65207.1	167,017/5.1899	8.4047/100	1/0.9358	(K)MTLRNRRGKAIRGA(D)
Venom acid phosphatase Acph-1-like/XP_003706107.1	45,598/5.7832	8.4047/100	1/3.0612	(I)LFAQRDIGLELP(E)
Phospholipase A1/PA1_VESGE	33,218/8.5664	8.4047/99.99	1/4.0	(T)NFINLSKALVDK(D)
Phospholipase B1/XP_00656550.1	46,606/8.2764	7.7436/51.63	1/2.7027	(D)SPFNLLIWRAI(R)
Teratocyte released chitinase/AAX69085.1	54,061/5.4902	8.4042/99.95	1/2.0284	(I)GAPTRGPGAA(G)
Venom protein Ci-35a/CBM69265.1	21,486/8.3701	8.4042/99.95	1/5.3197	(−)FLPMIAKLLGGLL(−)
Thr-6 bradykinin-scoliid wasp/A61057	1073/12.4028	0.1913/80.78	1/88.8889	(−)GRPPGFSPFR(I)

3.3. Vespid chemotactic peptide precursor

The vespid chemotactic peptide cDNA sequences of *V. crabro* (321 bp) and *V. analis* (390 bp) encoded peptides of 65 amino acids and were identified to be most similar to that of *V. magnifica* (82% and 91% amino acid sequence identity, respectively). The amino acid sequence identity between the vespid chemotactic peptide precursors of the two social hornets was 83.1% and that of the mature peptide region was 38.5%. Among the 13 amino acid residues in the mature region, eight amino acid residues were non-conserved (positions 4, 6, 7, 8, 9, 10, 11 and 12). The positively charged hydrophilic amino acid Lys (position 8) in *V. crabro* was substituted with the hydrophobic amino acid Leu in *V. analis* (Fig. 5). A phylogenetic tree showed that the vespid chemotactic peptide of *V. crabro* was closely related to that of *Eumenes pomiformis* and *Orancistrocerus drewseni*, whereas that of *V. analis* was clustered into another branch. Vespid chemotactic peptides are known to have antimicrobial activity and to cause cellular chemotactic responses (Tsukamoto et al., 1987; Xu et al., 2006). The relative expression level of vespid chemotactic peptide, as judged by FPKM values, was approximately 16-fold higher in *V. crabro* compared to *V. analis*. This finding implies that the chemotactic potential in *V. crabro* venom is likely greater than that of *V. analis* venom.

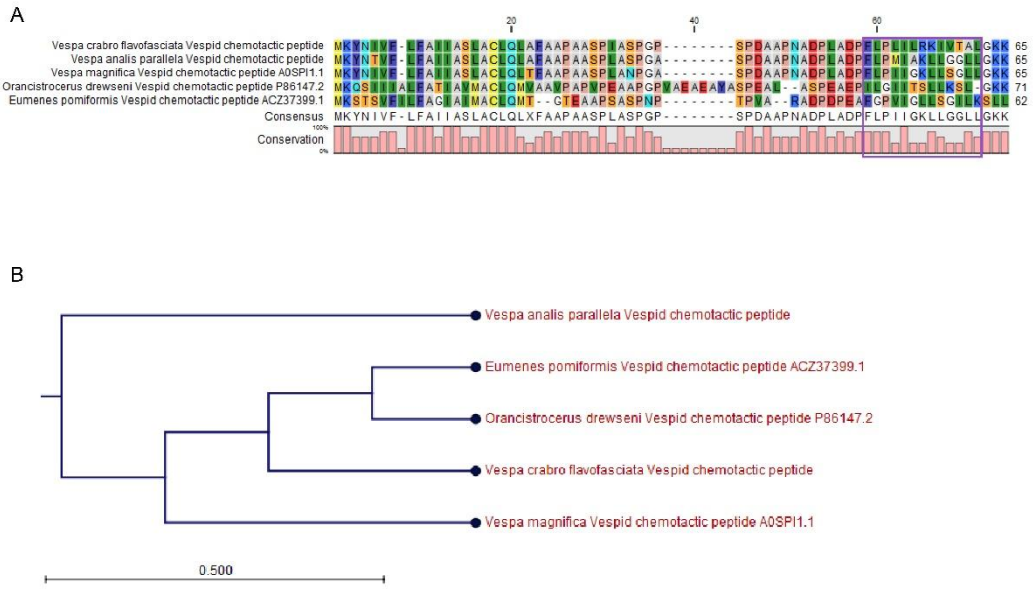


Figure 5. Amino acid alignments of the vespid chemotactic peptide. A: Alignment of amino acid sequences from *V. crabro* and *V. analis* with the genera Vespa, Orancistrocerus and Eumenes. The functional domain is indicated by the purple box. B: Phylogenetic analysis of vespid chemotactic peptides.

3.4. Prepromastoparan

Prepromastoparan was the most abundantly transcribed gene in the two hornet species. The cDNA sequences of *V. crabro* and *V. analis*, which encode 60-amino acid peptides, were identified as prepromastoparans of *Vespa ducalis* (96%) and *V. analis* (100%), respectively. Amino acid sequence alignments revealed highly conserved regions for the signal sequence, prosequence and the last amino acid glycine, which is presumably responsible for C-terminal amidation (Lee et al., 2007). The phylogenetic analysis showed that the mastoparans from *V. crabro* and *V. analis* were more closely clustered together than those from other Vespa wasps, suggesting their closely related taxonomic status. Of the 15 amino acid residues composing the mature mastoparan peptide, differences between *V. crabro* and *V. analis* were found in 9 amino acid residues (positions 1, 2, 3, 6, 8, 9, 10, 13 and 14), of which 4 residues are non-conservative substitutions. In particular, the hydrophobic amino acid residue Ala (position 8) in *V. crabro* was substituted with the acidic amino acid residue Asp in *V. analis*. This substantial difference in amino acid composition suggests that the mature mastoparans of *V. crabro* and *V. analis* likely have considerably different biological activities (Fig. 6). Considering that mastoparan is known as an inducer of the mitochondrial permeability transition (PT) and that the induction of PT causes mitochondria to rupture, leading to apoptosis and necrosis (Yamamoto et al., 2014), the mastoparan-induced

cytotoxicity of these two closely related hornets may be quite different. The relative expression level of prepromastoparan, as judged by FPKM values, was approximately 112-fold higher in *V. crabro* venom compared to *V. analis*, suggesting that *V. crabro* venom is more cytotoxic.

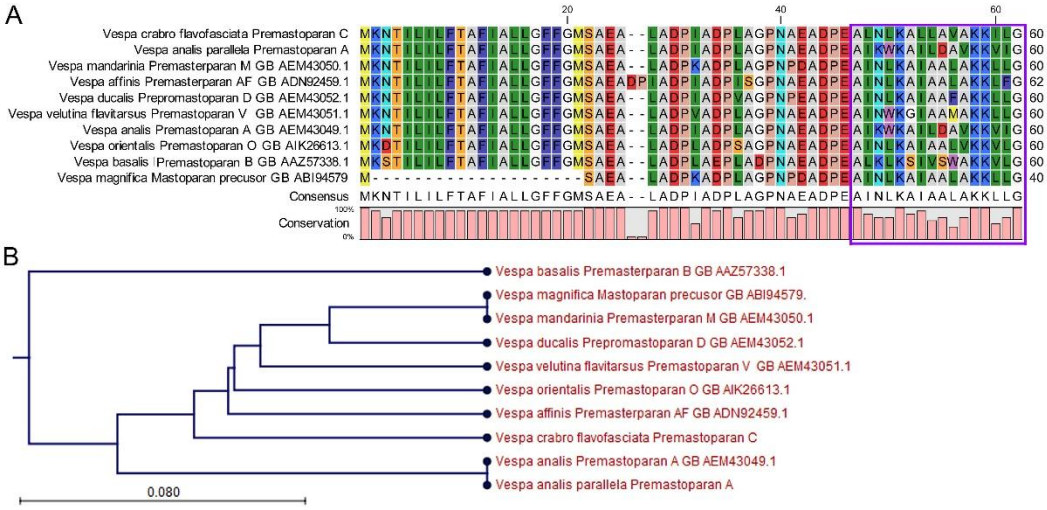


Figure 6. Amino acid sequence alignments of the prepromastoparan. A: Predicted amino acid sequences of the two prepromastoparans identified in this study were aligned with previously predicted prepromastoparan sequences from the genus Vespa. Functional mastoparan peptides are indicated in the pink boxes. B: The phylogenetic analysis of prepromastoparan from the genus Vespa revealed that prepromastoparan from *V. crabro* and *V. analis* are closely clustered.

3.5. Venom allergen 5

The cDNA sequences of venom allergen 5 from *V. crabro* and *V. analis* encoded 225 amino acid residues, and the open reading frame (ORF) was highly similar (96% and 95%, respectively) to that of *Vespa magnifica*. The deduced amino acid sequences of venom allergen 5 from various Vespid wasps showed a high homology, particularly within the sterol carrier protein (SCP)-like extracellular protein domain (SCP-euk). The amino acid sequence identity was 91.1% between *V. crabro* and *V. analis*, suggesting that venom allergen 5 has diverged less compared to mastoparan between these two hornets. The phylogenetic tree of venom allergen 5 genes from several wasp species indicated that the venom allergen 5 peptides of *V. crabro* and *V. analis* were grouped with other Vespid wasps, and clearly separated from those of other genera, such as Vespula and Dolichovespula (Fig. 7).

Venom allergen 5 proteins, belonging to the cysteine-rich anti-microbial peptides (CAP) family, are a common allergen family found in the venoms of Vespid wasps and fire ants and induce acute hypersensitivity responses in humans (An et al., 2012; King and Spangfort, 2000). As there were no significant differences in the amino acid sequences of venom allergen 5 genes between *V. crabro* and *V. analis*, their allergenic capacities are likely not noticeably different each other. The relative expression level of venom allergen 5, as judged by FPKM

values, was remarkably higher in *V. crabro* venom (approximately 902-fold) compared to *V. analis* venom. This finding implies that the allergenic potential of *V. crabro* venom is likely greater than that of *V. analis* venom.

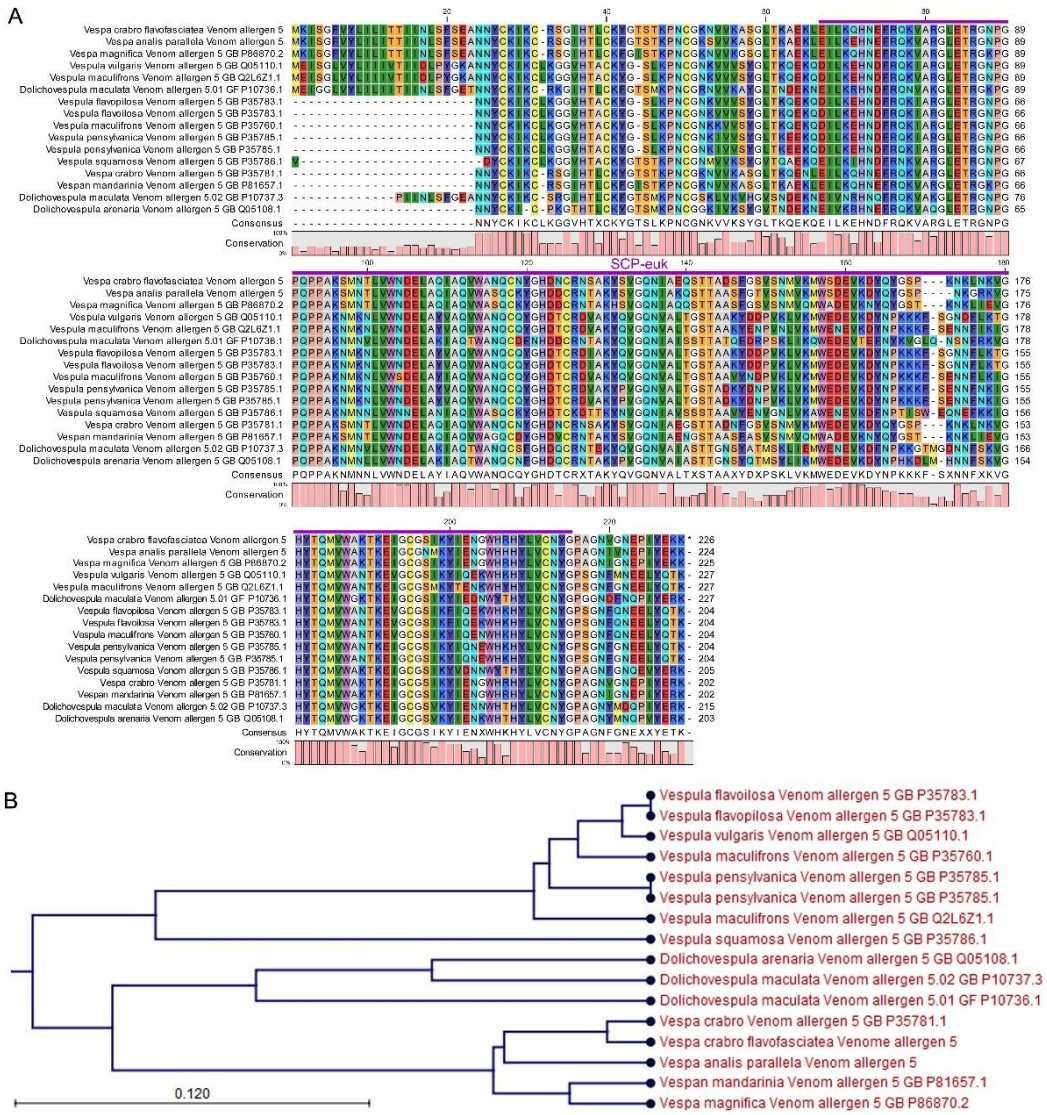


Figure 7. Amino acid sequence alignments of the venom allergen 5. The amino acid sequences of venom allergen 5 from two hornet species were compared with other venom allergen 5 proteins from the genera Vespa, Vespula and Dolichovespula. A: Venom allergen 5 from various wasps shared high homology within the SCP-like extracellular protein domain (SCP-euk). B: Phylogenetic analyses of venom allergen 5 proteins were carried out according to the genus.

3.6. Vespakinin

The vespakinin cDNA sequences of both *V. crabro* and *V. analis* encoded 55-amino acid-long peptides with high degrees of identity to the vespakinins of *Vespa tropica* and *Vespa magnifica* (95% and 96%, respectively). The overall amino acid sequence of vespakinin was identical between *V. crabro* and *V. analis*, except for a four-amino acid deletion (position 49–52) in *V. analis* and one amino acid substitution (Lys in *V. crabro* vs. Glu in *V. analis*) in the functional peptide region. These two vespakinins were also clustered within a clade, indicating their close relationship (Fig. 8). Vespakinins are commonly found in the venom of wasps and have similar amino acid sequences and pharmacological properties to mammalian bradykinin (Rochat and Martin-Eauclaire, 2000). It has been reported that vespakinins cause hypertension in mammals and result in contraction and relaxation of isolated smooth muscle preparations (Mendes and Palma, 2006). Two kinds of G protein coupled receptors, the B1 and B2 receptors, cause pharmacological effects and the wasp kinins are B2 receptor activators (Mortari et al., 2007). Wasps use vespakinins as a defense system by producing pain in vertebrate predators and use it to paralyze their prey (Konno et al., 2002). The relatively higher level of expression of vespakinin in V. crabro venom (161-fold), as judged by FPKM values, suggests that the pharmacological potential of *V. crabro* venom is likely greater compared to that of *V. analis* venom.

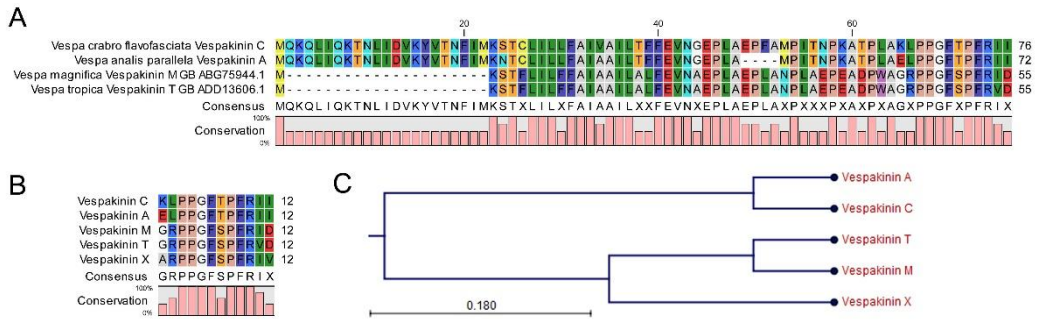


Figure 8. Amino acid sequence alignments of the vespakinin. A: The predicted amino acid sequences of vespakinin from *V. crabro* and *V. analis* were highly similar. B: The functional peptides of vespakinin showed high levels of amino acid identity. C: The vespakinins identified in this study were analyzed by constructing phylogenetic tree with previously identified vespakinins.

3.7. Hyaluronidase

The hyaluronidase cDNA sequences of *V. crabro* (1331 bp) and *V. analis* (1334 bp) were most similar to that of *V. magnifica*, with amino acid sequence identities of 97% and 93%, respectively. Highly conserved amino acid sequences containing Asp and Glu are obvious candidates for catalytic activity. The Asp residue has a significant role in stabilization of the catalytic nucleophile and the Glu residue likely functions as a proton donor for the hydroxyl leaving group (Zhang et al., 2009). The –DFE- motif in the active site is extremely well conserved not only in the two social hornets but also in other genera, suggesting that the hyaluronidases from *V. crabro* and *V. analis* would have hydrolysis activity similar to that of other hyaluronidases. Phylogenetic analysis with other

wasps showed a high sequence homology between the closely clustered hyaluronidases from *V. crabro* and *V. analis* (Fig. 9).

Hyaluronidase is a glycoside hydrolase which cleaves the β-1,4-glycosidic bondage between N-acetylglucosamine and d-glucuronic acid of hyaluronic acid (An et al., 2012). Hyaluronidase catalyzes the hydrolysis of hyaluronic acid, a major constituent of the extracellular matrix (ECM), thereby increasing tissue permeability and allowing venom components to more easily spread through the tissues of the host (Kemparaju and Girish, 2006). Since hyaluronidase is known to be capable of causing severe immunological reactions (Dotimas and Hider, 1987), the presence of this enzyme in the venoms of both hornets would contribute, along with the venom allergen 5, to their allergenic potentials. Nevertheless, overall expression level of hyaluronidase was significantly lower than that of venom allergen 5 in *V. crabro* venom (4543-fold), whereas the difference was much smaller in *V. analis* venom (2.0-fold). This finding suggests that the relative allergenic potential of hyaluronidase is higher in *V. analis* venom compared to *V. crabro* venom.

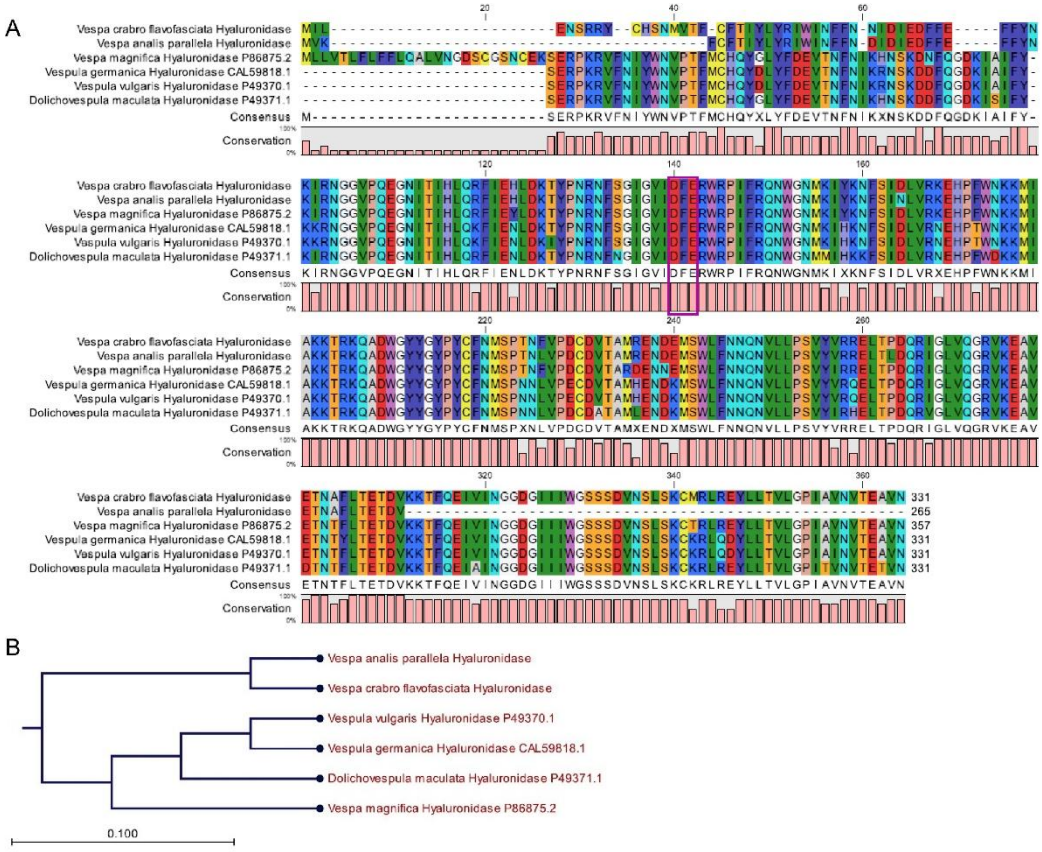


Figure 9. Amino acid sequence alignments of the hyaluronidase. A: Alignments of amino acid sequences of hyaluronidase from *V. crabro* and *V. analis* with that from the genera Vespa, Vespula and Dolichovespula. The active site is indicated by the purple box. B: The result of phylogenetic tree construction showed a close relationship between the hyaluronidases from *V. crabro* and *V. analis*.

3.8. Phospholipase A1

The cDNA sequences of phospholipase A1 from *V. crabro* and *V. analis* encoded 333- and 334-amino acid proteins that matched well with phospholipase A1 of *Vespa affinis* (92% and 93% sequence identity, respectively). All of the extracellular phospholipase A1s, including those from *V. crabro* and *V. analis*, belong to the pancreatic lipase gene family and contain conserved catalytic regions containing Ser-His-Asp (Aoki et al., 2007; Carriere et al., 1998). The amino acid sequence identity between the pancreas lipase-like domains of phospholipase A1 of the two social hornets was 93.4%, and eleven cysteine residues were extremely well conserved, not only between the two hornet species but also among other genera such as Vespa, Vespula, Dolichovespula and Polybia (Fig. 10). Phospholipase A, one of the major allergens, hydrolyses phospholipids, the major biological membrane component, resulting in the release of lysophospholipids and free fatty acids. The reaction products released from membranes by phospholipase A1 is known to cause fatal anaphylaxis and local inflammatory reactions in allergic patients (King et al., 2003). The relative expression level of phospholipase A1, as judged by FPKM values, was much higher in *V. crabro* venom (approximately 108-fold) compared to *V. analis* venom, suggesting that the allergenic potential of *V. crabro* venom is likely greater than that of *V. analis* venom.

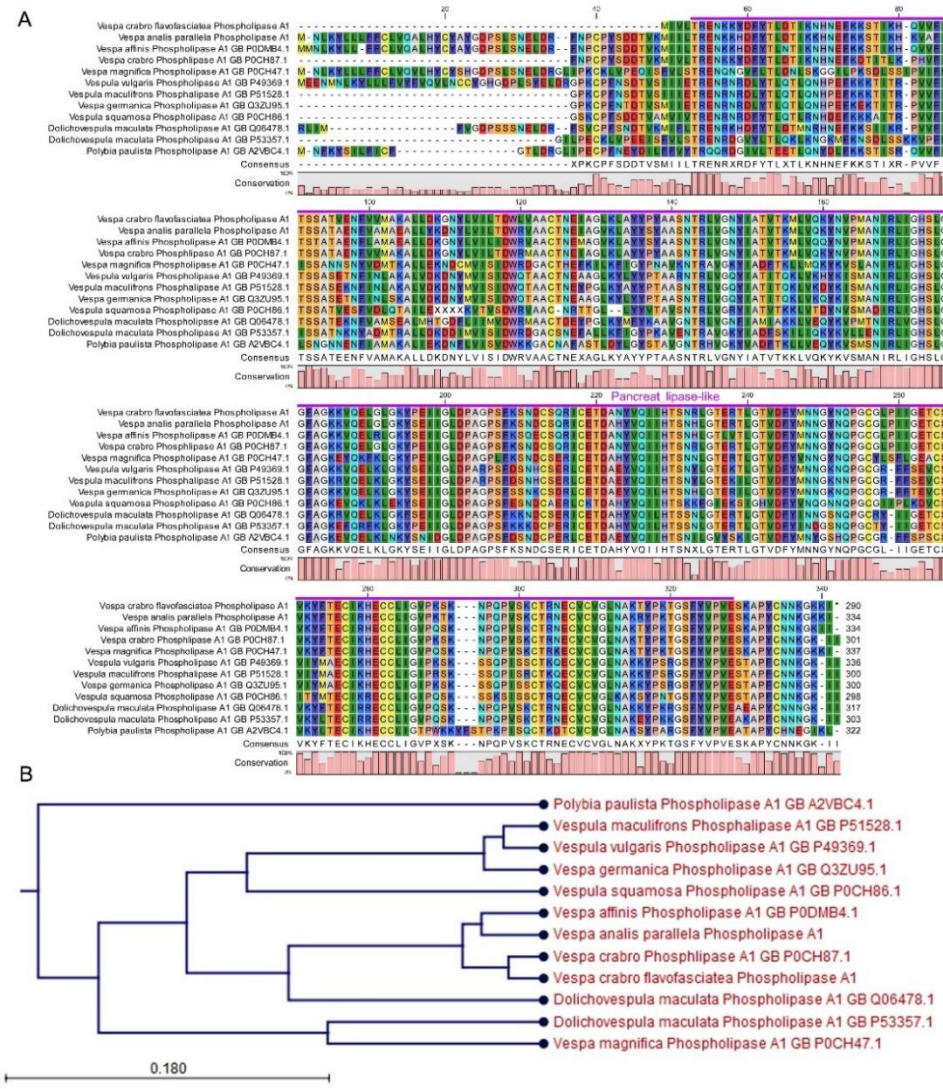


Figure 10. The amino acid sequences of phospholipase A1 from the two wasps were aligned with phospholipase A1 from the genera Vespa, Vespula, Dolichovespula and Polybia. A: The pancreas lipase-like domain showed high homology among the amino acid sequences of phospholipase A1 from various wasps. B: The result of a phylogenetic analysis revealed a close relationship between phospholipase A1 from *V. crabro* and *V. analis*, as evidenced by their close branch positions.

3.9. Defensin

The cDNA sequences of defensin from *V. crabro* and *V. analis* encoded 101-amino acid peptides, which displayed the highest sequence similarity to the defensin 1 of *Apis mellifera* (63% and 61%, respectively). Defensins from the two social hornet species showed a highly identical clustering pattern in the phylogenetic analysis and defensin 2 had five highly conserved cysteine residues (Fig. 11). The amino acid sequence identity between the defensins from the two different social hornet species was high (96%). Cysteine-rich peptides, such as defensins, have been characterized as anti-microbial peptides (AMPs) in invertebrates, especially in insects (Dimarcq et al., 1998). Although there is sequence diversity among species, defensins have a conserved structure, including one α-helix and one β-sheet composed of two antiparallel strands (Cornet et al., 1995). It has been reported that *A. mellifera* encodes two defensin genes, defensin 1 and defensin 2 (Fujiwara et al., 1990). Defensin 1 shows antimicrobial activity against Gram-positive bacteria at low concentrations, while the biochemical function of defensin 2 has not yet been identified. Based on FPKM values, *V. analis* appears to produce more (approximately 2.2-fold) defensin in its venom compared with *V. crabro*.

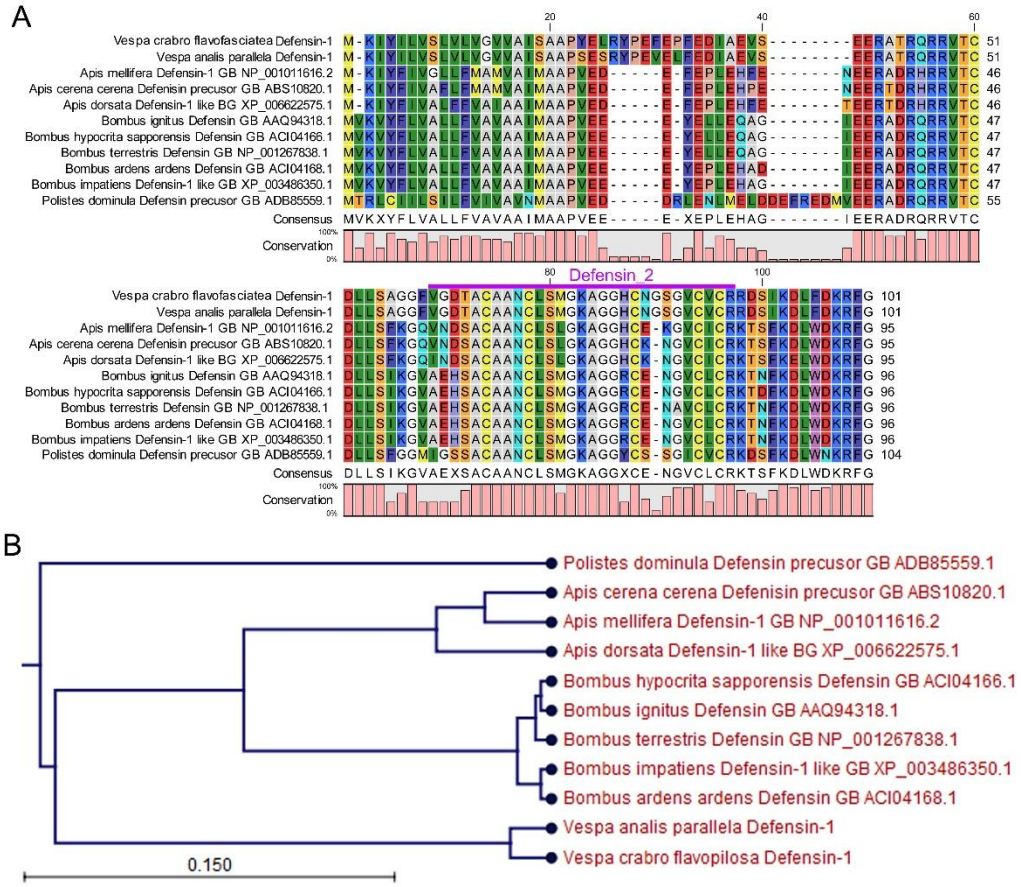


Figure 11. Amino acid sequence alignments of the defensin 1. Defensin 1 from two social wasps was compared with defensin 1 from the genera Apis, Bombus and Polistes. A: Defensin 2 domains were highly conserved in defensin 1 from two wasps. B: Clustering of defensin 1 peptides according to their genera.

4. Conclusions

Our findings reveal that the overall components of *V. crabro* and *V. analis* venoms are similar, but that their expression profile and level differ considerably. The comparison of venom gland transcriptomes suggests that *V. crabro* likely produces venom that is more highly enriched in major venom components, thereby with potentially higher toxicity compared with *V. analis* venom.

This chapter was published as Yoon et al., 2015a, b.

CHAPTER III

Characterization of Biological Activities of Main Venom Peptides in Aculeate Hymenoptera

Abstract

To examine the differences in the potential toxicity and bioactivity of 10 venom peptides (mastoparan, vespid chemotactic peptide (VCP), vespakinin (Vespk) and bombolitin) from social wasps (*Vespa analis*, *V. crabro*, *V. mandarina* and *Parapolybia varia*) and bumble bees (*Bombus ardens*, *B. consobrinus*, *B. terrestris* and *B. ussurensis*), differential toxicological and pharmacological activities of synthesized venom peptides were investigated. Hemolytic, anti-microbial, and anti-tumor activities of synthesized *V. analis* mastoparan were higher than those of *V. crabro* mastoparan. *Pv*VCP, *Pv*Vespk, and *Vm*Vespk showed little to low hemolytic activities. Only *Vm*VCP showed hemolytic activity at a high concentration. Among the four peptides tested, *Vm*VCP showed both anti-microbial and anti-fungal activities, whereas *Pv*VCP showed only anti-fungal activity to *Candida albicans*. Interestingly, *Pv*VCP showed significantly stronger anti-tumor activities to two ovarian cancer cell lines compared with *Vm*VCP. Vespks only showed anti-tumor activity to SK-OV-3 cells but not to NIH-OVCAR-3 cells. Among the four bombolitins tested, bombolitin T showed the highest hemolytic and anti-tumor activities. All bombolitins exhibited strong anti-microbial and anti-fungal activities, and bombolitin A specifically possessed the highest anti-microbial activity against the Gram-negative bacteria *Escherichia coli*.

These differential bioactivities are likely due to the amino acid sequence differences in the mature peptides.

To design selective anti-tumor peptides with reduced hemolytic activity, we used bombolitin T as a model and modified its amino acid composition to be similar to that of mitoparan, which has high anti-tumor activity against astrocytoma and bladder carcinoma cells (Jones et al., 2008). To determine the correlation between anti-tumor or hemolytic activity and the physicochemical properties of bombolitin T, principal component analysis (PCA) was conducted. The lowest hemolytic activity was observed with the addition of a hydrophobic amino acid (Ile^{14}) to the C-terminal end as well as the substitution of Ser^{10} and Leu^{12} with a hydrophobic amino acid (Leu) and positively charged amino acid (Lys), respectively. This finding implies that the balanced substitution of hydrophobic and positively charged amino acids could affect the cytotoxicity. Finally, the combination of amino acid substitution and a selective increase in anti-tumor activity with reduction of hemolytic activity was suggested and discussed.

1. Introduction

The mastoparan, an amphiphilic tetradecapeptide, is a major peptide in social wasp venoms and shows various bioactivities (Liu et al., 2007). Sequence analysis of premastoparan cDNA revealed that non-conservative substitutions were found at four different sites of the 15 amino acid residues consisting of the mature mastoparan peptide between *V. crabro* and *V. analis*, among which the hydrophobic amino acid residue Ala (position 8) in *V. crabro* was substituted with the acidic amino acid residue Asp in *V. analis* (Yoon et al., 2015b). Such substantial variation at the critical amino acid residues suggests that the mature mastoparans likely exhibit substantial differences in their biological activities between *V. crabro* and *V. analis*. Considering that mastoparan is known as an inducer of the mitochondrial permeability transition (PT) and that the induction of PT causes mitochondria to rupture, leading to apoptosis and necrosis (Yamamoto et al., 2014), the mastoparan-induced cytotoxicity of these two closely related hornets may be quite different. In addition to the cytotoxic property, mastoparan is known to possess a variety of bioactivities, including antimicrobial activity, antitumor activity and antiviral activity (Sample et al., 2013).

The genes encoding premastoparans were the most and second most abundantly transcribed in the venom glands of *V. crabro* and *V. analis*,

respectively (Yoon et al., 2015a, b). They showed anti-microbial, anti-fungal, anti-tumor, and hemolytic activities (Yoon et al., 2015b). Along with premastoparan, vespid chemotactic precursor (VCP) and vespakinin (Vespk) have been identified as the top three genes most prevalently transcribed in the venom glands of *V. crabro* and *V. analis*.

The lesser paper wasp *Parapolybia varia* is a mid-sized social wasp species widely distributed in the Middle East, the Indo-Papuan region, and East Asia (Katada et al., 2007; Saito-Morooka et al., 2015). Despite its abundance, little information is available on its venom composition and functional venom peptides. In particular, no information regarding Vespks from the genus Parapolybia has been reported yet. The amino acid sequences of venom peptides even in closely related species show variances, and changes of only a few amino acid residues in peptides can significantly alter the bio-physical and chemical properties of peptides (Kaas and Craik, 2015).

Bombolitin is a toxic peptide originally isolated from the venom of the bumblebee Megabombus pennsylvanicus (Argiolas and Pisano, 1985) and is the most abundant component of bumblebee venom (Choo et al., 2010a; Choo et al., 2010b; Choo et al., 2011; Qiu et al., 2012; Xin et al., 2009). Bombolitin belongs to the families of the mast cell degranulating peptides and the α-pore forming toxins (α-PFTs). Functional peptides of these two families have cytolytic, anti-

microbial, anti-fungal and anti-tumor activities (Konno et al., 2001a; Konno et al., 2006; Mendes et al., 2004; Souza et al., 2005; Stocklin et al., 2010; Turillazzi et al., 2006; Xu et al., 2006; Yoon et al., 2016; Yoon et al., 2015a). Bombolitin possesses an amphipathic α-helical structure in lipid-membrane-mimicking environments, which is similar to melittin, one of the most investigated peptides from bee venom (Argiolas and Pisano, 1985; Choo et al., 2010a; Javkhlantugs et al., 2011). Bombolitin exhibits various biological activities, including hemolytic, anti-microbial and anti-fungal activities (Argiolas and Pisano, 1985; Battistutta et al., 1994; Choo et al., 2010a; Hamuro et al., 1999; Kini and Evans, 1989; Qiu et al., 2012; Stocklin et al., 2010). However, no anti-tumor activity of bombolitin has been investigated to date.

The goal of this study was to examine the differences in the potential bioactivity and toxicity of mastoparans, VCPs, Vespks and bombolitins of aculeate bees/wsps. To this end, mature mastoparans, VCPs, Vespks and bombolitins were synthesized and their differential toxicological and pharmacological properties were investigated and discussed.

In an attempt to reduce the hemolytic activity of bombolitin, we used bombolitin T as a model and modified its amino acid composition to be similar to that of mitoparan, which has high anti-tumor activity against astrocytoma and bladder carcinoma cells (Jones et al., 2008). To determine the correlation between

anti-tumor or hemolytic activity and the physicochemical properties of bombolitin T, principal component analysis (PCA) was conducted. Finally, the combination of amino acid substitution and a selective increase in anti-tumor activity with reduction of hemolytic activity was suggested and discussed.

2. Materials and Methods

2.1. Peptide synthesis

Mature peptides of mastoparan, vespid chemotactic peptide (VCP), vespakinin (Vespk) and bombolitin were synthesized through GenScript (GenScript, Piscataway, NJ, USA) and stored at -80°C until used for biological activity assays. Lyophilized peptides were dissolved in a 1% dimethyl sulfoxide (DMSO) solution at various concentrations and used for biological activity assays.

2.2. Amino acid sequence analysis

The deduced amino acid sequences of mastoparan A, mastoparan C, *Pv*VCP, *Vm*VCP, *Pv*Vespk, *Vm*Vespk, bombolitins A, C, T and U were obtained from the transcriptome data of *V. analis*, *V. crabro*, *V. mandarina*, *P. varia*, *B. ardens*, *B. consobrinus*, *B. terrestris* and *B. ussurensis* venoms. The mature peptide sequences of the venom peptides were aligned with those of other bees/wasps

using CLC Main Workbench 7 (CLC bio, Waltham, MA, USA) to determine the conserved amino acid residues.

2.3. Circular dichroism spectrum characterization

The Protean program (DNAStar, Madison, Wisconsin, USA) was used to predict the secondary structure of mastoparan from *V. analis* and *V. crabro*. The circular dichroism (CD) spectra of VCP, Vespk and bombolitin were measured in 10 mM sodium phosphate buffer at pH 7.2 (SPB), 30 mM sodium dodecyl sulfate (SDS), and 50% trifluoroethanol (TFE) to identify the secondary structures. The spectra were recorded from 180 to 260 nm on a JASCO J-1500 spectropolarimeter (JASCO, Easton, MD, USA) at 298 K using a quartz cuvette of 0.1 and averaged by three scans. The protein sample concentration was 0.1 mg/ml. The data were recorded at a scan speed of 200 nm/min, bandwidth of 1.0 nm with 1 sec response and 0.1 nm resolution. The molar ellipticity and the percentages of α-helical, β-sheet, turn and random structures of the synthesized venom peptides were calculated.

2.4. Hemolytic activity

Human red blood cells (RBCs) that were purchased from Korean Red Cross Blood Services were used to conduct hemolytic activity assays of the synthesized

venom peptide. RBCs were washed three times with the same volume of phosphate buffered saline (PBS), centrifuged at 960 rpm for 15 min at room temperature and re-suspended with PBS at 2% concentration. Ten microliters of synthetic peptide dissolved in 1% DMSO to various concentrations (10 to 200 μM) were incubated with 90 μl of washed RBCs for 30 min at 37°C. Each incubated mixture was centrifuged at 960 rpm for 15 min at room temperature, and the optical density (OD) value of the supernatant was measured at 540 nm using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). The relative hemolytic activity was determined by establishing the activity of 0.1% DMSO as a blank and setting that of 0.1% Triton X-100 as 100%. Assays were conducted with three replications, and the protocol was approved by the Institutional Review Board (IRB# E1611/003-005, Seoul National University IRB).

2.5. Anti-microbial activity

Gram-negative *Escherichia coli* (ATCC 11775), Gram-positive *Staphylococcus aureus* (ATCC 12600), Gram-positive yeast *Candida albicans* (ATCC 10231), and gray mold *Botrytis cinerea* were used for examining the anti-microbial activities of venom peptides. Luria–Bertani (LB) broth, brain heart infusion (BHI) broth, and potato-dextrose (PD) broth were used to culture *E. coli*,

S. aureus, and *C. albicans*, respectively. The bacterial and fungal cultures were incubated with various concentrations of synthetic peptides (0.05-0.1 mM) in a shaking incubator (150 rpm) overnight at 37°C, and then the OD of each culture was measured using a microplate reader (VersaMax) to identify the minimal inhibition concentration (MIC). *B. cinerea* was incubated for 5 days at 25°C on PD agar, and 20 μl of synthesized venom peptides were applied in various concentrations (0.1-2 mM) overnight. The MICs were determined by measuring the size of the inhibition zone on the fungi-incubated agar. A top agar assay was conducted with the same bacteria and yeast used in the broth inhibition assay. After incubating *E. coli* in LB broth to 0.5 OD_{600}, 1 ml of liquid culture was mixed with 10 ml of 0.7% agar-containing LB and then poured into a plate. The same method was used to prepare *S. aureus* with BHI broth and nutrient agar, and *C. albicans* with PD broth and PD agar. After the agar plates hardened, 20 μL aliquot of various concentrations of synthesized peptides was applied on the plates and the inhibition zone was measured after incubating at 37°C overnight.

2.6. Anti-tumor activity

A human ovarian adenocarcinoma cell line (SK-OV-3; ATCC HTB-77) and a drug-resistant human ovarian adenocarcinoma cell line (NIH-OVCAR-3; ATCC HTB-161) were used for anti-tumor assays. Ovarian tumor cells (1×10^4

cells/plate) were cultured for 1 day, and various concentrations (2.5 to 100 μM) of synthetic peptides dissolved in DMSO were added to the cell plates. Non-treated and 0.001% DMSO-treated plates were used as controls. The survivorship of tumor cells was measured by an MTT assay. After incubating with various concentrations of peptides for 24 h and 72 h, the cells were additionally incubated for 4 h at 37°C in a CO_2 incubator, and then OD was measured at 540 nm.

2.7. Principal component analysis (PCA)

To determine the correlation between the anti-tumor or hemolytic activity and the structural properties (percentage of positively charged amino acids (PPA), net charge (NC), isoelectric point (pI), α-helicity in lipid-membrane-mimicking buffers and hydrophobicity (HP)) of bombolitins, principal component analysis (PCA) was conducted using XLSTAT (Addinsoft, New York, NY, USA) of the bombolitins together with vespid chemotactic peptides of Parapolybia varia and Vespa mandarinia (Yoon et al., 2016), which are also α-PFTs, as these peptides had been analyzed under the same conditions.

2.8. Peptide substitution and hemolytic activity assay

To analyze the factors affecting the hemolytic activity, the structural properties of bombolitin T, which had the highest hemolytic and anti-tumor activities, were

modified to be similar to those of mitoparan, a group of mastoparan-like peptides known to have strong anti-tumor activity (Jones et al., 2008). Composition of five amino acid residues at the N-terminal strand of bombolitin T was different from those of other three bombolitins. Bombolitins A, C and U possessed two positively charged amino acids (Lys^4 and Lys^5), whereas bombolitin T had hydrophobic amino acid (Leu^4) and Gly^5. To match the size of mature mitoparan, five amino acids of bombolitin T were removed and used as a template. Four residues of the template were replaced by four different amino acids (Ala, Leu, Lys and Ile) to make them identical to those of mature mitoparan at the corresponding positions, thereby modifying the structural properties of bombolitin T, including its hydrophobicity, net charge and isoelectric point (pI). The cDNA fragments for a total of 14 substituted peptides were synthesized with primers (Table 1) using the Oligo DNA Synthesis Kit (Macrogen, Rockville, MD, USA) and cloned with a pET-30a expression vector (Merck Millipore, Billerica, MA, USA). Modified peptides were expressed using the PURExpress In Vitro Protein Synthesis Kit (New England Biolabs, Whitby, Ontario, Canada) from the peptide cDNAs, and their hemolytic activities were evaluated.

Table 1. Primers for peptide substitution using bombolitin T

Name	Sequence	Tm (°C)	%GC
Bomb T-F	GATCCATGTTGTTAGGAAAAATAGGAAAAATATTGTCTCACTTGAAATAAA	73.19	27
Bomb T-R	AGCTTTTATTTCAAGTGAGACAATATTTTTCCTATTTTTCCTAACAACATG		
T1-F	GATCCATGTTGTTAGGAAAAATAGGAGCAATATTGTCTCACTTGAAATAAA	75.5	31
T1-R	AGCTTTTATTTCAAGTGAGACAATATTGCTCCTATTTTTCCTAACAACATG		
T2-F	GATCCATGTTGTTAGGAAAAATAGGAGCAATATTGTTGCACTTGAAATAAA	75.5	31
T2-R	AGCTTTTATTTCAAGTGCAACAATATTGCTCCTATTTTTCCTAACAACATG		
T3-F	GATCCATGTTGTTAGGAAAAATAGGAGCAATATTGTCTCACAAAAAATAAA	74.7	29
T3-R	AGCTTTTATTTTTTGTGAGACAATATTGCTCCTATTTTTCCTAACAACATG		
T4-F	GATCCATGTTGTTAGGAAAAATAGGAGCAATATTGTCTCACTTGAAAATCTAAA	76.4	54
T4-R	AGCTTTTAGATTTTCAAGTGAGACAATATTGCTCCTATTTTTCCTAACAACATG		
T5-F	GATCCATGTTGTTAGGAAAAATAGGAGCAATATTGCTTCACTTGAAAATCTAAA	76.4	31
T5-R	AGCTTTTAGATTTTCAAGTGAAGCAATATTGCTCCTATTTTTCCTAACAACATG		
T6-F	GATCCATGTTGTTAGGAAAAATAGGAGCAATATTGTCTCACAAAAAAATCTAAA	76	30
T6-R	AGCTTTTAGATTTTTTTGTGAGACAATATTGCTCCTATTTTTCCTAACAACATG		
T7-F	GATCCATGTTGTTAGGAAAAATAGGAGCAATATTGTTGCACAAAAAATAAA	74.7	29
T7-R	AGCTTTTATTTTTTGTGCAACAATATTGCTCCTATTTTTCCTAACAACATG		
T8-F	GATCCATGTTGTTAGGAAAAATAGGAAAAATATTGTTGCACTTGAAATAAA	73.9	27
T8-R	AGCTTTTATTTCAAGTGCAACAATATTTTTCCTATTTTTCCTAACAACATG		
T9-F	GATCCATGTTGTTAGGAAAAATAGGAAAAATATTGTTGCACAAAAAATAAA	73.1	25
T9-R	AGCTTTTATTTTTTGTGCAACAATATTTTTCCTATTTTTCCTAACAACATG		
T10-F	GATCCATGTTGTTAGGAAAAATAGGAAAAATATTGTTGCACTTGAAAATCTAAA	75.2	28
T10-R	AGCTTTTAGATTTTCAAGTGCAACAATATTTTTCCTATTTTTCCTAACAACATG		
T11-F	GATCCATGTTGTTAGGAAAAATAGGAAAAATATTGCTTCACAAAAAAATCTAAA	74.4	26
T11-R	AGCTTTTAGATTTTTTTGTGAAGCAATATTTTTCCTATTTTTCCTAACAACATG		
T12-F	GATCCATGTTGTTAGGAAAAATAGGAAAAATATTGTCTCACAAAAAATAAA	73.1	25
T12-R	AGCTTTTATTTTTTGTGAGACAATATTTTTCCTATTTTTCCTAACAACATG		
T13-F	GATCCATGTTGTTAGGAAAAATAGGAAAAATATTGTCTCACAAAAAAATCTAAA	74.4	26
T13-R	AGCTTTTAGATTTTTTTGTGAGACAATATTTTTCCTATTTTTCCTAACAACATG		
T14-F	GATCCATGTTGTTAGGAAAAATAGGAAAAATATTGTCTCACTTGAAAATCTAAA	75.2	28
T14-R	AGCTTTTAGATTTTCAAGTGAGACAATATTTTTCCTATTTTTCCTAACAACATG		

2.9. Statistical analysis

Statistical significances of biological activity mean values were analyzed by Tukey's HSD test using Graph Pad Prism 3 software. Less than 0.05 of P values were considered as statistically significant.

3. Results

3.1. Structural characterization of venom peptides

The amino acid sequences of the mature mastoparans from *V. crabro* and *V. analis* showed 35.7% homology and shared an amphipathic α-helix conformation, which is essential for augmentation of antibiotic activity (Berkowitz et al., 1990). While *V. crabro* mastoparan possessed three positively charged amino acids (Lysine; position 4, 11 and 12), *V. analis* mastoparan possessed four positively charged amino acids (Lysine; position 2, 4, 11 and 12) and one negatively charged amino acid (Aspartate; position 8).

The predicted molecular masses of deduced *Pv*VCP and *Pv*Vespk were 7.72 and 4.12 kDa, and their estimated pI values were 3.96 and 12.11, respectively. Single-signal peptide regions of *Pv*VCP and *Pv*Vespk were predicted at N-terminal strand 1-25 and 1-21 amino acid residues, respectively. One putative N-glycosylation site was predicted at sequences 3-6 (-NISL-) of Vespk within the predicted signal peptide, but none of it was identified in VCP. The mature functional peptide regions from *Pv*VCP and *Pv*Vespk were present at the C-terminal ends, which are indicated by red boxes.

Mature *Pv*VCP peptides comprised a tridecapeptide (FWPGLILKGLGAL)

including multiple hydrophobic and one positively charged residues. In contrast, mature *Pv*Vespk peptides contained dodecapeptide (GRPPGFSPFRSG) including two positively charged residues. A precursor of *Pv*VCP showed several repeats of AXP (X = leucine, glutamic acid, glycine or aspartic acid) residues, which were also identified in the precursors of bioactive peptides of other wasp species (Xu et al., 2006; Yoon et al., 2015b; Yu et al., 2007; Zhou et al., 2006).

The sizes of the mature functional peptide regions of bombolitins were all 18 amino acids. The predicted molecular masses of the deduced bombolitins A, C, T and U were 2.02, 2, 1.93 and 1.94 kDa, and their estimated pI values were 10.98, 10.73, 10.99 and 10.98, respectively. Their net charge was 3.1, except for that of bombolitin C (2.1), and the percentage of hydrophobicity was 50%, except for that of bombolitin U (55.56%).

Mature bombolitin peptides comprised octadecapeptides including multiple hydrophobic residues. Bombolitin A, T and U possessed four positively charged amino acids, while bombolitin C had three. The amino acid alignment revealed that only bombolitin T possessed a different pattern of sequences from the other three peptides at the N-terminal strand. Bombolitins A, C and U had two conserved positively charged amino acids (Lys) at consecutive positions (K^4 and K^5), whereas bombolitin T had these residues at dispersed positions (K^9 and K^{12})

(Fig. 1).

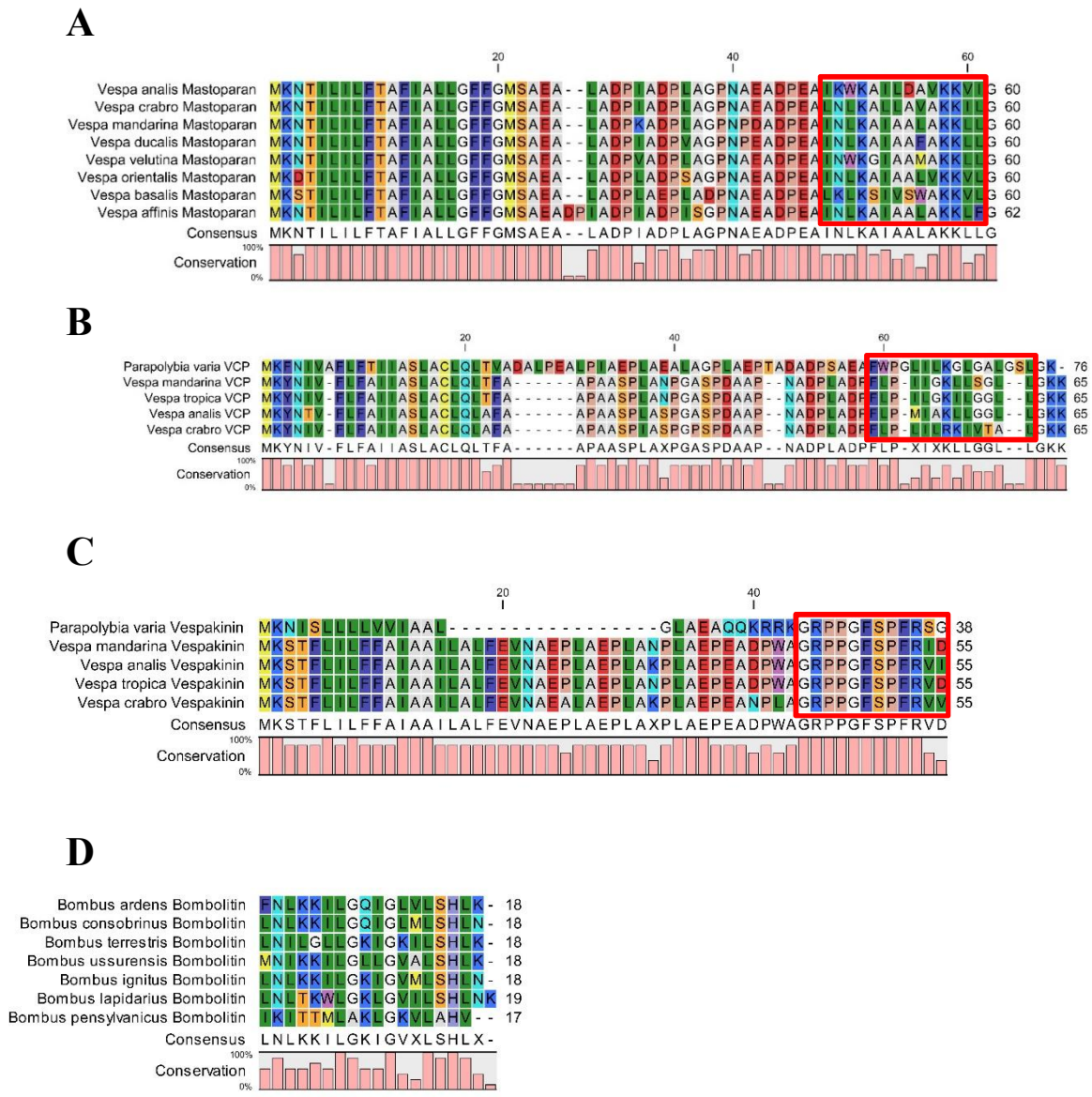


Figure 1. Amino acid sequence alignment of Mastoparan (A), VCP (B) and Vespakinin (C) precursors from Vespid wasps. Red box shows mature peptide regions. Sequence alignment of mature peptide region of bombolitins (D) from *Bombus ardens*, *B. consobrinus*, *B. terrestris*, *B. ussurensis*, *B. ignitus*, *B. lapidarius* and *B. pensylvanicus*.

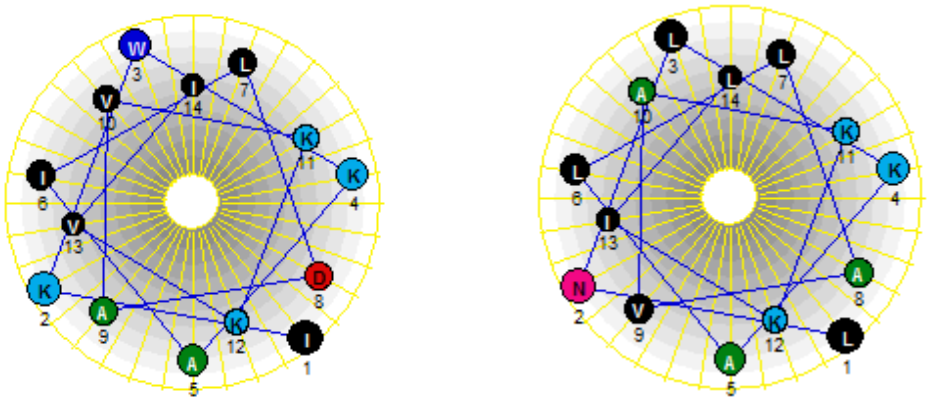
3.2. Characterization of secondary structure

An analysis based on structure modeling using the helical wheel mode revealed that the mastoparans from the two social hornets possess amphipathic characteristics showing segregation of the hydrophobic and hydrophilic amino acids (Fig. 2). Peptides from *V. crabro* and *V. analis* had the same net charge (+ 3) at pH 7. The biological activity of mastoparan is known to be due to the interaction with cell membrane surfaces via positively charged amino acids in an amphipathic α-helix conformation (Etzerodt et al., 2011). It has also been reported that amplification of the net positive charge plays a significant role in improving antimicrobial activity by strengthening the interaction between the venom protein and the negatively charged microbial cell membrane (Bessalle et al., 1992). Likewise, a net positive charge would be crucial for hemolytic or antitumor activity because of the negatively charged membranes of cells (D'Errico et al., 2011; Jiang et al., 2009) (Fig. 2).

The secondary structures of peptides were examined by performing CD spectrometry in aqueous and hydrophobic solutions. The CD spectra of peptides in 10 mM SPB, 30 mM SDS, and 50% TFE were obtained. The results indicated that all peptides were mainly unordered structures in 10 mM SPB. In contrast, they showed distinctive secondary structures in 30 mM SDS and 50% TFE solution. VCPs formed high contents of α-helix structure (> 29.0%) with variable

β-sheet structure contents (0.2% ~ 27.6%) in the hydrophobic environment. In contrast, Vespks showed high contents of β-sheet structure (> 39.0%) with little to no α-helix structures in the hydrophobic environment.

All bombolitins showed high content of α-helix structures (> 45.5%) with no β-sheet structure contents in the hydrophobic environments. These findings imply that the four bombolitins were composed of α-helix conformations in lipid-membrane environments (Table 1).



Mastoparan (*V. analis*) **Mastoparan (*V. crabro*)**

Figure 2. Predicted secondary structure of mastoparans from two social hornets. Helical wheel analysis revealed hydrophobic and hydrophilic amino acid segregation, thereby demonstrating the amphipathic characteristics of mastoparan. A different number of positively charged amino acids was also seen between mastoparans from the two social hornet species.

Table 2. Percentage of α-helical, β-sheet, turn and random structure of VCP, Vespk and Bombolitin in 30 mM sodium dodecyl sulfate (SDS) and 50% trifluoroethanol (TFE)

Peptides	30 mM SDS				50% TFE			
	α-helix	β-sheet	Turn	Random	α-helix	β-sheet	Turn	Random
*Pv*VCP	31.2	0.2	27.6	41.1	29.4	24.7	10.6	35.2
*Vm*VCP	38.2	11.2	18.7	32	30	27.6	9.2	33.2
*Pv*Vespk	0	46	0	54	0.1	43.7	4.5	51.6
*Vm*Vespk	0	45.8	0	54.2	0.8	39	7	53.2
Bombolitin A	63	0	16	21	47.8	0	17.8	34.4
Bombolitin C	78.8	0	10.5	10.7	51.5	0	15.7	32.8
Bombolitin T	61.7	0	17.5	20.7	51.8	0	15	33.2
Bombolitin U	63.6	0	16.2	20.2	45.5	0	17.4	37.1

3.3. Hemolytic activity

One of the known problems caused by functional venom peptides is the lysis of blood cells, which can possibly induce anaphylaxis (Lee and Vadas, 2011). Thus, we examined whether synthesized venom peptides could lyse human red blood cells.

The hemolytic activity assay using synthesized mastoparan revealed that *V. crabro* mastoparan had only limited activity against human erythrocytes over a concentration range of 10–200 μM. In contrast, *V. analis* mastoparan showed a 7-fold higher relative activity at 200 μM, suggesting its higher cytotoxic potential compared with *V. crabro* mastoparan.

*Pv*VCP showed a 12.4-fold lower hemolytic activity than that of *Vm*VCP at 200 μM concentration. In contrast, both *Pv*Vespk and *Vm*Vespk exhibited very low hemolytic activity even at 200 μM concentration. These results suggested that, except for *Vm*VCP, the other peptides examined in this study had very low levels of hemolytic activity and might not be involved in inducing anaphylaxis.

All of the bombolitins exhibited extremely high hemolytic activities showing over 100% of activity at 200 μM concentration. Specifically, bombolitin T showed the highest hemolytic activity (2.3-fold higher than that of 0.1% Triton X-100) among the peptides at a concentration of 200 μM, and bombolitin C exhibited the lowest activity at the same concentration (1.3-fold higher than that of 0.1% Triton

X-100). These results suggested that all of the bombolitins had high levels of hemolytic activity and might be involved in causing serious anaphylaxis (Table 2).

3.4. Anti-microbial activity

Mastoparans from both hornet species at concentrations over 0.5 mM showed strong antimicrobial activities against *S. aureus*. The same range of concentrations (over 0.5 mM) of the peptides also showed antimicrobial activity against *B. cinerea*. *V. crabro* mastoparan showed an expected minimal inhibitory concentration (MIC) of over 1 mM against Escherichia coli. *V. analis* mastoparan exhibited higher antimicrobial activity, as its expected MIC against *E. coli* was 0.5 mM. The MIC of *V. crabro* mastoparan against *C. albicans* was estimated to be over 0.1 mM, whereas that of *V. analis* mastoparan was expected to be over 0.5 mM. Likewise, the top agar assay against all bacteria and fungi showed an inhibition zone at concentrations of mastoparans over 0.5 mM.

*Pv*VCP only showed anti-fungal activities to *C. albicans*. *Pv*VCP did not inhibit the growth of *E. coli*, *S. aureus*, and *B. cinerea*. In contrast, *Vm*VCP exhibited anti-microbial and anti-fungal activities above 0.5 mM concentrations except to *B. cinerea*. The growth of *B. cinerea* was not inhibited by even the highest test concentrations of *Pv*VCP and *Vm*VCP. *Pv*Vespk and *Vm*Vespk did not show any anti-microbial or anti-fungal activities even at high concentrations.

These results suggested that *Pv*VCP and *Pv*Vespk have little to no anti-fungal activities.

All of the peptides exhibited anti-microbial activity against *S. aureus* and *C. albicans* at concentrations of 0.5 mM. In particular, bombolitin T had high activity against *S. aureus* at a concentration of 0.1 mM. The growth of *E. coli* was inhibited at 0.5 mM of bombolitin A, whereas other bombolitins began to show inhibition at concentrations of 1 mM. All four bombolitins showed anti-fungal activities against *B. cinerea* at the same concentration (2 mM) following 33 h of incubation. Four peptides also exhibited strong anti-microbial activities in top agar assays by showing an inhibition zone at concentrations over 0.25 mM. These results suggested that the four bombolitins had considerable levels of anti-microbial and anti-fungal activity (Table 2).

3.5. Anti-tumor activity

The treatment of SK-OV-3 cells with 100 μM of *V. crabro* mastoparan resulted in a slightly decreasing tendency in the relative survival rate of cells over a period of 24 to 72 h post-treatment. In contrast, the use of 100 μM caused a dramatic decrease in the survival rate of SK-OV-3 cells at 24 h post-treatment. Although the survival rate of SK-OV-3 increased after 72 h, the survival rate at 100 μM of *V. analis* mastoparan was still extremely low compared with the survival rates of

cells exposed to lower concentrations, thereby displaying a typical dose-dependency. Similar results were also obtained using NIH-OVCAR-3 cells. When 100 μM of *V. crabro* mastoparan was treated, the survival rate of NIH-OVCAR-3 was slightly decreased at 24 h post-treatment and the rate was further decreased by 0.8-fold until 72 h post-treatment. The treatment with *V. analis* mastoparan resulted in a large reduction in the relative survival rates of NIH-OVCAR-3 cells at 24 h post-treatment at concentrations over 100 μM. The rate was further increased 1.4-fold until 72 h post-treatment at the same concentration (100 μM). These results clearly demonstrate the differential antitumor activity of the mastoparans from the two hornets. The relatively greater antitumor activity associated with *V. analis* mastoparan is also likely due to the differences in amino acid sequence between the two species. Since *V. crabro* mastoparan showed antitumor activity at a lower concentration than the concentration required for hemolytic activity, it may have higher selective toxicity against tumor cells.

*Pv*VCP showed significantly stronger anti-tumor activities to SK-OV-3 and NIH-OVCAR-3 lines at concentrations higher than 10 μM compared with the same concentration of VmVCP when applied for 24 h and 72 h. In contrast, *Pv*Vespk showed a significantly stronger anti-tumor activity to SK-OV-3 at 24 h post-treatment compared with those of *Vm*Vespk. The anti-tumor activities of *Pv*Vespk were similar to those of *Vm*Vespk under other conditions. Taken together,

*Pv*VCP and *Pv*Vespk had significantly stronger anti-tumor activities compared with those of *Vm*VCP and *Vm*Vespk, respectively.

The growth of two types of tumor cells (SK-OV-3 and NIH-OVCAR-3) was totally inhibited at 25 μM concentrations of bombolitins A, C and U after 72 h of treatment, whereas bombolitin T exhibited a stronger anti-tumor activity by showing growth inhibition at 10 μM. Bombolitin T showed 12.5-fold higher anti-tumor activity against SK-OV-3 cells, as judged by half maximal inhibitiory concentration (IC_{50}) value after 72 h of treatment, than did the vespid chemotactic peptide from *P. varia* (*Pv*VCP), a member of a group of α-PFTs known to have strong anti-tumor activity (Yoon et al., 2016). The other three bombolitins also showed approximately 5-fold higher anti-tumor activity than that of *Pv*VCP. Bombolitin T exhibited about 2.5- and 2.4-fold lower IC_{50} values for the two tumor cell types than those of the other three bombolitins after 72 h of treatment. This implies that bombolitin T had significantly higher anti-tumor activity compared with those of other venom peptides (Table 2).

Table 3. Biological activities of mastoparan, VCP, Vespk and bombolitin. Anti-microbial activity against Gram-negative *Escherichia coli* (E), Gram-positive *Staphylococcus aureus* (S), Gram-positive *Candida albicans* (C) and gray mold *Botrytis cinerea* (B). Anti-tumor activity against ovarian tumor cells (SK-OV-3 and NIH-OVCAR-3). Hemolytic activity against human red blood cells (RBCs).

Peptides	Anti-microbial activity, MIC (μM)				Antitumor activity				Hemolytic activity,
					IC_{50} (M) (24 h)		IC_{50} (M) (72 h)		EC_{50} (μM)
	E	S	C	B	SK-OV-3	NIH-OVCAR-3	SK-OV-3	NIH-OVCAR-3	RBC
Mastoparan A	500*	500**	500*	500	>50**	>50**	>50**	ni**	>200**
Mastoparan C	>1000**	500**	100*	500	ni**	ni**	ni**	ni**	ne
*Pv*VCP	ni	ni	1000**	ni	>100**	>100**	>100**	>100**	ne
*Vm*VCP	>1000	500*	1000	ni	>100**	>100**	>100**	>100**	>200
*Pv*Vespk	ni	ni	ni	ni	ni**	ni**	>100**	ni**	ne
*Vm*Vespk	ni	ni	ni	ni	>100**	ni**	>100**	ni**	ne
Bombolitin A	500**	500**	500**	2000	17.8**	16.4**	19.1**	20.9**	54.1**
Bombolitin C	1000**	500*	500*	2000	16**	16.4**	19.6**	21.0**	61.2**
Bombolitin T	1000**	100*	500**	2000	7.3**	8.5**	8.0**	8.8**	12.6**
Bombolitin U	1000**	500**	500**	2000	16.8**	16.4**	20.7**	20.7**	48.6**

* $P < 0.01$
** $P < 0.0001$

3.6. Analysis of factors affecting hemolytic activity

Six structural properties of venomic peptides were arranged in a matrix and PCA analysis was conducted. The first and second principal components (PC1 and PC2) explained 80.35 and 13.44% of the total variance, respectively (Fig. 3). PC1 correlated in high degree with PPA, NC, pI and α-helicities, but only hydrophobicity showed negative correlations (Table 4). The result of score plot showed four Bombus species were grouped along with positive value of PC1 and *P. varia* and *V. mandarina* were independently distributed in accordance twith negative value of PC1 and each side of PC2 as an outlier (Fig. 4). This implies that four Bombus species have different structural properties compared to *P. varia* and *V. mandarina*.

Coefficients for PC1 had significant influences on anti-tumor activities but not on hemolytic activity (Table 5). Anti-tumor and hemolytic activities showed strong correlation with each other. These results suggest that structural properties except hydrophobicity might affect anti-tumor activity of venom peptides and anti-tumor and hemolytic activities interact each other.

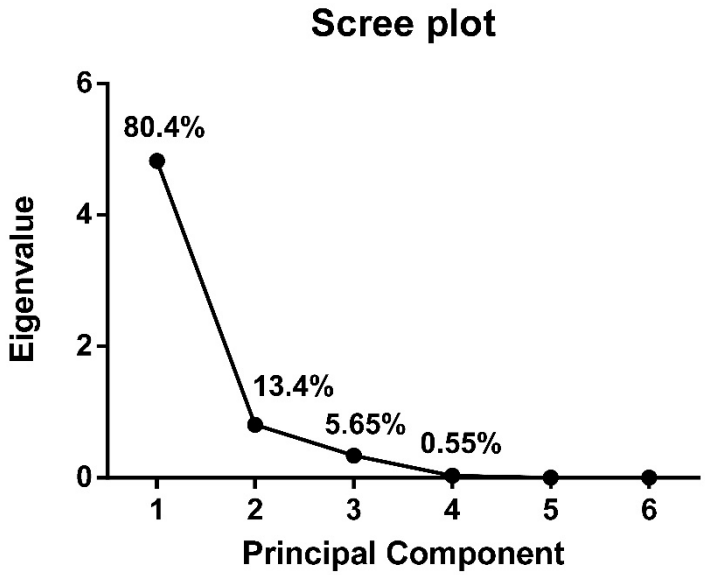


Figure 3. The scree test for structural properties of venomic peptides.

Table 4. Factors loading of principal components for structural properties of venomic peptides.

	Eigenvectors					
	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**
PPA	0.444542	0.010601	0.37158	-0.10346	0.0535	-0.80662
NC	0.435622	-0.01456	0.499772	-0.07544	0.537477	0.515439
pI	0.452588	0.050407	0.171233	-0.11002	-0.81716	0.288885
HP	-0.2392	0.931218	0.270602	0.042129	-0.02471	-0.00198
α-helicity (SDS)	0.399988	0.317442	-0.63657	-0.54692	0.186267	0.013868
α-helicity (TFE)	0.436545	0.170875	-0.32309	0.818902	0.072157	-0.00625

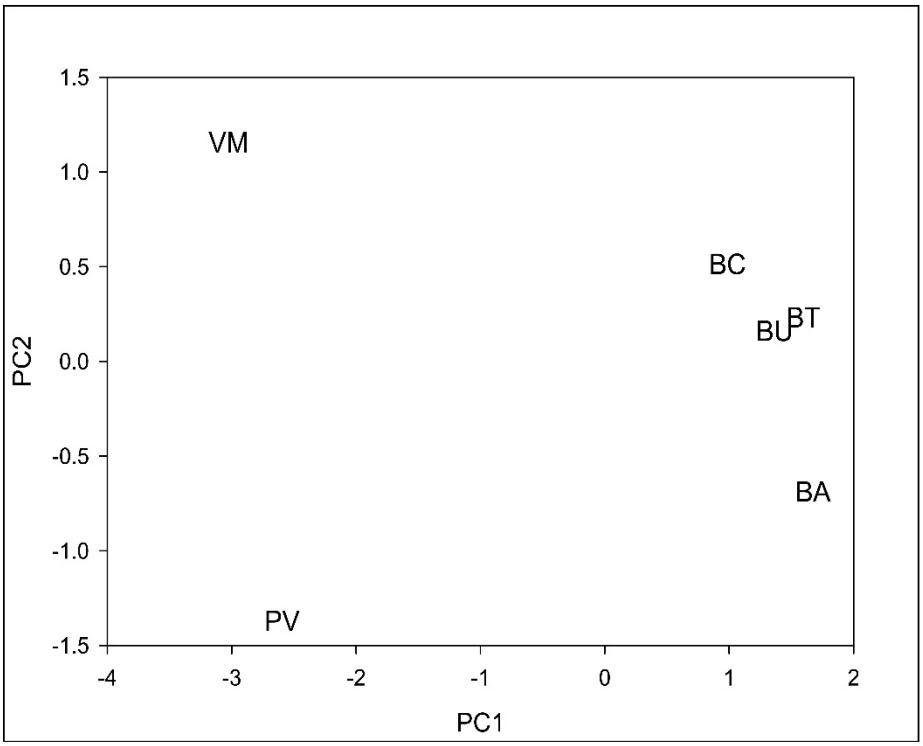


Figure 4. Score plot of PC1 *vs.* PC2 for six venomic peptides.
Abbreviations: PV, *P. varia* VCP; VM, *V. mandarina* VCP; BA, *B. ardens* bombolitin; BC, *B. consobrinus* bombolitin; BT, *B. terrestris* bombolitin; BU, *B. ussurensis* bombolitin

Table 5. Correlation coefficients of PC1 and PC2 with anti-tumor and hemolytic activities.

	Pearson Correlation Coefficients, N = 6				
	PC1	**PC2**	**IC_{50} (SK)**	**IC_{50} (NIH)**	**EC_{50}**
PC1	1	0	0.80021	0.85513	0.51859
		1	0.0559	0.03	0.2918
PC2	0	1	0.10729	0.11219	0.15746
	1		0.8397	0.8324	0.7658
IC50 (SK)	0.80021	0.10729	1	0.99365	0.92324
	0.0559	0.8397		<0.0001	0.0086
IC50 (NIH)	0.85513	0.11219	0.99365	1	0.87885
	0.03	0.8324	<0.0001		0.0211
EC50	0.51859	0.15746	0.92324	0.87885	1

0.2918	0.7658	0.0086	0.0211	

3.7. Characterization of amino acid-substituted bombolitins

Among 14 substituted peptides, two showed comparatively lower hemolytic activities, though not at a statistically significant level, than the bombolitin T template (Table 3). T6 and T14 exhibited 1.14- and 1.12-fold lower half maximal effective concentration (EC_{50}) values than the template. Six peptides showed higher EC_{50} values than the template, and T11 specifically possessed the highest value (1.1-fold higher than the template) among all of the peptides. To observe the factors that affect hemolytic activity, we calculated the hydrophobicity, net charge and pI values. The net charge and pI values of T6 and T14 were the same as those of the template, but their hydrophobicities were higher than that of the template (50.0 and 57.1, respectively). T6 and T14 had the same sequence composition except for one amino acid. T11 and T12, which possessed higher and lower hydrophobicities than the template, respectively, exhibited high cytotoxicity, implying that hydrophobicity is not the main factor influencing the hemolytic activity of modified bombolitins. Taken together, these results indicate that structural properties including net charge and pI values and the positions of amino acids might affect the biological activity of bombolitins (Table 3).

Table 6. Structural properties and hemolytic activity of amino acid substituted bombolitin from *B. terrestris*.

Peptides	Sequences	Remarks	Hydrophobicity	Net charge	pI	EC_{50} (μM)
Bomb T	LLGKIGKILSHLK		46.15	+4	11.1	18.91
T1	LLGKIG**A**ILSHLK	K^7 → A	53.85	+3	10.8	18.91
T2	LLGKIG**A**IL**L**HLK	K^7, S^{10} → A, L	61.54	+3	10.8	19.04
T3	LLGKIG**A**ILSH**K**K	K^7, L^{12} → A, K	46.15	+4	11.1	18.54
T4	LLGKIG**A**ILSHLK**I**	K^7 → A, I^{14}	57.14	+3	10.8	19.12
T5	LLGKIG**A**IL**L**HLK**I**	K^7, S^{10} → A, L, I^{14}	64.29	+3	10.8	18.19
T6	LLGKIG**A**ILSH**K**K**I**	K^7, L^{12} → A, K, I^{14}	50	+4	11.1	16.53
T7	LLGKIG**A**IL**L**H**K**K	K^7, S^{10}, L^{12} → A, L, K	53.85	+4	11.1	19.15
T8	LLGKIGKIL**L**HLK	S^{10} → L	53.85	+4	11.1	17.73
T9	LLGKIGKIL**L**H**K**K	S^{10}, L^{12} → L, K	46.15	+5	11.28	19.39
T10	LLGKIGKIL**L**HLK**I**	S^{10} → L, I^{14}	57.14	+4	11.1	16.85
T11	LLGKIGKIL**L**H**K**K**I**	S^{10}, L^{12} → L, K, I^{14}	50	+5	11.28	19.93
T12	LLGKIGKILSH**K**K	L^{12} → K	38.46	+5	11.28	19.55
T13	LLGKIGKILSH**K**K**I**	L^{12} → K, I^{14}	42.86	+5	11.28	17.07
T14	LLGKIG**A**IL**L**H**K**K**I**	K^7, S^{10}, L^{12} → A, L, K, I^{14}	57.14	+4	11.1	16.81

Substituted amino acids are described in bold.
* $P < 0.05$, ** $P < 0.0001$

4. Discussion

V. analis mastoparan exhibited significantly greater antimicrobial and antitumor activities than *V. crabro* mastoparan. Such differential bioactivities appear due to the amino acid sequence differences in the mature peptides. In particular, the additional Lys residue present in *V. analis* mastoparan may contribute to the higher levels of bioactivity as proposed by secondary structure prediction. The selective antitumor activity of *V. analis* mastoparan merits further characterization of the structure (amino acid composition)-activity relationship.

*Pv*VCP and *Pv*Vespk showed very low to no hemolytic activities. In contrast, *Vm*VCP exhibited hemolytic activity at a higher concentration. Studies in cell lytic peptides have suggested that they have an amphiphilic α-helix structure that allows peptides to selectively bind and disrupt the more negatively charged membrane of prokaryotes and tumor cells than normal eukaryotic cells (Hansel et al., 2007; Orsolic, 2012). Pore-forming toxins (PFTs) such as mastoparan, melittin, VCP, and Vespk can be classified into α-PFTs and β-PFTs depending on whether their secondary structure comprises α-helices or β-sheets, respectively (Dal Peraro and van der Goot, 2016; Gouaux, 1997; Iacovache et al., 2010; Lesieur et al., 1997). Because *Pv*VCP and *Vm*VCP showed 29.4–31.2% and 30–38.2% of α-helical structure in 30 mM SDS and 50% TFE solutions, respectively, they appear

to be categorized into α-PFTs. In contrast, *Pv*Vespk and *Vm*Vespk, which were not composed of *α*-helical structures in both lipid-membrane-mimicking solutions as judged by CD spectra, likely belong to *β*-PFTs. Thus, the more selective anti-tumor activity of *Pv*VCP compared with *Pv*Vespk is likely to be due to its high percentage of *α*-helix folding.

Polybia-MPI, a mastoparan-like *α*-PFT from the Brazilian wasp *Phylloscartes paulista* venom, is known to have highly selective anti-tumor activities against several types of cancer cells, including bladder and prostate cancer cells (Wang et al., 2008). Most of the *α*-PFTs exhibiting anti-tumor-activity, including polybia-MPI, *V. crabro* mastoparan, *V. analis* mastoparan, *Pv*VCP, and *Vm*VCP, possessed one to four positively charged amino acids and + 1 to + 3 of net charge at pH 7. Since tumor cells cause anionic phosphatidylserine externalization (Lee et al., 2013), these cationic peptides can easily bind to their membranes rather than those of normal cells (Moreno and Giralt, 2015). Nevertheless, because polybia-MPI has a lower number of positively charged amino acids than *V. analis* mastoparan and a lower net charge (+ 1) compared with other cytotoxic peptides, the positive charge property associated with *α*-PFTs may not be the major determining factor for anti-tumor-activity. The high anti-tumor activity found in some *α*-PFTs might be due to their high percentage of *α*-helix folding, as observed in polybia-MPI, which consists of 74.1% and 56.2% of *α*-helical structure in 30 mM SDS and 50%

TFE solutions, respectively (Wang et al., 2008). A positive correlation has been reported between α-helicity and the hemolytic or anti-tumor activity of α-helical antibiotic peptides on lipid membranes but not with anti-microbial activity (Shin et al., 2001).

Venomic peptides, which are classified as α-PFTs, including the mastoparans of *V. analis* and *V. crabro* (Yoon et al., 2015a), the vespid chemotactic peptide of *V. mandarinia* (Yoon et al., 2016) and bombolitins (Yoon et al., 2017), exhibited strong anti-microbial activities against Gram-positive bacteria and yeast at concentrations over 0.5 mM. Only *V. analis* mastoparan and bombolitin A inhibited the growth of Gram-negative *E. coli* at the same concentration. Gram-positive bacteria are composed of plasma membranes and peptidoglycan cell walls, but the same bilayers of Gram-negative bacteria are covered with a lipopolysaccharide (LPS) outer membrane. Since the LPS outer membrane controls anti-microbial peptide binding and insertion into Gram-negative bacteria, this might be the reason for selective anti-microbial activity between Gram-positive and Gram-negative bacteria (Papo and Shai, 2005). The aromatic amino acid Phe1 of bombolitin A might also contribute to its high anti-microbial activity against gram-negative *E. coli* by providing potential anti-microbial activity due to its relationship with LPS outer membrane permeabilization (Datta et al., 2016).

The strongest biological activities of bombolitin T could be explained by its

relatively higher hydrophobicity in the N-terminal strand compared to the other bombolitins. The N-terminal strands with seven amino acids of bombolitins A, C and U showed conserved and similar patterns in each position of the sequence. The characteristic of bombolitin T sequence that differs from the others is the position of the positively charged amino acids Lys^{9} and Lys^{12}. In the other bombolitins, these two amino acids were positioned at Lys^{4} and Lys^{5}. Mastoparans including Parapolybia-MP and Polybia-MP, which have the same positions of lysine as bombolitins A, C and U, showed high anti-microbial and hemolytic activity. HR2, which is a mast cell degranulating peptide originated from *V. orientalis*, possessed lysine positions (Lys^{8} and Lys^{11}) similar to those of bombolitin T and exhibited extremely high hemolytic activity (de Souza et al., 2011). Protonectarina-MP possessed the same positions of lysine (Lys^{4}, Lys^{11} and Lys^{12}) as mitoparan and exhibited high anti-microbial and hemolytic activity. Although there are three known mechanisms of pore formation by anti-microbial peptides (Ludtke et al., 1996; Sansom, 1991; Shai, 1999), the pore formation mechanism in mastoparans is not well known. This high cytotoxicity might be explained by difference in the position of the positively charged amino acid lysine (from 4^{th} to 12^{th}), which results in high hydrophobic surface and contributes to interactions with the acyl chains of the phospholipids in biological membranes (de Souza et al., 2011).

Taken together, further study for investigating correlations between anti-tumor and hemolytic activity might be useful for designing selective anti-tumor peptides by modifying amino acids.

There have been many attempts to modify the structural properties of functional peptides to design selective anti-microbial and anti-tumor peptides. Structural properties including α-helicity (Yoon et al., 2016), amphipathicity (Kondejewski et al., 1999), hydrophobicity (Dathe et al., 1996; Dhondikubeer et al., 2012; Kiyota et al., 1996; Sitaram et al., 1995; Subbalakshmi et al., 1999; Wieprecht et al., 1997), net positive charge (Jiang et al., 2008) and balanced substitution with hydrophobic and positively charged amino acids (Zhao et al., 2013) are known to affect cytotoxicity. Since the high hemolytic activity associated with bombolitins could be a major obstacle for the use of these peptides as a new lead for anti-tumor agents, we used PCA to determine the structural properties associated with the hemolytic activity of bombolitins and to search for an efficient way to reduce the cytotoxicity of bombolitins.

The secondary structure of the hybrid peptide that is derived from cecropin and melittin is related to hemolytic activity (Shin et al., 1997). Although the authors could not exactly explain the relationships between peptide secondary structures and hemolytic activity, their study showed that α-helicity is closely related to cytotoxicity. Although bombolitin T only had the highest α-helicity

among the four bombolitins in the 50% TFE buffer, the percentages of α-helix conformation between the four bombolitins were not extremely different. In our previous study, the vespid chemotactic peptides of *P. varia* and *V. mandarinia* (*Vm*VCP) had 31.2% and 38.2% α-helicity, respectively, but only *Vm*VCP showed high hemolytic activity, implying that α-helicity could not be the sole factor affecting hemolysis in vespid chemotactic peptides (Yoon et al., 2016). Bombolitin T and bombolitin C, which had the lowest and the highest α-helicity in 30 mM SDS buffer, respectively, exhibited the lowest and the highest IC_{50} and EC_{50} values against tumor and human red blood cells. This implies that α-helicity has a positive relationship with anti-tumor and hemolytic activities in bombolitins and that the anti-tumor and hemolytic activities of bombolitins are sensitive to α-helicity. The analysis of α-helicity by CD spectrometry is important in identification of the relationships between the secondary structures and the biological activity of modified bombolitins.

Another study reported that amphipathicity in an anti-microbial peptide that consisted of highly amphipathic β-sheet conformations had a correlation with high anti-microbial activity and low hemolytic activity, because it gave the peptides a high affinity to the outer membranes of Gram-negative microorganisms (Kondejewski et al., 1999). Since the PCA results showed that there was a positive relationship between hemolytic activity and the percentage of positively charged

amino acids and that only hydrophobic amino acids had negative relationships with biological activities, four residues of bombolitin T were replaced by positively charged and hydrophobic amino acids. The negative value of hydrophobicity could be explained by the fact that hydrophobicity is not extremely different between 6 peptides, resulting in a reduction of specificity in the analysis. In terms of the hemolytic activity of the amino acid-substituted bombolitins (Table 3), modified bombolitins with lower (T12) or higher (T11) hydrophobicities than the template exhibited higher cytotoxicity than the template peptide. This also suggests that the hydrophobicity of bombolitins is not a primary factor for minimal cytotoxicity. T2, T4, T7, T9, T11 and T12, which had higher EC_{50} values than the template, showed a pattern of increasing hemolytic activity with increasing net positive charge. This can be explained by the fact that the increase in net charge destabilizes the formation of transmembrane channels or pores in human red blood cells, and increasing the net positive charge beyond the optimum level stabilizes the pores or channels by increasing the hydrophilicity of the pores (Jiang et al., 2008). This implies that hemolytic activity is sensitive to the net charge. A recent study on modification of anti-microbial peptides to improve their anti-microbial and hemolytic activities reported that the balanced substitution of a positively charged amino acid (Arg) and a hydrophobic amino acid (Trp) can efficiently kill bacteria but minimize hemolysis (Zhao et al., 2013).

Positively charged amino acids could facilitate the initial anchoring and absorption of peptide on the negatively charged lipid bilayer surface, whereas hydrophobic amino acids could interact with the hydrophobic tails of lipids for membrane insertion (Zhao et al., 2013).

T11 has optimized and balanced positions of amino acids for low cytotoxicity. The substitution of Ser^{10} and Leu^{12} with a hydrophobic amino acid (Leu) and a positively charged amino acid (Lys) could reduce hemolytic activity. The addition of hydrophobic amino acid (Ile^{14}) might also affect hemolytic activity because a hydrophobic tail results in reduced cytotoxicity (Dhondikubeer et al., 2012).

5. Conclusions

In this study, to investigate the pharmacological and toxicological properties of venom peptides including mastoparan, VCP, Vespk and bombolitin in detail, we isolated peptides from social wasps *V. analis*, *V. crabro*, *V. mandarina* and *P. varia* and bumble bees *B. ardens*, *B. consobrinus*, *B. terrestris* and *B. ussurensis* and evaluated their biological activities with a particular emphasis on their anti-tumor activity. Among the venom peptides tested, bombolitin T showed the highest hemolytic and anti-tumor activities. In an attempt to reduce the hemolytic activity of bombolitin, we used bombolitin T as a model and modified its amino acid composition to be similar to that of mitoparan, which has high anti-tumor activity. To determine the correlation between anti-tumor or hemolytic activity and the physicochemical properties of bombolitin T, principal component analysis (PCA) was conducted. The lowest hemolytic activity was observed with the addition of a hydrophobic amino acid (Ile^{14}) to the C-terminal end as well as the substitution of Ser^{10} and Leu^{12} with a hydrophobic amino acid (Leu) and positively charged amino acid (Lys), respectively. This finding implies that the balanced substitution of hydrophobic and positively charged amino acids could affect the cytotoxicity. Taken together, this work provides a description of newly found, strong anti-tumor peptides in the venom of bumblebees and useful tools for designing

selective anti-tumor agents for future therapeutic applications.

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KOREAN ABSTRACT

Aculeata 벌류의 독성분 동정 및 특성분석

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초록

Aculeata 벌류/말벌류의 독액 성분과 발현 양상을 탐색하기 위해 독성 특이 전사체 분석을 실행하였고 FPKM 값은 하우스키핑 유전자인 α-tubulin의 평균 전사량을 통해 일반화하였다. Hyaluronidase, phospholipase A2, metalloendopeptidase 등과 같은 독액 성분이 사회성 말벌과 단독생활형 사냥벌에서 공통적으로 발견되었다. 먹이의 불활성화와 생리조절 같은 기능을 하는 다양한 생리활성물질들이 단독생활형 사냥벌에서 검출될 것으로 예상되었지만, 이 예상을 일반화할 만큼의 충분한 양의 독선전사체 정보를 얻을 수 없었다. 그럼에도 불구하고, pompolidotoxin과 dendrotoxin-like peptide와 같은 신경독성 펩타이드와 insulin-like peptide binding protein과 같은 단백질이 단독생활형 사냥벌에서 특이적으로 검출되었다. 반대로, venom allergen 5, venom acid phosphatase, phospholipases와 같은 몇몇 유전자들은 사회성 말벌에서 상대적으로 빈번히 검출되었다. 뒤영벌류의 독선 전사체 분석을 통해 알레르기 유발 항원과 고통 유발 물질이 거의 검출되지 않은 사실을 보았을때, 뒤영벌류의

독액은 사회성 말벌과 단독생활형 사냥벌의 독액에 비해 상대적으로 낮은 독성을 지닐것으로 생각된다.

말벌과 좀말벌의 독액 특성과 독성 정도의 차이를 탐색하기 위해 독액 성분과 독선 특이 전사특성을 분석하고 비교하였다. 두 종의 독선 전사체에서 총 35개의 독액 특이 유전자가 동정되었으나, 말벌과 좀말벌의 전사 양상은 차이를 보였다. 질량분광학을 이용하여 주요 독액 성분들을 동정하고 확인하였다. 대부분의 주요 독액 성분들은 말벌에서 더욱 빈번히 발현되었고, muscle LIM protein, troponin, paramyosin, calponin 등과 같은 소수 독선유전자들은 좀말벌 내에서 더 높은 전사량을 보여주었다. 독선 특이 전사체 비교를 통해, 높은 빈도로 주요 독액을 내포하고 있는 말벌의 독성이 좀말벌에 비교해 잠재적으로 높은 독성을 가질것으로 생각된다.

말벌, 좀말벌, 뱀허물쌍살벌, 장수말벌, 좀뒤영벌, 황토색뒤영벌, 서양뒤영벌, 그리고 우수리뒤영벌에서 발견된 Mastoparan, Vespid chemotactic peptide (VCP), vespakinin (Vespk), bombolitin의 잠재적 독성과 생리활성의 차이를 탐색하기 위해 합성된 독액 펩타이드들의 독성학적/약학적인 활성을 분석하였다. 합성된 좀말벌 mastoparan의 용혈성, 항균성, 항암성이 말벌보다 높았는데 이러한 활성차이는 mature peptide의 아미노산 서열이 다르기 때문일 것으로 생각된다. 뱀허물쌍살벌 VCP, Vespk 그리고 장수말벌 Vespk은 용혈성을 보이지 않았으며, 장수말벌 VCP만 높은 농도에서 용혈성을 보였다. 장수말벌 VCP는 항균성과 항진균성을 보였고, 뱀허물쌍살벌 VCP는 *Candida albicans*에 대해서만 항진균성을 보였다. 뱀허물쌍살벌 VCP는 두 난소암 세포에 대해서 장수말벌 VCP보다 높은 항암성을 보였고, Vespk들은 NIH-OVCAR-3 세포보다 SK-OV-3 세포에 대한 항암성을 보였다. 4종의 bombolitin 중 bombolitin T가 가장 높은 용혈성과 항암성을 보여주었다. 모든 bombolitin은 강한 항균성과 항진균성을 보였는데, 특히 bombolitin A는 그람음성 세균인 *Escherichia coli*에 대해서 가장 높은 항균성을 보였다.

낮은 용혈성의 특이적인 항암성을 지닌 펩타이드를 만들기 위해, bombolitin T를 선정하여 아미노산 서열 치환을 수행하였다. C-terminal 말단에 소수성 아미노산인 Ile^{14}을 붙이고, Ser^{10}과 Leu^{12}를 각각 소수성 아미노산인 Leu과 양전하를 띄는 아미노산인 Lys으로 치환하였을 때 가장 낮은 용혈성을 보였다. 이는 소수성 아미노산과 양전하를 띄는 아미노산의 균형적인 치환이 세포독성에 영향을 미치기 때문인 것으로 사료된다.

검색어: 독선, 독액 펩타이드, 전사 프로필, RNA 시퀀싱, 원이색성, 생리활성, 주성분분석, 펩타이드 치환

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연구를 시작하게 해주시고 항상 곁에서 가르쳐주신 김길하 교수님, 이시혁 교수님, 고영호 교수님께 진심으로 감사의 인사를 전해드립니다.
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열정적이며 청렴하신 저의 지도교수님 이시혁 교수님을 알게 된 것은 제 인생의 가장 큰 행운이라 생각합니다.
더욱 멋진 연구를 통해 보답하겠습니다.

저희 가족들 그 누구보다 사랑합니다.
앞으로 가족이 될 인진이에게도 항상 고맙고 사랑한다는 말을 전합니다.

2018년 8월
윤경재 올림